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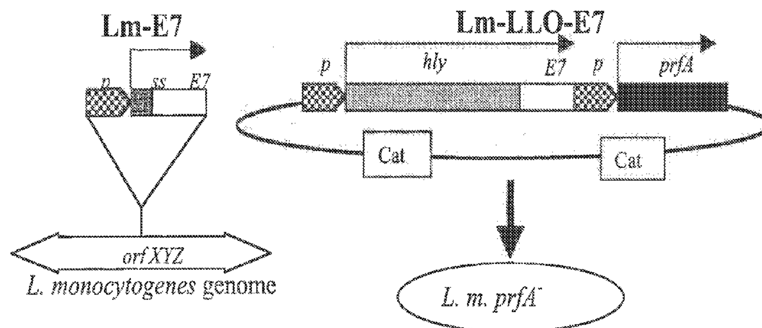

Figure 1A Figure 1B

FIGURE 1

(57) Abstract: This invention provides a system of providing and creating personalized immunotherapeutic compositions for a subject having a disease or condition, including therapeutic immunotherapy delivery vectors and methods of making the same comprising gene expression constructs expressing peptides associated with one or more neo-epitopes or peptides containing mutations that are specific to a subject's cancer or unhealthy tissue. A delivery vector of this invention includes bacterial vectors including *Listeria* bacterial vectors; or viral vectors, peptide immunotherapy vectors; or DNA immunotherapy vectors, comprising one or more fusion proteins comprising one or more peptides comprising one or more neo-epitopes present in disease-bearing biological samples obtained from the subject. This invention also provides methods of using the same for inducing an immune response against a disease or condition, including a tumor or cancer, or an infection, or an autoimmune disease or an organ transplant rejection in the subject.

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PERSONALIZED DELIVERY VECTOR-BASED IMMUNOTHERAPY AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Application No. 62/166,591, filed May 26, 2015, U.S. Application No. 62/174,692, filed June 12, 2015, and U.S. Application No. 62/218,936, filed, September 15, 2015, each of which is herein incorporated by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING SUBMITTED AS A TEXT FILE VIA EFS WEB

[002] The Sequence Listing written in file SEQLIST_ST25.txt is 488 kb, was created on May 25, 2016, and is hereby incorporated by reference.

FIELD OF INTEREST

[003] This invention provides a personalized immunotherapy composition for a subject having a disease or condition, including therapeutic immunotherapy delivery vectors and methods of making the same comprising gene expression constructs expressing peptides associated with one or more neo-epitopes or peptides containing mutations that are specific to a subject's cancer or unhealthy tissue. A delivery vector of this invention includes bacterial vectors; or viral vectors, or peptide immunotherapy vectors; or DNA immunotherapy vectors including *Listeria* bacterial vectors comprising one or more fusion proteins comprising one or more peptides comprising one or more neo-epitopes present in disease-bearing biological samples obtained from the subject. This invention also provides methods of using the same for inducing an immune response against a disease or condition, including a tumor or cancer, or an infection, or an autoimmune disease or an organ transplant rejection in the subject.

BACKGROUND OF THE INVENTION

[004] Before personalized medicine, most patients with a specific type and stage of cancer received the same treatment. However, it has become clear to doctors and patients that some treatments worked well for certain patients and not as well for others. Thus, there is a need to develop effective, personalized immunotherapies effective for a particular tumor. Personalized treatment strategies may be more effective for an individual and cause fewer side effects than would be expected with standard treatments.

[005] Tumors develop due to mutations in a person's DNA, which can cause the production of mutated or abnormal proteins, comprising potential neo-epitopes not present within the corresponding normal protein produced by the host. Some of these neo-epitopes may stimulate T-cell responses and mediate the destruction of early-stage cancerous cells by the immune system so that clinical evidence of a cancer does not develop. In cases of established cancer, however, the immune response has been insufficient. A large body of data has been generated regarding the development of therapeutic immunotherapies that target natural sequence tumor-associated, over-expressed or inappropriately expressed biomarkers in cancer. However demonstration of clear clinical benefit associated with these treatments has proven quite difficult with only one therapeutic immunotherapy being approved by the FDA at the time of this writing. . A major reason for this is that as part of central tolerance that develops in all individuals, any T cells that have high binding affinity toward natural sequence peptides are identified as self-antigens and these self-reactive clones are eliminated by the thymus early in life, or otherwise inactivated through mechanisms of tolerance to prevent auto-immunity.

[006] Neo-epitopes are potentially immunogenic epitopes present within a protein associated with a disease that result from a change in the DNA that occurs later in life, such as an acquired mutation or genomic change caused by changes in the DNA of certain cells. For example a cancer, wherein the specific "neo-epitope" is not present within the corresponding normal protein associated with cells (in the same individual) that do not harbor the acquired DNA abnormality which results in the neoepitope expressed in a subjects cells that are not diseased or comprising a disease-bearing tissue therein. Neo-epitopes may be challenging to identify, however doing so and developing treatments that target them would be advantageous for use within a personalized treatment strategy. The specific acquired DNA abnormality(s) are very individual to both the specific patient's diseased cells as well as the particular epitope that their immune system might recognize. Because these factors vary from person to person, a personalized approach must be employed to target the multiple neoepitopes, which may number in the thousands, that occur in a person with disease like a cancer or pre-malignant condition.

[007] *Listeria monocytogenes* (Lm) is a Gram-positive facultative intracellular pathogen that can cause listeriosis. In its intercellular lifecycle, *Lm* enters host cells through by phagocytosis or by active invasion of non-phagocytic cells. Following internalization, Lm may mediate its escape from the membrane bound phagosome/ vacuole by secretion of several bacterial virulence factors, primarily the pore-forming protein listeriolysin O (LLO), enabling the bacteria

to enter the host cell cytoplasm. In the cytoplasm Lm replicates and spreads to adjacent cells based on the mobility facilitated by the bacterial actin-polymerizing protein (ActA) along with other virulence factors. In the cytoplasm, Lm-secreted proteins and ultimately Lm structural proteins are degraded by the proteasome and processed into peptides that can associate with MHC class I molecules in the endoplasmic reticulum. These MHC-peptide complexes are transported to the cell surface and can be presented to and recognized by target-specific T cells. This unique characteristic makes it a very attractive T cell generating vector in that tumor antigen can be presented with MHC class I molecules to activate tumor-specific cytotoxic T lymphocytes (CTLs). CTLs are the primary target-specific effector cells that kill other cells in the body like cancer cells or cells that harbor an intracellular infection.

[008] In addition, once internalized, Lm is also processed in the phagolysosomal compartment and its peptides presented on MHC Class II which can generate antigen specific CD4-T cell responses which can assits CTLs in target-directed killing of cabcerous or infected cells.

[009] In addition, since the vector is a live bacteria its composition can stimulate a number of triggers of innate immunity which includes several external, intercellular, and cytosolic molecular pattern receptors, including PAMPs, DAMPS, and TLR's. For example, recognition of peptidoglycan by nuclear oligomerization domain-like receptors and Lm DNA by DNA sensors, AIM2 and STING, and activate inflammatory and immune-modulatory cascades. This combination of inflammatory responses and efficient delivery of antigens to the MHC I and MHC II pathways makes Lm a powerful immunotherapy vector in treating, protecting against, and inducing an immune response against a tumor.

[0010] Targeting neo-epitopes specific to a subject's cancer as a component of a *Listeria* based immunotherapy vector that additionally stimulates T-cell response and can also be used in combination with other therapies, may provide an immunotherapy that is both personalized to a subject's cancer and effective in the treatment of the cancer. The fusion of a highly immunogenic peptide antigen to a targeted peptide can significantly increase the immunogenicity of the target antigen or the ability of immunotherapies to stimulate T cells that have escaped tolerance mechanisms, may have a particular potential as immunotherapies.

[0011] The present invention provides personalized immunotherapy compositions and uses thereof for targeting potential neo-epitopes within abnormal or unhealthy tissue of a subject, wherein the immunotherapy comprises the use of a recombinant *Listeria* immunotherapy as a delivery and immunotherapeutic vector for expressing peptides and/or fusion polypeptides comprising said neo-epitopes in order to enhance an immune response targeting these neo-

epitopes. The personalized immunotherapies created may effectively treat, prevent, prolong life, or reduce the incidence of a disease, for example cancer in a subject. Further, recombinant *Listeria* of the present invention may effectively be used in combination with other anti-disease or anti-cancer therapies.

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SUMMARY OF THE INVENTION

[0012] In one aspect, the present invention relates to a system for providing a personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

a. an attenuated *Listeria* strain delivery vector; and

10 b. a plasmid vector for transforming said *Listeria* strain, said plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes or potential neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition;

15 wherein transforming said *Listeria* strain with said vector creates a personalized immunotherapy system targeted to said subject's disease or condition.

[0013] In one aspect, the present invention relates to a system for providing a personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

20 a. a delivery vector; and optionally

b. a plasmid vector for transforming said delivery vector, said plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said

25 subject having said disease or condition.

[0014] In a related aspect, said delivery vector comprises a bacterial delivery vector. In another related aspect said delivery vector comprises a viral vector delivery vector. In another related aspect said delivery vector comprises a peptide immunotherapy delivery vector. In another related aspect, said delivery vector comprises a DNA plasmid immunotherapy

30 delivery vector.

[0015] In a related aspect, the disease or condition comprises an infectious disease, an autoimmune disease, an organ rejection of a transplant, or a tumor, or cancer, or dysplastic cells or tissue. In another aspect, the adaptive immune response is facilitated and enhanced by

an innate immune response triggered by the administration of the live, attenuated immunotherapy agents to a person as treatment. In another related aspect, the immune response is an adaptive immune response. In yet another related aspect, the immune response is a T-cell immune response. In another related aspect, an attenuated, recombinant *Listeria* is cultivated, cryopreserved, optionally lyophilized and spray-dried, and administered as a form of treatment to the subject either alone or in combination with other potentially beneficial treatments for their disease. The treatment can include repeated administrations.

[0016] In another aspect, the present invention relates to a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample;
b. screening peptides comprising said one or more neo-epitopes for an immunogenic response;
c. transforming an attenuated *Listeria* strain with a vector comprising a nucleic acid sequence that encodes a one or more peptides comprising said one or more immunogenic neo-epitopes;
d. and, alternatively storing said attenuated recombinant *Listeria* for administering to said subject at a pre-determined period or administering said attenuated recombinant *Listeria* strain to said subject, wherein said attenuated recombinant *Listeria* strain is administered as part of an immunogenic composition.

[0017] In a related aspect, the invention relates to a recombinant attenuated *Listeria* strain comprising the following:

a. a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises an immunogenic polypeptide or fragment thereof fused to one or more peptides comprising one or more neo-epitopes provided herein; or,
b. a minigene nucleic acid construct comprising a first open reading frame encoding a chimeric protein, wherein said chimeric protein comprises:
    i. a bacterial secretion signal sequence,
    ii. a ubiquitin (Ub) protein,

iii. one or more peptides comprising one or more neo-epitopes provided herein; and

wherein the signal sequence, the ubiquitin, and the one or more peptides in i.-iii. are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

[0018] In a related aspect, the bacterial sequence is a *Listerial* sequence, wherein in some embodiments, said *Listerial* sequence is an hly signal sequence or an actA signal sequence.

[0019] In another related aspect, the present invention relates to an immunogenic composition comprising an attenuated recombinant *Listeria* strain provided herein, and a pharmaceutically acceptable carrier.

[0020] In another related aspect, the composition comprises one or more attenuated *Listeria* strains, wherein each attenuated *Listeria* strain expresses one or more different peptides comprising one or more neo-epitopes. In another aspect, each attenuated *Listeria* expresses a range of neo-epitopes.

[0021] In a related aspect, the process provided herein allows the generation of a personalized enhanced anti-disease, or anti-infectious disease, anti-autoimmune disease, anti-rejection of an organ transplant, or anti-tumor or anticancer immune response in said subject having a disease or condition.

[0022] In another related aspect, the process provided herein allows personalized treatment or prevention of said disease, or said infection, said autoimmune disease, said rejection of an organ transplant, or said tumor or cancer in a subject.

[0023] In another related aspect, the process provided herein increases survival time in said subject having said disease or condition, or said infection, or said autoimmune disease, or said organ transplant rejection, or said tumor or cancer.

[0024] In one aspect, the present invention relates to a recombinant attenuated *Listeria* strain, wherein the *Listeria* strain comprises a nucleic acid sequence comprising one or more open reading frames encoding one or more peptides comprising one or more personalized neo-epitopes, wherein the neo-epitope(s) comprises immunogenic epitopes present in a disease or condition bearing tissue or cell of a subject having the disease or condition.

[0025] In one aspect, the present invention relates to a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of: (a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one

or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample; (b) transforming an attenuated Listeria strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said attenuated recombinant Listeria for administering to said subject at a pre-determined period or administering a composition comprising said attenuated recombinant Listeria strain to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; optionally, (c) obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell from said T-cell immune response and characterizing specific peptides comprising one or more neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells , wherein said one or more neo-epitopes are immunogenic; (d) screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and, (e) transforming a second attenuated recombinant Listeria strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes; and, alternatively storing said second attenuated recombinant Listeria for administering to said subject at a pre-determined period or administering a second composition comprising said second attenuated recombinant Listeria strain to said subject, wherein said process creates a personalized immunotherapy for said subject.

[0026] A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of: (a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample; (b) transforming a vector with a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a., or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject, and wherein said administering results in the generation of a

personalized T-cell immune response against said disease or said condition; and optionally, (c) obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell from said T-cell immune response and characterizing specific peptides comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells; (d) screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and, (e) transforming a second vector with a nucleic acid sequence comprising one or more open reading frames encoding one or more peptides comprising said one or more immunogenic neo-epitopes or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes identified in c.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period, or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject, wherein said process creates a personalized immunotherapy for said subject.

[0027] In one aspect, the present invention relates to a recombinant attenuated *Listeria* strain comprising: (a) a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises an immunogenic polypeptide or fragment thereof fused to one or more peptides comprising one or more neo-epitopes provided herein; or, (b) a minigene nucleic acid construct comprising one or more open reading frames encoding a chimeric protein, wherein the chimeric protein comprises: (i) a bacterial secretion signal sequence, (ii) a ubiquitin (Ub) protein, (iii) one or more peptides comprising one or more neo-epitopes provided herein; and, wherein the signal sequence, the ubiquitin and one or more peptides in (a)-(c) are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus, wherein the neo-epitope(s) comprise immunogenic epitopes present in a disease or condition bearing tissue or cell of a subject having the disease or condition.

[0028] In a related aspect, administrating the *Listeria* strain to a subject having said disease or condition generates an immune response targeted to the subject's disease or condition.

[0029] In a related aspect, the strain is a personalized immunotherapy vector for said subject targeted to said subject's disease or condition.

[0030] In a related aspect, the neo-epitope sequences are tumor specific, metastases specific, bacterial infection specific, viral infection specific, and any combination thereof.

[0031] In a related aspect, one or more neo-epitope comprises between about 5 to 50 amino

acids.

[0032] In a related aspect, the neo-epitopes are determined using exome sequencing or transcriptome sequencing of the disease-bearing tissue or cell.

[0033] In a related aspect, one or more neo-epitope(s) are screened for immunosuppressive epitopes, wherein immunosuppressive epitopes are excluded from the nucleic acid molecule.

[0034] In a related aspect, one or more neo-epitope(s) are codon optimized for expression and secretion according to the *Listeria* strain.

[0035] In a related aspect, one or more peptides are each fused to an immunogenic polypeptide or fragment thereof.

[0036] In a related aspect, the immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, an ActA-PEST2 (LA-242) fusion, or a PEST amino acid sequence.

[0037] In a related aspect, the disease or condition is an infectious disease, an autoimmune disease, or a tumor or a cancer, or dysplasia.

[0038] In a related aspect, the infectious disease comprises a viral or bacterial infection.

[0039] In a related aspect, one or more neo-epitopes comprise an infectious disease-associated-specific epitope.

[0040] In a related aspect, the attenuated *Listeria* comprises a mutation in one or more endogenous genes.

[0041] In a related aspect, the *Listeria* strain further comprises a nucleic acid construct comprising one or more open reading frames encoding one or more one or more immunomodulatory molecule(s).

[0042] In a related aspect, a personalized immunotherapy composition comprising one or more *Listeria* strain(s) as disclosed in any of the above.

[0043] In a related aspect, a personalized immunotherapy composition elicits an immune response targeted against one or more neo-epitopes.

[0044] In a related aspect, the composition comprises a combination of the *Listeria* strains, wherein the combination comprises a plurality of the neo-epitopes that are administered on the same day.

[0045] In a related aspect the combination comprises a pleurality of the Listeria strains that are administered on different days or in alternating sequence wherein the combination of strains administered on different days comprises a pleurality of the neo-epitopes.

[0046] In a related aspect, the composition comprises a combination of the *Listeria* strains, wherein the combination comprises a plurality of the neo-epitopes that are administered on

the same day.

[0047] In a related aspect, the combination comprises all of the neo-epitopes identidied in the patient that can be expressed in this system.

[0048] In a related aspect, the combination comprises all or a pleurality of of the neo-epitopes described as clonal.

[0049] In a related aspect, the combination comprises all or a pleurality of the neo-epitopes that are also represented in the transcriptome based on RNA sequencing.

[0050] In a related aspect, the composition comprises a combination of a plurality of the *Listeria* strains, wherein each strain comprises the nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising at least one unique the neo-epitope.

[0051] In a related aspect, the composition comprises a combination of the *Listeria* strains, wherein the combination comprises a plurality of the neo-epitopes.

[0052] In a related aspect, the combination comprises up to about 500 of the neo-epitopes.

[0053] In a related aspect, the combination further comprises one or more recombinant attenuated *Listeria* strain delivery vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more epitopes, wherein the epitope(s) comprise immunogenic epitope(s) present in a disease-bearing tissue or cell of the subject having the disease or condition, wherein administrating the *Listeria* strain generates a immunotherapy targeted to the subject's disease or condition.

[0054] In a related aspect, the composition, as disclosed in any of the above, further comprising an adjuvant.

[0055] In a related aspect, administering the composition to the subject generates a personalized enhanced anti-disease, or anti-condition immune response in the subject.

[0056] In a related aspect of the present invention, a DNA immunotherapy comprising the personalized immunotherapy composition as described in any of the above.

[0057] In a related aspect of the present invention, a peptide immunotherapy comprising the personalized immunotherapy composition as described in any of the above.

[0058] In a related aspect of this invention, a pharmaceutical composition of the present invention comprising the immunotherapy or personalized immunotherapy composition as described in any of the above and a pharmaceutical carrier.

[0059] In a related aspect of this invention, a method of inducing an immune response to at least one neo-epitope present in a disease or condition bearing tissue or cell in a subject having the disease or condition, the method comprising the step of administering the

personalized immunotherapy composition or immunotherapy as described in any of the above to the subject.

[0060] In a related aspect of this invention, a method of inducing a targeted immune response in a subject having a disease or condition, comprising administering to the subject the immunogenic composition or immunotherapy as described in any of the above, wherein administrating the *Listeria* strain generates a personalized immunotherapy targeted to the subject's disease or condition.

[0061] In a related aspect of this invention, a method of treating, suppressing or inhibiting disease or condition in a subject, the method comprising the step of administrating a personalized immunotherapy composition or immunotherapy as described in any of the above, for targeting the disease or condition.

[0062] In yet another embodiment, the disease or condition is an infectious disease, autoimmune disease, organ transplantation rejection, a tumor or a cancer.

[0063] In a related aspect of the present invention, a method of increasing the ratio of T effector cells to regulatory T cells (Tregs) in the lymphoid tissue or systemic circulation, and tumor, or diseased or dysplastic tissue of a subject, wherein the T effector cells are targeted to a neo-epitope present within a disease or condition bearing tissue of a subject, the method comprising the step of administering to the subject personalized immunotherapy composition or immunotherapy as described in any of the above.

[0064] In a related aspect of the present invention, a method for increasing antigen-specific T-cells in a subject, wherein the antigen or a peptide fragment thereof comprises one or more neo-epitopes, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

[0065] In a related aspect of the present invention, a method for increasing survival time of a subject having a tumor or suffering from cancer, or suffering from an infectious disease, comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

[0066] In a related aspect of the present invention, a method of protecting a subject from a cancer, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

[0067] In a related aspect of the present invention, a method of inhibiting or delaying the onset of cancer in a subject, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

[0068] In a related aspect of the present invention, a method of reducing tumor or metastases size in a subject, the method comprising the step of administrating to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

5 [0069] In a related aspect of the present invention, a method of protecting a subject from an infectious disease, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as described in any of the above.

[0070] According to another embodiment of the present invention, a method as described

10 above is disclosed, additionally comprising the steps of creating the personalized immunotherapy composition, wherein the creating comprises the steps of:

(a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein the comparing

15 identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within one or more ORFs from the disease-bearing sample;

(b) transforming an attenuated *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising one or more neo-epitopes identified

20 in a.; and, alternatively storing the attenuated recombinant *Listeria* for administering to the subject at a pre-determined period or administering a composition comprising the attenuated recombinant *Listeria* strain to the subject, and wherein the administering results in the generation of a personalized T-cell immune response against the disease or the condition; optionally,

25 (c) obtaining a second biological sample from the subject comprising a T-cell clone or T-infiltrating cell from the T-cell immune response and characterizing specific peptides comprising one or more neo-epitopes bound by MHC Class I or MHC Class II molecules on the T cells , wherein one or more neo-epitopes are immunogenic;

(d) screening for and selecting a nucleic acid construct encoding one or more peptides

30 comprising one or more immunogenic neo-epitope identified in (c); and,

(e) transforming a second attenuated recombinant *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising one or more immunogenic neo-epitopes; and, alternatively storing the second attenuated recombinant *Listeria* for administering to the subject at a pre-determined period or

administering a second composition comprising the second attenuated recombinant *Listeria* strain to the subject,

wherein the process creates a personalized immunotherapy for the subject.

[0071] In one embodiment, the invention relates to an immunogenic mixture of compositions comprising one or more recombinant *Listeria* strains produced by the process disclosed herein. In another embodiment, each of said *Listeria* in said mixture comprises a nucleic acid molecule encoding a fusion polypeptide or chimeric protein comprising one or more neo-epitopes. In another embodiment, each *Listeria* in said mixture expresses 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes. In another embodiment, each mixture comprises 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 recombinant Listeria strains.

[0072] In one embodiment, the invention relates to a method of eliciting a personalized anti-tumor response in a subject, the method comprising the step of concomitantly or sequentially administering to said subject an immunogenic mixture composition disclosed herein. In another embodiment, disclosed herein is a method of preventing or treating a tumor in a subject, the method comprising the step of concomitantly or sequentially administering to said subject the immunogenic mixture of compositions disclosed herein. In one embodiment, the invention relates to a nucleic acid construct encoding a chimeric protein comprising the following elements: a N-terminal truncated LLO (tLLO) fused to a first neo-epitope amino acid sequence, wherein said first neo-epitope AA sequence is operatively linked to a second neo-epitope AA sequence via a linker sequence, wherein said second neo-epitope AA sequence is operatively linked to at least one additional neo-epitope amino acid sequence via a linker sequence, and wherein a last neo-epitope is operatively linked to a histidine tag at the C-terminus via a linker sequence.

[0073] In another embodiment, the invention relates to a system for creating personalized immunotherapy for a subject, comprising: at least one processor; and at least one storage medium containing program instructions for execution by said processor, said program instructions causing said processor to execute steps comprising:

a. Receiving output data containing all neo-antigens and the human leukocyte antigen (HLA) type of the subject;
b. Scoring the hydrophobicity of each epitope and removing epitopes that score above a certain threshold;
c. Numerically rate the remaining neo-antigens based its ability to bind to

subject HLA and on its predictive MHC binding score;

d. Inserting the amino acid sequence of each epitope into a plasmid;

e. Scoring the hydrophobicity of each construct and removing any constructs that score above a certain threshold;

5 f. Translating the amino acid sequence of each construct into the corresponding DNA sequence, starting with the highest scored construct;

g. Inserting additional epitopes into the plasmid construct in order of ranking until a predetermined upper limit is reached;

h. Adding a DNA sequence tag to the end of the construct in order to

10 measure the immunotheraputic response in a subject; and

i. Optimizing the epitope and DNA sequence tag for expression and secretion in *Listeria monocytogenes*.

[0074] Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however,

15 that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

20 [0075] The subject matter regarded as the invention is particularly pointed out and distinctly claimed in the concluding portion of the specification. The invention, however, both as to organization and method of operation, together with objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:

25 [0076] **Figs. 1A and 1B.** Lm-E7 and Lm-LLO-E7 (ADXS11-001) use different expression systems to express and secrete E7. Lm-E7 was generated by introducing a gene cassette into the orfZ domain of the L. monocytogenes genome **(Fig. 1A)**. The hly promoter drives expression of the hly signal sequence and the first five amino acids (AA) of LLO followed by HPV-16 E7. (**Fig. 1B**), Lm-LLO-E7 was generated by transforming the prfA- strain XFL-7

30 with the plasmid pGG-55. pGG-55 has the hly promoter driving expression of a non-hemolytic fusion of LLO-E7. pGG-55 also contains the prfA gene to select for retention of the plasmid by XFL-7 in vivo.

[0077] **Fig. 2**. Lm-E7 and Lm-LLO-E7 secrete E7. Lm-Gag (lane 1), Lm-E7 (lane 2), Lm-

LLO-NP (lane 3), Lm-LLO-E7 (lane 4), XFL-7 (lane 5), and 10403S (lane 6) were grown overnight at 37°C in Luria-Bertoni broth. Equivalent numbers of bacteria, as determined by OD at 600 nm absorbance, were pelleted and 18 ml of each supernatant was TCA precipitated. E7 expression was analyzed by Western blot. The blot was probed with an anti-E7 mAb, followed by HRP-conjugated anti-mouse (Amersham), then developed using ECL detection reagents.

[0078] **Fig. 3.** Tumor immunotherapeutic efficacy of LLO-E7 fusions. Tumor size in millimeters in mice is shown at 7, 14, 21, 28 and 56 days post tumor-inoculation. Naive mice: open-circles; Lm-LLO-E7: filled circles; Lm-E7: squares; Lm-Gag: open diamonds; and Lm-LLO-NP: filled triangles.

[0079] **Fig. 4.** Splenocytes from Lm-LLO-E7-immunized mice proliferate when exposed to TC-1 cells. C57BL/6 mice were immunized and boosted with Lm-LLO-E7, Lm-E7, or control rLm strains. Splenocytes were harvested 6 days after the boost and plated with irradiated TC-1 cells at the ratios shown. The cells were pulsed with ^{3}H thymidine and harvested. Cpm is defined as (experimental cpm) - (no-TC-1 control).

[0080] **Figs. 5A and 5B.** (**Fig. 5A**) Western blot demonstrating that Lm-ActA-E7 secretes E7. Lane 1: Lm-LLO-E7; lane 2: Lm-ActA-E7.001; lane 3; Lm-ActA-E7-2.5.3; lane 4: Lm-ActA-E7-2.5.4. (**Fig. 5B**) Tumor size in mice administered Lm-ActA-E7 (rectangles), Lm-E7 (ovals), Lm-LLO-E7 (X), and naive mice (non-vaccinated; solid triangles).

[0081] **Figs. 6A-6C.** (**Fig. 6A**) schematic representation of the plasmid inserts used to create 4 LM immunotherapies. Lm-LLO-E7 insert contains all of the *Listeria* genes used. It contains the hly promoter, the first 1.3 kb of the hly gene (which encodes the protein LLO), and the HPV-16 E7 gene. The first 1.3 kb of hly includes the signal sequence (ss) and the PEST region. Lm-PEST-E7 includes the hly promoter, the signal sequence, and PEST and E7 sequences but excludes the remainder of the truncated LLO gene. Lm-ΔPEST-E7 excludes the PEST region, but contains the hly promoter, the signal sequence, E7, and the remainder of the truncated LLO. Lm-E7epi has only the hly promoter, the signal sequence, and E7. (**Fig. 6B**) Top panel: *Listeria* constructs containing PEST regions induce tumor regression. Bottom panel: Average tumor sizes at day 28 post-tumor challenge in 2 separate experiments. (**Fig. 6C**) *Listeria* constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes in the spleen. Average and SE of data from 3 experiments are depicted.

[0082] **Figs. 7A and 7B.** (**Fig. 7A**) Induction of E7-specific IFN-gamma-secreting CD8$^+$ T cells in the spleens and the numbers penetrating the tumors, in mice administered TC-1 tumor cells and subsequently administered Lm-E7, Lm-LLO-E7, Lm-ActA-E7, or no

immunotherapy (naive). (**Fig. 7B**) Induction and penetration of E7 specific $CD8^+$ cells in the spleens and tumors of the mice described for (**Fig. 7A**).

[0083] **Figs. 8A and 8B.** *Listeria* constructs containing PEST regions induce a higher percentage of E7-specific lymphocytes within the tumor. (**Fig. 8A**) representative data from 1 experiment. (**Fig. 8B**) average and SE of data from all 3 experiments.

[0084] **Fig. 9.** Data from Cohorts 1 and 2 indicting the efficacy observed in the patients in the clinical trial presented in Example 6.

[0085] **Figs. 10A and 10B.** (**Fig. 10A**) Schematic representation of the chromosomal region of the *Lmdd*-143 and *LmddA*-143 after *klk3* integration and *actA* deletion; (**Fig. 10B**) The *klk3* gene is integrated into the *Lmdd* and *LmddA* chromosome. PCR from chromosomal DNA preparation from each construct using *klk3* specific primers amplifies a band of 714 bp corresponding to the *klk3* gene, lacking the secretion signal sequence of the wild type protein.

[0086] **Figs.11A-11D.** (**Fig. 11A**) Map of the pADV134 plasmid. (**Fig. 11B**) Proteins from *LmddA*-134 culture supernatant were precipitated, separated in a SDS-PAGE, and the LLO-E7 protein detected by Western-blot using an anti-E7 monoclonal antibody. The antigen expression cassette consists of *hly* promoter, ORF for truncated LLO and human PSA gene (*klk3*). (**Fig. 11C**) Map of the pADV142 plasmid. (**Fig. 11D**) Western blot showed the expression of LLO-PSA fusion protein using anti-PSA and anti-LLO antibody.

[0087] **Figs. 12A and 12B.** (**Fig. 12A**) Plasmid stability *in vitro* of LmddA-LLO-PSA if cultured with and without selection pressure (D-alanine). Strain and culture conditions are listed first and plates used for CFU determination are listed after. (**Fig. 12B**) Clearance of LmddA-LLO-PSA *in vivo* and assessment of potential plasmid loss during this time. Bacteria were injected i.v. and isolated from spleen at the time point indicated. CFUs were determined on BHI and BHI + D-alanine plates.

[0088] **Figs. 13A and 13B.** (**Fig. 13A**) *In vivo* clearance of the strain LmddA-LLO-PSA after administration of 10^8 CFU in C57BL/6 mice. The number of CFU were determined by plating on BHI/str plates. The limit of detection of this method was 100 CFU. (**Fig. 13B**) Cell infection assay of J774 cells with 10403S, LmddA-LLO-PSA and XFL7 strains.

[0089] **Figs. 14A-14E.** (**Fig. 14A**) PSA tetramer-specific cells in the splenocytes of naïve and LmddA-LLO-PSA immunized mice on day 6 after the booster dose. (**Fig. 14B**) Intracellular cytokine staining for IFN-γ in the splenocytes of naïve and LmddA-LLO-PSA immunized mice were stimulated with PSA peptide for 5 h. Specific lysis of EL4 cells pulsed with PSA peptide with in vitro stimulated effector T cells from LmddA-LLO-PSA

immunized mice and naïve mice at different effector/target ratio using a caspase based assay (**Fig. 14C**) and a europium based assay (**Fig. 14D**). Number of IFNγ spots in naïve and immunized splenocytes obtained after stimulation for 24 h in the presence of PSA peptide or no peptide (**Fig. 14E**).

5 [0090] **Figs. 15A-15C.** Immunization with *LmddA*-142 induces regression of Tramp-C1-PSA (TPSA) tumors. Mice were left untreated (n=8) (**Fig. 15A**) or immunized i.p. with *LmddA*-142 ($1x10^8$ CFU/mouse) (n=8) (**Fig. 15B**) or Lm-LLO-PSA (n=8), (**Fig. 15C**) on days 7, 14 and 21. Tumor sizes were measured for each individual tumor and the values expressed as the mean diameter in millimeters. Each line represents an individual mouse.

10 [0091] **Figs. 16A and 16B.** (**Fig. 16A**) Analysis of PSA-tetramer$^+$CD8$^+$ T cells in the spleens and infiltrating T-PSA-23 tumors of untreated mice and mice immunized with either an *Lm* control strain or *LmddA*-LLO-PSA (*LmddA*-142). (**Fig. 16B**) Analysis of CD4$^+$ regulatory T cells, which were defined as CD25$^+$FoxP3$^+$, in the spleens and infiltrating T-PSA-23 tumors of untreated mice and mice immunized with either an *Lm* control strain or

15 *LmddA*-LLO-PSA.

[0092] **Figs. 17A and 17B.** (**Fig. 17A**) Schematic representation of the chromosomal region of the Lmdd-143 and LmddA-143 after *klk3* integration and *actA* deletion; (**Fig. 17B**) The *klk3* gene is integrated into the Lmdd and LmddA chromosome. PCR from chromosomal DNA preparation from each construct using *klk3* specific primers amplifies a band of 760 bp

20 corresponding to the *klk3* gene.

[0093] **Figs. 18A-C.** (**Fig. 18A**) Lmdd-143 and LmddA-143 secretes the LLO-PSA protein. Proteins from bacterial culture supernatants were precipitated, separated in a SDS-PAGE and LLO and LLO-PSA proteins detected by Western-blot using an anti-LLO and anti-PSA antibodies; (**Fig. 18B**) LLO produced by *Lmdd*-143 and *LmddA*-143 retains hemolytic

25 activity. Sheep red blood cells were incubated with serial dilutions of bacterial culture supernatants and hemolytic activity measured by absorbance at 590nm; (**Fig. 18C**) *Lmdd*-143 and *LmddA*-143 grow inside the macrophage-like J774 cells. J774 cells were incubated with bacteria for 1 hour followed by gentamicin treatment to kill extracellular bacteria. Intracellular growth was measured by plating serial dilutions of J774 lysates obtained at the

30 indicated timepoints. *Lm* 10403S was used as a control in these experiments.

[0094] **Fig. 19.** Immunization of mice with *Lmdd*-143 and *LmddA*-143 induces a PSA-specific immune response. C57BL/6 mice were immunized twice at 1-week interval with $1x10^8$ CFU of *Lmdd*-143, *LmddA*-143 or *LmddA*-142 and 7 days later spleens were harvested.

Splenocytes were stimulated for 5 hours in the presence of monensin with 1 μM of the PSA_{65-74} peptide. Cells were stained for CD8, CD3, CD62L and intracellular IFN-γ and analyzed in a FACS Calibur cytometer.

[0095] **Figs. 20A and 20B.** Construction of ADXS31-164. (**Fig. 20A**) Plasmid map of pAdv164, which harbors *bacillus subtilis dal* gene under the control of constitutive *Listeria* p60 promoter for complementation of the chromosomal *dal-dat* deletion in *LmddA* strain. It also contains the fusion of truncated $LLO_{(1-441)}$ to the chimeric human Her2/neu gene, which was constructed by the direct fusion of 3 fragments the Her2/neu: EC1 (aa 40-170), EC2 (aa 359-518) and ICI (aa 679-808). (**Fig. 20B**) Expression and secretion of tLLO-ChHer2 was detected in *Lm*-LLO-ChHer2 (Lm-LLO-138) and *LmddA*-LLO-ChHer2 (ADXS31-164) by western blot analysis of the TCA precipitated cell culture supernatants blotted with anti-LLO antibody. A differential band of ~104 KD corresponds to tLLO-ChHer2. The endogenous LLO is detected as a 58 KD band. *Listeria* control lacked ChHer2 expression.

[0096] **Figs. 21A-21C.** Immunogenic properties of ADXS31-164 (**Fig. 21A**) Cytotoxic T cell responses elicited by Her2/neu *Listeria*-based immunotherapies in splenocytes from immunized mice were tested using NT-2 cells as stimulators and 3T3/neu cells as targets. Lm-control was based on the *LmddA* background that was identical in all ways but expressed an irrelevant antigen (HPV16-E7). (**Fig. 21B**) IFN-γ secreted by the splenocytes from immunized FVB/N mice into the cell culture medium, measured by ELISA, after 24 hours of *in vitro* stimulation with mitomycin C treated NT-2 cells. (**Fig. 21C**) IFN-γ secretion by splenocytes from HLA-A2 transgenic mice immunized with the chimeric immunotherapy, in response to *in vitro* incubation with peptides from different regions of the protein. A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide groups constituted the negative controls as listed in the Fig. legend. IFN-γ secretion was detected by an ELISA assay using cell culture supernatants harvested after 72 hours of co-incubation. Each data point was an average of triplicate data +/- standard error. * P value < 0.001.

[0097] **Fig. 22.** Tumor Prevention Studies for *Listeria*-ChHer2/neu Immunotherapies Her2/neu transgenic mice were injected six times with each recombinant *Listeria*-ChHer2 or a control *Listeria* immunotherapy. Immunizations started at 6 weeks of age and continued every three weeks until week 21. Appearance of tumors was monitored on a weekly basis and expressed as percentage of tumor free mice. *$p<0.05$, N = 9 per group.

[0098] **Fig. 23.** Effect of immunization with ADXS31-164 on the % of Tregs in Spleens.

FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each immunotherapy at one week intervals. Spleens were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. Dot-plots of the Tregs from a representative experiment showing the frequency of $CD25^+$/$FoxP3^+$ T cells, expressed as percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells across the different treatment groups.

[0099] **Figs. 24A and 24B.** Effect of immunization with ADXS31-164 on the % of tumor infiltrating Tregs in NT-2 tumors. FVB/N mice were inoculated s.c. with 1 x 10^6 NT-2 cells and immunized three times with each immunotherapy at one week intervals. Tumors were harvested 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti CD3, CD4, CD25 and FoxP3 antibodies. (**Fig. 24A**). Dot-plots of the Tregs from a representative experiment. (**Fig. 24B**). Frequency of $CD25^+$/$FoxP3^+$ T cells, expressed as percentages of the total $CD3^+$ or $CD3^+CD4^+$ T cells (left panel) and intratumoral CD8/Tregs ratio (right panel) across the different treatment groups. Data is shown as mean±SEM obtained from 2 independent experiments.

[00100] **Figs. 25A-25C.** Vaccination with ADXS31-164 can delay the growth of a breast cancer cell line in the brain. Balb/c mice were immunized thrice with ADXS31-164 or a control *Listeria* immunotherapy. EMT6-Luc cells (5,000) were injected intracranially in anesthetized mice. (**Fig. 25A**) *Ex vivo* imaging of the mice was performed on the indicated days using a Xenogen X-100 CCD camera. (**Fig. 25B**) Pixel intensity was graphed as number of photons per second per cm2 of surface area; this is shown as average radiance. (**Fig. 25C**) Expression of Her2/neu by EMT6-Luc cells, 4T1-Luc and NT-2 cell lines was detected by Western blots, using an anti-Her2/neu antibody. J774.A2 cells, a murine macrophage like cell line was used as a negative control.

[00101] **Figs. 26A-C** represents a schematic map of a recombinant *Listeria* protein minigene construct. (**Fig. 26A**) represents a construct producing the ovalbumin derived SIINFEKL peptide (SEQ ID NO: 75). (**Fig. 26B**) represents a comparable recombinant protein in which a GBM derived peptide has been introduced in place of SIINFEKL by PCR cloning. (**Fig. 26C**) represents a construct designed to express 4 separate peptide antigens from a strain of *Listeria*.

[00102] **Fig. 27.** A schematic representation showing the cloning of the different ActA PEST regions in the plasmid backbone pAdv142 (see Fig. 11C) to create plasmids pAdv211, pAdv223 and pAdv224 is shown in (**Fig. 27**). This schematic shows different ActA coding regions were cloned in frame with Listeriolysin O signal sequence in the backbone plasmid

pAdv142, restricted with XbaI and XhoI.

[00103] **Figs. 28A-B.** (**Fig. 28A**) Tumor regression study using TPSA23 as transplantable tumor model. Three groups of eight mice were implanted with 1 x 10^6 tumor cells on day 0 and were treated on day 6, 13 and 20 with 10^8 CFU of different therapies: *Lm*ddA142, *Lm*ddA211, *Lm*ddA223 and *Lm*ddA224. Naïve mice did not receive any treatment. Tumors were monitored weekly and mice were sacrificed if the average tumor diameter was 14-18 mm. Each symbol in the graph represents the tumors size of an individual mouse. The experiment was repeated twice and similar results were obtained. (**Fig. 28B**) The percentage survival of the naïve mice and immunized mice at different days of the experiment.

[00104] **Figs. 29A-B.** PSA specific immune responses were examined by tetramer staining (**Fig. 29A**) and intracellular cytokine staining for IFN-γ (**Fig. 29B**). Mice were immunized three times at weekly intervals with 10^8 CFU of different therapies: *Lm*ddA142 (ADXS31-142), *Lm*ddA211, *Lm*ddA223 and *Lm*ddA224. For immune assays, spleens were harvested on day 6 after the second boost. Spleens from 2 mice/group were pooled for this experiment. (A) PSA specific T cells in the spleen of naïve, *Lm*ddA142, *Lm*ddA211, *Lm*ddA223 and *Lm*ddA224 immunized mice were detected using PSA-epitope specific tetramer staining. Cells were stained with mouse anti-CD8 (FITC), anti-CD3 (Percp-Cy5.5), anti-CD62L (APC) and PSA tetramer-PE and analyzed by FACS Calibur. (**Fig. 29B**) Intracellular cytokine staining to detect the percentage of IFN-γ secreting CD8+ CD62Llow cells in the naïve and immunized mice after stimulation with 1 μM of PSA specific, H-2Db peptide (HCIRNKSVIL) for 5 h.

[00105] **Figs. 30A-C.** TPSA23, tumor model was used to study immune response generation in C57BL6 mice by using ActA/PEST2 (LA229) fused PSA and tLLO fused PSA. Four groups of five mice were implanted with 1 x 10^6 tumor cells on day 0 and were treated on day 6 and 14 with 10^8 CFU of different therapies: *Lm*ddA274, *Lm*ddA142 (ADXS31-142) and *Lm*ddA211. Naïve mice did not receive any treatment. On Day 6 post last immunization, spleen and tumor was collected from each mouse. (**Fig. 30A**) Table shows the tumor volume on day 13 post immunization. PSA specific immune responses were examined by pentamer staining in spleen (**Fig. 30B**) and in tumor (**Fig. 30C**). For immune assays, spleens from 2 mice/group or 3 mice/group were pooled and tumors from 5 mice/group was pooled. Cells were stained with mouse anti-CD8 (FITC), anti-CD3 (Percp-Cy5.5), anti-CD62L (APC) and PSA Pentamer-PE and analyzed by FACS Calibur.

[00106] **Figs. 31A-31C.** SOE mutagenesis strategy. Decreasing/lowering the virulence of LLO was achieved by mutating the 4th domain of LLO. (**Figs. 31A-31B**). This domain

contains a cholesterol binding site allowing it to bind to membranes where it oligomerizes to form pores. **Fig. 31C** Shows fragments of full length LLO (rLLO529). Recombinant LLO, rLLO493, represents a LLO N-terminal fragment spanning from amino acids 1- 493 (including the signal sequence). Recombinant LLO, rLLO482, represents an N-terminal LLO fragment (including a deletion of the cholesterol binding domain, amino acids 483-493) spanning from amino acids 1- 482 (including the signal sequence). Recombinant LLO, rLLO415, represents an N-terminal LLO fragment (including a deletion of the cholesterol binding domain, amino acids 483-493) spanning from amino acids 1- 415 (including the signal sequence). Recombinant LLO, rLLO59-415, represents an N-terminal LLO fragment that spans from amino acids 59-415 (excluding the cholesterol binding domain). Recombinant LLO, rLLO416-529, represents a N-terminal LLO fragment that spans from amino acids 416-529 and includes the cholesterol binding domain.

[00107] **Figs. 32A and 32B**. Expression of mutant LLO proteins by Coomassie staining is shown in **Fig. 32A** and by Western blot in **Fig. 32B**.

[00108] **Figs. 33A and 33B.** Histograms present data showing hemolytic activity of mutant LLO (mutLLO and ctLLO) proteins at pH 5.5 (Fig. 33A) and 7.4 (Fig. 33B).

[00109] **Fig. 34.** A plasmid map of a PAK6 construct (7605 bp), wherein PAK6 is expressed as a fusion protein with tLLO. Schematic map of the plasmid for PAK6. The plasmid contains both *Listeria* (Rep R) and *Escherichia coli* (p15) origin of replication. The black arrow represents the direction of transcription. *Bacillus subtilis dal* gene complements the synthesis of D-alanine. The antigen expression cassette consists of *hly* promoter, ORF for truncated LLO and human PAK6 gene.

[00110] **Fig. 35.** A nucleic acid sequences of PAK6 as set forth in SEQ ID NO: 102.

[00111] **Fig. 36.** An amino acid sequence of PAK6 as set forth in SEQ ID NO: 103.

[00112] **Fig. 37A.** General overview of the tumor sequencing and DNA generation work stream.

[00113] **Fig. 37B.** General overview of DNA cloning and immunotherapy manufacturing work stream.

[00114] **Fig. 38.** Diagram of a cluster of fully enclosed single use cell growth systems arranged for parallel manufacturing of personalized immunotherapy compositions.

[00115] **Fig. 39.** Detailed diagram of the inoculation and fermentation segments of fully enclosed single use cell growth system.

[00116] **Fig. 40.** Detailed diagram of the concentration segment of fully enclosed single use cell growth system.

[00117] **Fig. 41.** Detailed diagram of the diafiltration segment of fully enclosed single use cell growth system.

[00118] **Fig. 42.** Detailed diagram of the product dispensation segment of fully enclosed single use cell growth system.

5 [00119] **Fig. 43A.** Diagram of the process of using a serial selection of neo-epitopes in order to improve efficiency of immunotherapy.

[00120] **Fig. 43B.** Diagram of the process of using a parallel selection multiple neo-epitopes.

[00121] **Fig. 44.** Flow chart of a process (manual or automated) that generates the DNA sequence of a personalized plasmid vector comprising one or more neo-epitopes for use in a

10 delivery vector, e.g., *Listeria monocytogenes* using output data containing all neo-antigens and patient HLA types.

[00122] **Fig. 45** shows the effects of moving the SIINFEKL tag on 25D detection. The SIINFEKL tag identifies a secreted neo-epitope whetehr the tag is located at the C-terminus, the N-terminus, or in between.

15 [00123] **Fig. 46A** shows the timeline for B16F10 tumor experiments, including treatments with *Lm* Neo constructs.

[00124] **Fig. 46B** shows tumor regression with *Lmdd*A274, *Lm*-Neo-12, and *Lm*-Neo-20, with PBS used as a negative control.

[00125] **Fig. 46C** compares survival of mice with B16F10 tumors following treatment with

20 *Lmdd*A274, *Lm*-Neo-12, or *Lm*-Neo-20, with PBS used as a negative control.

[00126] **Fig. 47A-C** show expression and secretion levels for PSA-Survivin-SIINFEKL (**Fig. 47A**), PSA-Survivin without SIINFEKL (**Fig. 47B**), and Neo 20-SIINFEKL (**Fig. 47C**).

[00127] **Fig. 48** shows CD8 T-cell response to the Neo 20 antigen (with C-terminal SIINFEKL tag) or a negative control. The graph indicates the percent SIINFEKL-specific

25 CD8 T-cell response for each condition.

[00128] **Fig. 49A** shows tumor regression with *Lmdd*A274, *Lm*-Neo-12, *Lm*-Neo-20, and Lm-Neo 30, with PBS used as a negative control.

[00129] **Fig. 49B** compares survival of mice with B16F10 tumors following treatment with *Lmdd*A274, *Lm*-Neo-12, *Lm*-Neo-20, and Lm-Neo 30, with PBS used as a negative control.

30 [00130] **Fig. 50** shows the effects of randomizing the order of neo-epitopes within a construct or breaking down the combination of neo-epitopes into subcombinations of neo-epitopes and randomizing those subcombinations to modify secretion.

[00131] **Fig. 51** shows the relative CD8 cell response in mice immunized with lung neo-epitope constructs.

[00132] It will be appreciated that for simplicity and clarity of illustration, elements shown in the Figs. have not necessarily been drawn to scale. For example, the dimensions of some of the elements may be exaggerated relative to other elements for clarity. Further, where considered appropriate, reference numerals may be repeated among the Figs. to indicate corresponding or analogous elements.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[00133] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details, as embodied herein. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

[00134] In one embodiment, provided herein is a system for providing a personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

a. an attenuated *Listeria* strain delivery vector; and
b. a plasmid vector for transforming said *Listeria* strain, said plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition;

wherein transforming said *Listeria* strain with said plasmid vector creates a personalized immunotherapy system targeted to said subject's disease or condition.

[00135] In one embodiment, the present invention provides a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample;
b. screening peptides comprising said one or more neo-epitopes for an immunogenic response;

c. transforming an attenuated *Listeria* strain with a plasmid vector comprising a nucleic acid sequence that encodes a one or more peptides comprising said one or more immunogenic neo-epitopes; and
d. alternatively storing said attenuated recombinant *Listeria* for administering to said subject at a pre-determined period or administering said attenuated recombinant *Listeria* strain to said subject, wherein said attenuated recombinant *Listeria* strain is administered as part of an immunogenic composition.

[00136] In another embodiment, provided herein is a system for providing a personalized immunotherapy for a subject having a disease or condition, comprising the following components:

a. a disease-bearing biological sample obtained from said subject having said disease or condition;
b. a healthy biological sample, wherein said healthy biological sample is obtained from said human subject having said disease or condition or another healthy human subject;
c. a screening assay or screening tool and associated digital software for comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from said disease-bearing biological sample with open reading frames in nucleic acid sequences extracted from said healthy biological sample, and for identifying mutations in said ORFs encoded by said nucleic acid sequences of said disease-bearing sample, wherein said mutations comprise one or more neo-epitopes;
    i. wherein said associated digital software comprises access to a sequence database that allows screening of said mutations within said ORFs for identification of T-cell epitope(s) or immunogenic potential, or any combination thereof;
d. a nucleic acid cloning and expression kit for cloning and expressing a nucleic acid encoding one or more peptides comprising said one or more neo-epitopes from said disease-bearing sample;
e. an immunogenic assay for testing the T-cell immunogenecity of candidate peptides comprising one or more neo-epitopes;
f. an attenuated *Listeria* delivery vector for transforming with a plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding said identified immunogenic peptides comprising one or more immunogenic neo-epitopes of step (e),

wherein once transformed, said *Listeria* is stored or is administered to said human subject in (a) as part of an immunogenic composition.

[00137] In another embodiment, an infectious disease, an organ transplant rejection, or a tumor or cancer.

5 [00138] In one embodient, the present invention relates to a system for providing a personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

c. a delivery vector; and optionally
d. a plasmid vector for transforming said delivery vector, said plasmid vector comprising
10 a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition.

[00139] In one embodiment, provided herein is a recombinant attenuated *Listeria* strain,
15 wherein the *Listeria* strain comprises a nucleic acid sequence comprising one or more open reading frames encoding one or more peptides comprising one or more personalized neo-epitopes, wherein the neo-epitopes comprise immunogenic epitopes present in a disease- or condition-bearing tissue or cell of a subject having the disease or condition.

[00140] In one embodiment, provided herein is a recombinant attenuated *Listeria* strain
20 comprising: (a) a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises an immunogenic polypeptide or fragment thereof fused to one or more peptides comprising one or more neo-epitopes provided herein; or (b) a minigene nucleic acid construct comprising one or more open reading frames encoding a chimeric protein, wherein the chimeric protein
25 comprises: (i) a bacterial secretion signal sequence; (ii) a ubiquitin (Ub) protein; and (iii) one or more peptides comprising one or more neo-epitopes provided herein; wherein the signal sequence, the ubiquitin, and the one or more peptides in (i)-(iii) are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus, wherein the neo-epitopes comprise immunogenic epitopes present in a disease- or condition-bearing tissue or
30 cell of a subject having the disease or condition.

[00141] In another embodiment, administrating the *Listeria* strain to a subject having said disease or condition generates an immune response targeted to the subject's disease or condition.

[00142] In another embodiment, the strain is a personalized immunotherapy vector for said

subject targeted to said subject's disease or condition.

[00143] In another embodiment, the peptides comprise at least two different neo-epitope amino acid sequences.

[00144] In another embodiment, the peptides comprise one or more neo-epitope repeats of the same amino acid sequence.

[00145] In another embodiment, the *Listeria* strain comprises one neo-epitope.

[00146] In another embodiment, the *Listeria* strain comprises the neo-epitopes in the range of about 1-100. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, 5-15, 5-20, 5-25, 15-20, 15-25, 15-30, 15-35, 20-25, 20-35, 20-45, 30-45, 30-55 ,40-55, 40-65, 50-65, 50-75, 60-75, 60-85, 70-85, 70-95, 80-95, 80-105 or 95-105. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 50-100. Alternatively, the *Listeria* strain comprises up to about 100 neo-epitopes. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 1-100, 5-100, 5-75, 5-50, 5-40, 5-30, 5-20, 5-15 or 5-10. Alternatively, the *Listeria* strain comprises the neo-epitopes in the range of about 1-100, 1-75, 1-50, 1-40, 1-30, 1-20, 1-15 or 1-10.

[00147] In another embodiment, the *Listeria* strain comprises above about 100 neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 10 neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 20 neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 30 neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 40 neo-epitopes. In another embodiment, the *Listeria* strain comprises up to about 50 neo-epitopes. Alternatively, the *Listeria* strain comprises about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 neo-epitopes.

[00148] In one embodiment described herein, incorporation of amino acids in the range of about 5-30 amino acids flanking each side of a detected mutation in a neo-epitope are generated. Additionally or alternatively, varying sizes of neo-epitope inserts are inserted in the range of about 8-27 amino acids in length. Additionally or alternatively, varying sizes of neo-epitope inserts are inserted in the range of about 5-50 amino acids in length.

Additionally or alternatively, varying sizes of neo-epitope inserts (i.e., a peptide encoding a neo-epitope) are inserted in the range of 10-30, 10-40, 15-30, 15-40, or 15-25 amino acids in length. In another embodiment each neo-epitope insert is 1-10, 10-20, 20-30, or 30-40 amino acids long. In another embodiment, the neo-epitope insert is 1-100, 5-100, 5-75, 5-50, 5-40, 5-30, 5-20, 5-15 or 5-10 amino acids long. In yet another embodiment, the neo-epitope amino acid sequence is 1-100, 1-75, 1-50, 1-40, 1-30, 1-20, 1-15 or 1-10. In another embodiment, each neo-epitope insert is 21 amino acids in length or is a “21-mer” neo-epitope sequence. In yet another embodiment, the neo-epitope amino acid insert is about 8-11 or 11-16 amino acids long.

[00149] In another embodiment, the neo-epitope sequences are tumor-specific, metastasis-specific, bacterial-infection-specific, viral-infection-specific, and any combination thereof. Additionally or alternatively, the neo-epitope sequences are inflammation-specific, immune-regulation-molecule-epitope-specific, T-cell-specific, an autoimmune-disease-specific, *Graft-versus-host disease*-(*GvHD*)-specific, and any combination thereof.

[00150] In another embodiment, one or more neo-epitopes comprise linear neo-epitopes. Additionally or alternatively, one or more neo-epitopes comprise a solvent-exposed epitope. In another embodiment, one or more neo-epitopes comprise conformational neo-epitopes.

[00151] In another embodiment, one or more neo-epitopes comprise a T-cell epitope.

[00152] In one embodiment, disclosed herein is a nucleic acid construct encoding a chimeric protein comprising the following elements: an immunogenic polypeptide fused to a first neo-epitope amino acid (AA) sequence, wherein said first neo-epitope AA sequence is operatively linked to a second neo-epitope AA sequence via a linker sequence, wherein said second neo-epitope AA sequence is operatively linked to at least one additional neo-epitope amino acid sequence via a linker sequence. Optionally, the immunogenic polypeptide is an N-terminal truncated LLO (tLLO). Optionally, the last neo-epitope is operatively linked to a tag, such as a histidine tag at the C-terminus, via a linker sequence. Optionally, the nucleic acid construct comprises at least 1 stop codon (e.g., 2 stop codons) following the sequence encoding the tag. In one embodiment, disclosed herein is a nucleic acid construct encoding a chimeric protein comprising the following elements: a N-terminal truncated LLO (tLLO) fused to a first neo-epitope amino acid (AA) sequence, wherein said first neo-epitope AA sequence is operatively linked to a second neo-epitope AA sequence via a linker sequence, wherein said second neo-epitope AA sequence is operatively linked to at least one additional neo-epitope amino acid

sequence via a linker sequence, and wherein a last neo-epitope is operatively linked to a histidine tag at the C-terminus via a linker sequence. Optionally, the histidine tag is a 6X histidine tag. In another embodiment, said elements are arranged or are operatively linked from N-terminus to C-terminus. In another embodiment, each nucleic acid construct comprises at least 1 stop codon following the sequence encoding said 6X histidine (HIS) tag. In another embodiment, each nucleic acid construct comprises 2 stop codons following the sequence encoding said 6X histidine (HIS) tag. In another embodiment, said 6X histidine tag is operatively linked at the N-terminus to a SIINFEKL peptide. In another embodiment, said linker is a 4X glycine linker.

[00153] In another embodiment, the nucleic acid construct comprises at least one additional neo-epitope amino acid sequence. In another embodiment, the nucleic acid construct comprises 2-10 additional neo-epitopes, 10-15 additional neo-epitopes, 10-25 additional neo-epitopes, 25-40 additional neo-epitopes, or 40-60 additional neo-epitopes. In another embodiment, the nucleic acid construct comprises about 1-10, about 10-30, about 30-50, about 50-70, about 70-90, or up to about 100 neo-epitopes. For example, the nucleic acid construct can comprise about 5-100 neo-epitopes, or about 15-35 neo-epitopes.

[00154] In another embodiment each neo-epitope amino acid sequence is 1-10, 10-20, 20-30, or 30-40 amino acids long. In another embodiment, the neo-epitope amino acid sequence is 1-100, 5-100, 5-75, 5-50, 5-40, 5-30, 5-20, 5-15 or 5-10 amino acids long. In yet another embodiment, the neo-epitope amino acid sequence is 1-100, 1-75, 1-50, 1-40, 1-30, 1-20, 1-15 or 1-10. In another embodiment, each neo-epitope amino acid sequence is 21 amino acids in length or is a "21-mer" neo-epitope sequence. In yet another embodiment, the neo-epitope amino acid sequence is about 8-11 or 11-16 amino acids long.

[00155] In another embodiment, the nucleic acid construct encodes a recombinant polypeptide, chimeric protein, or fusion polypeptide comprising an N-terminal truncated LLO fused to a 21 amino acid sequence of a neo-epitope flanked by a linker sequence and followed by at least one second neo epitope flanked by another linker and terminated by a SIINFEKL-6xHis tag-and 2 stop codons closing the open reading frame: p*Hly*-tLLO-21mer #1-4x glycine linker G1-21mer #2-4x glycine linker G2-…-SIINFEKL-6xHis tag-2x stop codon. In another embodiment, expression of the above construct is driven by an *hly* promoter.

[00156] In another embodiment, the nucleic acid sequence comprises one or more linker sequences incorporated between at least one first neo-epitope and at least one second neo-epitope. In another embodiment, the nucleic acid sequence comprises at least two different

linker sequences incorporated between at least one first neo-epitope and at least one second neo-epitope to at least one third epitope. In another embodiment, one or more linker(s) is a 4xglycine linker selected from a group comprising nucleotide sequences as set forth in SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, and SEQ ID NO: 86.

[00157] In another embodiment, the nucleic acid sequence comprises at least one sequence encoding a TAG fused to the encoded peptide. In another embodiment, the TAG comprises the amino acid sequence as set forth in SEQ ID NO: 87.

[00158] In another embodiment, the one or more neo-epitopes each comprises between about 8 to 27 amino acids. Alternatively, the one or more neo-epitopes each comprises between about 5 to 50 amino acids. In another embodiment, the one or more neo-epitopes each comprises about 21 amino acids

[00159] In another embodiment, the neo-epitopes are determined using exome sequencing or transcriptome sequencing of the disease-bearing tissue or cell.

[00160] In another embodiment, the neo-epitopes comprise a nucleic acid sequence encoding a selected amino acid mutation in comparison to a matching biological sample amino acid sequences, flanked by about 10 amino acids on its N-terminus and about 10 amino acids on its C-terminus.

[00161] In another embodiment, one or more neo-epitopes, peptides comprising the immunogenic epitopes, or both, are hydrophilic.

[00162] In another embodiment, one or more neo-epitopes, peptides comprising the immunogenic epitopes, or both are up to 1.6 on the Kyte Doolittle hydropathy plot.

[00163] In another embodiment, one or more neo-epitope(s) are screened for immunosuppressive epitopes, wherein immunosuppressive epitopes are excluded from the nucleic acid molecule.

[00164] In another embodiment, one or more neo-epitope(s) are codon optimized for expression and secretion according to the *Listeria* strain.

[00165] In one embodiment, the nucleic acid sequence encoding a neo-epitope, therapeutic polypeptide or nucleic acid is optimized for increased levels of one or more neo-epitope or nucleic acid expression, or, in another embodiment, for increased duration of therapeutic polypeptide comprising one or more neo-epitopes or nucleic acid expression, or, in another

embodiment, a combination thereof. Additionally or alternatively, the nucleic acid sequence encoding a neo-epitope, therapeutic polypeptide or nucleic acid is optimized for increased levels of translation, secretion, transcription, and any combination thereof.

[00166] Additionally or alternatively, the nucleic acid sequence encoding a neo-epitope, therapeutic polypeptide or nucleic acid is optimized for nucleic acid sequence encoding a neo-epitope, therapeutic polypeptide or nucleic acid is optimized for decreased levels of secondary structures possibilities possibly formed in the oligonucleotide sequence, or alternatively optimized to prevent attachment of any enzyme that may modify the sequence.

[00167] In one embodiment, the term "optimized" refers to a desired change, which, in one embodiment, is a change in synthetic gene expression comprising one or more neo-epitopes as described in the present invention, and, in another embodiment, in protein expression. In one embodiment, optimized gene expression is optimized regulation of gene expression. In another embodiment, optimized gene expression is an increase in gene expression. According to this aspect and in one embodiment, a 2-fold through 1000-fold increase in gene expression compared to wild-type is contemplated. In another embodiment, a 2-fold to 500-fold increase in gene expression, in another embodiment, a 2-fold to 100-fold increase in gene expression, in another embodiment, a 2-fold to 50-fold increase in gene expression, in another embodiment, a 2-fold to 20-fold increase in gene expression, in another embodiment, a 2-fold to 10-fold increase in gene expression, in another embodiment, a 3-fold to 5-fold increase in gene expression is contemplated.

[00168] In another embodiment, optimized gene expression may be an increase in gene expression under particular environmental conditions. In another embodiment, optimized gene expression may comprise a decrease in gene expression, which, in one embodiment, may be only under particular environmental conditions.

[00169] In another embodiment, optimized synthetic gene expression is an increased duration of gene expression. According to this aspect and in one embodiment, a 2-fold through 1000-fold increase in the duration of gene expression compared to wild-type is contemplated. In another embodiment, a 2-fold to 500-fold increase in the duration of gene expression, in another embodiment, a 2-fold to 100-fold increase in the duration of gene expression, in another embodiment, a 2-fold to 50-fold increase in the duration of gene expression, in another embodiment, a 2-fold to 20-fold increase in the duration of gene expression, in another embodiment, a 2-fold to 10-fold increase in the duration of gene expression, in another embodiment, a 3-fold to 5-fold increase in the duration of gene expression is contemplated. In another embodiment, the increased duration of gene

expression is compared to gene expression in non-vector-expressing controls, or alternatively, compared to gene expression in wild-type-vector-expressing controls.

[00170] Expression in bacterial cells is hampered, in one embodiment, by transcriptional silencing, low mRNA half-life, secondary structure formation, attachment sites of oligonucleotide binding molecules such as repressors and inhibitors, and availability of rare tRNAs pools. The source of many problems in bacterial expressions is found within the original sequence. The optimization of RNAs may include modification of cis acting elements, adaptation of its GC-content, modifying codon bias with respect to non-limiting tRNAs pools of the bacterial cell, and voiding internal homologous regions.

[00171] Therefore, in one embodiment, when relying on carefully designed synthetic sequences, stable messages with prolonged half-lives, high level protein production within the host can be expected.

[00172] Thus, in one embodiment, optimizing a sequence entails adapting the codon usage to the codon bias of host genes, which in one embodiment, are *Listeria monocytogenes* genes; adjusting regions of very high (> 80%) or very low (< 30%) GC content; avoiding one or more of the following cis-acting sequence motifs: internal TATA-boxes, chi-sites and ribosomal entry sites; AT-rich or GC-rich sequence stretches; repeat sequences and RNA secondary structures; (cryptic) splice donor and acceptor sites, branch points; or a combination thereof. In one embodiment, a gene is optimized for expression in *Homo sapiens* cells. In still another embodiment, optimizing expression entails adding sequence elements to flanking regions of a gene and/or elsewhere in the expression vector.

[00173] In one embodiment, the formulations and methods of the present invention provide a nucleic acid optimized for increased expression levels, duration, or a combination thereof of a therapeutic polypeptide comprising one or more neo-epitope encoded by said nucleic acid.

[00174] In another embodiment, one or more neo-epitope(s) allow for MHC class II epitope presentation.

[00175] In another embodiment, the *Listeria* strain expresses and secretes one or more peptides comprising one or more neo-epitopes.

[00176] In another embodiment, the *Listeria* strain expresses and secretes one or more peptides comprising one or more neo-epitopes during infection of the subject.

[00177] In another embodiment, the *Listeria* strain comprises a plurality of the nucleic acid sequence molecules.

[00178] In one embodiment, the nucleic acid construct encoding the fusion polypeptides disclosed herein is a plasmid insert. In another embodiment, the insert comprises a first open reading frame encoding said fusion polypeptide. In another embodiment, the fusion polypeptide comprises an immunogenic polypeptide or fragment thereof fused to one or more peptides comprising one or more neo-epitopes disclosed herein. In an embodiment, this insert may be on a plasmid, or at least partially integrated into the genome. In another embodiment the insert can be designed as a minigene nucleic acid construct comprising one or more open reading frames encoding a chimeric protein, the chimeric protein includes: a bacterial secretion signal sequence, an ubiquitin (Ub) protein, and one or more peptides comprising one or more neo-epitopes provided herein. In another embodiment, the signal sequence, said ubiquitin and one or more peptides are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

[00179] In another embodiment, the *Listeria* strain comprises the nucleic acid sequence in a minigene nucleic acid construct comprising one or more open reading frames encoding a chimeric protein, wherein the chimeric protein comprises: (a) a bacterial secretion signal sequence, (b) a ubiquitin (Ub) protein, (c) one or more peptides comprising one or more neo-epitopes provided herein; and, wherein the signal sequence, the ubiquitin, and the one or more peptides in (a)-(c) are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

[00180] In another embodiment, the nucleic acid molecule is in a bacterial artificial chromosome in the recombinant *Listeria* strain.

[00181] In another embodiment, the nucleic acid molecule is in a plasmid in the recombinant *Listeria* strain.

[00182] In another embodiment, the plasmid is an integrative plasmid.

[00183] In another embodiment, the plasmid is an extrachromosomal multicopy plasmid.

[00184] In another embodiment, the plasmid is stably maintained in the *Listeria* strain in the absence of antibiotic selection.

[00185] In another embodiment, the plasmid does not confer antibiotic resistance upon the recombinant *Listeria*.

[00186] In another embodiment, the one or more peptides are each fused to an immunogenic polypeptide or fragment thereof. For example, each of the one or more peptides can be fused

to different immunogenic polypeptides or fragments thereof, or the combination of the one or more peptides can be fused to an immunogenic polypeptide or fragment thereof (e.g., an immunogenic polypeptide linked to a first neo-epitope, which is linked to a second neo-epitope, which is linked to a third neo-epitope, and so forth).

5 [00187] In another embodiment, the one or more peptides comprising one or more immunogenic neo-epitopes are fused concomitantly to an immunogenic polypeptide or fragment thereof.

[00188] In another embodiment, the immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, an ActA-PEST2 10 fusion, or a PEST amino acid sequence.

[00189] In another embodiment, the ActA-PEST2 fusion protein is set forth in SEQ ID NO: 16.

[00190] In another embodiment, the tLLO protein is set forth in SEQ ID NO: 3.

[00191] In another embodiment, the actA is set forth in SEQ ID NO: 12-13 and 15-18.

15 [00192] In another embodiment, the PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

[00193] In another embodiment, the mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

[00194] In another embodiment, the mutation comprises a substitution of residue C484, 20 W491, or W492 of SEQ ID NO: 2, or any combination thereof.

[00195] In another embodiment, the mutation comprises a substitution of 1-11 amino acid(s) within the CBD set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein the non-LLO peptide comprises a peptide comprising a neo-epitope.

[00196] In another embodiment, the mutation comprises a deletion of 1-11 amino acid(s) 25 within the CBD as set forth in SEQ ID NO: 68.

[00197] In another embodiment, the one or more peptides comprise a heterologous antigen or a self-antigen associated with said disease. In another embodiment, the heterologous antigen or the self-antigen is a tumor-associated antigen or a fragment thereof.

[00198] In another embodiment, the neo-epitope or fragment thereof comprises a Human 30 Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a

chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

[00199] In another embodiment, the tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

[00200] In another embodiment, the tumor or cancer comprises a metastasis of the tumor or cancer.

[00201] In another embodiment, the disease or condition is an infectious disease, an autoimmune disease, or a tumor or a cancer.

[00202] In another embodiment, the infectious disease comprises a viral or bacterial infection.

[00203] In another embodiment, one or more neo-epitopes comprise an infectious disease-associated-specific epitope.

[00204] In another embodiment, the infections disease is an infectious viral disease.

[00205] In another embodiment, the infections disease is an infectious bacterial disease.

[00206] In another embodiment, the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris,

BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

[00207] In another embodiment, the attenuated *Listeria* comprises a mutation in one or more endogenous genes.

[00208] In another embodiment, the endogenous gene mutation is selected from an actA gene mutation, a prfA mutation, an actA and inlB double mutation, a dal/dal gene double mutation, or a dal/dat/actA gene triple mutation, or a combination thereof.

[00209] In another embodiment, the mutation comprises an inactivation, truncation, deletion, replacement or disruption of the gene or genes.

[00210] In another embodiment, the vector further comprises an open reading frame or a second nucleic acid sequence comprising an open reading frame encoding a metabolic enzyme.

[00211] In another embodiment, the metabolic enzyme encoded by the open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

[00212] In another embodiment, the *Listeria* is *Listeria* monocytogenes.

[00213] In another embodiment, the *Listeria* strain further comprises a nucleic acid construct comprising one or more open reading frames encoding one or more one or more immunomodulatory molecule(s).

[00214] In another embodiment, the immunomodulatory molecule is expressed and secreted from said *Listeria* strain, wherein said molecule is selected from a group comprising Interferon gamma, a cytokine, a chemokine, a T-cell stimulant, and any combination thereof.

[00215] In one embodiment, a personalized immunotherapy composition disclosed herein comprises one or more delivery vectors as disclosed herein. In one embodiment, a personalized immunotherapy composition disclosed herein comprises one or more *Listeria* strain(s) as disclosed in any of the above. In another embodiment, a personalized immunotherapy composition comprises a mixture of 1-2, 1-5, 1-10, 1-20 or 1-40 recombinant delivery vectors, each vector expressing one or more neo-epitopes. In another embodiment, the mixture comprises 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 delivery vectors. In another embodiment, a personalized immunotherapy composition comprises a mixture of 1-2, 1-5, 1-10, 1-20 or 1-40 recombinant delivery vectors, each vector expressing one or more neo-epitopes in the context of a fusion protein with a truncated LLO protein, a truncated ActA protein or a PEST amino acid sequence. In one embodiment, the individual delivery vectors present in the mixture of delivery vectors are administered concomitantly to a subject as part of a therapy. In another embodiment, the individual delivery vectors present in the mixture of delivery vectors are administered sequentially to a subject as part of a therapy.

[00216] In one embodiment, disclosed herein is an immunogenic mixture of compositions comprising one or more recombinant delivery vectors produced by the process disclosed herein. In another embodiment, each of said delivery vector in said mixture comprises a nucleic acid molecule encoding a fusion polypeptide or chimeric protein comprising one or more neo-epitopes. In another embodiment, each delivery vector in said mixture expresses 1-

5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes. In another embodiment, each mixture comprises 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 delivery vectors. In another embodiment, the mixture comprises a plurality of delivery vectors, each delivery vector comprising a different set of one or more neo-epitopes. A first set of neo-epitopes can be different from a second set if it includes one neo-epitope that the second set does not. Likewise, a first set of neo-epitopes can be different from a second set if it does not include a neo-epitopes that the second set does include. For example, a first set and a second set of neo-epitopes can include one or more of the same neo-epitopes and can still be different sets, or a first set can be different from a second set of neo-epitopes by virtue of not including any of the same neo-epitopes

[00217] In one embodiment, disclosed herein is an immunogenic mixture of compositions comprising one or more recombinant *Listeria* strains produced by the process disclosed herein. In another embodiment, each of said Listeria in said mixture comprises a nucleic acid molecule encoding a fusion polypeptide or chimeric protein comprising one or more neo-epitopes. In another embodiment, each Listeria in said mixture expresses 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes. In another embodiment, each mixture comprises 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 recombinant Listeria strains. In another embodiment, the mixture comprises a plurality of recombinant *Listeria* strains, each *Listeria* strain comprising a different set of one or more neo-epitopes. A first set of neo-epitopes can be different from a second set if it includes one neo-epitope that the second set does not. Likewise, a first set of neo-epitopes can be different from a second set if it does not include a neo-epitopes that the second set does include. For example, a first set and a second set of neo-epitopes can include one or more of the same neo-epitopes and can still be different sets, or a first set can be different from a second set of neo-epitopes by virtue of not including any of the same neo-epitopes.

[00218] In one embodiment, disclosed herein is a method of eliciting a personalized anti-tumor response in a subject, the method comprising the step of concomitantly or sequentially administering to said subject an immunogenic mixture composition disclosed herein. In another embodiment, disclosed herein is a method of preventing or treating a tumor in a subject, the method comprising the step of concomitantly or sequentially administering to said subject the immunogenic mixture of compositions disclosed herein. In one embodiment, a composition comprising at least one recombinant *Listeria* strain selected from said mixture

of compositions may be administered simultaneously (i.e., in the same medicament), concurrently (i.e., in separate medicaments administered one right after the other in any order) or sequentially in any order with at least another recombinant *Listeria* strain selected from said mixture of compositions. Sequential administration is particularly useful when a drug substance comprising a recombinant *Listeria* strain disclosed herein is in different dosage forms (one agent is a tablet or capsule and another agent is a sterile liquid) and/or are administered on different dosing schedules, e.g., one composition from said mixture of compositions comprising one *Listeria* strain is administered at least daily and another that is administered less frequently, such as once weekly, once every two weeks, or once every three weeks.

[00219] In another embodiment, the personalized immunotherapy composition elicits an immune response targeted against one or more neo-epitopes.

[00220] In another embodiment, the composition comprises a plurality or combination of *Listeria* strains, wherein each strain comprises the nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising at least one unique the neo-epitope.

[00221] In another embodiment, the composition comprises a combination of the *Listeria* strains, wherein the combination comprises a plurality of the neo-epitopes.

[00222] It will be appreciated by a skilled artisan that the term "plurality" may encompass an integer above 1. In one embodiment, the term refers to a range of 1-10, 10-20, 20-30, 30-40, 40-50, 60-70, 70-80, 80-90, or 90-100.

[00223] In another embodiment, the combination comprises up to about 300 the neo-epitopes.

[00224] In another embodiment, the combination comprises a range of about 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes.

[00225] In one embodiment, the combination comprises a range of about 8-27 epitopes per vector. In another embodiment, the combination comprises a range of about 21 epitopes per vector. In another embodiment, the combination comprises a range of about 1-5, 1-10 , 1-20, 1-30, 1-50, 1-60, 1-70, 1-80, 1-90, 1-100, 1-110, 1-150, 1-200, 1-250, 1-300, or 1-500 epitopes per vector.

[00226] In one embodiment all epitopes are neo-epitopes. In another embodiment, at least

one epitope per vector is a neo-epitope.

[00227] In one embodiment, determination of a number of constructs vs. mutational burden in a delivery vector is performed to determine efficiency of expression and secretion of neo-epitopes. In another embodiment, ranges of linear neo-epitopes are tested, starting with about 50 epitopes per vector. In another embodiment, ranges of linear neo-epitopes are tested, starting with about 1-5, 5-10, 10-20, 20-50, 50-70, 70-90, 90-110, 110-150, 150-200, 200-250, 300-350, or 400-500 epitopes per vector. In one embodiment, constructs include at least one neo-epitope per vector.

[00228] In one embodiment, the number of vectors to be used is determined considering the efficiency of translation and secretion of multiple epitopes from a single vector, and the multiplicity of infection (MOI) needed for each Lm vector harboring specific neo-epitopes, or in reference to the number of neo-epitopes.

[00229] In one embodiment, the number of vectors to be used (e.g. a *Listeria* vector) is determined by taking into consideration predefining groups of: known tumor-associated mutations found in circulating tumor cells; known cancer "driver" mutations; and/or known chemotherapy resistance mutations and giving these priority in the 21 amino acid sequence peptide selection (see Example 30). In another embodiment, this can be accomplished by screening identified mutated genes against the COSMIC (Catalogue of somatic mutations in cancer, cancer.Sanger.ac.uk) or Cancer Genome Analysis or other similar cancer-associated gene database. Further, and in another embodiment, screening for immunosuppressive epitopes (T-reg epitopes, IL-10 inducing T helper epitopes, etc.) is utilized to de-select or to avoid immunosuppressive influences on the vector. In another embodiment, selected codons are codon optimized to efficient translation and secretion according to specific the specific delivery vector (e.g. *Listeria* strain). Example for codons optimized for *L. monocytogenes* as known in the art is presented in table 8 herein.

[00230] In another embodiment, the combination comprises at least two different neo-epitopes amino acid sequences.

[00231] In another embodiment, the combination comprises the neo-epitopes in the range of about 1-5, 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, or 90-100.

[00232] In another embodiment, the combination comprises the neo-epitopes in the range of about 50-100.

[00233] In another embodiment, the combination comprises up to about 100 the neo-epitopes.

[00234] In another embodiment, the combination comprises above about 100 the neo-epitopes.

5 [00235] In another embodiment, the combination comprises up to about 10 the neo-epitopes.

[00236] In another embodiment, the combination comprises up to about 20 the neo-epitopes.

[00237] In another embodiment, the combination comprises up to about 50 the neo-epitopes.

[00238] In another embodiment, the combination comprises about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35,
10 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 the neo-epitopes.

[00239] In another embodiment, the combination comprises the neo-epitopes in the range of about 5-15, 5-20, 5-25, 15-20, 15-25, 15-30, 15-35, 20-25, 20-35, 20-45, 30-45, 30-55 ,40-55, 40-65, 50-65, 50-75, 60-75, 60-85, 70-85, 70-95, 80-95, 80-105 or 95-105.

[00240] In another embodiment, the combination comprises about 51, 52, 53, 54, 55, 56, 57,
15 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 the neo-epitopes.

[00241] In another embodiment, the combination further comprises one or more recombinant attenuated *Listeria* strain delivery vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more one or more immunomodulatory
20 molecule(s).

[00242] In another embodiment, the immunomodulatory molecule is expressed and secreted from the *Listeria* strain, wherein the molecule is selected from a group comprising Interferon gamma, a cytokine, a chemokine, a T-cell stimulant, and any combination thereof.

[00243] In another embodiment, the combination further comprises one or more recombinant
25 attenuated *Listeria* strain delivery vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more epitopes, wherein the epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of the subject having the disease or condition, wherein administrating the *Listeria* strain generates a immunotherapy targeted to the subject's disease or condition.

30 [00244] In another embodiment, the composition, as disclosed in any of the above, further

comprising an adjuvant.

[00245] In another embodiment, the adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

5 [00246] In another embodiment, administering the composition to the subject generates a personalized enhanced anti-disease, or anti-condition immune response in the subject.

[00247] In another embodiment, the immune response comprises an anti-cancer or anti-tumor response.

[00248] In another embodiment, the immune response comprises an anti-infectious disease
10 response.

[00249] In another embodiment, the infectious disease comprises a viral infection.

[00250] In another embodiment, the infectious disease comprises a bacterial infection.

[00251] In another embodiment, the personalized immunotherapy increases survival time in the subject having the disease or condition.

15 [00252] In another embodiment, the personalized immunotherapy reduces tumor size or metastases size in the subject having the disease or condition.

[00253] In another embodiment, the personalized immunotherapy protects against metastases in the subject having the disease or condition.

[00254] In another embodiment of the present invention, a DNA immunotherapy comprising
20 the personalized immunotherapy composition as disclosed in any of the above.

[00255] In another embodiment of the present invention, a peptide immunotherapy comprising the personalized immunotherapy composition as disclosed in any of the above.

[00256] In another embodiment, the immunotherapy further comprises an adjuvant, cytokine, chemokine, or combination thereof.

25 [00257] In another embodiment of this invention, a pharmaceutical composition of the present invention comprising the immunotherapy or personalized immunotherapy composition as disclosed in any of the above and a pharmaceutical carrier.

[00258] In another embodiment of this invention, a method of inducing an immune response to at least one neo-epitope present in a disease or condition bearing tissue or cell in a subject

having the disease or condition, the method comprising the step of administering the personalized immunotherapy composition or immunotherapy as disclosed in any of the above to the subject.

[00259] In another embodiment of this invention, a method of inducing a targeted immune response in a subject having a disease or condition, comprising administering to the subject the immunogenic composition or immunotherapy as disclosed in any of the above, wherein administrating the *Listeria* strain generates a personalized immunotherapy targeted to the subject's disease or condition.

[00260] In another embodiment of this invention, a method of treating, suppressing or inhibiting disease or condition in a subject, the method comprising the step of administrating a personalized immunotherapy composition or immunotherapy as disclosed in any of the above, for targeting the disease or condition.

[00261] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the step of administrating the composition or immunotherapy orally or parenterally.

[00262] In another embodiment administrating parenterally comprises intravenous administration, subcutaneous administration, or intramuscular administration.

[00263] In yet another embodiment, the disease or condition is an infectious disease, autoimmune disease, organ transplantation rejection, a tumor or a cancer.

[00264] In another embodiment, the tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

[00265] In another embodiment, the infectious disease comprises a viral or bacterial infection.

[00266] In another embodiment, the infectious disease is caused by one of the following

pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

[00267] In another embodiment of the present invention, provided is a method of increasing the ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, wherein the T effector cells are targeted to a neo-epitope present within a disease or condition bearing tissue of a subject, the method comprising the step of administering to the subject personalized immunotherapy composition or immunotherapy as disclosed in any of the above.

[00268] In another embodiment of the present invention, provided is a method for increasing antigen-specific T-cells in a subject, wherein the antigen or a peptide fragment thereof comprises one or more neo-epitopes, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of
5 the above.

[00269] In another embodiment of the present invention, provided is a method for increasing survival time of a subject having a tumor or suffering from cancer, or suffering from an infectious disease, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of the
10 above.

[00270] In another embodiment of the present invention, provided is a method of protecting a subject from a cancer, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of the above.

15 [00271] In another embodiment of the present invention, provided is a method of inhibiting or delaying the onset of cancer in a subject, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of the above.

[00272] In another embodiment of the present invention, provided is a method of reducing
20 tumor or metastasis size in a subject, the method comprising the step of administrating to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of the above.

[00273] According to another embodiment of the present invention, the tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or
25 tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a
30 bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

[00274] In another embodiment of the present invention, provided is a method of protecting

a subject from an infectious disease, the method comprising the step of administering to the subject a personalized immunotherapy composition or immunotherapy as disclosed in any of the above.

[00275] In another embodiment of the present invention, the infectious disease comprises a viral or bacterial infection.

[00276] In another embodiment of the present invention, the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

[00277] In another embodiment of the administering results in the generation of a personalized T-cell immune response against the disease or the condition.

[00278] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the steps of creating the personalized immunotherapy composition, wherein the creating comprises the steps of:

(a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein the comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within one or more ORFs from the disease-bearing sample;

(b) transforming an attenuated *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising one or more neo-epitopes identified in a.; and, alternatively storing the attenuated recombinant *Listeria* for administering to the subject at a pre-determined period or administering a composition comprising the attenuated recombinant *Listeria* strain to the subject, and wherein the administering results in the generation of a personalized T-cell immune response against the disease or the condition; optionally,

(c) obtaining a second biological sample from the subject comprising a T-cell clone or T-infiltrating cell from the T-cell immune response and characterizing specific peptides comprising one or more neo-epitopes bound by MHC Class I or MHC Class II molecules on the T cells , wherein one or more neo-epitopes are immunogenic;

(d) screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

(e) transforming a second attenuated recombinant *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising one or more immunogenic neo-epitopes; and, alternatively storing the second attenuated recombinant *Listeria* for administering to the subject at a pre-determined period or administering a second composition comprising the second attenuated recombinant *Listeria* strain to the subject,

wherein the process creates a personalized immunotherapy for the subject.

[00279] In one embodiment, the one or more neo-epitopes comprise a plurality of neo-

epitopes. Optionally, step (b) can further comprise one or more iterations of randomizing the order of the one or more peptides comprising the plurality of neo-epitopes within the nucleic acid sequence of step (b). Such randomizing can include, for example, randomizing the order of the entire set of one or more peptides comprising the plurality of neo-epitopes, or can comprise randomizing the order of a subset of the one or more peptides comprising a subset of the plurality of neo-epitopes. For example, if the nucleic acid sequence comprises 20 peptides (ordered 1-20) comprising 20 neo-epitopes, the randomizing can comprise randomizing the order of all 20 peptides or can comprise randomizing the order of only a subset of the peptides (e.g., peptides 1-5 or 6-10). Such randomization of the order can facilitate secretion and presentation of the neo-epitopes and of each individual region.

[00280] In another embodiment, the step of comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, further comprises using of a screening assay or screening tool and associated digital software for comparing one or more ORFs in nucleic acid sequences extracted from the disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from the healthy biological sample, wherein the associated digital software comprises access to a sequence database that allows screening of mutations within the ORFs in the nucleic acid sequences extracted from the disease-bearing biological sample for identification of immunogenic potential of the neo-epitopes.

[00281] In another embodiment of the invention the method as disclosed in any of the above, additionally comprises the step of screening one or more neo-epitopes, peptide comprising one or more neo-epitopes, or both, for hydrophobicity and hydrophilicity.

[00282] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprises the step of selecting one or more neo-epitopes, peptides comprising one or more neo-epitopes, or both, that are hydrophilic.

[00283] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprises the step of selecting one or more neo-epitopes, peptides comprising one or more neo-epitopes, or both, that are up to 1.6 in the Kyte Doolittle hydropathy plot.

[00284] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the step of codon optimizing one or more neo-

epitopes or peptides comprising one or more neo-epitopes for expression and secretion according to the specific *Listeria* strain.

[00285] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the step of screening one or more neo-epitope(s) for immunosuppressive epitopes.

[00286] According to another embodiment of the present invention, the biological sample is tissue, cells, blood or sera.

[00287] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprises the step of obtaining the disease-bearing biological sample from the subject having the disease or condition.

[00288] According to another embodiment of the present invention as disclosed herein, additionally comprises the step of obtaining the healthy biological sample from the subject having the disease or condition.

[00289] According to another embodiment, the step of obtaining a second biological sample from the subject comprises obtaining a biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of the second composition comprising the attenuated recombinant *Listeria* strain.

[00290] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the steps of: (a) identifying, isolating and expanding T cell clones or T-infiltrating cells that respond against the disease; and, (b) screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules to which a T-cell receptor on the T cells binds to.

[00291] In another embodiment, the step of screening for and identifying comprises T-cell receptor sequencing, multiplex based flow cytometry, or high-performance liquid chromatography.

[00292] In another embodiment, the sequencing comprises using associated digital software and database.

[00293] According to another embodiment of the present invention, a method as described above is disclosed, additionally comprising the step of determining the sequencing of the nucleic acid sequences using exome sequencing or transcriptome sequencing.

[00294] In one embodiment, a fusion polypeptide or chimeric protein disclosed herein is expressed and secreted by a recombinant *Listeria* disclosed herein. In another embodiment, the fusion polypeptide, or chimeric protein disclosed herein comprises a C-terminal SIINFEKL-S-6xHIS tag. In another embodiment, the fusion polypeptide, or chimeric protein disclosed herein is expressed and secreted by a recombinant *Listeria* disclosed herein. In another embodiment, secretion of the antigen, or polypeptides (fusion or chimeric) disclosed herein is detected using a protein, molecule or antibody (or fragment thereof) that specifically binds to a polyhistidine (His) tag. In another embodiment, the fusion polypeptide, or chimeric protein disclosed herein is expressed and secreted by a recombinant *Listeria* disclosed herein. In another embodiment, secretion of the antigen, or polypeptides (fusion or chimeric) disclosed herein is detected using an antibody, protein or molecule that binds a SIINFEKL-S-6xHIS tag. In another embodiment, the fusion polypeptide of chimeric protein disclosed herein comprise any other tag know in the art, including, but not limited to chitin binding protein (CBP), maltose binding protein (MBP), and glutathione-S-transferase (GST), thioredoxin (TRX) and poly(NANP).

[00295] In one embodiment, each neo-epitope is connected with a linker sequence to the following neo-epitope encoded on the same vector. In one embodiment, the linker is 4Xglycine DNA sequence. It will be appreciated by a skilled artisan that other linker sequences known in the art may be used in the methods and compositions disclosed herein (see for e.g. Reddy Chichili, V. P., Kumar, V. and Sivaraman, J. (2013), Linkers in the structural biology of protein–protein interactions. Protein Science, 22: 153–167, which is incorporated by reference herein in its entirety). In yet another embodiment the linker is selected from a group comprising SEQ ID NO: 1-11, SEQ ID NO 76-86 accordingly, and any combination thereof.

[00296] In one embodiment, the final neo-epitope in an insert is fused to a TAG sequence followed by a stop codon. It will be appreciated by a skilled artisan that a TAG may allow easy detection of the fusion polypeptide or chimeric protein during for example secretion from the Lm vector or when testing construct for affinity to specific T-cells, or presentation by antigen presenting cells.

[00297] In one embodiment, about 10 flanking amino acids on each side of the detected mutation are incorporated to accommodate class1 MHC-1 presentation, in order to provide at least some of the different HLA T-cell receptor (TCR) reading frames.

[00298] Table 7 herein shows a sample list of 50 neo-epitope peptides wherein each mutation is indicated by a Bolded amino acid letter and is flanked by 10 amino acids on each side providing an 21 amino acid peptide neo-epitope. In one embodiment, if there are more usable 21 amino acid peptides than can fit into a single plasmid, different 21 amino acid peptides are designated into 1st, 2nd, etc. construct by priority rank as needed/desired. In another embodiment, the priority of assignment to one of multiple vectors composing the entire set of desired neo-epitopes are determined based on factors like relative size, priority of transcription, and/or overall hydrophobicity of the translated polypeptide.

[00299] In one embodiment different linker sequences are distributed between the neo-epitopes for minimizing repeats. In another embodiment, distributing different linker sequences between the neo-epitopes reduce secondary structures thereby allowing efficient transcription, translation, secretion, maintenance, or stabilization of the plasmid including the insert within the Lm recombinant vector strain population.

[00300] In one embodiment, disclosed herein is a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample;

b. transforming an attenuated *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said attenuated recombinant *Listeria* for administering to said subject at a pre-determined period or administering a composition comprising said attenuated recombinant *Listeria* strain to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; optionally,

c. Obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell from said T-cell immune response and characterizing specific peptides comprising one or more neo-epitopes bound by MHC Class I orMHC Class II molecules on said T cells, wherein said one or more neo-epitopes are immunogenic;

d. Screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

e. Transforming a second attenuated recombinant *Listeria* strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes; and, alternatively storing said second attenuated recombinant *Listeria* for administering to said subject at a pre-determined period or administering a second composition comprising said second attenuated recombinant *Listeria* strain to said subject,

wherein said process creates a personalized immunotherapy for said subject.

[00301] In one embodiment, disclosed herein is a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample;

b. transforming a vector with a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a., or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; and optionally,

c. Obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell from said T-cell immune response and characterizing specific peptides comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells;

d. Screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

e. Transforming a second vector with a nucleic acid sequence comprising one or more open reading frames encoding one or more peptides comprising said one or more immunogenic neo-epitopes or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes identified in c.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period, or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject,

wherein said process creates a personalized immunotherapy for said subject.

[00302] In one embodiment, disclosed herein is a process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORFs from the disease-bearing sample;

b. transforming a vector with a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a., or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence comprising one or more ORFs encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; and optionally,

c. Obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell or blood or tissue specimen whereby response to potential neo-epitope peptides can be identified and selected based on increased or changed T-cell immune response and characterizing by reacting with specific peptides comprising one or more

immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells, wherein said one or more neo-epitopes are immunogenic or by PCR based deep sequencing of the T cell receptor specificity and evaluation of increased Tcell responses associated with neo-epitopes;

5 d. Screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

e. Transforming a second vector with a nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes, or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid

10 sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes identified in c.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period, or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject,

15 wherein said process creates a personalized immunotherapy for said subject.

[00303] In another embodiment, provided herein is a system for providing a personalized immunotherapy for a subject having a disease or condition, comprising the following components:

g. a disease-bearing biological sample obtained from said subject having said disease

20 or condition;

h. a healthy biological sample, wherein said healthy biological sample is obtained from said human subject having said disease or condition or another healthy human subject;

i. a screening assay or screening tool and associated digital software for comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from said disease-

25 bearing biological sample with open reading frames in nucleic acid sequences extracted from said healthy biological sample, and for identifying mutations in said ORFs encoded by said nucleic acid sequences of said disease-bearing sample, wherein said mutations comprise one or more neo-epitopes;

i. wherein said associated digital software comprises access to a sequence database

30 that allows screening of said mutations within said ORFs for identification of T-cell epitope(s) or immunogenic potential, or any combination thereof;

j. a nucleic acid cloning and expression kit for cloning and expressing a nucleic acid encoding one or more peptides comprising said one or more neo-epitopes from said disease-

bearing sample;

k. an immunogenic assay for testing the T-cell immunogenecity and/or binding of candidate peptides comprising one or more neo-epitopes;

l. analytic equipment, and associated software for sequencing and analyzing nucleic acid sequences, peptide amino acid sequences and T-cell receptor amino acid sequences.

m. an attenuated *Listeria* delivery vector for transforming with a plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding said identified immunogenic peptides comprising one or more immunogenic neo-epitopes of step (e),

i. wherein once transformed, said *Listeria* is stored or is administered to said human subject in (a) as part of an immunogenic composition; or

n. a delivery vector; and optionally

o. a vector for transforming said delivery vector, said vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition.

[00304] In another embodiment, said one or more peptides are encoded by one or more open reading frames (ORFs) in said nucleic acid sequence.

[00305] In another embodiment, a disease is an infectious disease, or a tumor or cancer.

[00306] In another embodiment, said delivery vector comprises a bacterial delivery vector. In another related aspect said delivery vector comprises a viral vector delivery vector. In another related aspect said delivery vector comprises a peptide immunotherapy delivery vector. In another related aspect, said delivery vector comprises a DNA immunotherapy delivery vector.

[00307] In one embodiment, provided herein is a process for creating a personalized immunotherapy, the process comprising the steps of:

a. obtaining a disease-bearing biological sample from a subject having said disease or condition;

b. extracting nucleic acids from said disease-bearing sample;

c. obtaining a healthy biological sample from said subject in step (a) or from a different individual of the same species;

d. extracting nucleic acids from said healthy sample;

e. sequencing the extracted nucleic acid from steps (b) and (d);

f. comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from said disease-bearing biological sample with open reading frames in nucleic acid sequences extracted from said healthy biological sample, and for identifying mutated nucleic acid sequences within said ORFs of said disease-bearing sample, wherein said ORFs encodes a peptide comprising one or more neo-epitopes;

g. identifying mutated sequences within said ORFs in said disease-bearing sample, wherein said ORFs encodes a peptide comprising one or more neo-epitopes;

a. wherein said neo-epitopes are identified using methods well known in the art, including, but not limited to T-cell receptor (TCR) sequencing, or whole exome sequencing.

h. expressing said one or more peptides comprising said identified mutated nucleic acid sequences;

i. screening each peptide comprising said one or more neo-epitopes for an immunogenic T-cell response, wherein the presence of an immunogenic T-cell response correlates with presence of one or more neo-epitopes comprising a T-cell epitope;

j. identifying and selecting a nucleic acid sequence that encodes a one or more immunogenic peptides comprising one or more immunogenic neo-epitopes that are T-cell epitopes, and transforming an attenuated *Listeria* strain with a plasmid vector comprising said sequence;

k. culturing and characterizing said attenuated *Listeria* strain to confirm expression and secretion of said one or more immunogenic peptides; and,

l. storing said attenuated *Listeria* for administering to said subject at a pre-determined period or administering said attenuated *Listeria* strain to said subject, wherein said attenuated *Listeria* strain is administered as part of an immunogenic composition.

[00308] In another embodiment, the process of obtaining a second biological sample from said subject comprises obtaining a biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of said second composition comprising said attenuated recombinant *Listeria* strain.

[00309] In another embodiment, the process of characterizing specific peptides comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells comprises the steps of:

a. Identifying, isolating and expanding T cell clones or T-infiltrating cells that respond against said disease;

b. Screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules
5 to which a T-cell receptor on said T cells binds to.

[00310] In another embodiment, a screening step for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules comprises contacting said T-cells with said one or more peptides. In another embodiment, said screening step for and identifying comprises performing T-cell
10 receptor sequencing, multiplex based flow cytometry, or high-performance liquid chromatography to determine peptide specificity. It will be well appreciated by a skilled artisan that methods for determining peptides that bind to T-cell receptors are well known in the art.

[00311] In one embodiment, the step of comparing in a system or a process of creating a
15 personalized immunotherapy provided herein, comprises a use of a screening assay or screening tool and associated digital software for comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from said disease-bearing biological sample with open reading frames in nucleic acid sequences extracted from said healthy biological sample, and for identifying mutated nucleic acid sequences within said ORFs of said disease-bearing
20 sample that encode or are comprised within a peptide comprising one or more neo-epitopes. In another embodiment, the associated digital software comprises access to a sequence database that allows screening of said disease-bearing nucleic acid sequences within said ORFs or the corresponding digitally translated amino acid sequence encoding said peptide comprising one or more neo-epitopes for identification of a T-cell epitope or immunogenic
25 potential, or any combination thereof.

[00312] In one embodiment, a step of screening for an immunogenic T-cell response in the system or process of creating a personalized immunotherapy provided comprises use of an immune response assay well known in the art, including for example T-cell proliferation assays, *in vitro* tumor regression assays using T-cells activated with said neo-epitope and co-
30 incubated with tumor cells using a ^{51}Cr-release assay or a ^{3}H-thymidine assay, an ELISA assay, an ELIspot assay, and a FACS analysis. (See for example US Patent No. 8,771,702, which is incorporated herein in its entirety).

[00313] In another embodiment, the bacterial sequence is a *Listerial* sequence, wherein in some embodiments, said *Listeria* sequence is an hly signal sequence or an actA signal sequence.

[00314] In another embodiment, the disease is a localized disease. In another embodiment, the disease is a tumor or cancer. In another embodiment, the tumor or cancer is a solid tumor or cancer. In another embodiment, the tumor or cancer is a liquid tumor or cancer. In another embodiment, an abnormal or unhealthy biological sample comprises a tumor, or a cancer, or a portion thereof.

[00315] In one embodiment, the disease is an infectious disease. In another embodiment, the infectious disease is an infectious viral disease or an infectious bacterial disease. In another embodiment, a neo-epitope identified by the process provided herein is an infectious disease-associated-specific epitope.

[00316] In another embodiment, a neo-epitope comprises a unique tumor or cancer neo-epitope. In another embodiment, a neo-epitope comprises a cancer-specific or tumor-specific epitope. In another embodiment, a neo-epitope is immunogenic. In another embodiment, a neo-epitope is recognized by T-cells. In another embodiment, a peptide comprising one or more neo-epitopes activates a T-cell response against a tumor or cancer, wherein said response is personalized to said subject.

[00317] In another embodiment, a neo-epitope comprises a unique tumor or cancer neo-epitope. In another embodiment, a neo-epitope comprises a unique epitope related to an infectious disease. In one embodiment, the infectious disease epitope directly correlates with the disease. In an alternate embodiment, the infectious disease epitope is associated with the infectious disease.

[00318] In another embodiment, the process provided herein allows the generation of a personalized enhanced anti-disease, or anti-infection, or anti-infectious disease, or anti-tumor immune response in said subject having a disease. In another embodiment, the process provided herein allows personalized treatment or prevention of said disease, or said infection or infectious disease, or said tumor or cancer in a subject. In another embodiment, the process provided herein increases survival time in said subject having said disease, or said infection or infectious disease, or said tumor or cancer.

[00319] In one embodiment, the present invention provides an immunogenic composition comprising a recombinant *Listeria* strain provided herein, and a pharmaceutically acceptable

carrier. In another embodiment, provided herein are one or more immunogenic compositions comprising one or more recombinant *Listeria* strains, wherein each *Listeria* strain expresses one or more different peptides comprising one or more different neo-epitopes. In another embodiment each *Listeria* expresses a range of neo-epitopes. In another embodiment, each peptide comprises one or more neo-epitopes that are T-cell epitopes. In one embodiment, provided herein is a method of eliciting targeted, personalized anti-tumor T cell response in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain provided herein, wherein the *Listeria* strain expresses one or more neo-epitopes. In another embodiment, a *Listeria* strain comprises one of the following: a nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises an immunogenic polypeptide or fragment thereof fused to a peptide comprising one or more neo-epitopes associated with cancer disease; or, a minigene nucleic acid construct comprising a first open reading frame encoding a chimeric protein, wherein said chimeric protein comprises a *Listerial* secretion signal sequence, an ubiquitin (Ub) protein, and one or more peptides each comprising one or more neo-epitopes associated with a tumor or a cancer, wherein said signal sequence, said ubiquitin and said one or more peptides are respectively arranged in tandem, or are operatively linked, from the amino terminus to the carboxy terminus.

[00320] In another embodiment, the fusion peptides are further linked to a HIS tag or a SIINFEKL tag. In another embodiment, the tag sequence comprises a C-terminal SIINFEKL and 6 His amino acids. In another embodiment, the tag sequence is an amino acid or nucleic acid sequence that allows for easy detection of the neo-epitope. In another embodiment, the tag sequence is an amino acid or nucleic acid sequence that for confirmation of secretion of a neo-epitope disclosed herein. It will be appreciated by a skilled artisan that the sequences for the tags may be incorporated into the fusion peptide sequences on the plasmid or phage vector. These tags may be expressed and the antigenic epitopes presented allowing a clinician to follow the immunogenicity of the secreted peptide by following immune responses to these “tag” sequence peptides. Such immune response can be monitored using a number of reagents including but not limited to, monoclonal antibodies and DNA or RNA probes specific for these tags.

[00321] In another embodiment, a method of this invention is increasing the ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, wherein said T

effector cells are targeted to a neo-epitope present within abnormal or unhealthy tissue of a subject, for example a tumor tissue or a cancer, the method comprising the step of administering to the subject an immunogenic composition comprising a recombinant *Listeria* strain provided herein.

[00322] In another embodiment, a method of this invention is for increasing antigen-specific T-cells in a subject, wherein said antigen or a peptide fragment thereof comprises one or more neo-epitopes, the method comprising the step of administering to the subject an immunogenic composition comprising a recombinant *Listeria* strain provided herein.

[00323] In another embodiment, a method of this invention is for increasing survival time of a subject having a tumor or suffering from cancer, or suffering from an infectious disease, the method comprising the step of administering to the subject an immunogenic composition comprising a recombinant *Listeria* strain provided herein.

[00324] In another embodiment, a method of this invention is treating a tumor or a cancer or an infection or an infectious disease in a subject, the method comprising the step of administering to the subject an immunogenic composition comprising a recombinant *Listeria* strain provided herein.

I. Personalizing immunotherapy

[00325] In one embodiment, a process of this invention creates a personalized immunotherapy. In another embodiment, a process of creating a personalized immunotherapy for a subject having a disease or condition comprises identifying and selecting neo-epitopes within mutated and variant antigens (neo-antigens) that are specific to said patient's disease. In another embodiment, a process for creating a personalized immunotherapy for a subject is in order to provide a treatment for said subject. In another embodiment, personalized immunotherapy may be used to treat such diseases as cancer, autoimmune disease, organ transplantation rejection, bacterial infection, viral infection, and chronic viral illnesses such as HIV.

[00326] A step in a process of creating a personalized immunotherapy is, in one embodiment, to obtain an abnormal or unhealthy biological sample, from a subject having a disease or condition. As used herein, the term "abnormal or unhealthy biological sample" is used interchangeably with "disease-bearing biological sample" or "disease-bearing sample" having all the same meanings and qualities. In one embodiment, a biological sample is a tissue, cells, blood, any sample obtained from a subject that comprises lymphocytes, any

sample obtained from a subject that comprises disease-bearing cells, or any sample obtained from a subject that is healthy but is also comparable to a disease-bearing sample that is obtained from the same subject or similar individual.

[00327] In one embodiment, an abnormal or unhealthy biological sample comprises a tumor tissue or a cancer tissue or a portion thereof. In another embodiment, a tumor or cancer may be a solid tumor. In another embodiment, a tumor or cancer is not a solid tumor or cancer, for example a blood cancer or a breast cancer wherein a tumor does not form.

[00328] In another embodiment, a tumor sample relates to any sample such as a bodily sample derived from a patient containing or being expected of containing tumor or cancer cells. The bodily sample may be any tissue sample such as blood, a tissue sample obtained from the primary tumor or from tumor metastases or any other sample containing tumor or cancer cells. In yet another embodiment, a bodily sample is blood, cells from saliva, or cells from cerebrospinal fluid. In another embodiment, a tumor sample relates to one or more isolated tumor or cancer cells such as circulating tumor cells (CTCs) or a sample containing one or more isolated tumor or cancer cells such as circulating tumor cells (CTCs). In another embodiment, a tumor or a cancer comprises a breast cancer or tumor. In another embodiment, a tumor or a cancer comprises is a cervical cancer or tumor. In another embodiment, a tumor or a cancer comprises a Her2 containing tumor or cancer. In another embodiment, a tumor or a cancer comprises melanoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a pancreatic tumor or cancer. In another embodiment, a tumor or a cancer comprises an ovarian tumor or cancer. In another embodiment, a tumor or a cancer comprises a gastric tumor or cancer. In another embodiment, a tumor or a cancer comprises a carcinomatous lesion of the pancreas. In another embodiment, a tumor or a cancer comprises a pulmonary adenocarcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a glioblastoma multiforme tumor or cancer. In another embodiment, a tumor or a cancer comprises a colorectal adenocarcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a pulmonary squamous adenocarcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a gastric adenocarcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises an ovarian surface epithelial neoplasm (e.g. a benign, proliferative or malignant variety thereof) tumor or cancer. In another embodiment, a tumor or a cancer comprises an oral squamous cell carcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a non-small-cell lung carcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises an

endometrial carcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a bladder tumor or cancer. In another embodiment, a tumor or a cancer comprises a head and neck tumor or cancer. In another embodiment, a tumor or a cancer comprises a prostate carcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises a gastric adenocarcinoma tumor or cancer. In another embodiment, a tumor or a cancer comprises an oropharyngeal tumor or cancer. In another embodiment, a tumor or a cancer comprises a lung tumor or cancer. In another embodiment, a tumor or a cancer comprises an anal tumor or cancer. In another embodiment, a tumor or a cancer comprises a colorectal tumor or cancer. In another embodiment, a tumor or a cancer comprises an esophageal tumor or cancer. In another embodiment, a tumor or a cancer comprises a mesothelioma tumor or cancer.

[00329] In another embodiment, an abnormal or unhealthy biological sample comprises non-tumor or cancerous tissue. In another embodiment, an abnormal or unhealthy biological sample comprises cells isolated from a blood sample, cells from saliva, or cells from cerebral spinal fluid. In another embodiment, an abnormal or unhealthy biological sample comprises a sample of any tissue or portion thereof that is considered abnormal or unhealthy.

[00330] In one embodiment, other non-tumor or non-cancerous diseases, including infectious diseases from which a disease-bearing biological sample can be obtained for analysis according to the process provided herein, are encompassed by the present invention. In another embodiment, an infectious disease comprises a viral infection. In another embodiment, an infectious disease comprises a chronic viral infection. In another embodiment, an infectious disease comprises a chronic viral illness such as HIV. In another embodiment, an infectious disease comprises a bacterial infection. In another embodiment, the infectious disease is a parasitic infection.

[00331] In one embodiment, the infectious disease is one caused by, but not limited to, any one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral

hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola , Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic *E.coli,* Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Trichomonas vaginalis, or any other infectious disease known in the art that is not listed herein.

[00332] In one embodiment, pathogenic protozoans and helminths infections include*:* amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; pneumocystis carinii; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

[00333] In another embodiment, the infectious disease is a livestock infectious disease. In another embodiment, livestock diseases can be transmitted to man and are called “zoonotic diseases.” In another embodiment, these diseases include, but are not limited to, Foot and mouth disease, West Nile Virus, rabies, canine parvovirus, feline leukemia virus, equine influenza virus, infectious bovine rhinotracheitis (IBR), pseudorabies, classical swine fever (CSF), IBR, caused by bovine herpesvirus type 1 (BHV-1) infection of cattle, and pseudorabies (Aujeszky's disease) in pigs, toxoplasmosis, anthrax, vesicular stomatitis virus, rhodococcus equi, Tularemia, Plague (Yersinia pestis), trichomonas.

[00334] In one embodiment, other non-tumor or non-cancerous diseases, including

autoimmune diseases from which a disease-bearing biological sample can be obtained for analysis according to the process provided herein, are encompassed by the present invention. It will be appreciated by the skilled artisan that the term “autoimmune disease” refers to a disease or condition arising from immune reactions directed against an individual's own tissues, organs or manifestation thereof or resulting condition therefrom. As used herein the term "autoimmune disease" includes cancers and other disease states where the antibodies that are directed towards self-tissues are not necessarily involved in the disease condition but are still important in diagnostics. Further, in one embodiment, it refers to a condition that results from, or is aggravated by, the production of autoantibodies by B cells of antibodies that are reactive with normal body tissues and antigens. In other embodiments, the autoimmune disease is one that involves secretion of an autoantibody that is specific for an epitope from a self-antigen (e.g. a nuclear antigen).

[00335] In an effort to treat a subject having an autoimmune disease, in one embodiment, this invention comprises systems and methods to identify auto-reactive neo-epitopes, wherein said system or process comprises methods to immunize a subject having an autoimmune disease against these auto-reactive neo-epitopes, in order to induce tolerance mediated by antibodies or immunosuppressor cells, for examples Tregs or MDSCs.

[00336] In one embodiment, an autoimmune disease comprises a systemic autoimmune disease. The term "systemic autoimmune disease" refers to a disease, disorder or a combination of symptoms caused by autoimmune reactions affecting more than one organ. In another embodiment, a systemic autoimmune disease includes, but is not limited to, Anti-GBM nephritis (Goodpasture's disease), Granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MP A), systemic lupus erythematosus (SLE), polymyositis (PM) or Celiac disease.

[00337] In one embodiment, an autoimmune disease comprises a connective tissue disease. The term "connective tissue disease" refers to a disease, condition or a combination of symptoms caused by autoimmune reactions affecting the connective tissue of the body. In another embodiment, a connective tissue disease includes, but is not limited to, systemic lupus erythematosus (SLE), polymyositis (PM), systemic sclerosis or mixed connective tissue disease (MCTD).

[00338] In one embodiment, other non-tumor or non-cancerous diseases, including organ transplantation rejection from which a disease-bearing biological sample can be obtained for

analysis according to the process provided herein, are encompassed by the present invention. In another embodiment, the rejected organ is a solid organ, including but not limited to a heart, a lung, a kidney, a liver, pancreas, intestine, stomach, testis, cornea, skin, heart valve, a blood vessel, or bone. In another embodiment, the rejected organs include but are not limited to a blood tissue, bone marrow, or islets of Langerhans cells.

[00339] In an effort to treat a transplant subject having a rejection of the transplanted organ or is experiencing graft v. host disease (GVhD), in one embodiment, this invention comprises systems and methods to identify auto-reactive neo-epitopes, wherein said system or process comprises methods to immunize a subject having an autoimmune disease against these auto-reactive neo-epitopes, in order to induce tolerance mediated by antibodies or immunosuppressor cells, for examples Tregs or MDSCs.

[00340] Samples may be obtained using routine biopsy procedures well known in the art. Biopsies may comprise the removal of cells or tissues from a subject by skilled medical personnel, for example a pathologist. There are many different types of biopsy procedures. The most common types include: (1) incisional biopsy, in which only a sample of tissue is removed; (2) excisional biopsy, in which an entire lump or suspicious area is removed; and (3) needle biopsy, in which a sample of tissue or fluid is removed with a needle. When a wide needle is used, the procedure is called a core biopsy. When a thin needle is used, the procedure is called a fine-needle aspiration biopsy.

[00341] In one embodiment, a sample of this invention is obtained by incisional biopsy. In another embodiment a sample is obtained by an excisional biopsy. In another embodiment, a sample is obtained using a needle biopsy. In another embodiment, a needle biopsy is a core biopsy. In another embodiment, a biopsy is a fine-needle aspiration biopsy. In another embodiment, a sample is obtained from as part of a blood sample. In another embodiment, a sample is obtained as part of a cheek swab. In another embodiment, a sample is obtained as part of a saliva sampling. In another embodiment, a biological sample comprises all or part of a tissue biopsy. In another embodiment, a tissue biopsy is taken and cells from that tissue sample are collected, wherein the cells comprise a biological sample of this invention. In another embodiment, a sample of this invention is obtained as part of a cell biopsy. In another embodiment, multiple biopsies may be taken from the same subject. In another embodiment, biopsies from the same subject may be collected from the same tissue or cells. In another embodiment, biopsies from the same subject may be collected from a different tissue of cell source within the subject.

[00342] In one embodiment, a biopsy comprises a bone marrow tissue. In another embodiment, a biopsy comprises a blood sample. In another embodiment, a biopsy comprises a biopsy of gastrointestinal tissue, for example esophagus, stomach, duodenum, rectum, colon and terminal ileum. In another embodiment, a biopsy comprises lung tissue. In another embodiment, a biopsy comprises prostate tissue. In another embodiment, a biopsy comprises liver tissue. In another embodiment, a biopsy comprises nervous system tissue, for example a brain biopsy, a nerve biopsy, or a meningeal biopsy. In another embodiment, a biopsy comprises urogenital tissue, for example a renal biopsy, an endometrial biopsy or a cervical conization. In another embodiment, a biopsy comprises a breast biopsy. In another embodiment, a biopsy comprises a lymph node biopsy. In another embodiment, a biopsy comprises a muscle biopsy. In yet another embodiment, a biopsy comprises a skin biopsy. In another embodiment, a biopsy comprises a bone biopsy. In another embodiment, a disease-bearing sample pathology of each sample is examined to confirm a diagnosis of the diseased tissue. In another embodiment, a healthy sample is examined to confirm a diagnosis of the health tissue.

[00343] In one embodiment, normal or a healthy biological sample is obtained from the subject. In another embodiment, the normal or healthy biological sample is a non-tumor sample which relates to any sample such as a bodily sample derived from a subject. The sample may be any tissue sample such as healthy cells obtained from a biological sample provided herein. In another embodiment, the normal or healthy biological sample is obtained from another individual which in one embodiment, is a related individual. In another embodiment, another individual is of the same species as the subject. In another embodiment, another individual is a healthy individual not containing or not being expected of containing a disease-bearing biological sample. In another embodiment, another individual is a healthy individual not containing or not being expected of containing tumor or cancer cells. It will be appreciated by a skilled artisan that the healthy individual may be screened using methods known in the art for the presence of a disease in order to determine that he or she is healthy. A disease-bearing biological sample and a healthy biological sample can both be obtained from the same tissue (e.g., a tissue section containing both tumor tissue and surrounding normal tissue). Preferably, healthy biological samples consist essentially or entirely of normal, healthy cells and can be used in comparison to a disease-bearing biological sample (e.g., a sample thought to comprise cancer cells or a particular type of cancer cells). Preferably, the samples are of the same type (e.g., both blood or both sera). For example, if

the disease-bearing biological sample comprises cells, preferably the cells in the healthy biological sample have the same tissue origin as the disease-bearing cells in the disease-bearing biological sample (e.g., lung or brain) and arise from the same cell type (e.g., neuronal, epithelial, mesenchymal, hematopoietic).

5 [00344] In another embodiment, the normal or healthy biological sample is obtained at the same time. The terms “normal or healthy biological sample” and “reference sample” or “reference tissue” are used interchangeably throughout, having all the same meanings and qualities. In another embodiment, a "reference" may be used to correlate and compare the results obtained in from a tumor specimen. In another embodiment, a "reference" can be
10 determined empirically by testing a sufficiently large number of normal specimens from the same species. In another embodiment, the normal or healthy biological sample is obtained at a different time, wherein the time may be such that the normal of healthy sample is obtained prior to obtaining the abnormal or healthy sample or afterwards. Methods of obtaining comprise those used routinely in the art for biopsy or blood collection. In another
15 embodiment, a sample is a frozen sample. In another embodiment, a sample is comprised as a tissues paraffin embedded (FFPE) tissue block.

[00345] In one embodiment, following obtaining said normal or healthy biological sample, said sample is processed for extracting nucleic acids using techniques and methodologies well known in the art. In another embodiment, nucleic acids extracted comprise DNA. In another
20 embodiment, nucleic acids extracted comprise RNA. In another embodiment, RNA is mRNA. In another embodiment, a next generation sequencing (NGS) library is prepared. Next-generation sequencing libraries may be constructed and may undergo exome or targeted gene capture. In another embodiment, a cDNA expression library is made using techniques known in the art, for example see US20140141992, which is hereby incorporated in full.

25 [00346] A process of this invention for creating a personalized immunotherapy may comprise use of the extracted nucleic acid from the abnormal or unhealthy sample and the extracted nucleic acid from the normal or healthy reference sample in order to identify somatic mutations or sequence differences present in the abnormal or unhealthy sample as compared with the normal or healthy sample, wherein these sequence having somatic
30 mutations or differences encode an expressed amino acid sequence. In one embodiment, a peptide expressing said somatic mutations or sequence differences, may in certain embodiments, be referred to throughout as “neo-epitopes.”

[00347] It will be appreciated by a skilled artisan that the term "neo-epitope" may also refer to an epitope that is not present in a reference sample, such as a normal non-cancerous or germline cell or tissue but is found in disease-bearing tissues, for example in a cancer cell. This includes, in another embodiment, situations wherein in a normal non-cancerous or germline cell a corresponding epitope is found, however, due to one or more mutations in a cancer cell the sequence of the epitope is changed so as to result in the neo-epitope. In another embodiment, a neo-epitope comprises a mutated epitope. In another embodiment, a neo-epitope has non-mutated sequence on either side of the epitope. In one embodiment, a neo-epitope is a linear epitope. In another embodiment, a neo-epitope is considered solvent-exposed and therefore accessible to T-cell antigen receptors.

[00348] In another embodiment, one or more peptides provided herein do not comprise one or more immunosuppressive T-regulatory neo-epitopes. In another embodiment, a neo-epitope identified and used by the methods provided herein does not comprise an immunosuppressive epitope. In another embodiment, a neo-epitope identified and used by the methods provided herein does not activate T-regulatory (T-reg) cells.

[00349] In another embodiment, a neo-epitope is immunogenic. In another embodiment, a neo-epitope comprises a T-cell epitope. In another embodiment, a neo-epitope comprises an adaptive immune response epitope.

[00350] In another embodiment, a neo-epitope comprises a single mutation. In another embodiment, a neo-epitope comprises at least 2 mutations. In another embodiment, a neo-epitope comprises at least 2 mutations. In another embodiment, a neo-epitope comprises at least 3 mutations. In another embodiment, a neo-epitope comprises at least 4 mutations. In another embodiment, a neo-epitope comprises at least 5 mutations. In another embodiment, a neo-epitope comprises at least 6 mutations. In another embodiment, a neo-epitope comprises at least 7 mutations. In another embodiment, a neo-epitope comprises at least 8 mutations. In another embodiment, a neo-epitope comprises at least 9 mutations. In another embodiment, a neo-epitope comprises at least 10 mutations. In another embodiment, a neo-epitope comprises at least 20 mutations. In another embodiment, a neo-epitope comprises 1-10, 11-20, 20-30, and 31-40 mutations.

[00351] In another embodiment, a neo-epitope is associated with said disease or condition of said subject. In another embodiment, a neo-epitope is causative of said disease or condition of said subject. In another embodiment, a neo-epitope is present within said disease bearing

biological sample. In another embodiment, a neo-epitope is present within said disease bearing biological tissue but is not causative or associated with said disease or condition.

[00352] In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises one neo-epitope. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises two neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 3 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 4 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 5 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 6 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 7 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 8 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 9 neo-epitopes. In another embodiment, a peptide, a polypeptide or a fusion peptide of this invention comprises 10 or more neo-epitopes.

[00353] In one embodiment, a step towards identifying neo-epitopes comprises sequencing the extracted nucleic acids obtained from the abnormal or unhealthy biological sample and sequencing the extracted nucleic acids obtained from the normal or healthy biological reference sample. In another embodiment, the entire genome is sequenced. In another embodiment, the exome is sequenced. In yet another embodiment, the transcriptome is sequenced. In another embodiment, a neo-epitope is identified using T-cell receptor sequencing.

[00354] In another embodiment, a neo-epitope comprises a neo-epitope known in the art, a disclosed in Pavlenko M, Leder C, Roos AK, Levitsky V, Pisa P. (2005) Identification of an immunodominant H-2D(b)-restricted CTL epitope of human PSA. Prostate. 15;64(1):50-9 (PSA neo-epitope); Maciag PC, Seavey MM, Pan ZK, Ferrone S, Paterson Y. (2008) Cancer immunotherapy targeting the high molecular weight melanoma-associated antigen protein results in a broad antitumor response and reduction of pericytes in the tumor vasculature. Cancer Res. 1;68(19):8066-75 (HMW-MAA epitope in HLA-A2 mice); Zhang KQ, Yang F, Ye J, Jiang M, Liu Y, Jin FS, Wu YZ. (2012) A novel DNA/peptide combined immunotherapy induces PSCA-specific cytotoxic T-lymphocyte responses and suppresses tumor growth in experimental prostate cancer. Urology.;79(6):1410.e7-13. doi: 10.1016/j.urology.2012.02.011. Epub 2012 Apr 17 (HLA-A2 epitope PSCA); Kouiavskaia

DV, Berard CA, Datena E, Hussain A, Dawson N, Klyushnenkova EN, Alexander RB. (2009)Vaccination with agonist peptide PSA: 154-163 (155L) derived from prostate specific antigen induced CD8 T-cell response to the native peptide PSA: 154-163 but failed to induce the reactivity against tumor targets expressing PSA: a phase 2 study in patients with recurrent prostate cancer .J Immunother.;32(6):655-66 (HLA-A2 epitope PSA).

[00355] The term "genome" relates to the total amount of genetic information in the chromosomes of an organism. The term "exome" refers to the coding regions of a genome. The term "transcriptome" relates to the set of all RNA molecules.

[00356] A nucleic acid is according to one embodiment, deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), more preferably RNA, most preferably *in vitro* transcribed RNA (Γv RNA) or synthetic RNA. Nucleic acids include according to the invention genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. In another embodiment, a nucleic acid may be present as a single-stranded or double- stranded and linear or covalently circularly closed molecule. A nucleic acid may, in another embodiment, be isolated. The term "isolated nucleic acid" means, according to the invention, that the nucleic acid (i) was amplified *in vitro*, for example via polymerase chain reaction (PCR), (ii) was produced recombinantly by cloning, (iii) was purified, for example, by cleavage and separation by gel electrophoresis, or (iv) was synthesized, for example, by chemical synthesis. A nucleic can be employed for introduction into, i.e. transfection of, cells, in particular, in the form of RNA which can be prepared by *in vitro* transcription from a DNA template. The RNA can moreover be modified before application by stabilizing sequences, capping, and polyadenylation.

[00357] It would be understood by a skilled artisan that the term "mutation" may encompass a change of or difference in the nucleic acid sequence (nucleotide substitution, addition or deletion, early termination or stop) compared to a reference sequence. For example a change or difference present in the abnormal sample not found in the normal sample. A "somatic mutation" can occur in any of the cells of the body except the germ cells (sperm and egg) and therefore are not passed on to children. These alterations can (but do not always) cause cancer or other diseases. In one embodiment, a mutation is a non-synonymous mutation. The term "non-synonymous mutation" refers to a mutation, preferably a nucleotide substitution, which does result in an amino acid change such as an amino acid substitution in the translation product.

[00358] In the case of an abnormal sample being a tumor or cancer tissue, in one embodiment, a mutation may comprise a “cancer mutation signature.” The term "cancer mutation signature" refers to a set of mutations which are present in cancer cells when compared to non-cancerous reference cells. Included are pre-cancerous or dysplastic tissue, and somatic mutations of same.

[00359] Digital karyotyping is a technique used to analyze chromosomes in order to look for any major chromosomal anomaly which may cause a genetic condition. In one embodiment, digital karyotyping may be used to focus on regions of a chromosome for sequencing and comparative analysis. In another embodiment, digital karyotyping is performed virtually analyzing short sequences of DNA from specific loci all over the genome, which are isolated and enumerated.

[00360] Any suitable sequencing method can be used according to the invention. In one embodiment, next Generation Sequencing (NGS) technologies is used. Third Generation Sequencing methods might substitute for the NGS technology in the future to speed up the sequencing step of the method. For clarification purposes: the terms "Next Generation Sequencing" or "NGS" in the context of the present invention mean all novel high throughput sequencing technologies which, in contrast to the "conventional" sequencing methodology known as Sanger chemistry, read nucleic acid templates randomly in parallel along the entire genome by breaking the entire genome into small pieces. Such NGS technologies (also known as massively parallel sequencing technologies) are able to deliver nucleic acid sequence information of a whole genome, exome, transcriptome (all transcribed sequences of a genome) or methylome (all methylated sequences of a genome) in very short time periods, e.g. within 1-2 weeks, preferably within 1-7 days or most preferably within less than 24 hours and allow, in principle, single cell sequencing approaches. Multiple NGS platforms which are commercially available or which are mentioned in the literature can be used in the context of the present invention e.g. those described in detail in Zhang et al. 2011: The impact of next-generation sequencing on genomics. J. Genet Genomics 38 (3), 95-109; or in Voelkerding et al. 2009: Next generation sequencing: From basic research to diagnostics. Clinical chemistry 55, 641-658. Non-limiting examples of such NGS technologies/platforms include:

[00361] 1) The sequencing-by-synthesis technology known as pyrosequencing implemented e.g. in the GS-FLX 454 Genome Sequencer™ of Roche-associated company 454 Life Sciences (Branford, Connecticut), first described in Ronaghi et al. 1998: A sequencing method based on real-time pyrophosphate". Science 281 (5375), 363-365. This technology

uses an emulsion PCR in which single-stranded DNA binding beads are encapsulated by vigorous vortexing into aqueous micelles containing PCR reactants surrounded by oil for emulsion PCR amplification. During the pyrosequencing process, light emitted from phosphate molecules during nucleotide incorporation is recorded as the polymerase synthesizes the DNA strand.

[00362] 2) The sequencing-by-synthesis approaches developed by Solexa (now part of Illumina Inc., San Diego, California) which is based on reversible dye-terminators and implemented e.g. in the Illumina Solexa Genome Analyzer™ and in the Illumina HiSeq 2000 Genome Analyzer™. In this technology, all four nucleotides are added simultaneously into oligo-primed cluster fragments in flow-cell channels along with DNA polymerase. Bridge amplification extends cluster strands with all four fluorescently labeled nucleotides for sequencing.

[00363] 3) Sequencing-by-ligation approaches, e.g. implemented in the SOLid™ platform of Applied Biosystems (now Life Technologies Corporation, Carlsbad, California). In this technology, a pool of all possible oligonucleotides of a fixed length are labeled according to the sequenced position. Oligonucleotides are annealed and ligated; the preferential ligation by DNA ligase for matching sequences results in a signal informative of the nucleotide at that position. Before sequencing, the DNA is amplified by emulsion PCR. The resulting bead, each containing only copies of the same DNA molecule, are deposited on a glass slide. As a second example, the Polonator™ G.007 platform of Dover Systems (Salem, New Hampshire) also employs a sequencing-by-ligation approach by using a randomly arrayed, bead -based, emulsion PCR to amplify DNA fragments for parallel sequencing.

[00364] 4) Single-molecule sequencing technologies such as e.g. implemented in the PacBio RS system of Pacific Biosciences (Menlo Park, California) or in the HeliScope™ platform of Helicos Biosciences (Cambridge, Massachusetts). The distinct characteristic of this technology is its ability to sequence single DNA or RNA molecules without amplification, defined as Single-Molecule Real Time (SMRT) DNA sequencing. For example, HeliScope uses a highly sensitive fluorescence detection system to directly detect each nucleotide as it is synthesized. A similar approach based on fluorescence resonance energy transfer (FRET) has been developed from Visigen Biotechnology (Houston, Texas). Other fluorescence-based single-molecule techniques are from U.S. Genomics (GeneEngine™) and Genovoxx (AnyGene™).

[00365] 5) Nano-technologies for single-molecule sequencing in which various nano structures are used which are, e.g., arranged on a chip to monitor the movement of a polymerase molecule on a single strand during replication. Non-limiting examples for approaches based on nano-technologies are the GridON™ platform of Oxford Nanopore Technologies (Oxford, UK), the hybridization-assisted nano-pore sequencing (HANS™) platforms developed by Nabsys (Providence, Rhode Island), and the proprietary ligase-based DNA sequencing platform with DNA nanoball (DNB) technology called combinatorial probe-anchor ligation (cPAL™).

[00366] 6) Electron microscopy based technologies for single-molecule sequencing, e.g. those developed by LightSpeed Genomics (Sunnyvale, California) and Halcyon Molecular (Redwood City, California)

[00367] 7) Ion semiconductor sequencing which is based on the detection of hydrogen ions that are released during the polymerisation of DNA. For example, Ion Torrent Systems (San Francisco, California) uses a high-density array of micro-machined wells to perform this biochemical process in a massively parallel way. Each well holds a different DNA template. Beneath the wells is an ion-sensitive layer and beneath that a proprietary Ion sensor.

[00368] In some embodiments, DNA and RNA preparations serve as starting material for NGS. Such nucleic acids can be easily obtained from samples such as biological material, e.g. from fresh, flash-frozen or formalin-fixed paraffin embedded tumor tissues (FFPE) or from freshly isolated cells or from CTCs which are present in the peripheral blood of patients. Normal non-mutated genomic DNA or RNA can be extracted from normal, somatic tissue, however germline cells are preferred in the context of the present invention. Germline DNA or RNA is extracted from peripheral blood mononuclear cells (PBMCs) in patients with non-hematological malignancies. Although nucleic acids extracted from FFPE tissues or freshly isolated single cells are highly fragmented, they are suitable for NGS applications.

[00369] Several targeted NGS methods for exome sequencing are described in the literature (for review see e.g. Teer and Mullikin 2010: Human Mol Genet 19 (2), R145-51), all of which can be used in conjunction with the present invention. Many of these methods (described e.g. as genome capture, genome partitioning, genome enrichment etc.) use hybridization techniques and include array-based (e.g. Hodges et al. 2007: Nat. Genet. 39, 1522-1527) and liquid-based (e.g. Choi et al. 2009: Proc. Natl. Acad. Sci USA 106, 19096-19101) hybridization approaches. Commercial kits for DNA sample preparation and

subsequent exome capture are also available: for example, Illumina Inc. (San Diego, California) offers the TruSeq™ DNA Sample Preparation Kit and the Exome Enrichment Kit TruSeq™ Exome Enrichment Kit.

[00370] In the context of the present invention, the term "RNA" relates to a molecule which comprises at least one ribonucleotide residue and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β-D-ribofuranosyl group. The term "RNA" comprises double-stranded RNA, single-stranded RNA, isolated RNA such as partially or completely purified RNA, essentially pure RNA, synthetic RNA, and recombinantly generated RNA such as modified RNA which differs from naturally occurring RNA by addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

[00371] According to the present invention, the term "RNA" includes and preferably relates to "mRNA". The term "mRNA" means "messenger- RNA" and relates to a "transcript" which is generated by using a DNA template and encodes a peptide or polypeptide. Typically, an mRNA comprises a 5'-UTR, a protein coding region, and a 3'-UTR. mRNA only possesses limited half-life in cells and in vitro. In the context of the present invention, mRNA may be generated by in vitro transcription from a DNA template. The in vitro transcription methodology is known to the skilled person. For example, there is a variety of in vitro transcription kits commercially available.

[00372] In one embodiment, DNA and RNA from a biological sample (disease and/or normal) obtained from human tissue (or any non-human animal) are extracted in triplicates. In another embodiment, the disease sample is a tumor sample and said sample provides the source of neo-antigens/neo-epitopes. In another embodiment, a source of neo-antigens is from sequencing metastases or circulating tumor cells. It will be appreciated by a skilled artisan that these may contain additional mutations that are not resident in the initial biopsy but could be included in the vector to specifically target cytotoxic T cells (CTC's) or metastases that have mutated differently than the primary biopsy that was sequenced.

[00373] In one embodiment, triplicates of each sample obtained according to the disclosure herein are sequenced by DNA exome sequencing. Following a whole exome sequencing a VCF file output data or other suitable file is obtained and is presented in the FASTA format or any other suitable format known in the art. In one embodiment, the term “VCF” or Variant Call Format is a file format used by the 1000 Genomes project to encode SNPs and other structural genetic variants. The format is further described on the 1000 Genomes project Web site (www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/VCF%20%28Variant%20Call%20Format%29%20version%204.0/encoding-structural-variants). VCF calls are available at EBI / NCBI. In one embodiment, the presentation places the non-synonymous mutation in the center and shows 10-15 amino acids on either side of the mutation encoded amino acid. Frame shift mutations will display the entire sequence of the mutated peptide that is encoded until a stop codon with the surrounding 10-15 amino acids. In one embodiment, extracting the relevant information from a VCF or other suitable file and putting it in FASTA or other suitable format allows for direct input of the 21mer neo-epitope sequences into both hydropathy testing and MHC binding affinity scripts.

[00374] In one embodiment, the hydrophobicity is scaled using the Kyte-Doolittle (Kyte J, Doolittle RF (May 1982). "A simple method for displaying the hydropathic character of a protein". J. Mol. Biol. 157 (1): 105–32) or other suitable hydropathy plot or other appropriate scale including, but not limited those disclosed by Rose et.al (Rose, G.D. and Wolfenden, R. (1993) Annu. Rev. Biomol. Struct., 22, 381–415.); Kallol M. Biswas, Daniel R. DeVido, John G. Dorsey(2003) Journal of Chromatography A,1000, 637–655, Eisenberg D (July 1984). Ann. Rev. Biochem. 53: 595–623.); Abraham D.J., Leo A.J. Proteins: Structure, Function and Genetics 2:130-152(1987); Sweet R.M., Eisenberg D. J. Mol. Biol. 171:479-488(1983); Bull H.B., Breese K. Arch. Biochem. Biophys. 161:665-670(1974); Guy H.R. Biophys J. 47:61-70(1985); Miyazawa S., et al., Macromolecules 18:534-552(1985); Roseman M.A. J. Mol. Biol. 200:513-522(1988); Wolfenden R.V., et al. Biochemistry 20:849-855(1981); Wilson K.J; Biochem. J. 199:31-41(1981); Cowan R., Whittaker R.G. Peptide Research 3:75-80(1990); Aboderin A.A. Int. J. Biochem. 2:537-544(1971); Eisenberg D. et al., J. Mol. Biol. 179:125-142(1984); Hopp T.P., Woods K.R. Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828(1981); Manavalan P., Ponnuswamy P.K. Nature 275:673-674(1978).; Black S.D., Mould D.R. Anal. Biochem. 193:72-82(1991); Fauchere J.-L., Pliska V.E. Eur. J. Med. Chem. 18:369-375(1983); Janin J. Nature 277:491-492(1979); Rao M.J.K., Argos P.

Biochim. Biophys. Acta 869:197-214(1986); Tanford C. J. Am. Chem. Soc. 84:4240-4274(1962); Welling G.W., et al., FEBS Lett. 188:215-218(1985); Parker J.M.R. et al., Biochemistry 25:5425-5431(1986); Cowan R., Whittaker R.G. Peptide Research 3:75-80(1990), all of which are incorporated by reference herein in their entirety. In another embodiment, all epitopes scoring on the scale-appropriate measure to have an unsatisfactorily high level of hydrophobicity to be efficiently secreted are moved from the listing or are de-selected. In another embodiment, all epitopes scoring on the Kyte-Doolittle plot to have an unsatisfactorily high level of hydrophobicity to be efficiently secreted, such as 1.6 or above, are moved from the listing or are de-selected. In another embodiment, each neo-antigen's ability to bind to subject HLA is rated using the Immune Epitope Database (IEDB) analysis resource which includes: netMHCpan, ANN, SMMPMBEC. SMM, CombLib_Sidney2008, PickPocket, netMHCcons. Other sources include TEpredict (tepredict.sourceforge.net/help.html) or alternative MHC binding measurement scales available in the art.

[00375] In another embodiment, disclosed herein is a system for creating personalized immunotherapy for a subject, comprising: at least one processor; and at least one storage medium containing program instructions for execution by said processor, said program instructions causing said processor to execute steps comprising:

a. Receiving output data containing all neo-antigens/neo-epitopes and the human leukocyte antigen (HLA) type of the subject;
b. Scoring the hydrophobicity of each epitope and removing epitopes that score above a certain threshold;
c. Numerically rate the remaining neo-antigens based its ability to bind to subject HLA and on its predictive MHC binding score;
d. Inserting the amino acid sequence of each epitope into a plasmid;
e. Scoring the hydrophobicity of each construct and removing any constructs that score above a certain threshold;
f. Translating the amino acid sequence of each construct into the corresponding DNA sequence, starting with the highest scored construct;
g. Inserting additional epitopes into the plasmid construct in order of ranking until a predetermined upper limit is reached;
h. Adding a DNA sequence tag to the end of the construct in order to measure the immunotheraputic response in a subject; and

i. Optimizing the epitope and DNA sequence tag for expression and secretion in *Listeria monocytogenes*.

[00376] In one embodiment, once a neo-epitope is identified, the neo-epitope is scored by the Kyte and Doolittle hydropathy index 21 amino acid window, wherein in another embodiment, neo-epitopes scoring above a specific cutoff (around 1.6) are excluded as they are unlikely to be secretable by *Listeria monocytogenes*. In another embodiment, the cut off is selected from the following ranges 1.2-1.4, 1.4-1.6, 1.6-1.8, 1.8-2.0, 2.0-2.2 2.2-2.5, 2.5-3.0, 3.0-3.5, 3.5-4.0, or 4.0-4.5. In one embodiment, embodiment the cutoff score used to determine what epitopes are moved from the list or are de-selected is 1.6. In another embodiment, the cutoff is 1.4, 1.5, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.3, 2.5, 2.6, 2.7, 2.8, 2.9, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, or 4.5. In another embodiment, the cut off varies depending on the genus of the delivery vector being used. In another embodiment, the cut off varies depending on the species of the delivery vector being used.

[00377] In one embodiment, the neo-epitope is scored by the Kyte and Doolittle hydropathy index 21 amino acid sliding window. In another embodiment, the sliding window size is selected from the group comprising 9, 11, 13, 15, 17, 19, and 21 amino acids. In another embodiment, the sliding window size is 9-11 amino acids, 11-13 amino acids, 13-15 amino acids, 15-17 amino acids, 17-19 amino acids or 19-21 amino acids.

[00378] In another embodiment, wherein the DNA sequence tag of step h. is SIINFEKL-6xHis or a substitute tag sequence available in the art. In another embodiment, neo-antigens known to have immunosuppressive properties are removed from consideration before step a. above. In one embodiment, these immunosuppressive epitopes are as presented in the sequence or are artificially created as a result of the splicing together of epitope sequences and linkers.

[00379] In one embodiment, an output FASTA file obtained by the process disclosed herein (see e.g., Example 30 herein) is used to design patient-specific constructs, either manually or by programmed script. In another embodiment, the programmed script automates the creation of the personalized plasma construct containing one or more neo-epitopes for each subject using a series of protocols (**Fig. 44**). The output FASTA file is inputted and after running the protocols, the DNA sequence of a LM vector including one or more neo-epitopes is outputted. The software program is useful for creating personalized immunotherapy for each subject.

[00380] In one embodiment, the nucleic acid sequences from disease-bearing and healthy samples are compared in order to identify neo-epitopes. Neo-epitopes comprise amino acid sequences changes within ORF sequences. As used herein, the term "sequence change" with respect to peptides or proteins relates to amino acid insertion variants, amino acid addition variants, amino acid deletion variants and amino acid substitution variants, preferably amino acid substitution variants. All these sequence changes according to the invention may potentially create new epitopes.

[00381] In one embodiment, amino acid insertion variants comprise insertions of single or two or more amino acids in a particular amino acid sequence. In another embodiment, amino acid addition variants comprise amino- and/or carboxy-terminal fusions of one or more amino acids, such as 1, 2, 3, 4 or 5, or more amino acids. In another embodiment, amino acid deletion variants are characterized by the removal of one or more amino acids from the sequence, such as by removal of 1, 2, 3, 4 or 5, or more amino acids. In another embodiment, amino acid substitution variants are characterized by at least one residue in the sequence being removed and another residue being inserted in its place.

[00382] All samples are analyzed for novel genetic sequencing within ORFs. Methods for comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from said disease-bearing biological sample and healthy biological sample comprise the use of screening assays or screening tools and associated digital software. Methods for performing bioinformatics analyses are known in the art, for example, see US Publication Nos. US 2013/0210645, US 2014/0045881, and International Publication WO 2014/052707, which are each incorporated in full in this application.

[00383] Human tumors typically harbor a remarkable number of somatic mutations. Yet, identical mutations in any particular gene are rarely found across tumors (and are even at low frequency for the most common driver mutations). Thus, in one embodiment, a process of this invention comprehensively identifying patient-specific tumor mutations provides a target for a personalized immunotherapy.

[00384] In one embodiment, mutations identifying from a disease-bearing sample may be presented on major histocompatibility complex class I molecules (MHCI). In one embodiment, a peptides containing a neo-epitope mutation is immunogenic and is recognized as a ‘non-self’ neo-antigens by the adaptive immune system. In another embodiment, use of one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion

polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate a T-cell immune responses to said disease or condition. In another embodiment, use of one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate an adaptive immune responses to a disease or condition.

[00385] In another embodiment, one or more neo-epitope sequencse comprised in a peptide, a polypeptide, or a fusion polypeptide is used to provide a therapeutic anti-tumor or anti-cancer T-cell immune response. In another embodiment, use of one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate an anti-tumor or anti-cancer adaptive immune response. In another embodiment, one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide is used to provide a therapeutic anti-autoimmune disease T-cell immune response. In another embodiment, use of one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate an anti-autoimmune disease adaptive immune response. In another embodiment, a one or more neo-epitope sequence comprised in a peptide, a polypeptide, or a fusion polypeptide is use to provide a therapeutic anti-infectious disease T-cell immune response. In another embodiment, use of one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate an anti-infectious disease adaptive immune response. In another embodiment, one or more neo-epitope sequences comprised in a peptide, a polypeptide, or a fusion polypeptide is used to provide a therapeutic anti-organ transplantation rejection T-cell immune response. In another embodiment, use of a one or more neo-epitope sequence comprised in a peptide, a polypeptide, or a fusion polypeptide provides a targeting immunotherapy, which may, in certain embodiments therapeutically activate an anti-organ transplantation rejection adaptive immune response.

[00386] In another embodiment, wherein the presence of an immunogenic response correlates with a presence of one or more immunogenic neo-epitopes. In another embodiment, a recombinant *Listeria* comprises nucleic acid encoding neo-epitopes comprising T-cell epitopes, or adaptive immune response epitopes, or any combination thereof.

[00387] In one embodiment, the process comprises screening each amino acid sequence comprising one or more neo-epitopes for an immunogenic response, wherein the presence of an immunogenic response correlates with one or more neo-epitopes comprising an immunogenic epitope. In another embodiment, one or more immunogenic neo-epitopes is comprised in a peptide. In another embodiment, one or more immunogenic neo-epitopes is comprised in a polypeptide. In another embodiment, one or more immunogenic neo-epitopes is comprised in a fusion-polypeptide. In another embodiment, one or more immunogenic neo-epitopes is comprised fused to a ubiquitin polypeptide.

[00388] In another embodiment, the process comprises screening each amino acid sequence comprising one or more neo-epitopes for an immunogenic T-cell response, wherein the presence of an immunogenic T-cell response correlates with one or more neo-epitopes comprising a T-cell epitope. In another embodiment, the process comprises screening each amino acid sequence comprising one or more neo-epitopes for an adaptive immune response, wherein the presence of an adaptive immune response correlates with one or more neo-epitopes comprising an adaptive immune response epitope.

[00389] In one embodiment, a step of screening for an immunogenic T-cell response in the system or process of creating a personalized immunotherapy provided comprises use of an immune response assay well known in the art, including for example T-cell proliferation assays, *in vitro* tumor regression assays using T-cells activated with said neo-epitope and co-incubated with tumor cells using a ^{51}Cr-release assay or a ^{3}H-thymidine assay, an ELISA assay, an ELIspot assay, and a FACS analysis. (See for example US Patent No. 8,771,702, and European Patent No. EP_1774332_B1, which are incorporated herein in their entirety). In another embodiment, a step for screening for an immunogenic response examines a non-T-cell response. In another embodiment, a step of screening for a non-T-cell response in the system or process of creating a personalized immunotherapy provided comprises use of an immune response assay well known in the art, including for example an assay similar to those above for T-cells, except that examining cytokine production focuses on a different subset of cytokines, namely, IL-10 and IL-1β. (See for example US Patent No. 8962319 and EP 177432, both of which are incorporated in full herein. For example, a T-cell immune response may be assayed by a ^{51}Cr release assay, comprising the steps of immunizing mice with a immunotherapy comprising one or more neo-epitopes, followed by harvesting spleens about ten days post-immunization, wherein splenocytes may then be established in culture with irradiated TC-1 cells (100:1, splenocytes:TC-1) as feeder cells; stimulated in vitro for 5 days,

then used in a standard ^{51}Cr release assay, using a peptide/polypeptide comprising one or more neo-epitopes as the target.

[00390] In another embodiment, a step for screening for an immune response comprises use of an HLA-A2 transgenic mouse, for example as disclosed in US Patent Application Publication No.: US-2011-0129499, which is incorporated in full herein.

[00391] In one embodiment, the process comprises selecting a nucleic acid sequence that encodes an identified T-cell neo epitope or encodes a peptide comprising said identified T-cell neo-epitope, and transforming said sequence into a recombinant attenuated *Listeria* strain. In one embodiment, the process comprises selecting a nucleic acid sequence that encodes an identified adaptive immune response neo-epitope or encodes a peptide comprising said identified adaptive immune response neo-epitope, and transforming said sequence into a recombinant attenuated *Listeria* strain.

[00392] In one embodiment, the system or process described herein comprises culturing and characterizing said *Listeria* strain to confirm expression and secretion of said T-cell neo-epitope. In one embodiment, the system or process described herein comprises culturing and characterizing said *Listeria* strain to confirm expression and secretion of said adaptive immune response neo-epitope. In one embodiment, the system or process described herein comprises culturing and characterizing said *Listeria* strain to confirm expression and secretion of said one or more peptides.

[00393] In one embodiment, the system or process of this invention comprises storing said *Listeria* for administrating to said subject at a pre-determined period or administering said *Listeria* to said subject, wherein said *Listeria* strain is administered as part of an immunogenic composition.

II. Recombinant *Listeria* strains

[00394] In one embodiment, a recombinant *Listeria* strain of the present invention comprises a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein the fusion polypeptide comprises a truncated listeriolysin O (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to one or more peptides comprising one or more neo-epitopes. It will be understood by a skilled artisan that one or more peptides provided herein which comprise one or more epitopes may be immunogenic to start with and their immunogenicity may be enhanced by fusing with or mixing with an immunogenic polypeptide such as a tLLO, a truncated ActA

protein or a PEST amino acid sequence. . In another embodiment, a recombinant *Listeria* strain of the present invention comprises a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence. In one embodiment, the recombinant *Listeria* strain is attenuated.

[00395] In one embodiment, one or more peptides comprising one or more immunogenic neo-epitopes provided herein are each fused to an immunogenic polypeptide or fragment thereof.

[00396] In another embodiment, a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence is not fused to a heterologous antigen or a fragment thereof. In another embodiment, a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence is not fused to one or more peptides provided herein.

[00397] In another embodiment, one or more peptides comprising one or more immunogenic neo-epitopes provided herein are mixed with an immunogenic polypeptide or fragment thereof as part of an immunogenic composition.

[00398] In one embodiment, a truncated listeriolysin O (LLO) protein comprises a putative PEST sequence. In one embodiment, a truncated actA protein comprises a PEST-containing amino acid sequence. In another embodiment, a truncated actA protein comprises a putative PEST-containing amino acid sequence.

[00399] In one embodiment, a PEST amino acid (AA) sequence comprises a truncated LLO sequence. In another embodiment, the PEST amino acid sequence is KENSISSMAPPASPPASPKTPIEKKHADEIDK (SEQ ID NO: 1). In another embodiment, fusion of an antigen to other LM PEST AA sequences from *Listeria* will also enhance immunogenicity of the antigen.

[00400] The N-terminal LLO protein fragment of methods and compositions of the present invention comprises, in another embodiment, SEQ ID No: 3. In another embodiment, the fragment comprises an LLO signal peptide. In another embodiment, the fragment comprises SEQ ID No: 4. In another embodiment, the fragment consists approximately of SEQ ID No: 4. In another embodiment, the fragment consists essentially of SEQ ID No: 4. In another embodiment, the fragment corresponds to SEQ ID No: 4. In another embodiment, the fragment is homologous to SEQ ID No: 4. In another embodiment, the fragment is homologous to a fragment of SEQ ID No: 4. In one embodiment, a truncated LLO used

excludes of the signal sequence. In another embodiment, the truncated LLO comprises a signal sequence. It will be clear to those skilled in the art that any truncated LLO without the activation domain, and in particular without cysteine 484, are suitable for methods and compositions of the present invention. In another embodiment, fusion of a heterologous antigen to any truncated LLO, including the PEST AA sequence, SEQ ID NO: 1, enhances cell mediated and anti-tumor immunity of the antigen.

[00401] The LLO protein utilized to construct immunotherapies of the present invention has, in another embodiment, the sequence: MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSMAPPASPPASPKTPIEKKHADE IDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQ VVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNA TKSNVNNAVNTLVERWNEKYAQAYPNVSAKIDYDDEMAYSESQLIAKFGTAFKAV NNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK NNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDPEGNEIVQHKNWSENNK SKLAHFTSSIYLPGNARNINVYAKECTGLAWEWWRTVIDDRNLPLVKNRNISIWGTT LYPKYSNKVDNPIE (GenBank Accession No. P13128; SEQ ID NO: 2; nucleic acid sequence is set forth in GenBank Accession No. X15127). The first 25 AA of the proprotein corresponding to this sequence are the signal sequence and are cleaved from LLO when it is secreted by the bacterium. Thus, in this embodiment, the full length active LLO protein is 504 residues long. In another embodiment, the above LLO fragment is used as the source of the LLO fragment incorporated in a immunotherapy of the present invention.

[00402] In another embodiment, the N-terminal fragment of an LLO protein utilized in compositions and methods of the present invention has the sequence: MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKKHADE IDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQ VVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNA TKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFKAV NNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK NNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYD (SEQ ID NO: 3).

[00403] In another embodiment, the LLO fragment corresponds to about AA 20-442 of an LLO protein utilized herein.

[00404] In another embodiment, the LLO fragment has the sequence:
MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKKHADE
IDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQ
VVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNA
TKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFKAV
NNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF
KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK
NNSEYIETTSKAYTD (SEQ ID NO: 4).

[00405] In another embodiment, the terms “N-terminal truncated LLO protein,” “N-terminal LLO fragment,” “truncated LLO protein,” “ΔLLO” or their grammatical equivalents are used interchangeably herein and refers to a fragment of LLO that is non-hemolytic. In another embodiment, the terms refer to an LLO fragment that comprises a putative PEST sequence.

[00406] In another embodiment, the LLO fragment is rendered non-hemolytic by deletion or mutation of the activation domain. In another embodiment, the LLO fragment is rendered non-hemolytic by deletion or mutation of region comprising cysteine 484. In another embodiment, the LLO is rendered non-hemolytic by a deletion or mutation of the cholesterol binding domain (CBD) as detailed in US Patent No. 8,771,702, which is incorporated by reference herein.

[00407] In one embodiment, the present invention provides a recombinant protein or polypeptide comprising a listeriolysin O (LLO) protein, wherein said LLO protein comprises a mutation of residues C484, W491, W492, or a combination thereof of the cholesterol-binding domain (CBD) of said LLO protein. In one embodiment, said C484, W491, and W492 residues are residues C484, W491, and W492 of SEQ ID NO: 2, while in another embodiment, they are corresponding residues as can be deduced using sequence alignments, as is known to one of skill in the art. In one embodiment, residues C484, W491, and W492 are mutated. In one embodiment, a mutation is a substitution, in another embodiment, a deletion. In one embodiment, the entire CBD is mutated, while in another embodiment, portions of the CBD are mutated, while in another embodiment, only specific residues within the CBD are mutated.

[00408] In one embodiment, the present invention provides a recombinant protein or polypeptide comprising a mutated LLO protein or fragment thereof, wherein the mutated LLO protein or fragment thereof contains a substitution of a non-LLO peptide for a mutated region of the mutated LLO protein or fragment thereof, the mutated region comprising a residue selected from C484, W491, and W492. In another embodiment, the LLO fragment is an N-terminal LLO fragment. In another embodiment, the LLO fragment is at least 492 amino acids (AA) long. In another embodiment, the LLO fragment is 492-528 AA long. In another embodiment, the non-LLO peptide is 1-50 amino acids long. In another embodiment, the mutated region is 1-50 amino acids long. In another embodiment, the non-LLO peptide is the same length as the mutated region. In another embodiment, the non-LLO peptide has a length different from the mutated region. In another embodiment, the substitution is an inactivating mutation with respect to hemolytic activity. In another embodiment, the recombinant protein or polypeptide exhibits a reduction in hemolytic activity relative to wild-type LLO. In another embodiment, the recombinant protein or polypeptide is non-hemolytic.

[00409] As provided herein, a mutant LLO protein was created wherein residues C484, W491, and W492 of LLO were substituted with alanine residues (Example 25). The mutated LLO protein, mutLLO, could be expressed and purified in an *E. coli* expression system (Example 27) and exhibited substantially reduced hemolytic activity relative to wild-type LLO (Example 28).

[00410] In another embodiment, the present invention provides a recombinant protein or polypeptide comprising (a) a mutated LLO protein, wherein the mutated LLO protein contains an internal deletion, the internal deletion comprising the cholesterol-binding domain of the mutated LLO protein; and (b) a heterologous peptide of interest. In another embodiment, the sequence of the cholesterol-binding domain is set forth in SEQ ID NO: 101. In another embodiment, the internal deletion is an 11-50 amino acid internal deletion. In another embodiment, the internal deletion is inactivating with regard to the hemolytic activity of the recombinant protein or polypeptide. In another embodiment, the recombinant protein or polypeptide exhibits a reduction in hemolytic activity relative to wild-type LLO.

[00411] In another embodiment, the present invention provides a recombinant protein or polypeptide comprising (a) a mutated LLO protein, wherein the mutated LLO protein contains an internal deletion, the internal deletion comprising a fragment of the cholesterol-binding domain of the mutated LLO protein; and (b) a heterologous peptide of interest. In another embodiment, the internal deletion is a 1-11 amino acid internal deletion. In another

embodiment, the sequence of the cholesterol-binding domain is set forth in SEQ ID NO: 101. In another embodiment, the internal deletion is inactivating with regard to the hemolytic activity of the recombinant protein or polypeptide. In another embodiment, the recombinant protein or polypeptide exhibits a reduction in hemolytic activity relative to wild-type LLO.

[00412] The mutated region of methods and compositions of the present invention comprises, in another embodiment, residue C484 of SEQ ID NO: 2. In another embodiment, the mutated region comprises a corresponding cysteine residue of a homologous LLO protein. In another embodiment, the mutated region comprises residue W491 of SEQ ID NO: 2. In another embodiment, the mutated region comprises a corresponding tryptophan residue of a homologous LLO protein. In another embodiment, the mutated region comprises residue W492 of SEQ ID NO: 2. In another embodiment, the mutated region comprises a corresponding tryptophan residue of a homologous LLO protein. Methods for identifying corresponding residues of a homologous protein are well known in the art, and include, for example, sequence alignment.

[00413] In another embodiment, the mutated region comprises residues C484 and W491. In another embodiment, the mutated region comprises residues C484 and W492. In another embodiment, the mutated region comprises residues W491 and W492. In another embodiment, the mutated region comprises residues C484, W491, and W492.

[00414] In another embodiment, the mutated region of methods and compositions of the present invention comprises the cholesterol-binding domain of the mutated LLO protein or fragment thereof. For example, a mutated region consisting of residues 470-500, 470-510, or 480-500 of SEQ ID NO: 2 comprises the CBD thereof (residues 483-493). In another embodiment, the mutated region is a fragment of the CBD of the mutated LLO protein or fragment thereof. For example, as provided herein, residues C484, W491, and W492, each of which is a fragment of the CBD, were mutated to alanine residues (Example 25). Further, as provided herein, a fragment of the CBD, residues 484-492, was replaced with a heterologous sequence from NY-ESO-1 (Example 26). In another embodiment, the mutated region overlaps the CBD of the mutated LLO protein or fragment thereof. For example, a mutated region consisting of residues 470-490, 480-488, 490-500, or 486-510 of SEQ ID NO: 2 comprises the CBD thereof. In another embodiment, a single peptide may have a deletion in the signal sequence and a mutation or substitution in the CBD. Each possibility represents a separate embodiment of the present invention.

[00415] The length of the mutated region is, in another embodiment, 1-50 AA. In another embodiment, the length is 1-11 AA. In another embodiment, the length is 2-11 AA. In another embodiment, the length is 3-11 AA. In another embodiment, the length is 4-11 AA. In another embodiment, the length is 5-11 AA. In another embodiment, the length is 6-11 AA. In another embodiment, the length is 7-11 AA. In another embodiment, the length is 8-11 AA. In another embodiment, the length is 9-11 AA. In another embodiment, the length is 10-11 AA. In another embodiment, the length is 1-2 AA. In another embodiment, the length is 1-3 AA. In another embodiment, the length is 1-4 AA. In another embodiment, the length is 1-5 AA. In another embodiment, the length is 1-6 AA. In another embodiment, the length is 1-7 AA. In another embodiment, the length is 1-8 AA. In another embodiment, the length is 1-9 AA. In another embodiment, the length is 1-10 AA. In another embodiment, the length is 2-3 AA. In another embodiment, the length is 2-4 AA. In another embodiment, the length is 2-5 AA. In another embodiment, the length is 2-6 AA. In another embodiment, the length is 2-7 AA. In another embodiment, the length is 2-8 AA. In another embodiment, the length is 2-9 AA. In another embodiment, the length is 2-10 AA. In another embodiment, the length is 3-4 AA. In another embodiment, the length is 3-5 AA. In another embodiment, the length is 3-6 AA. In another embodiment, the length is 3-7 AA. In another embodiment, the length is 3-8 AA. In another embodiment, the length is 3-9 AA. In another embodiment, the length is 3-10 AA. In another embodiment, the length is 11-50 AA. In another embodiment, the length is 12-50 AA. In another embodiment, the length is 11-15 AA. In another embodiment, the length is 11-20 AA. In another embodiment, the length is 11-25 AA. In another embodiment, the length is 11-30 AA. In another embodiment, the length is 11-35 AA. In another embodiment, the length is 11-40 AA. In another embodiment, the length is 11-60 AA. In another embodiment, the length is 11-70 AA. In another embodiment, the length is 11-80 AA. In another embodiment, the length is 11-90 AA. In another embodiment, the length is 11-100 AA. In another embodiment, the length is 11-150 AA. In another embodiment, the length is 15-20 AA. In another embodiment, the length is 15-25 AA. In another embodiment, the length is 15-30 AA. In another embodiment, the length is 15-35 AA. In another embodiment, the length is 15-40 AA. In another embodiment, the length is 15-60 AA. In another embodiment, the length is 15-70 AA. In another embodiment, the length is 15-80 AA. In another embodiment, the length is 15-90 AA. In another embodiment, the length is 15-100 AA. In another embodiment, the length is 15-150 AA. In another embodiment, the length is 20-25 AA. In another embodiment, the length is 20-30 AA. In another embodiment, the length is 20-35 AA. In another embodiment, the length is 20-40 AA. In another embodiment,

the length is 20-60 AA. In another embodiment, the length is 20-70 AA. In another embodiment, the length is 20-80 AA. In another embodiment, the length is 20-90 AA. In another embodiment, the length is 20-100 AA. In another embodiment, the length is 20-150 AA. In another embodiment, the length is 30-35 AA. In another embodiment, the length is 30-40 AA. In another embodiment, the length is 30-60 AA. In another embodiment, the length is 30-70 AA. In another embodiment, the length is 30-80 AA. In another embodiment, the length is 30-90 AA. In another embodiment, the length is 30-100 AA. In another embodiment, the length is 30-150 AA. Each possibility represents another embodiment of the present invention.

[00416] The substitution mutation of methods and compositions of the present invention is, in another embodiment, a mutation wherein the mutated region of the LLO protein or fragment thereof is replaced by an equal number of heterologous AA. In another embodiment, a larger number of heterologous AA than the size of the mutated region is introduced. In another embodiment, a smaller number of heterologous AA than the size of the mutated region is introduced. Each possibility represents another embodiment of the present invention.

[00417] In another embodiment, the substitution mutation is a point mutation of a single residue. In another embodiment, the substitution mutation is a point mutation of 2 residues. In another embodiment, the substitution mutation is a point mutation of 3 residues. In another embodiment, the substitution mutation is a point mutation of more than 3 residues. In another embodiment, the substitution mutation is a point mutation of several residues. In another embodiment, the multiple residues included in the point mutation are contiguous. In another embodiment, the multiple residues are not contiguous.

[00418] The length of the non-LLO peptide that replaces the mutated region of recombinant protein or polypeptides of the present invention is, in another embodiment, 1-50 AA. In another embodiment, the length is 1-11 AA. In another embodiment, the length is 2-11 AA. In another embodiment, the length is 3-11 AA. In another embodiment, the length is 4-11 AA. In another embodiment, the length is 5-11 AA. In another embodiment, the length is 6-11 AA. In another embodiment, the length is 7-11 AA. In another embodiment, the length is 8-11 AA. In another embodiment, the length is 9-11 AA. In another embodiment, the length is 10-11 AA. In another embodiment, the length is 1-2 AA. In another embodiment, the length is 1-3 AA. In another embodiment, the length is 1-4 AA. In another embodiment, the length is 1-5 AA. In another embodiment, the length is 1-6 AA. In another embodiment, the length is 1-7 AA. In another embodiment, the length is 1-8 AA. In another embodiment, the

length is 1-9 AA. In another embodiment, the length is 1-10 AA. In another embodiment, the length is 2-3 AA. In another embodiment, the length is 2-4 AA. In another embodiment, the length is 2-5 AA. In another embodiment, the length is 2-6 AA. In another embodiment, the length is 2-7 AA. In another embodiment, the length is 2-8 AA. In another embodiment, the length is 2-9 AA. In another embodiment, the length is 2-10 AA. In another embodiment, the length is 3-4 AA. In another embodiment, the length is 3-5 AA. In another embodiment, the length is 3-6 AA. In another embodiment, the length is 3-7 AA. In another embodiment, the length is 3-8 AA. In another embodiment, the length is 3-9 AA. In another embodiment, the length is 3-10 AA. In another embodiment, the length is 11-50 AA. In another embodiment, the length is 12-50 AA. In another embodiment, the length is 11-15 AA. In another embodiment, the length is 11-20 AA. In another embodiment, the length is 11-25 AA. In another embodiment, the length is 11-30 AA. In another embodiment, the length is 11-35 AA. In another embodiment, the length is 11-40 AA. In another embodiment, the length is 11-60 AA. In another embodiment, the length is 11-70 AA. In another embodiment, the length is 11-80 AA. In another embodiment, the length is 11-90 AA. In another embodiment, the length is 11-100 AA. In another embodiment, the length is 11-150 AA. In another embodiment, the length is 15-20 AA. In another embodiment, the length is 15-25 AA. In another embodiment, the length is 15-30 AA. In another embodiment, the length is 15-35 AA. In another embodiment, the length is 15-40 AA. In another embodiment, the length is 15-60 AA. In another embodiment, the length is 15-70 AA. In another embodiment, the length is 15-80 AA. In another embodiment, the length is 15-90 AA. In another embodiment, the length is 15-100 AA. In another embodiment, the length is 15-150 AA. In another embodiment, the length is 20-25 AA. In another embodiment, the length is 20-30 AA. In another embodiment, the length is 20-35 AA. In another embodiment, the length is 20-40 AA. In another embodiment, the length is 20-60 AA. In another embodiment, the length is 20-70 AA. In another embodiment, the length is 20-80 AA. In another embodiment, the length is 20-90 AA. In another embodiment, the length is 20-100 AA. In another embodiment, the length is 20-150 AA. In another embodiment, the length is 30-35 AA. In another embodiment, the length is 30-40 AA. In another embodiment, the length is 30-60 AA. In another embodiment, the length is 30-70 AA. In another embodiment, the length is 30-80 AA. In another embodiment, the length is 30-90 AA. In another embodiment, the length is 30-100 AA. In another embodiment, the length is 30-150 AA.

[00419] In another embodiment, the length of the LLO fragment of methods and

compositions of the present invention is at least 484 AA. In another embodiment, the length is over 484 AA. In another embodiment, the length is at least 489 AA. In another embodiment, the length is over 489. In another embodiment, the length is at least 493 AA. In another embodiment, the length is over 493. In another embodiment, the length is at least 500 AA. In another embodiment, the length is over 500. In another embodiment, the length is at least 505 AA. In another embodiment, the length is over 505. In another embodiment, the length is at least 510 AA. In another embodiment, the length is over 510. In another embodiment, the length is at least 515 AA. In another embodiment, the length is over 515. In another embodiment, the length is at least 520 AA. In another embodiment, the length is over 520. In another embodiment, the length is at least 525 AA. In another embodiment, the length is over 520. When referring to the length of an LLO fragment herein, the signal sequence is included. Thus, the numbering of the first cysteine in the CBD is 484, and the total number of AA residues is 529.

[00420] In another embodiment, the present invention provides a recombinant protein or polypeptide, or an attenuated *Listeria* strain provided herein comprising the same, comprising (a) a mutated LLO protein, wherein the mutated LLO protein contains an internal deletion, the internal deletion comprising the cholesterol-binding domain of the mutated LLO protein; and (b) peptide comprising one or more epitopes provided herein. In another embodiment, the sequence of the cholesterol-binding domain is set forth in SEQ ID NO: 101. In another embodiment, the internal deletion is a 1-11, 1-50 or an 11-50 amino acid internal deletion. In another embodiment, the internal deletion is inactivating with regard to the hemolytic activity of the recombinant protein or polypeptide. In another embodiment, the recombinant protein or polypeptide exhibits a reduction in hemolytic activity relative to wild-type LLO.

[00421] In another embodiment, a peptide of the present invention is a fusion peptide. In another embodiment, “fusion peptide” refers to a peptide or polypeptide comprising two or more proteins linked together by peptide bonds or other chemical bonds. In another embodiment, the proteins are linked together directly by a peptide or other chemical bond. In another embodiment, the proteins are linked together with one or more AA (e.g. a “spacer”) between the two or more proteins.

[00422] As provided herein, a mutant LLO protein was created wherein residues C484, W491, and W492 of LLO were substituted with a CTL epitope from the antigen NY-ESO-1 (Example 26). The mutated LLO protein, mutLLO, could be expressed and purified in an *E. coli* expression system (Example 2 7) and exhibited substantially reduced hemolytic activity

relative to wild-type LLO (Example 28). It will be appreciated by a skilled artisan that any neo-epitope identified by the methods or processes provided herein can be used for substituting or replacing the CBD of LLO.

[00423] The length of the internal deletion of methods and compositions of the present invention is, in another embodiment, 1-50 AA. In another embodiment, the length is 1-11 AA. In another embodiment, the length is 2-11 AA. In another embodiment, the length is 3-11 AA. In another embodiment, the length is 4-11 AA. In another embodiment, the length is 5-11 AA. In another embodiment, the length is 6-11 AA. In another embodiment, the length is 7-11 AA. In another embodiment, the length is 8-11 AA. In another embodiment, the length is 9-11 AA. In another embodiment, the length is 10-11 AA. In another embodiment, the length is 1-2 AA. In another embodiment, the length is 1-3 AA. In another embodiment, the length is 1-4 AA. In another embodiment, the length is 1-5 AA. In another embodiment, the length is 1-6 AA. In another embodiment, the length is 1-7 AA. In another embodiment, the length is 1-8 AA. In another embodiment, the length is 1-9 AA. In another embodiment, the length is 1-10 AA. In another embodiment, the length is 2-3 AA. In another embodiment, the length is 2-4 AA. In another embodiment, the length is 2-5 AA. In another embodiment, the length is 2-6 AA. In another embodiment, the length is 2-7 AA. In another embodiment, the length is 2-8 AA. In another embodiment, the length is 2-9 AA. In another embodiment, the length is 2-10 AA. In another embodiment, the length is 3-4 AA. In another embodiment, the length is 3-5 AA. In another embodiment, the length is 3-6 AA. In another embodiment, the length is 3-7 AA. In another embodiment, the length is 3-8 AA. In another embodiment, the length is 3-9 AA. In another embodiment, the length is 3-10 AA. In another embodiment, the length is 11-50 AA. In another embodiment, the length is 12-50 AA. In another embodiment, the length is 11-15 AA. In another embodiment, the length is 11-20 AA. In another embodiment, the length is 11-25 AA. In another embodiment, the length is 11-30 AA. In another embodiment, the length is 11-35 AA. In another embodiment, the length is 11-40 AA. In another embodiment, the length is 11-60 AA. In another embodiment, the length is 11-70 AA. In another embodiment, the length is 11-80 AA. In another embodiment, the length is 11-90 AA. In another embodiment, the length is 11-100 AA. In another embodiment, the length is 11-150 AA. In another embodiment, the length is 15-20 AA. In another embodiment, the length is 15-25 AA. In another embodiment, the length is 15-30 AA. In another embodiment, the length is 15-35 AA. In another embodiment, the length is 15-40 AA. In another embodiment, the length is 15-60 AA. In another embodiment, the length is

15-70 AA. In another embodiment, the length is 15-80 AA. In another embodiment, the length is 15-90 AA. In another embodiment, the length is 15-100 AA. In another embodiment, the length is 15-150 AA. In another embodiment, the length is 20-25 AA. In another embodiment, the length is 20-30 AA. In another embodiment, the length is 20-35 AA. In another embodiment, the length is 20-40 AA. In another embodiment, the length is 20-60 AA. In another embodiment, the length is 20-70 AA. In another embodiment, the length is 20-80 AA. In another embodiment, the length is 20-90 AA. In another embodiment, the length is 20-100 AA. In another embodiment, the length is 20-150 AA. In another embodiment, the length is 30-35 AA. In another embodiment, the length is 30-40 AA. In another embodiment, the length is 30-60 AA. In another embodiment, the length is 30-70 AA. In another embodiment, the length is 30-80 AA. In another embodiment, the length is 30-90 AA. In another embodiment, the length is 30-100 AA. In another embodiment, the length is 30-150 AA.

[00424] In another embodiment, the mutated LLO protein of the present invention that comprises an internal deletion is full length except for the internal deletion. In another embodiment, the mutated LLO protein comprises an additional internal deletion. In another embodiment, the mutated LLO protein comprises more than one additional internal deletion. In another embodiment, the mutated LLO protein is truncated from the C-terminal end.

[00425] In another embodiment, the internal deletion of methods and compositions of the present invention comprises the CBD of the mutated LLO protein or fragment thereof. For example, an internal deletion consisting of residues 470-500, 470-510, or 480-500 of SEQ ID NO: 2 comprises the CBD thereof (residues 483-493). In another embodiment, the internal deletion is a fragment of the CBD of the mutated LLO protein or fragment thereof. For example, residues 484-492, 485-490, and 486-488 are all fragments of the CBD of SEQ ID NO: 2. In another embodiment, the internal deletion overlaps the CBD of the mutated LLO protein or fragment thereof. For example, an internal deletion consisting of residues 470-490, 480-488, 490-500, or 486-510 of SEQ ID NO: 2 comprises the CBD thereof.

[00426] In another embodiment, a truncated LLO fragment comprises the first 441 AA of the LLO protein. In another embodiment, the LLO fragment comprises the first 420 AA of LLO. In another embodiment, the LLO fragment is a non-hemolytic form of the wild-type LLO protein.

[00427] In another embodiment, the LLO fragment consists of about residues 1-25. In

another embodiment, the LLO fragment consists of about residues 1-50. In another embodiment, the LLO fragment consists of about residues 1-75. In another embodiment, the LLO fragment consists of about residues 1-100. In another embodiment, the LLO fragment consists of about residues 1-125. In another embodiment, the LLO fragment consists of about residues 1-150. In another embodiment, the LLO fragment consists of about residues 1175. In another embodiment, the LLO fragment consists of about residues 1-200. In another embodiment, the LLO fragment consists of about residues 1-225. In another embodiment, the LLO fragment consists of about residues 1-250. In another embodiment, the LLO fragment consists of about residues 1-275. In another embodiment, the LLO fragment consists of about residues 1-300. In another embodiment, the LLO fragment consists of about residues 1-325. In another embodiment, the LLO fragment consists of about residues 1-350. In another embodiment, the LLO fragment consists of about residues 1-375. In another embodiment, the LLO fragment consists of about residues 1-400. In another embodiment, the LLO fragment consists of about residues 1-425.

[00428] In another embodiment, the LLO fragment contains residues of a homologous LLO protein that correspond to one of the above AA ranges. The residue numbers need not, in another embodiment, correspond exactly with the residue numbers enumerated above; e.g. if the homologous LLO protein has an insertion or deletion, relative to an LLO protein utilized herein, then the residue numbers can be adjusted accordingly. In another embodiment, the LLO fragment is any other LLO fragment known in the art.

[00429] Methods for identifying corresponding residues of a homologous protein are well known in the art, and include, for example, sequence alignment. In one embodiment, a homologous LLO refers to identity to an LLO sequence (e.g. to one of SEQ ID No: 2-4) of greater than 70%. In another embodiment, a homologous LLO refers to identity to one of SEQ ID No: 2-4 of greater than 72%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 75%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 78%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 80%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 82%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 83%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 85%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 87%. In another embodiment, a homologous refers to identity to

one of SEQ ID No: 2-4 of greater than 88%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 90%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 92%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 93%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 95%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 96%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 97%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 98%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of greater than 99%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 2-4 of 100%.

[00430] The terms "PEST amino acid sequence," "PEST sequence," "PEST sequence peptide," "PEST peptide," or "PEST sequence-containing protein or peptide," are used interchangeably herein. It will be appreciated by the skilled artisan that these terms may encompass a truncated LLO protein, which in one embodiment is an N-terminal LLO, or in another embodiment, a truncated ActA protein. PEST sequence peptides are known in the art and are described in US Patent Serial No. 7,635,479, and in US Patent Publication Serial No. 2014/0186387, both of which are hereby incorporated in their entirety herein.

[00431] In another embodiment, a PEST sequence of prokaryotic organisms can be identified routinely in accordance with methods such as described by Rechsteiner and Roberts (TBS 21:267-271,1996) for *L. monocytogenes*. Alternatively, PEST amino acid sequences from other prokaryotic organisms can also be identified based by this method. Other prokaryotic organisms wherein PEST amino acid sequences would be expected to include, but are not limited to, other *Listeria* species. For example, the *L. monocytogenes* protein ActA contains four such sequences. These are KTEEQPSEVNTGPR (SEQ ID NO: 5), KASVTDTSEGDLDSSMQSADESTPQPLK (SEQ ID NO: 6), KNEEVNASDFPPPPTDEELR (SEQ ID NO: 7), and RGGIPTSEEFSSLNSGDFTDDENSETTEEEIDR (SEQ ID NO: 8). Also Streptolysin O from *Streptococcus* sp. contain a PEST sequence. For example, *Streptococcus pyogenes* Streptolysin O comprises the PEST sequence KQNTASTETTTTNEQPK (SEQ ID NO: 9) at amino acids 35-51 and *Streptococcus equisimilis* Streptolysin O comprises the PEST-like sequence KQNTANTETTTTNEQPK (SEQ ID NO: 10) at amino acids 38-54. Further, it is believed that the PEST sequence can be embedded within the antigenic protein.

Thus, for purposes of the present invention, by “fusion” when in relation to PEST sequence fusions, it is meant that the antigenic protein comprises both the antigen and the PEST amino acid sequence either linked at one end of the antigen or embedded within the antigen. In other embodiments, a PEST sequence or PEST containing polypeptide is not part of a fusion protein, nor does the polypeptide include a heterologous antigen.

[00432] The terms “nucleic acid sequence,” “nucleic acid molecule,” “polynucleotide,” or “nucleic acid construct” are used interchangeably herein, and may refer to a DNA or RNA molecule, which may include, but is not limited to, prokaryotic sequences, eukaryotic mRNA, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. The term also refers to sequences that include any of the known base analogs of DNA and RNA. The terms may also refer to a string of at least two base-sugar-phosphate combinations. The term may also refer to the monomeric units of nucleic acid polymers. RNA may be, in one embodiment, in the form of a tRNA (transfer RNA), snRNA (small nuclear RNA), rRNA (ribosomal RNA), mRNA (messenger RNA), anti-sense RNA, small inhibitory RNA (siRNA), micro RNA (miRNA) and ribozymes. The use of siRNA and miRNA has been described (Caudy AA et al, Genes & Devel 16: 2491-96 and references cited therein). DNA may be in form of plasmid DNA, viral DNA, linear DNA, or chromosomal DNA or derivatives of these groups. In addition, these forms of DNA and RNA may be single, double, triple, or quadruple stranded. The terms may also include, artificial nucleic acids that may contain other types of backbones but the same bases. In one embodiment, the artificial nucleic acid is a PNA (peptide nucleic acid). PNA contain peptide backbones and nucleotide bases and are able to bind, in one embodiment, to both DNA and RNA molecules. In another embodiment, the nucleotide is oxetane modified. In another embodiment, the nucleotide is modified by replacement of one or more phosphodiester bonds with a phosphorothioate bond. In another embodiment, the artificial nucleic acid contains any other variant of the phosphate backbone of native nucleic acids known in the art. The use of phosphothiorate nucleic acids and PNA are known to those skilled in the art, and are described in, for example, Neilsen PE, Curr Opin Struct Biol 9:353-57; and Raz NK et al Biochem Biophys Res Commun. 297:1075-84. The production and use of nucleic acids is known to those skilled in art and is described, for example, in Molecular Cloning, (2001), Sambrook and Russell, eds. and Methods in Enzymology: Methods for molecular cloning in eukaryotic cells (2003) Purchio and G. C. Fareed.

[00433] In another embodiment, a nucleic acid molecule provided herein is expressed from

an episomal or plasmid vector. In another embodiment, the plasmid is stably maintained in the recombinant *Listeria* immunotherapy strain in the absence of antibiotic selection. In another embodiment, the plasmid does not confer antibiotic resistance upon the recombinant *Listeria*.

5 [00434] In one embodiment, an immunogenic polypeptide or fragment thereof provided herein is an ActA protein or fragment thereof. In one embodiment, an ActA protein comprises the sequence set forth in SEQ ID NO: 11:
MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTGPRYETA
REVSSRDIEELEKSNKVKNTNKADLIAMLKAKAEKGPNNNNNNGEQTGNVAINEEA
10 SGVDRPTLQVERRHPGLSSDSAAEIKKRRKAIASSDSELESLTYPDKPTKANKRKVAK
ESVVDASESDLDSSMQSADESTPQPLKANQKPFFPKVFKKIKDAGKWVRDKIDENPE
VKKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPTPSE
PSSFEFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIMRETAPSLD
SSFTSGDLASLRSAINRHSENFSDFPLIPTEEELNGRGGRPTSEEFSSLNSGDFTDDENS
15 ETTEEEIDRLADLRDRGTGKHSRNAGFLPLNPFISSPVPSLTPKVPKISAPALISDITKK
APFKNPSQPLNVFNKKTTTKTVTKKPTPVKTAPKLAELPATKPQETVLRENKTPFIEK
QAETNKQSINMPSLPVIQKEATESDKEEMKPQTEEKMVEESESANNANGKNRSAGIE
EGKLIAKSAEDEKAKEEPGNHTTLILAMLAIGVFSLGAFIKIIQLRKNN (SEQ ID NO: 11).

20 [00435] The first 29 AA of the proprotein corresponding to this sequence are the signal sequence and are cleaved from ActA protein when it is secreted by the bacterium. In one embodiment, an ActA polypeptide or peptide comprises the signal sequence, AA 1-29 of SEQ ID NO: 11 above. In another embodiment, an ActA polypeptide or peptide does not include the signal sequence, AA 1-29 of SEQ ID NO: 11 above.

25 [00436] In one embodiment, a truncated ActA protein comprises an N-terminal fragment of an ActA protein. In another embodiment, a truncated ActA protein is an N-terminal fragment of an ActA protein. In one embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 12:
MRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTGPRYETA
30 REVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKGPNINNNNSEQTENAAINEEAS
GADRPAIQVERRHPGLPSDSAAEIKKRRKAIASSDSELESLTYPDKPTKVNKKKVAKE
SVADASESDLDSSMQSADESSPQPLKANQQPFFPKVFKKIKDAGKWVRDKIDENPEV
KKAIVDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFNAPATSEP

SSFEFPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEFPPPPTEDELEIIRETASSLDSS FTRGDLASLRNAINRHSQNFSDFPPIPTEEELNGRGGRP (SEQ ID NO: 12).

[00437] In another embodiment, the ActA fragment comprises the sequence set forth in SEQ ID NO: 12.

5 [00438] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 13:
MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEKTEEQPSEVNTG PRYETAREVSSRDIKELEKSNKVRNTNKADLIAMLKEKAEKG (SEQ ID NO: 13).

[00439] In another embodiment, the ActA fragment is any other ActA fragment known in
10 the art. In another embodiment, the ActA fragment is an immunogenic fragment.

[00440] In another embodiment, an ActA protein comprises the sequence set forth in SEQ ID NO: 14:

M G L N R F M R A M M V V F I T A N C I T I N P D I I F A A T D S E D S S L N T D E
W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D I E E L E K S N K V K N T
15 N K A D L I A M L K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D
R P T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S L T Y P D
K P T K A N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L K A N
Q K P F F P K V F K K I K D A G K W V R D K I D E N P E V K K A I V D K S A G L I
D Q L L T K K K S E E V N A S D F P P P P T D E E L R L A L P E T P M L L G F N A P
20 T P S E P S S F E F P P P P T D E E L R L A L P E T P M L L G F N A P A T S E P S S F E
F P P P P T E D E L E I M R E T A P S L D S S F T S G D L A S L R S A I N R H S E N F
S D F P L I P T E E E L N G R G G R P T S E E F S S L N S G D F T D D E N S E T T E E
E I D R L A D L R D R G T G K H S R N A G F L P L N P F I S S P V P S L T P K V P K I
S A P A L I S D I T K K A P F K N P S Q P L N V F N K K T T T K T V T K K P T P V K
25 T A P K L A E L P A T K P Q E T V L R E N K T P F I E K Q A E T N K Q S I N M P S L
P V I Q K E A T E S D K E E M K P Q T E E K M V E E S E S A N N A N G K N R S A G
I E E G K L I A K S A E D E K A K E E P G N H T T L I L A M L A I G V F S L G A F I K
I I Q L R K N N (SEQ ID NO: 14). The first 29 AA of the proprotein corresponding to this sequence are the signal sequence and are cleaved from ActA protein when it is secreted by
30 the bacterium. In one embodiment, an ActA polypeptide or peptide comprises the signal sequence, AA 1-29 of SEQ ID NO: 154. In another embodiment, an ActA polypeptide or peptide does not include the signal sequence, AA 1-29 of SEQ ID NO: 14.

[00441] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 15:

A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G (SEQ ID NO: 15). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 15 is referred to as ActA/PEST1. In another embodiment, a truncated ActA comprises from the first 30 to amino acid 122 of the full length ActA sequence. In another embodiment, SEQ ID NO: 15 comprises from the first 30 to amino acid 122 of the full length ActA sequence. In another embodiment, a truncated ActA comprises from the first 30 to amino acid 122 of SEQ ID NO: 14. In another embodiment, SEQ ID NO: 15 comprises from the first 30 to amino acid 122 of SEQ ID NO: 14.

[00442] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 16:

A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D K (SEQ ID NO: 16). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 16 is referred to as ActA/PEST2. In another embodiment, a truncated ActA as set forth in SEQ ID NO: 16 is referred to as LA229. In another embodiment, a truncated ActA comprises from amino acid 30 to amino acid 229 of the full length ActA sequence. In another embodiment, SEQ ID NO: 16 comprises from about amino acid 30 to about amino acid 229 of the full length ActA sequence. In another embodiment, a truncated ActA comprises from about amino acid 30 to amino acid 229 of SEQ ID NO: 14. In another embodiment, SEQ ID NO: 16 comprises from amino acid 30 to amino acid 229 of SEQ ID NO: 14.

[00443] In another embodiment, a truncated ActA sequence disclosed herein is further fused to an hly signal peptide at the N-terminus. In another embodiment, the truncated ActA fused to hly signal peptide comprises SEQ ID NO: 138:

M K K I M L V F I T L I L V S L P I A Q Q T E A S R A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P T L Q V E R R H

P G L S SD S A A E I K K R R K A I A S S D S E L E S L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D K.

[00444] In another embodiment, a truncated ActA fused to hly signal peptide is encoded by a sequence comprising SEQ ID NO: 139:

Atgaaaaaaataatgctagtttttattacacttatattagttagtctaccaattgcgcaacaaactgaagca***tctaga***gcgacagatagcg aagattccagtctaaacacagatgaatgggaagaagaaaaaacagaagagcagccaagcgaggtaaatacgggaccaagatacga aactgcacgtgaagtaagttcacgtgatattgaggaactagaaaaatcgaataaagtgaaaaatacgaacaaagcagacctaatagca atgttgaaagcaaaagcagagaaaggtccgaataacaataataacaacggtgagcaaacaggaaatgtggctataaatgaagaggct tcaggagtcgaccgaccaactctgcaagtggagcgtcgtcatccaggtctgtcatcggatagcgcagcggaaattaaaaaaagaaga aaagccatagcgtcgtcggatagtgagcttgaaagccttacttatccagataaaccaacaaaagcaaataagagaaaagtggcgaaag agtcagttgtggatgcttctgaaagtgacttagattctagcatgcagtcagcagacgagtctacaccacaacctttaaaagcaaatcaa aaccatttttccctaaagtatttaaaaaaataaaagatgcggggaaatgggtacgtgataaa (SEQ ID NO: 139). In another embodiment, SEQ ID NO: 139 comprises a sequence encoding a linker region (see bold, italic text) that is used to create a unique restriction enzyme site for XbaI so that different polypeptides, heterologous antigens, etc. can be cloned after the signal sequence. Hence, it will be appreciated by a skilled artisan that signal peptidases act on the sequences before the linker region to cleave signal peptide.

[00445] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 17:

A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N N N N N N G E Q T G N V A I N E E A S G V D R P T L Q V E R R H P G L S S D S A A E I K K R R K A I A S S D S E L E S L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q S A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D K I D E N P E V K K A I V D K S A G L I D Q L L T K K K S E E V N A S D F P P P P T D E E L R L A L P E T P M L L G F N A P T P S E P S S F E F P P P P T D E E L R L A L P E T P M L L G F N A P A T S E P S S (SEQ ID NO: 17). In another embodiment, a truncated ActA as set forth in SEQ ID NO: 17 is referred to as ActA/PEST3. In another embodiment, this truncated ActA comprises from the first 30 to amino acid 332 of the full length ActA sequence. In another embodiment, SEQ ID NO: 17 comprises from the first 30 to amino acid 332 of the full length ActA sequence. In another embodiment, a truncated ActA comprises from about the first 30 to amino acid 332 of SEQ ID NO: 14. In another embodiment, SEQ ID NO: 17

comprises from the first 30 to amino acid 332 of SEQ ID NO: 14.

[00446] In another embodiment, a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 18:

A T D S E D S S L N T D E W E E E K T E E Q P S E V N T G P R Y E T A R E V S S R D
5 I E E L E K S N K V K N T N K A D L I A M L K A K A E K G P N N N N N N G E Q T G
N V A I N E E A S G V D R P T L Q V E R R H P G L S S D S A A E I K K R R K A I A S
S D S E L E S L T Y P D K P T K A N K R K V A K E S V V D A S E S D L D S S M Q S
A D E S T P Q P L K A N Q K P F F P K V F K K I K D A G K W V R D K I D E N P E V
K K A I V D K S A G L I D Q L L T K K K S E E V N A S D F P P P P T D E E L R L A L
10 P E T P M L L G F N A P T P S E P S S F E F P P P P T D E E L R L A L P E T P M L L G
F N A P A T S E P S S F E F P P P P T E D E L E I M R E T A P S L D S S F T S G D L A
S L R S A I N R H S E N F S D F P L I P T E E E L N G R G G R P T S E (SEQ ID NO: 18).
In another embodiment, a truncated ActA as set forth in SEQ ID NO: 18 is referred to as ActA/PEST4. In another embodiment, this truncated ActA comprises from the first 30 to
15 amino acid 399 of the full length ActA sequence. In another embodiment, SEQ ID NO: 18 comprises from the first 30 to amino acid 399 of the full length ActA sequence. In another embodiment, a truncated ActA comprises from the first 30 to amino acid 399 of SEQ ID NO: 14. In another embodiment, SEQ ID NO: 18 comprises from the first 30 to amino acid 399 of SEQ ID NO: 14.

20 [00447] In another embodiment, "truncated ActA" or "ΔActA" refers to a fragment of ActA that comprises a PEST domain. In another embodiment, the terms refer to an ActA fragment that comprises a PEST sequence.

In another embodiment, the recombinant nucleotide encoding a truncated ActA protein comprises the sequence set forth in SEQ ID NO: 19:

25 atgcgtgcgatgatggtggttttcattactgccaattgcattacgattaaccccgacataatatttgcagcgacagatagcgaagattctag
tctaaacacagatgaatgggaagaagaaaaaacagaagagcaaccaagcgaggtaaatacgggaccaagatacgaaactgcacgt
gaagtaagttcacgtgatattaaagaactagaaaaatcgaataaagtgagaaatacgaacaaagcagacctaatagcaatgttgaaaga
aaaagcagaaaaaggtccaaatatcaataataacaacagtgaacaaactgagaatgcggctataaatgaagaggcttcaggagccga
ccgaccagctatacaagtggagcgtcgtcatccaggattgccatcggatagcgcagcggaaattaaaaaaagaaggaaagccatagc
30 atcatcggatagtgagcttgaaagccttacttatccggataaaccaacaaaagtaaataagaaaaaagtggcgaaagagtcagttgcgg
atgcttctgaaagtgacttagattctagcatgcagtcagcagatgagtcttcaccacaacctttaaaagcaaaccaacaaccatttttccct
aaagtatttaaaaaaataaaagatgcggggaaatgggtacgtgataaaatcgacgaaaatcctgaagtaaagaaagcgattgttgataa
aagtgcagggttaattgaccaattattaaccaaaaagaaaagtgaagaggtaaatgcttcggacttcccgccaccacctacggatgaag

agttaagacttgctttgccagagacaccaatgcttcttggttttaatgctcctgctacatcagaaccgagctcattcgaatttccaccaccac
ctacggatgaagagttaagacttgctttgccagagacgccaatgcttcttggttttaatgctcctgctacatcggaaccgagctcgttcgaa
tttccaccgcctccaacagaagatgaactagaaatcatccgggaaacagcatcctcgctagattctagttttacaagaggggatttagcta
gtttgagaaatgctattaatcgccatagtcaaaatttctctgatttcccaccaatcccaacagaagaagagttgaacgggagaggcggta
gacca.

[00448] In another embodiment, the recombinant nucleotide has the sequence set forth in SEQ ID NO: 19. In another embodiment, the recombinant nucleotide comprises any other sequence that encodes a fragment of an ActA protein.

[00449] In another embodiment, the ActA fragment consists of about the first 100 AA of the ActA protein.

[00450] In another embodiment, the ActA fragment consists of about residues 1-25. In another embodiment, the ActA fragment consists of about residues 1-50. In another embodiment, the ActA fragment consists of about residues 1-75. In another embodiment, the ActA fragment consists of about residues 1-100. In another embodiment, the ActA fragment consists of about residues 1-125. In another embodiment, the ActA fragment consists of about residues 1-150. In another embodiment, the ActA fragment consists of about residues 1-175. In another embodiment, the ActA fragment consists of about residues 1-200. In another embodiment, the ActA fragment consists of about residues 1-225. In another embodiment, the ActA fragment consists of about residues 1-250. In another embodiment, the ActA fragment consists of about residues 1-275. In another embodiment, the ActA fragment consists of about residues 1-300. In another embodiment, the ActA fragment consists of about residues 1-325. In another embodiment, the ActA fragment consists of about residues 1-338. In another embodiment, the ActA fragment consists of about residues 1-350. In another embodiment, the ActA fragment consists of about residues 1-375. In another embodiment, the ActA fragment consists of about residues 1-400. In another embodiment, the ActA fragment consists of about residues 1-450. In another embodiment, the ActA fragment consists of about residues 1-500. In another embodiment, the ActA fragment consists of about residues 1-550. In another embodiment, the ActA fragment consists of about residues 1-600. In another embodiment, the ActA fragment consists of about residues 1-639. In another embodiment, the ActA fragment consists of about residues 30-100. In another embodiment, the ActA fragment consists of about residues 30-125. In another embodiment, the ActA fragment consists of about residues 30-150. In another embodiment, the ActA fragment consists of about residues 30-175. In another embodiment, the ActA fragment consists of about residues 30-200. In another

embodiment, the ActA fragment consists of about residues 30-225. In another embodiment, the ActA fragment consists of about residues 30-250. In another embodiment, the ActA fragment consists of about residues 30-275. In another embodiment, the ActA fragment consists of about residues 30-300. In another embodiment, the ActA fragment consists of about residues 30-325. In another embodiment, the ActA fragment consists of about residues 30-338. In another embodiment, the ActA fragment consists of about residues 30-350. In another embodiment, the ActA fragment consists of about residues 30-375. In another embodiment, the ActA fragment consists of about residues 30-400. In another embodiment, the ActA fragment consists of about residues 30-450. In another embodiment, the ActA fragment consists of about residues 30-500. In another embodiment, the ActA fragment consists of about residues 30-550. In another embodiment, the ActA fragment consists of about residues 1-600. In another embodiment, the ActA fragment consists of about residues 30-604.

[00451] In another embodiment, the ActA fragment contains residues of a homologous ActA protein that correspond to one of the above AA ranges. The residue numbers need not, in another embodiment, correspond exactly with the residue numbers enumerated above; e.g. if the homologous ActA protein has an insertion or deletion, relative to an ActA protein utilized herein, then the residue numbers can be adjusted accordingly. In another embodiment, the ActA fragment is any other ActA fragment known in the art.

[00452] In another embodiment, a homologous ActA refers to identity to an ActA sequence (e.g. to one of SEQ ID No: 11-18) of greater than 70%. In another embodiment, a homologous ActA refers to identity to one of SEQ ID No: 11-18 of greater than 72%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 75%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 78%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 80%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 82%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 83%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 85%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 87%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 88%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 greater than 90%. In another embodiment, a homologous refers to

identity to one of SEQ ID No: 11-18 of greater than 92%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 93%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 95%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 96%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 97%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 98%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of greater than 99%. In another embodiment, a homologous refers to identity to one of SEQ ID No: 11-18 of 100%.

[00453] It will be appreciated by the skilled artisan that the term “homology,” when in reference to any nucleic acid sequence provided herein may encompass a percentage of nucleotides in a candidate sequence that is identical with the nucleotides of a corresponding native nucleic acid sequence .

[00454] Homology is, in one embodiment, determined by computer algorithm for sequence alignment, by methods well described in the art. For example, computer algorithm analysis of nucleic acid sequence homology may include the utilization of any number of software packages available, such as, for example, the BLAST, DOMAIN, BEAUTY (BLAST Enhanced Alignment Utility), GENPEPT and TREMBL packages.

[00455] In another embodiment, “homology” refers to identity to a sequence selected from the sequences provided herein of greater than 68%. In another embodiment, “homology” refers to identity to a sequence selected from the sequences provided herein of greater than 70%. In another embodiment, “homology” refers to identity to a sequence selected from the sequences provided herein of greater than 72%. In another embodiment, the identity is greater than 75%. In another embodiment, the identity is greater than 78%. In another embodiment, the identity is greater than 80%. In another embodiment, the identity is greater than 82%. In another embodiment, the identity is greater than 83%. In another embodiment, the identity is greater than 85%. In another embodiment, the identity is greater than 87%. In another embodiment, the identity is greater than 88%. In another embodiment, the identity is greater than 90%. In another embodiment, the identity is greater than 92%. In another embodiment, the identity is greater than 93%. In another embodiment, the identity is greater than 95%. In another embodiment, the identity is greater than 96%. In another embodiment, the identity is greater than 97%. In another embodiment, the identity is greater than 98%. In another embodiment, the identity is greater than 99%. In another embodiment, the identity is 100%.

[00456] In another embodiment, homology is determined via determination of candidate sequence hybridization, methods of which are well described in the art (See, for example, "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., Eds. (1985); Sambrook et al., 2001, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and
5 Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y). For example methods of hybridization may be carried out under moderate to stringent conditions, to the complement of a DNA encoding a native caspase peptide. Hybridization conditions being, for example, overnight incubation at 42 ℃ in a solution comprising: 10-20 % formamide, 5 X SSC (150 mM NaCl, 15 mM trisodium
10 citrate), 50 mM sodium phosphate (pH 7. 6), 5 X Denhardt's solution, 10 % dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA .

[00457] In one embodiment, the recombinant *Listeria* strain provided herein lacks antibiotic resistance genes.

[00458] In one embodiment, the recombinant *Listeria* provided herein is capable of escaping
15 the phagolysosome.

[00459] In one embodiment, the *Listeria* genome comprises a deletion of the endogenous *actA* gene, which in one embodiment is a virulence factor. In one embodiment, the heterologous antigen or antigenic polypeptide is integrated in frame with LLO in the *Listeria* chromosome. In another embodiment, the integrated nucleic acid molecule is integrated in
20 frame with ActA into the *actA* locus. In another embodiment, the chromosomal nucleic acid encoding ActA is replaced by a nucleic acid molecule encoding an antigen.

[00460] In one embodiment, a peptide provided herein comprises one or more neo-epitopes. In one embodiment, a peptide provided herein is comprised by an antigen. In another embodiment, a peptide provided herein is an antigen fragment. In one embodiment, an
25 antigen provided herein comprises one or more neo-epitopes. In another embodiment, the antigen is a heterologous antigen or a self-antigen. In one embodiment, a heterologous antigen or self-antigen provided herein is a tumor-associated antigen. It will be appreciated by a skilled artisan that the term "heterologous" may refer to an antigen, or portion thereof, which is not naturally or normally expressed from a bacterium. In one embodiment, a
30 heterologous antigen comprises an antigen not naturally or normally expressed from a *Listeria* strain. In another embodiment, the tumor-associated antigen is a naturally occurring tumor-associated antigen. In another embodiment, the tumor-associated antigen is a synthetic

tumor-associated antigen. In yet another embodiment, the tumor-associated antigen is a chimeric tumor-associated antigen. In still another embodiment, the tumor-associated antigen comprises one or more neo-epitopes. In still another embodiment, the tumor-associated antigen is a neo-antigen.

[00461] In one embodiment, a recombinant *Listeria* provided herein comprises a nucleic acid molecule comprising a first open reading frame encoding recombinant polypeptide comprising one or more peptides, wherein said one or more peptides comprise one or more neo-epitopes. In another embodiment, the recombinant polypeptide further comprises a truncated LLO protein, a truncated ActA protein or PEST sequence fused to a peptide provided herein.

[00462] In another embodiment, the nucleic acid molecule provided herein comprises a first open reading frame encoding a recombinant polypeptide comprising a truncated LLO protein, a truncated ActA protein or a PEST sequence, wherein the truncated LLO protein, a truncated ActA protein or a PEST sequence peptide is not fused to a heterologous antigen. In another embodiment, the first open reading frame encodes a truncated LLO protein. In another embodiment, the first open reading frame encodes a truncated ActA protein. In another embodiment, the first open reading frame encodes a truncated LLO protein. In another embodiment, the first open reading frame encodes a truncated ActA protein. In another embodiment, the first open reading frame encodes a truncated LLO protein. In another embodiment, the first open reading frame encodes a truncated ActA protein consisting of an N-terminal ActA protein or fragment thereof.

[00463] It will be appreciated by a skilled artisan that the terms "antigen," "antigen fragment," "antigen portion," "heterologous protein," "heterologous protein antigen," "protein antigen," "antigen," "antigenic polypeptide," or their grammatical equivalents, which are used interchangeably herein, may refer to a polypeptide, peptide or recombinant peptide as described herein that is processed and presented on MHC class I and/or class II molecules present in a subject's cells leading to the mounting of an immune response when present in, or in another embodiment, detected by, the host. In one embodiment, the antigen may be foreign to the host. In another embodiment, the antigen might be present in the host but the host does not elicit an immune response against it because of immunologic tolerance. In another embodiment, the antigen is a neo-antigen comprising one or more neo-epitopes, wherein one or more neo-epitopes are T-cell epitopes. In another embodiment, the antigen or a peptide fragment thereof comprises one or more neo-epitopes that are T-cell epitopes.

[00464] In another embodiment, an antigen comprises at least one neo-epitope. In one embodiment, an antigen is a neo-antigen comprising at least one neo-epitope. In one embodiment, a neo-epitope is an epitope that has not been previously recognized by the immune system. Neo-antigens are often associated with tumor antigens and are found in oncogenic cells. Neo-antigens and, by extension, neo-antigenic determinants (neo-epitopes) may be formed when a protein undergoes further modification within a biochemical pathway such as glycosylation, phosphorylation or proteolysis. This, by altering the structure of the protein, can produce new or “neo” epitopes.

[00465] In one embodiment, a *Listeria* provided herein comprises a minigene nucleic acid construct, said construct comprising one or more open reading frames encoding a chimeric protein, wherein said chimeric protein comprises:

a. a bacterial secretion signal sequence;
b. a ubiquitin (Ub) protein;
c. one or more peptides comprising said one or more neo-epitopes; and,

wherein said signal sequence, said ubiquitin and said one or more peptides in a.-c. are respectively arranged in tandem, or are operatively linked, from the amino-terminus to the carboxy-terminus.

[00466] In another embodiment, a bacterial signal sequence provided herein is a *Listerial* signal sequences, which in another embodiment is an hly or an *actA* signal sequence. In another embodiment, the bacterial signal sequence is any other signal sequence known in the art. In another embodiment, a recombinant *Listeria* comprising a minigene nucleic acid construct further comprises two or more open reading frames linked by a Shine-Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. In another embodiment, a recombinant *Listeria* comprising a minigene nucleic acid construct further comprises one to four open reading frames linked by a Shine-Dalgarno ribosome binding site nucleic acid sequence between each open reading frame. In another embodiment, each open reading frame encodes a different peptide.

[00467] In another embodiment, provided herein is a recombinant attenuated *Listeria* strain comprising a recombinant nucleic acid construct comprising an open reading frame encoding a bacterial secretion signal sequence (SS), a ubiquitin (Ub) protein, and a peptide sequence. In another embodiment, the nucleic acid construct encodes a chimeric protein comprising a bacterial secretion signal sequence, a ubiquitin protein, and a peptide sequence. In one embodiment, the chimeric protein is arranged in the following manner (SS-Ub-Peptide).

[00468] In one embodiment, the nucleic acid construct comprises a codon that corresponds to the carboxy-terminus of the peptide moiety is followed by two stop codons to ensure termination of protein synthesis.

[00469] In one embodiment, a minigene nucleic acid construct provided in the compositions and methods described herein comprises an expression system that is designed to facilitate panels of recombinant proteins containing distinct peptide moieties at the carboxy terminus. This is accomplished, in one embodiment, by a PCR reaction utilizing a sequence encoding one of the bacterial secretion signal sequence-ubiquitin-peptide (SS-Ub-Peptide) constructs as a template. In one embodiment, using a primer that extends into the carboxy-terminal region of the Ub sequence and introducing codons for the desired peptide sequence at the 3' end of the primer, a new SS-Ub-Peptide sequence can be generated in a single PCR reaction (see Examples herein). The 5' primer encoding the bacterial promoter and the first few nucleotides of the bacterial secretion signal sequence may be the same for all the constructs. A schematic representation of this construct is provided in Figure 26A-C herein.

[00470] In one embodiment, nucleic acids encoding recombinant polypeptides provided herein also comprise a signal peptide or signal sequence. In one embodiment, the bacterial secretion signal sequence encoded by a nucleic acid constructs or nucleic acid molecule provided herein is a *Listeria* secretion signal sequence. In another embodiment, a fusion protein of methods and compositions of the present invention comprises an LLO signal sequence from Listeriolysin O (LLO). It will be appreciated by a skilled artisan that an antigen or a peptide comprising one or more neo-epitopes provided herein may be expressed through the use of a signal sequence, such as a *Listerial* signal sequence, for example, the hemolysin (*hly*) signal sequence or the *actA* signal sequence. Alternatively, for example, foreign genes can be expressed downstream from a *L. monocytogenes* promoter without creating a fusion protein. In another embodiment, the signal peptide is bacterial (*Listerial* or *non-Listerial*). In one embodiment, the signal peptide is native to the bacterium. In another embodiment, the signal peptide is foreign to the bacterium. In another embodiment, the signal peptide is a signal peptide from *Listeria monocytogenes*, such as a secA1 signal peptide. In another embodiment, the signal peptide is an Usp45 signal peptide from *Lactococcus lactis*, or a Protective Antigen signal peptide from *Bacillus anthracis*. In another embodiment, the signal peptide is a secA2 signal peptide, such the p60 signal peptide from *Listeria monocytogenes*. In addition, the recombinant nucleic acid molecule optionally comprises a third polynucleotide sequence encoding p60, or a fragment thereof. In another embodiment,

the signal peptide is a Tat signal peptide, such as a *B. subtilis* Tat signal peptide (e.g., PhoD). In one embodiment, the signal peptide is in the same translational reading frame encoding the recombinant polypeptide.

[00471] In another embodiment, the secretion signal sequence is from a *Listeria* protein. In another embodiment, the secretion signal is an $ActA_{300}$ secretion signal. In another embodiment, the secretion signal is an $ActA_{100}$ secretion signal.

[00472] In one embodiment, the nucleic acid construct comprises an open reading frame encoding a ubiquitin protein. In one embodiment, the ubiquitin is a full-length protein. It will be appreciated by the skilled artisan that the Ubiquitin in the expressed construct provided herein (expressed from the nucleic acid construct provided herein) is cleaved at the carboxy-terminus from the rest of the recombinant chimeric protein expressed from the nucleic acid construct through the action of hydrolases upon entry to the host cell cytosol. This liberates the amino-terminus of the peptide moiety, producing a peptide (length depends on the specific peptide) in the host cell cytosol.

[00473] In one embodiment, the peptide encoded by the nucleic acid constructs provided herein is 8-10 amino acids (AA) in length. In another embodiment, the peptide is 10-20 AA long. In another embodiment, the peptide is a 21-30 AA long. In another embodiment, the peptide is 31-50 AA long. In another embodiment, the peptide is 51-100 AA long.

[00474] In one embodiment, a nucleic acid molecule provided herein further comprises a second open reading frame encoding a metabolic enzyme. In another embodiment, the metabolic enzyme complements an endogenous gene that is lacking in the chromosome of the recombinant *Listeria* strain. In another embodiment, the metabolic enzyme complements an endogenous gene that is mutated in the chromosome of the recombinant *Listeria* strain. In another embodiment, the metabolic enzyme encoded by the second open reading frame is an alanine racemase enzyme (dal). In another embodiment, the metabolic enzyme encoded by the second open reading frame is a D-amino acid transferase enzyme (dat). In another embodiment, the *Listeria* strains provided herein comprise a mutation in the endogenous dal/dat genes. In another embodiment, the *Listeria* lacks the dal/dat genes.

[00475] In another embodiment, a nucleic acid molecule of the methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, the first open reading frame of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, the

second open reading frame of methods and compositions of the present invention is operably linked to a promoter/regulatory sequence. In another embodiment, each of the open reading frames are operably linked to a promoter/regulatory sequence.

[00476] "Metabolic enzyme" refers, in another embodiment, to an enzyme involved in synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme required for synthesis of a nutrient required by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a nutrient utilized by the host bacteria. In another embodiment, the term refers to an enzyme involved in synthesis of a nutrient required for sustained growth of the host bacteria. In another embodiment, the enzyme is required for synthesis of the nutrient.

[00477] In another embodiment, the recombinant *Listeria* is an attenuated auxotrophic strain. In another embodiment, the recombinant *Listeria* is an Lm-LLO-E7 strain described in US Patent No. 8,114,414, which is incorporated by reference herein in its entirety.

[00478] In one embodiment the attenuated strain is Lm dal(-)dat(-) (*Lmdd*). In another embodiment, the attenuated strains is Lm dal(-)dat(-)ΔactA (*LmddA*). *LmddA* is based on a *Listeria* immunotherapy vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for a desired heterologous antigen or truncated LLO expression *in vivo* and *in vitro* by complementation of *dal* gene.

[00479] In another embodiment the attenuated strain is *LmddA*. In another embodiment, the attenuated strain is LmΔactA. In another embodiment, the attenuated strain is LmΔPrfA. In another embodiment, the attenuated strain is LmΔPrfA*. In another embodiment, the attenuated strain is LmΔPlcB. In another embodiment, the attenuated strain is LmΔPlcA. In another embodiment, the strain is the double mutant or triple mutant of any of the above-mentioned strains. In another embodiment, this strain exerts a strong adjuvant effect which is an inherent property of *Listeria*-based immunotherapies. In another embodiment, this strain is constructed from the EGD *Listeria* backbone. In another embodiment, the strain used in the invention is a *Listeria* strain that expresses a non-hemolytic LLO.

[00480] In another embodiment, the *Listeria* strain is an auxotrophic mutant. In another embodiment, the *Listeria* strain is deficient in a gene encoding a vitamin synthesis gene. In another embodiment, the *Listeria* strain is deficient in a gene encoding pantothenic acid synthase.

[00481] In one embodiment, the generation of AA strains of *Listeria* deficient in D-alanine,

for example, may be accomplished in a number of ways that are well known to those of skill in the art, including deletion mutagenesis, insertion mutagenesis, and mutagenesis which results in the generation of frameshift mutations, mutations which cause premature termination of a protein, or mutation of regulatory sequences which affect gene expression. In another embodiment, mutagenesis can be accomplished using recombinant DNA techniques or using traditional mutagenesis technology using mutagenic chemicals or radiation and subsequent selection of mutants. In another embodiment, deletion mutants are preferred because of the accompanying low probability of reversion of the auxotrophic phenotype. In another embodiment, mutants of D-alanine which are generated according to the protocols presented herein may be tested for the ability to grow in the absence of D-alanine in a simple laboratory culture assay. In another embodiment, those mutants which are unable to grow in the absence of this compound are selected for further study.

[00482] In another embodiment, in addition to the aforementioned D-alanine associated genes, other genes involved in synthesis of a metabolic enzyme, as provided herein, may be used as targets for mutagenesis of *Listeria*.

[00483] In another embodiment, the metabolic enzyme complements an endogenous metabolic gene that is lacking in the remainder of the chromosome of the recombinant bacterial strain. In one embodiment, the endogenous metabolic gene is mutated in the chromosome. In another embodiment, the endogenous metabolic gene is deleted from the chromosome. In another embodiment, the metabolic enzyme is an amino acid metabolism enzyme. In another embodiment, the metabolic enzyme catalyzes a formation of an amino acid used for a cell wall synthesis in the recombinant *Listeria* strain. In another embodiment, the metabolic enzyme is an alanine racemase enzyme. In another embodiment, the metabolic enzyme is a D-amino acid transferase enzyme. Each possibility represents a separate embodiment of the methods and compositions as provided herein.

[00484] In one embodiment, the auxotrophic *Listeria* strain comprises an episomal expression vector comprising a metabolic enzyme that complements the auxotrophy of the auxotrophic *Listeria* strain. In another embodiment, the construct is contained in the *Listeria* strain in an episomal fashion. In another embodiment, the foreign antigen is expressed from a plasmid vector harbored by the recombinant *Listeria* strain. In another embodiment, the episomal expression plasmid vector lacks an antibiotic resistance marker. In one embodiment, an antigen of the methods and compositions as provided herein is fused to an polypeptide comprising a PEST sequence.

[00485] In another embodiment, the *Listeria* strain is deficient in an amino acid (AA) metabolism enzyme. In another embodiment, the *Listeria* strain is deficient in a D-glutamic acid synthase gene. In another embodiment, the *Listeria* strain is deficient in the *dat* gene. In another embodiment, the *Listeria* strain is deficient in the *dal* gene. In another embodiment, the *Listeria* strain is deficient in the *dga* gene. In another embodiment, the *Listeria* strain is deficient in a gene involved in the synthesis of diaminopimelic acid. *CysK*. In another embodiment, the gene is vitamin-B12 independent methionine synthase. In another embodiment, the gene is *trpA*. In another embodiment, the gene is *trpB*. In another embodiment, the gene is trpE. In another embodiment, the gene is *asnB*. In another embodiment, the gene is *gltD*. In another embodiment, the gene is *gltB*. In another embodiment, the gene is *leuA*. In another embodiment, the gene is *argG*. In another embodiment, the gene is *thrC*. In another embodiment, the *Listeria* strain is deficient in one or more of the genes described hereinabove.

[00486] In another embodiment, the *Listeria* strain is deficient in a synthase gene. In another embodiment, the gene is an AA synthesis gene. In another embodiment, the gene is *folP*. In another embodiment, the gene is dihydrouridine synthase family protein. In another embodiment, the gene is *ispD*. In another embodiment, the gene is *ispF*. In another embodiment, the gene is phosphoenolpyruvate synthase. In another embodiment, the gene is *hisF*. In another embodiment, the gene is *hisH*. In another embodiment, the gene is *fliI*. In another embodiment, the gene is ribosomal large subunit pseudouridine synthase. In another embodiment, the gene is *ispD*. In another embodiment, the gene is bifunctional GMP synthase/glutamine amidotransferase protein. In another embodiment, the gene is *cobS*. In another embodiment, the gene is *cobB*. In another embodiment, the gene is *cbiD*. In another embodiment, the gene is uroporphyrin-III C-methyltransferase/ uroporphyrinogen-III synthase. In another embodiment, the gene is *cobQ*. In another embodiment, the gene is *uppS*. In another embodiment, the gene is *truB*. In another embodiment, the gene is *dxs*. In another embodiment, the gene is *mvaS*. In another embodiment, the gene is *dapA*. In another embodiment, the gene is *ispG*. In another embodiment, the gene is *folC*. In another embodiment, the gene is citrate synthase. In another embodiment, the gene is *argJ*. In another embodiment, the gene is 3-deoxy-7-phosphoheptulonate synthase. In another embodiment, the gene is indole-3-glycerol-phosphate synthase. In another embodiment, the gene is anthranilate synthase/ glutamine amidotransferase component. In another embodiment, the gene is *menB*. In another embodiment, the gene is menaquinone-specific isochorismate

synthase. In another embodiment, the gene is phosphoribosylformylglycinamidine synthase I or II. In another embodiment, the gene is phosphoribosylaminoimidazole-succinocarboxamide synthase. In another embodiment, the gene is *carB*. In another embodiment, the gene is *carA*. In another embodiment, the gene is *thyA*. In another embodiment, the gene is *mgsA*. In another embodiment, the gene is *aroB*. In another embodiment, the gene is *hepB*. In another embodiment, the gene is *rluB*. In another embodiment, the gene is *ilvB*. In another embodiment, the gene is *ilvN*. In another embodiment, the gene is *alsS*. In another embodiment, the gene is *fabF*. In another embodiment, the gene is *fabH*. In another embodiment, the gene is pseudouridine synthase. In another embodiment, the gene is *pyrG*. In another embodiment, the gene is *truA*. In another embodiment, the gene is *pabB*. In another embodiment, the gene is an atp synthase gene (e.g. *atpC, atpD-2, aptG, atpA-2*, etc).

[00487] In another embodiment, the gene is *phoP*. In another embodiment, the gene is *aroA*. In another embodiment, the gene is *aroC*. In another embodiment, the gene is *aroD*. In another embodiment, the gene is *plcB*.

[00488] In another embodiment, the *Listeria* strain is deficient in a peptide transporter. In another embodiment, the gene is ABC transporter/ ATP-binding/permease protein. In another embodiment, the gene is oligopeptide ABC transporter/ oligopeptide-binding protein. In another embodiment, the gene is oligopeptide ABC transporter/ permease protein. In another embodiment, the gene is zinc ABC transporter/ zinc-binding protein. In another embodiment, the gene is sugar ABC transporter. In another embodiment, the gene is phosphate transporter. In another embodiment, the gene is ZIP zinc transporter. In another embodiment, the gene is drug resistance transporter of the EmrB/QacA family. In another embodiment, the gene is sulfate transporter. In another embodiment, the gene is proton-dependent oligopeptide transporter. In another embodiment, the gene is magnesium transporter. In another embodiment, the gene is formate/nitrite transporter. In another embodiment, the gene is spermidine/putrescine ABC transporter. In another embodiment, the gene is Na/Pi-cotransporter. In another embodiment, the gene is sugar phosphate transporter. In another embodiment, the gene is glutamine ABC transporter. In another embodiment, the gene is major facilitator family transporter. In another embodiment, the gene is glycine betaine/L-proline ABC transporter. In another embodiment, the gene is molybdenum ABC transporter. In another embodiment, the gene is techoic acid ABC transporter. In another embodiment, the gene is cobalt ABC transporter. In another embodiment, the gene is ammonium transporter.

In another embodiment, the gene is amino acid ABC transporter. In another embodiment, the gene is cell division ABC transporter. In another embodiment, the gene is manganese ABC transporter. In another embodiment, the gene is iron compound ABC transporter. In another embodiment, the gene is maltose/maltodextrin ABC transporter. In another embodiment, the
5 gene is drug resistance transporter of the Bcr/CflA family. In another embodiment, the gene is a subunit of one of the above proteins.

[00489] In one embodiment, provided herein is a nucleic acid molecule that is used to transform the *Listeria* in order to arrive at a recombinant *Listeria*. In another embodiment, the nucleic acid provided herein used to transform *Listeria* lacks a virulence gene. In another
10 embodiment, the nucleic acid molecule is integrated into the *Listeria* genome and carries a non-functional virulence gene. In another embodiment, the virulence gene is mutated in the recombinant *Listeria*. In yet another embodiment, the nucleic acid molecule is used to inactivate the endogenous gene present in the *Listeria* genome. In yet another embodiment, the virulence gene is an *actA* gene, an *inlA* gene, and *inlB* gene, an *inlC* gene, inlJ gene, a
15 *plbC* gene, a *bsh* gene, or a *prfA* gene. It is to be understood by a skilled artisan, that the virulence gene can be any gene known in the art to be associated with virulence in the recombinant *Listeria*.

[00490] In yet another embodiment the *Listeria* strain is an *inlA* mutant, an *inlB* mutant, an *inlC* mutant, an *inlJ* mutant, *prfA* mutant, *actA* mutant, a *dal/dat* mutant, a *prfA* mutant, a
20 *plcB* deletion mutant, or a double mutant lacking both *plcA* and *plcB* or *actA* and *inlB*. In another embodiment, the *Listeria* comprise a deletion or mutation of these genes individually or in combination. In another embodiment, the *Listeria* provided herein lack each one of genes. In another embodiment, the *Listeria* provided herein lack at least one and up to ten of any gene provided herein, including the *actA*, *prfA*, and *dal/dat* genes. In another
25 embodiment, the *prfA* mutant is a D133V *prfA* mutant.

[00491] In one embodiment, the live attenuated *Listeria* is a recombinant *Listeria*. In another embodiment, the recombinant *Listeria* comprises a mutation or a deletion of a genomic *internalin C* (*inlC*) gene. In another embodiment, the recombinant *Listeria* comprises a mutation or a deletion of a genomic *actA* gene and a genomic *internalin C* gene. In one
30 embodiment, translocation of *Listeria* to adjacent cells is inhibited by the deletion of the *actA* gene and/or the *inlC* gene, which are involved in the process, thereby resulting in unexpectedly high levels of attenuation with increased immunogenicity and utility as a immunotherapy backbone.

[00492] In one embodiment, the metabolic gene, the virulence gene, etc. is lacking in a chromosome of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the chromosome and in any episomal genetic element of the *Listeria* strain. In another embodiment, the metabolic gene, virulence gene, etc. is lacking in the genome of the virulence strain. In one embodiment, the virulence gene is mutated in the chromosome. In another embodiment, the virulence gene is deleted from the chromosome.

[00493] In one embodiment, the recombinant *Listeria* strain provided herein is attenuated. In another embodiment, the recombinant *Listeria* lacks the *actA* virulence gene. In another embodiment, the recombinant *Listeria* lacks the *prfA* virulence gene. In another embodiment, the recombinant *Listeria* lacks the *inlB* gene. In another embodiment, the recombinant *Listeria* lacks both, the *actA* and *inlB* genes. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA* gene. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *inlB* gene. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *inlC* gene. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA* and *inlB* genes. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA* and *inlC* genes. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA*, *inlB*, and *inlC* genes. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA*, *inlB*, and *inlC* genes. In another embodiment, the recombinant *Listeria* strain provided herein comprise an inactivating mutation of the endogenous *actA*, *inlB*, and *inlC* genes. In another embodiment, the recombinant *Listeria strain* provided herein comprise an inactivating mutation in any single gene or combination of the following genes: *actA, dal, dat, inlB, inlC, prfA, plcA, plcB*.

[00494] It will be appreciated by the skilled artisan that the term "mutation" and grammatical equivalents thereof, include any type of mutation or modification to the sequence (nucleic acid or amino acid sequence), and includes a deletion mutation, a truncation, an inactivation, a disruption, or a translocation. These types of mutations are readily known in the art.

[00495] In one embodiment, in order to select for an auxotrophic bacteria comprising a plasmid encoding a metabolic enzyme or a complementing gene provided herein, transformed

auxotrophic bacteria are grown on a media that will select for expression of the amino acid metabolism gene or the complementing gene. In another embodiment, a bacteria auxotrophic for D-glutamic acid synthesis is transformed with a plasmid comprising a gene for D-glutamic acid synthesis, and the auxotrophic bacteria will grow in the absence of D-glutamic acid, whereas auxotrophic bacteria that have not been transformed with the plasmid, or are not expressing the plasmid encoding a protein for D-glutamic acid synthesis, will not grow. In another embodiment, a bacterium auxotrophic for D-alanine synthesis will grow in the absence of D-alanine when transformed and expressing the plasmid of the present invention if the plasmid comprises an isolated nucleic acid encoding an amino acid metabolism enzyme for D-alanine synthesis. Such methods for making appropriate media comprising or lacking necessary growth factors, supplements, amino acids, vitamins, antibiotics, and the like are well known in the art, and are available commercially (Becton-Dickinson, Franklin Lakes, NJ). Each method represents a separate embodiment of the present invention.

[00496] In another embodiment, once the auxotrophic bacteria comprising the plasmid of the present invention have been selected on appropriate media, the bacteria are propagated in the presence of a selective pressure. Such propagation comprises growing the bacteria in media without the auxotrophic factor. The presence of the plasmid expressing an amino acid metabolism enzyme in the auxotrophic bacteria ensures that the plasmid will replicate along with the bacteria, thus continually selecting for bacteria harboring the plasmid. The skilled artisan, when equipped with the present disclosure and methods herein will be readily able to scale-up the production of the *Listeria* immunotherapy vector by adjusting the volume of the media in which the auxotrophic bacteria comprising the plasmid are growing.

[00497] The skilled artisan will appreciate that, in another embodiment, other auxotroph strains and complementation systems are adopted for the use with this invention.

[00498] In one embodiment, the N-terminal LLO protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the genes encoding the N-terminal LLO protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the N-terminal LLO protein fragment and heterologous antigen are operably attached via a linker peptide. In another embodiment, the N-terminal LLO protein fragment and heterologous antigen are attached via a heterologous peptide. In another embodiment, the N-terminal LLO protein fragment is N-terminal to the heterologous antigen. In another embodiment, the N-terminal LLO protein fragment is expressed and used alone, i.e., in unfused form. In another embodiment, an N-terminal LLO protein fragment is the N-

terminal-most portion of the fusion protein. In another embodiment, a truncated LLO is truncated at the C-terminal to arrive at an N-terminal LLO. In another embodiment, a truncated LLO is a non-hemolytic LLO.

[00499] In one embodiment, the N-terminal ActA protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the genes encoding the N-terminal ActA protein fragment and heterologous antigen are fused directly to one another. In another embodiment, the N-terminal ActA protein fragment and heterologous antigen are operably attached via a linker peptide. In another embodiment, the N-terminal ActA protein fragment and heterologous antigen are attached via a heterologous peptide. In another embodiment, the N-terminal ActA protein fragment is N-terminal to the heterologous antigen. In another embodiment, the N-terminal ActA protein fragment is expressed and used alone, i.e., in unfused form. In another embodiment, the N-terminal ActA protein fragment is the N-terminal-most portion of the fusion protein. In another embodiment, a truncated ActA is truncated at the C-terminal to arrive at an N-terminal ActA.

[00500] In one embodiment, the recombinant *Listeria* strain provided herein expresses the recombinant polypeptide. In another embodiment, the recombinant *Listeria* strain comprises a plasmid that encodes the recombinant polypeptide. In another embodiment, a recombinant nucleic acid provided herein is in a plasmid in the recombinant *Listeria* strain provided herein. In another embodiment, the plasmid is an episomal plasmid that does not integrate into the recombinant *Listeria* strain's chromosome. In another embodiment, the plasmid is an integrative plasmid that integrates into the *Listeria* strain's chromosome. In another embodiment, the plasmid is a multicopy plasmid.

[00501] In one embodiment, the heterologous antigen is a tumor-associated antigen. In one embodiment, the recombinant *Listeria* strain of the compositions and methods as provided herein express a heterologous antigenic polypeptide that is expressed by a tumor cell. In one embodiment, a tumor-associated antigen is a prostate specific antigen (PSA). In another embodiment, a tumor-associated antigen is a human papilloma virus (HPV) antigen. In yet another embodiment, a tumor-associated antigen is a Her2/neu chimeric antigen as described in US Patent Pub. No. US2011/014279, which is incorporated by reference herein in its entirety. In still another embodiment, a tumor-associated antigen is an angiogenic antigen.

[00502] In one embodiment, the peptide provided herein is an antigenic peptide. In another embodiment, the peptide provided herein is derived from a tumor antigen. In another

embodiment, the peptide provided herein is derived from an infectious disease antigen. In another embodiment, the peptide provided herein is derived from a self-antigen. In another embodiment, the peptide provided herein is derived from an angiogenic antigen.

[00503] In one embodiment, the antigen from which the peptide provided herein is derived from is derived from a fungal pathogen, bacteria, parasite, helminth, or viruses. In other embodiments, the antigen from which the peptide derived herein is selected from tetanus toxoid, hemagglutinin molecules from influenza virus, diphtheria toxoid, HIV gp120, HIV gag protein, IgA protease, insulin peptide B, *Spongospora subterranea* antigen, vibriose antigens, *Salmonella* antigens, pneumococcus antigens, respiratory syncytial virus antigens, *Haemophilus influenza* outer membrane proteins, Helicobacter pylori urease, *Neisseria meningitidis* pilins, N. gonorrhoeae pilins, the melanoma-associated antigens (TRP-2, MAGE-1, MAGE-3, gp-100, tyrosinase, MART-1, HSP-70, beta-HCG), human papilloma virus antigens E1 and E2 from type HPV-16, -18, -31, -33, -35 or -45 human papilloma viruses, the tumor antigens CEA, the ras protein, mutated or otherwise, the p53 protein, mutated or otherwise, Muc1, mesothelin, EGFRVIII or pSA.

[00504] In other embodiments, the peptide is derived from an antigen that is associated with one of the following diseases; cholera, diphtheria, Haemophilus, hepatitis A, hepatitis B, influenza, measles, meningitis, mumps, pertussis, small pox, pneumococcal pneumonia, polio, rabies, rubella, tetanus, tuberculosis, typhoid, Varicella-zoster, whooping cough, yellow fever, the immunogens and antigens from Addison's disease, allergies, anaphylaxis, Bruton's syndrome, cancer, including solid and blood borne tumors, eczema, Hashimoto's thyroiditis, polymyositis, dermatomyositis, type 1 diabetes mellitus, acquired immune deficiency syndrome, transplant rejection, such as kidney, heart, pancreas, lung, bone, and liver transplants, Graves' disease, polyendocrine autoimmune disease, hepatitis, microscopic polyarteritis, polyarteritis nodosa, pemphigus, primary biliary cirrhosis, pernicious anemia, coeliac disease, antibody-mediated nephritis, glomerulonephritis, rheumatic diseases, systemic lupus erthematosus, rheumatoid arthritis, seronegative spondylarthritides, rhinitis, sjogren's syndrome, systemic sclerosis, sclerosing cholangitis, Wegener's granulomatosis, dermatitis herpetiformis, psoriasis, vitiligo, multiple sclerosis, encephalomyelitis, Guillain-Barre syndrome, myasthenia gravis, Lambert-Eaton syndrome, sclera, episclera, uveitis, chronic mucocutaneous candidiasis, urticaria, transient hypogammaglobulinemia of infancy, myeloma, X-linked hyper IgM syndrome, Wiskott-Aldrich syndrome, ataxia telangiectasia, autoimmune hemolytic anemia, autoimmune thrombocytopenia, autoimmune neutropenia,

Waldenstrom's macroglobulinemia, amyloidosis, chronic lymphocytic leukemia, non-Hodgkin's lymphoma, malarial circumsporozite protein, microbial antigens, viral antigens, autoantigens, and lesteriosis.

[00505] In another embodiment, the antigen from which the peptide provided herein is derived is a tumor-associated antigen, which in one embodiment, is one of the following tumor antigens: a MAGE (Melanoma-Associated Antigen E) protein, e.g. MAGE 1, MAGE 2, MAGE 3, MAGE 4, a tyrosinase; a mutant ras protein; a mutant p53 protein; p97 melanoma antigen, a ras peptide or p53 peptide associated with advanced cancers; the HPV 16/18 antigens associated with cervical cancers, KLH antigen associated with breast carcinoma, CEA (carcinoembryonic antigen) associated with colorectal cancer, gp100, a MART1 antigen associated with melanoma, or the PSA antigen associated with prostate cancer. In another embodiment, the antigen for the compositions and methods as provided herein are melanoma-associated antigens, which in one embodiment are TRP-2, MAGE-1, MAGE-3, gp-100, tyrosinase, HSP-70, beta-HCG, or a combination thereof. Other tumor-associated antigens known in the art are also contemplated in the present invention.

[00506] In one embodiment, the peptide is derived from a chimeric Her2 antigen described in US patent application serial no. 12/945,386, which is hereby incorporated by reference herein in its entirety.

[00507] In another embodiment, the peptide is derived from an antigen selected from a HPV-E7 (from either an HPV16 or HPV18 strain), a HPV-E6 (from either an HPV16 or HPV18 strain), Her-2/neu, NY-ESO-1, telomerase (TERT, SCCE, CEA, LMP-1, p53, carboxic anhydrase IX (CAIX), PSMA, a prostate stem cell antigen (PSCA), a HMW-MAA, WT-1, HIV-1 Gag, Proteinase 3, Tyrosinase related protein 2, PSA (prostate-specific antigen), EGFR-III, survivin, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), LMP-1, p53, PSMA, PSCA, Muc1, PSA (prostate-specific antigen), or a combination thereof.

[00508] In one embodiment, a polypeptide expressed by the *Listeria* of the present invention may be a neuropeptide growth factor antagonist, which in one embodiment is [D-Arg1, D-Phe5, D-Trp7,9, Leu11] substance P, [Arg6, D-Trp7,9, NmePhe8]substance P(6-11). These and related embodiments are understood by one of skill in the art.

[00509] In one embodiment, the recombinant *Listeria* strain as provided herein comprises a nucleic acid molecule encoding a tumor associated antigen, wherein the antigen comprises an HPV-E7 protein. In one embodiment, the recombinant *Listeria* strain as provided herein

comprises a nucleic acid molecule encoding HPV-E7 protein.

[00510] In one embodiment, either a whole E7 protein or a fragment thereof is fused to a LLO protein or truncation or peptide thereof, an ActA protein or truncation or peptide thereof, or a PEST-like sequence-containing peptide to generate a recombinant polypeptide or peptide of the composition and methods of the present invention. The E7 protein that is utilized (either whole or as the source of the fragments) has, in another embodiment, the sequence MHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP (SEQ ID No: 20). In another embodiment, the E7 protein is a homologue of SEQ ID No: 20. In another embodiment, the E7 protein is a variant of SEQ ID No: 20. In another embodiment, the E7 protein is an isomer of SEQ ID No: 20. In another embodiment, the E7 protein is a fragment of SEQ ID No: 20. In another embodiment, the E7 protein is a fragment of a homologue of SEQ ID No: 20. In another embodiment, the E7 protein is a fragment of a variant of SEQ ID No: 20. In another embodiment, the E7 protein is a fragment of an isomer of SEQ ID No: 20.

[00511] In another embodiment, the sequence of the E7 protein is: MHGPKATLQDIVLHLEPQNEIPVDLLCHEQLSDSEEENDEIDGVNHQHLPARRAEPQRHTMLCMCCKCEARIELVVESSADDLRAFQQLFLNTLSFVCPWCASQQ (SEQ ID No: 21). In another embodiment, the E6 protein is a homologue of SEQ ID No: 21. In another embodiment, the E6 protein is a variant of SEQ ID No: 21. In another embodiment, the E6 protein is an isomer of SEQ ID No: 21. In another embodiment, the E6 protein is a fragment of SEQ ID No: 21. In another embodiment, the E6 protein is a fragment of a homologue of SEQ ID No: 21. In another embodiment, the E6 protein is a fragment of a variant of SEQ ID No: 21. In another embodiment, the E6 protein is a fragment of an isomer of SEQ ID No: 21.

[00512] In another embodiment, the E7 protein has a sequence set forth in one of the following GenBank entries: M24215, NC_004500, V01116, X62843, or M14119. In another embodiment, the E7 protein is a homologue of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a variant of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is an isomer of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a homologue of a sequence from one of the above GenBank entries. In another embodiment, the E7 protein is a fragment of a variant of a sequence from one of the

above GenBank entries. In another embodiment, the E7 protein is a fragment of an isomer of a sequence from one of the above GenBank entries.

[00513] In one embodiment the HPV antigen is an HPV 16. In another embodiment, the HPV is an HPV-18. In another embodiment, the HPV is selected from HPV-16 and HPV-18. In another embodiment, the HPV is an HPV-31. In another embodiment, the HPV is an HPV-35. In another embodiment, the HPV is an HPV-39. In another embodiment, the HPV is an HPV-45. In another embodiment, the HPV is an HPV-51. In another embodiment, the HPV is an HPV-52. In another embodiment, the HPV is an HPV-58. In another embodiment, the HPV is a high-risk HPV type. In another embodiment, the HPV is a mucosal HPV type.

[00514] In one embodiment, the HPV E6 is from HPV-16. In another embodiment, the HPV E7 is from HPV-16. In another embodiment, the HPV-E6 is from HPV-18. In another embodiment, the HPV-E7 is from HPV-18. In another embodiment, an HPV E6 antigen is utilized instead of or in addition to an E7 antigen in a composition or method of the present invention for treating or ameliorating an HPV-mediated disease, disorder, or symptom. In another embodiment, an HPV-16 E6 and E7 is utilized instead of or in combination with an HPV-18 E6 and E7. In such an embodiment, the recombinant *Listeria* may express the HPV-16 E6 and E7 from the chromosome and the HPV-18 E6 and E7 from a plasmid, or vice versa. In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed from a plasmid present in a recombinant *Listeria* provided herein. In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed from the chromosome of a recombinant *Listeria* provided herein. In another embodiment, the HPV-16 E6 and E7 antigens and the HPV-18 E6 and E7 antigens are expressed in any combination of the above embodiments, including where each E6 and E7 antigen from each HPV strain is expressed from either the plasmid or the chromosome.

[00515] In one embodiment, the recombinant *Listeria* strain as provided herein comprises a nucleic acid molecule encoding a tumor associated antigen, wherein the tumor associated antigen comprises a Her-2/neu peptide. In one embodiment, a tumor associated antigen comprises a Her-2/neu antigen. In one embodiment the Her-2/neu peptide comprises a chimeric Her-2/neu antigen (cHer-2).

[00516] In one embodiment, the attenuated auxotrophic *Listeria* immunotherapy strain is based on a *Listeria* immunotherapy vector which is attenuated due to the deletion of virulence gene *actA* and retains the plasmid for Her2/neu expression *in vivo* and *in vitro* by

complementation of *dal* gene. In one embodiment, the *Listeria* strain expresses and secretes a chimeric Her2/neu protein fused to the first 441 amino acids of listeriolysin O (LLO). In another embodiment, the *Listeria* is a *dal/dat/actA Listeria* having a mutation in the dal, dat and actA endogenous genes. In another embodiment, the mutation is a deletion, a truncation or an inactivation of the mutated genes. In another embodiment, *Listeria* strain exerts strong and antigen specific anti-tumor responses with ability to break tolerance toward HER2/neu in transgenic animals. In another embodiment, the *dal/dat/actA* strain is highly attenuated and has a better safety profile than previous *Listeria* immunotherapy generation, as it is more rapidly cleared from the spleens of the immunized mice. In another embodiment, the *Listeria* strain results in a longer delay of tumor onset in transgenic animals than *Lm*-LLO-ChHer2, the antibiotic resistant and more virulent version of this immunotherapy see USSN 12/945,386; US Publication No. 2011/0142791, which is incorporated by reference herein in its entirety). In another embodiment, the *Listeria* strain causes a significant decrease in intra-tumoral T regulatory cells (Tregs). In another embodiment, the lower frequency of Tregs in tumors treated with *LmddA* immunotherapies result in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* immunotherapies. In one embodiment, the present invention provides a recombinant polypeptide comprising an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof. In one embodiment, the present invention provides a recombinant polypeptide consisting of an N-terminal fragment of an LLO protein fused to a Her-2 chimeric protein or fused to a fragment thereof. In the embodiment, the heterologous antigen is a Her-2 chimeric protein or fragment thereof.

[00517] In another embodiment, the Her-2 chimeric protein of the methods and compositions of the present invention is a human Her-2 chimeric protein. In another embodiment, the Her-2 protein is a mouse Her-2 chimeric protein. In another embodiment, the Her-2 protein is a rat Her-2 chimeric protein. In another embodiment, the Her-2 protein is a primate Her-2 chimeric protein. In another embodiment, the Her-2 protein is a Her-2 chimeric protein of human or any other animal species or combinations thereof known in the art.

[00518] In another embodiment, a Her-2 protein is a protein referred to as "HER-2/neu," "Erbb2," "v-erb-b2," "c-erb-b2," "neu," or "cNeu."

[00519] In one embodiment, the Her2-neu chimeric protein, harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene, where, in another embodiment, the chimeric protein harbors 3

H2Dq and at least 17 of the mapped human MHC-class I epitopes of the Her2/neu antigen (fragments EC1, EC2, and IC1) (**Figure 20A**. In another embodiment, the chimeric protein harbors at least 13 of the mapped human MHC-class I epitopes (fragments EC2 and IC1). In another embodiment, the chimeric protein harbors at least 14 of the mapped human MHC-class I epitopes (fragments EC1 and IC1). In another embodiment, the chimeric protein harbors at least 9 of the mapped human MHC-class I epitopes (fragments EC1 and IC2). In another embodiment, the Her2-neu chimeric protein is fused to a non-hemolytic listeriolysin O (LLO). In another embodiment, the Her2-neu chimeric protein is fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O (LLO) protein and expressed and secreted by the *Listeria monocytogenes* attenuated auxotrophic strain *LmddA*. In another embodiment, the expression and secretion of the fusion protein tLLO-ChHer2 from the attenuated auxotrophic strain provided herein that expresses a chimeric Her2/neu antigen/LLO fusion protein is comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (**Figure 20B**).

[00520] In one embodiment, no CTL activity is detected in naïve animals or mice injected with an irrelevant *Listeria* immunotherapy (**Figure 21A**). While in another embodiment, the attenuated auxotrophic strain provided herein is able to stimulate the secretion of IFN-γ by the splenocytes from wild type FVB/N mice (**Figures 21B and 21C**).

[00521] In another embodiment, the Her-2 chimeric protein is encoded by the following nucleic acid sequence set forth in SEQ ID NO:22:

gagacccacctggacatgctccgccacctctaccagggctgccaggtggtgcagggaaacctggaactcacctacctgcccaccaa
tgccagcctgtccttcctgcaggatatccaggaggtgcagggctacgtgctcatcgctcacaaccaagtgaggcaggtcccactgca
gaggctgcggattgtgcgaggcacccagctctttgaggacaactatgccctggccgtgctagacaatggagacccgctgaacaatac
cacccctgtcacaggggcctccccaggaggcctgcgggagctgcagcttcgaagcctcacagagatcttgaaaggaggggtcttga
tccagcggaacccccagctctgctaccaggacacgattttgtggaagaatatccaggagtttgctggctgcaagaagatctttgggagc
ctggcatttctgccggagagctttgatggggacccagcctccaacactgccccgctccagccagagcagctccaagtgtttgagactc
tggaagagatcacaggttacctatacatctcagcatggccggacagcctgcctgacctcagcgtcttccagaacctgcaagtaatccg
gggacgaattctgcacaatggcgcctactcgctgaccctgcaagggctgggcatcagctggctggggctgcgctcactgagggaac
tgggcagtggactggccctcatccaccataacacccacctctgcttcgtgcacacggtgccctgggaccagctctttcggaacccgca
ccaagctctgctccacactgccaaccggccagaggacgagtgtgtgggcgagggcctggcctgccaccagctgtgcgcccgaggg
cagcagaagatccggaagtacacgatgcggagactgctgcaggaaacggagctggtggagccgctgacacctagcggagcgatg
cccaaccaggcgcagatgcggatcctgaaagagacggagctgaggaaggtgaaggtgcttggatctggcgcttttggcacagtcta
caagggcatctggatccctgatggggagaatgtgaaaattccagtggccatcaaagtgttgagggaaaacacatcccccaaagccaa

caaagaaatcttagacgaagcatacgtgatggctggtgtgggctccccatatgtctcccgccttctgggcatctgcctgacatccacggt gcagctggtgacacagcttatgccctatggctgcctcttagactaa (SEQ ID NO: 22).

[00522] In another embodiment, the Her-2 chimeric protein has the sequence:
E T H L D M L R H L Y Q G C Q V V Q G N L E L T Y L P T N A S L S F L Q D I Q E V
5 Q G Y V L I A H N Q V R Q V P L Q R L R I V R G T Q L F E D N Y A L A V L D N G D
P L N N T T P V T G A S P G G L R E L Q L R S L T E I L K G G V L I Q R N P Q L C Y
Q D T I L W K N I Q E F A G C K K I F G S L A F L P E S F D G D P A S N T A P L Q P
E Q L Q V F E T L E E I T G Y L Y I S A W P D S L P D L S V F Q N L Q V I R G R I L H
N G A Y S L T L Q G L G I S W L G L R S L R E L G S G L A L I H H N T H L C F V H T
10 V P W D Q L F R N P H Q A L L H T A N R P E D E C V G E G L A C H Q L C A R G Q
Q K I R K Y T M R R L L Q E T E L V E P L T P S G A M P N Q A Q M R I L K E T E L
R K V K V L G S G A F G T V Y K G I W I P D G E N V K I P V A I K V L R E N T S P K
A N K E I L D E A Y V M A G V G S P Y V S R L L G I C L T S T V Q L V T Q L M P Y
G C L L D (SEQ ID NO: 23).

15 [00523] In one embodiment, the Her2 chimeric protein or fragment thereof of the methods and compositions provided herein does not include a signal sequence thereof. In another embodiment, omission of the signal sequence enables the Her2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the signal sequence.

[00524] In another embodiment, the fragment of a Her2 chimeric protein of methods and
20 compositions of the present invention does not include a transmembrane domain (TM) thereof. In one embodiment, omission of the TM enables the Her-2 fragment to be successfully expressed in *Listeria*, due the high hydrophobicity of the TM.

[00525] Point mutations or amino-acid deletions in the oncogenic protein Her2/neu, have been reported to mediate treatment of resistant tumor cells, when these tumors have been
25 targeted by small fragment *Listeria*-based immunotherapies or trastuzumab (a monoclonal antibody against an epitope located at the extracellular domain of the Her2/neu antigen). Described herein is a chimeric Her2/neu based composition which harbors two of the extracellular and one intracellular fragments of Her2/neu antigen showing clusters of MHC-class I epitopes of the oncogene. This chimeric protein, which harbors 3 H2Dq and at least 17
30 of the mapped human MHC-class I epitopes of the Her2/neu antigen was fused to the first 441 amino acids of the *Listeria-monocytogenes* listeriolysin O protein and expressed and secreted by the *Listeria monocytogenes* attenuated strain *LmddA*.

[00526] In another embodiment, the tumor-associated antigen is an angiogenic antigen. In another embodiment, the angiogenic antigen is expressed on both activated pericytes and pericytes in tumor angiogeneic vasculature, which in another embodiment, is associated with neovascularization *in vivo*. In another embodiment, the angiogenic antigen is HMW-MAA. In another embodiment, the angiogenic antigen is one known in the art and are provided in WO2010/102140, which is incorporated by reference herein.

[00527] Protein and/or peptide homology for any amino acid sequence listed herein is determined, in one embodiment, by methods well described in the art, including immunoblot analysis, or via computer algorithm analysis of amino acid sequences, utilizing any of a number of software packages available, via established methods. Some of these packages may include the FASTA, BLAST, MPsrch or Scanps packages, and may employ the use of the Smith and Waterman algorithms, and/or global/local or BLOCKS alignments for analysis, for example.

[00528] In one embodiment, a plasmid comprising a minigene nucleic acid construct provided herein or a nucleic acid molecule encoding a fusion protein comprising an immunogenic polypeptide fused to one or more peptides provided herein is integrated into the *Listerial* chromosome using homologous recombination. Techniques for homologous recombination are well known in the art, and are described, for example, in Baloglu S, Boyle SM, et al. (Immune responses of mice to vaccinia virus recombinants expressing either *Listeria monocytogenes* partial listeriolysin or Brucella abortus ribosomal L7/L12 protein. Vet Microbiol 2005, 109(1-2): 11-7); and Jiang LL, Song HH, et al., (Characterization of a mutant *Listeria monocytogenes* strain expressing green fluorescent protein. Acta Biochim Biophys Sin (Shanghai) 2005, 37(1): 19-24). In another embodiment, homologous recombination is performed as described in United States Patent No. 6,855,320. In this case, a recombinant *Lm* strain that expresses E7 was made by chromosomal integration of the E7 gene under the control of the hly promoter and with the inclusion of the hly signal sequence to ensure secretion of the gene product, yielding the recombinant referred to as Lm-AZ/E7. In another embodiment, a temperature sensitive plasmid is used to select the recombinants. Each technique represents a separate embodiment of the present invention.

[00529] In another embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using transposon insertion. Techniques for transposon insertion are well known in the art, and are described, *inter alia*, by Sun et al. (Infection and Immunity 1990, 58: 3770-3778) in the construction of DP-L967. Transposon mutagenesis has the

advantage, in another embodiment, that a stable genomic insertion mutant can be formed but the disadvantage that the position in the genome where the foreign gene has been inserted is unknown.

[00530] In one embodiment, a vector provided herein is a vector known in the art, including a plasmid or a phage vector. In another embodiment, the construct or nucleic acid molecule is integrated into the *Listerial* chromosome using a phage vector comprising phage integration sites (Lauer P, Chow MY et al, Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J Bacteriol 2002; 184(15): 4177-86). In certain embodiments of this method, an integrase gene and attachment site of a bacteriophage (e.g. U153 or PSA listeriophage) is used to insert the heterologous gene into the corresponding attachment site, which may be any appropriate site in the genome (e.g. comK or the 3' end of the arg tRNA gene). In another embodiment, endogenous prophages are cured from the attachment site utilized prior to integration of the construct or heterologous gene. In another embodiment, this method results in single-copy integrants. In another embodiment, the present invention further comprises a phage based chromosomal integration system for clinical applications, where a host strain that is auxotrophic for essential enzymes, including, but not limited to, d-alanine racemase can be used, for example *Lm*dal(-)dat(-). In another embodiment, in order to avoid a "phage curing step," a phage integration system based on PSA is used. This requires, in another embodiment, continuous selection by antibiotics to maintain the integrated gene. Thus, in another embodiment, the current invention enables the establishment of a phage based chromosomal integration system that does not require selection with antibiotics. Instead, an auxotrophic host strain can be complemented.

[00531] In another embodiment, a vector provided herein is a delivery vector known in the art including a bacterial delivery vector, a viral vector delivery vector, a peptide immunotherapy delivery vector, and a DNA immunotherapy delivery vector. It will be appreciated by one skilled in the art that the term "delivery vectors" refers to a construct which is capable of delivering, and, within certain embodiments expressing, one or more neo-epitopes or peptides comprising one or more neo-epitopes in a host cell. Representative examples of such vectors include viral vectors, nucleic acid expression vectors, naked DNA, and certain eukaryotic cells (e.g., producer cells). In one embodiment, a delivery vector differs from a plasmid or phage vector. In another embodiment, a delivery vector and a plasmid or phage vector of this invention are the same. In another embodiment, a delivery

vector used in the methods and compositions disclosed herein is a *Listeria monocytogenes* strain.

[00532] In one embodiment of the methods and compositions as provided herein, the term "recombination site" or "site-specific recombination site" refers to a sequence of bases in a nucleic acid molecule that is recognized by a recombinase (along with associated proteins, in some cases) that mediates exchange or excision of the nucleic acid segments flanking the recombination sites. The recombinases and associated proteins are collectively referred to as "recombination proteins" see, e.g., Landy, A., (Current Opinion in Genetics & Development) 3:699-707; 1993).

[00533] A "phage expression vector," "phage vector," or "phagemid" refers to any phage-based recombinant expression system for the purpose of expressing a nucleic acid sequence of the methods and compositions as provided herein in vitro or in vivo, constitutively or inducibly, in any cell, including prokaryotic, yeast, fungal, plant, insect or mammalian cell. A phage expression vector typically can both reproduce in a bacterial cell and, under proper conditions, produce phage particles. The term includes linear or circular expression systems and encompasses both phage-based expression vectors that remain episomal or integrate into the host cell genome.

[00534] In one embodiment, the term "operably linked" as used herein means that the transcriptional and translational regulatory nucleic acid, is positioned relative to any coding sequences in such a manner that transcription is initiated. Generally, this will mean that the promoter and transcriptional initiation or start sequences are positioned 5' to the coding region.

[00535] In one embodiment, an "open reading frame" or "ORF" is a portion of an organism's genome which contains a sequence of bases that could potentially encode a protein. In another embodiment, the start and stop ends of the ORF are not equivalent to the ends of the mRNA, but they are usually contained within the mRNA. In one embodiment, ORFs are located between the start-code sequence (initiation codon) and the stop-codon sequence (termination codon) of a gene. Thus, in one embodiment, a nucleic acid molecule operably integrated into a genome as an open reading frame with an endogenous polypeptide is a nucleic acid molecule that has integrated into a genome in the same open reading frame as an endogenous polypeptide.

[00536] In one embodiment, the present invention provides a fusion polypeptide comprising

a linker sequence. In one embodiment, a "linker sequence" refers to an amino acid sequence that joins two heterologous polypeptides, or fragments or domains thereof. In general, as used herein, a linker is an amino acid sequence that covalently links the polypeptides to form a fusion polypeptide. A linker typically includes the amino acids translated from the remaining recombination signal after removal of a reporter gene from a display plasmid vector to create a fusion protein comprising an amino acid sequence encoded by an open reading frame and the display protein. As appreciated by one of skill in the art, the linker can comprise additional amino acids, such as glycine and other small neutral amino acids.

[00537] In one embodiment, “endogenous” as used herein describes an item that has developed or originated within the reference organism or arisen from causes within the reference organism. In another embodiment, endogenous refers to native.

[00538] “Stably maintained” refers, in another embodiment, to maintenance of a nucleic acid molecule or plasmid in the absence of selection (e.g. antibiotic selection) for 10 generations, without detectable loss. In another embodiment, the period is 15 generations. In another embodiment, the period is 20 generations. In another embodiment, the period is 25 generations. In another embodiment, the period is 30 generations. In another embodiment, the period is 40 generations. In another embodiment, the period is 50 generations. In another embodiment, the period is 60 generations. In another embodiment, the period is 80 generations. In another embodiment, the period is 100 generations. In another embodiment, the period is 150 generations. In another embodiment, the period is 200 generations. In another embodiment, the period is 300 generations. In another embodiment, the period is 500 generations. In another embodiment, the period is more than generations. In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vitro* (e.g. in culture). In another embodiment, the nucleic acid molecule or plasmid is maintained stably *in vivo*. In another embodiment, the nucleic acid molecule or plasmid is maintained stably both *in vitro* and *in vitro.*

[00539] In another embodiment, provided herein is a recombinant *Listeria* strain, comprising a nucleic acid molecule operably integrated into the *Listeria* genome as an open reading frame with an endogenous ActA sequence. In another embodiment, a recombinant *Listeria* strain of the methods and compositions as provided herein comprise an episomal expression plasmid vector comprising a nucleic acid molecule encoding fusion protein comprising an antigen fused to an ActA or a truncated ActA. In one embodiment, the expression and secretion of the antigen is under the control of an *actA* promoter and an actA signal sequence

and it is expressed as fusion to 1-233 amino acids of ActA (truncated ActA or tActA). In another embodiment, the truncated ActA consists of the first 390 amino acids of the wild type ActA protein as described in US Patent Serial No. 7,655,238, which is incorporated by reference herein in its entirety. In another embodiment, the truncated ActA is an ActA-N100 or a modified version thereof (referred to as ActA-N100*) in which a PEST motif has been deleted and containing the non-conservative QDNKR substitution as described in US Patent Publication Serial No. 2014/0186387.

[00540] In one embodiment, a fragment provided herein is a functional fragment. In another embodiment, a “functional fragment” is an immunogenic fragment that is capable of eliciting an immune response when administered to a subject alone or in a immunotherapy composition provided herein. In another embodiment, a functional fragment has biological activity as will be understood by a skilled artisan and as further provided herein.

[00541] In one embodiment, the *Listeria* strain provided herein is an attenuated strain. In another embodiment, the *Listeria* strain provided herein is a recombinant strain. In another embodiment, the *Listeria* strain provided herein is a live attenuated recombinant *Listeria* strain.

[00542] The recombinant *Listeria* strain of methods and compositions of the present invention is, in another embodiment, a recombinant *Listeria monocytogenes* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria seeligeri* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria grayi* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria ivanovii* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria murrayi* strain. In another embodiment, the *Listeria* strain is a recombinant *Listeria welshimeri* strain. In another embodiment, the *Listeria* strain is a recombinant strain of any other *Listeria* species known in the art.

[00543] In another embodiment, a recombinant *Listeria* strain of the present invention has been passaged through an animal host. In another embodiment, the passaging maximizes efficacy of the strain as a immunotherapy vector. In another embodiment, the passaging stabilizes the immunogenicity of the *Listeria* strain. In another embodiment, the passaging stabilizes the virulence of the *Listeria* strain. In another embodiment, the passaging increases the immunogenicity of the *Listeria* strain. In another embodiment, the passaging increases the virulence of the *Listeria* strain. In another embodiment, the passaging removes unstable sub-strains of the *Listeria* strain. In another embodiment, the passaging reduces the prevalence of

unstable sub-strains of the *Listeria* strain. In another embodiment, the *Listeria* strain contains a genomic insertion of the gene encoding the antigen-containing recombinant peptide. In another embodiment, the *Listeria* strain carries a plasmid comprising the gene encoding the antigen-containing recombinant peptide. In another embodiment, the passaging is performed as described herein. In another embodiment, the passaging is performed by any other method known in the art.

[00544] In another embodiment, a recombinant nucleic acid of the present invention is operably linked to a promoter/regulatory sequence that drives expression of the encoded peptide in the *Listeria* strain. Promoter/regulatory sequences useful for driving constitutive expression of a gene are well known in the art and include, but are not limited to, for example, the P_{hlyA}, P_{ActA}, and p60 promoters of *Listeria*, the *Streptococcus* bac promoter, the *Streptomyces griseus* sgiA promoter, and the *B. thuringiensis* phaZ promoter.

[00545] In another embodiment, inducible and tissue specific expression of the nucleic acid encoding a peptide of the present invention is accomplished by placing the nucleic acid encoding the peptide under the control of an inducible or tissue specific promoter/regulatory sequence. Examples of tissue specific or inducible promoter/regulatory sequences which are useful for this purpose include, but are not limited to the MMTV LTR inducible promoter, and the SV40 late enhancer/promoter. In another embodiment, a promoter that is induced in response to inducing agents such as metals, glucocorticoids, and the like, is utilized. Thus, it will be appreciated that the invention includes the use of any promoter/regulatory sequence, which is either known or unknown, and which is capable of driving expression of the desired protein operably linked thereto. It will be appreciated by a skilled artisan that the term "heterologous" encompasses a nucleic acid, amino acid, peptide, polypeptide, or protein derived from a different species than the reference species. Thus, for example, a *Listeria* strain expressing a heterologous polypeptide, in one embodiment, would express a polypeptide that is not native or endogenous to the *Listeria* strain, or in another embodiment, a polypeptide that is not normally expressed by the *Listeria* strain, or in another embodiment, a polypeptide from a source other than the *Listeria* strain. In another embodiment, heterologous may be used to describe something derived from a different organism within the same species. In another embodiment, the heterologous antigen is expressed by a recombinant strain of *Listeria*, and is processed and presented to cytotoxic T-cells upon infection of mammalian cells by the recombinant strain. In another embodiment, the heterologous antigen expressed by *Listeria* species need not precisely match the corresponding unmodified antigen

or protein in the tumor cell or infectious agent so long as it results in a T-cell response that recognizes the unmodified antigen or protein which is naturally expressed in the mammal. The term heterologous antigen may be referred to herein as “antigenic polypeptide”, “heterologous protein”, “heterologous protein antigen”, “protein antigen”, “antigen”, and the like.

[00546] It will be appreciated by the skilled artisan that the term “episomal expression vector” encompasses a nucleic acid plasmid vector which may be linear or circular, and which is usually double-stranded in form and is extrachromosomal in that it is present in the cytoplasm of a host bacteria or cell as opposed to being integrated into the bacteria’s or cell’s genome. In one embodiment, an episomal expression vector comprises a gene of interest. In another embodiment, episomal vectors persist in multiple copies in the bacterial cytoplasm, resulting in amplification of the gene of interest, and, in another embodiment, viral trans-acting factors are supplied when necessary. In another embodiment, the episomal expression vector may be referred to as a plasmid herein. In another embodiment, an “integrative plasmid” comprises sequences that target its insertion or the insertion of the gene of interest carried within into a host genome. In another embodiment, an inserted gene of interest is not interrupted or subjected to regulatory constraints which often occur from integration into cellular DNA. In another embodiment, the presence of the inserted heterologous gene does not lead to rearrangement or interruption of the cell's own important regions. In another embodiment, in stable transfection procedures, the use of episomal vectors often results in higher transfection efficiency than the use of chromosome-integrating plasmids (Belt, P.B.G.M., et al (1991) Efficient cDNA cloning by direct phenotypic correction of a mutant human cell line (HPRT2) using an Epstein-Barr virus-derived cDNA expression plasmid vector. Nucleic Acids Res. 19, 4861-4866; Mazda, O., et al. (1997) Extremely efficient gene transfection into lympho-hematopoietic cell lines by Epstein-Barr virus-based vectors. J. Immunol. Methods 204, 143-151). In one embodiment, the episomal expression vectors of the methods and compositions as provided herein may be delivered to cells in vivo, ex vivo, or in vitro by any of a variety of the methods employed to deliver DNA molecules to cells. The plasmid vectors may also be delivered alone or in the form of a pharmaceutical composition that enhances delivery to cells of a subject.

[00547] In one embodiment, the term "fused" refers to operable linkage by covalent bonding. In one embodiment, the term includes recombinant fusion (of nucleic acid sequences or open reading frames thereof). In another embodiment, the term includes chemical conjugation.

[00548] "Transforming," in one embodiment, refers to engineering a bacterial cell to take up a plasmid or other heterologous DNA molecule. In another embodiment, "transforming" refers to engineering a bacterial cell to express a gene of a plasmid or other heterologous DNA molecule. Each possibility represents a separate embodiment of the methods and compositions as provided herein.

[00549] In another embodiment, conjugation is used to introduce genetic material and/or plasmids into bacteria. Methods for conjugation are well known in the art, and are described, for example, in Nikodinovic J. et al (A second generation snp-derived Escherichia coli-Streptomyces shuttle expression vector that is generally transferable by conjugation. Plasmid. 2006 Nov;56(3):223-7) and Auchtung JM et al (Regulation of a Bacillus subtilis mobile genetic element by intercellular signaling and the global DNA damage response. Proc Natl Acad Sci U S A. 2005 Aug 30;102(35):12554-9). Each method represents a separate embodiment of the methods and compositions as provided herein.

[00550] In one embodiment, the term "attenuation," refers to a diminution in the ability of the bacterium to cause disease in an animal. In other words, the pathogenic characteristics of the attenuated *Listeria* strain have been lessened compared with wild-type *Listeria*, although the attenuated *Listeria* is capable of growth and maintenance in culture. Using as an example the intravenous inoculation of Balb/c mice with an attenuated *Listeria*, the lethal dose at which 50% of inoculated animals survive (LD_{50}) is preferably increased above the LD_{50} of wild-type *Listeria* by at least about 10-fold, more preferably by at least about 100-fold, more preferably at least about 1,000 fold, even more preferably at least about 10,000 fold, and most preferably at least about 100,000-fold. An attenuated strain of *Listeria* is thus one which does not kill an animal to which it is administered, or is one which kills the animal only when the number of bacteria administered is vastly greater than the number of wild type non-attenuated bacteria which would be required to kill the same animal. An attenuated bacterium should also be construed to mean one which is incapable of replication in the general environment because the nutrient required for its growth is not present therein. Thus, the bacterium is limited to replication in a controlled environment wherein the required nutrient is provided. The attenuated strains of the present invention are therefore environmentally safe in that they are incapable of uncontrolled replication.

Compositions

[00551] In one embodiment, compositions of the present invention are immunogenic

compositions. In one embodiment, compositions of the present invention induce a strong innate stimulation of interferon-gamma, which in one embodiment, has anti-angiogenic properties. In one embodiment, a *Listeria* of the present invention induces a strong innate stimulation of interferon-gamma, which in one embodiment, has anti-angiogenic properties (Dominiecki et al., Cancer Immunol Immunother. 2005 May;54(5):477-88. Epub 2004 Oct 6, incorporated herein by reference in its entirety; Beatty and Paterson, J. Immunol. 2001 Feb 15;166(4):2276-82, incorporated herein by reference in its entirety). In one embodiment, anti-angiogenic properties of *Listeria* are mediated by $CD4^+$ T cells (Beatty and Paterson, 2001). In another embodiment, anti-angiogenic properties of *Listeria* are mediated by $CD8^+$ T cells. In another embodiment, IFN-gamma secretion as a result of *Listeria* vaccination is mediated by NK cells, NKT cells, Th1 $CD4^+$ T cells, TC1 $CD8^+$ T cells, or a combination thereof.

[00552] In another embodiment, administration of compositions of the present invention induce production of one or more anti-angiogenic proteins or factors. In one embodiment, the anti-angiogenic protein is IFN-gamma. In another embodiment, the anti-angiogenic protein is pigment epithelium-derived factor (PEDF); angiostatin; endostatin; fms-like tyrosine kinase (sFlt)-1; or soluble endoglin (sEng). In one embodiment, a *Listeria* of the present invention is involved in the release of anti-angiogenic factors, and, therefore, in one embodiment, has a therapeutic role in addition to its role as a plasmid vector for introducing an antigen to a subject. Each *Listeria* strain and type thereof represents a separate embodiment of the present invention.

[00553] The immune response induced by methods and compositions as provided herein is, in another embodiment, a T cell response. In another embodiment, the immune response comprises a T cell response. In another embodiment, the response is a CD8+ T cell response. In another embodiment, the response comprises a $CD8^+$ T cell response. Each possibility represents a separate embodiment as provided herein.

[00554] In another embodiment, administration of compositions of the present invention increase the number of antigen-specific T cells. In another embodiment, administration of compositions activates co-stimulatory receptors on T cells. In another embodiment, administration of compositions induces proliferation of memory and/or effector T cells. In another embodiment, administration of compositions increases proliferation of T cells. Each possibility represents a separate embodiment as provided herein.

[00555] As used throughout, the terms "composition" and "immunogenic composition" are

interchangeable having all the same meanings and qualities. In one embodiment, an immunogenic composition provided herein comprising a recombinant *Listeria* strain and further comprising an antibody for concomitant or sequential administration of each component is also referred to as a "combination therapy". It is to be understood by a skilled artisan that a combination therapy may also comprise additional components, antibodies, therapies, etc. The term "pharmaceutical composition" refers, in some embodiments, to a composition suitable for pharmaceutical use, for example, to administer to a subject in need. In one embodiment, the present invention provides a pharmaceutical composition comprising the attenuated *Listeria* strain provided herein and a pharmaceutically acceptable carrier. In another embodiment, the present invention provides a pharmaceutical composition comprising the DNA immunotherapy provided herein and a pharmaceutically acceptable carrier. In another embodiment, the present invention provides a pharmaceutical composition comprising the vaccinia virus strain or virus-like particle provided herein and a pharmaceutically acceptable carrier. In another embodiment, the present invention provides a pharmaceutical composition comprising the peptide immunotherapy provided herein and a pharmaceutically acceptable carrier.

[00556] In another embodiment, the present invention provides a recombinant immunotherapy vector comprising a nucleotide molecule of the present invention. In another embodiment, the vector is an expression vector. In another embodiment, the expression vector is a plasmid. In another embodiment, the present invention provides a method for the introduction of a nucleotide molecule of the present invention into a cell. Methods for constructing and utilizing recombinant vectors are well known in the art and are described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Brent et al. (2003, Current Protocols in Molecular Biology, John Wiley & Sons, New York). In another embodiment, the vector is a bacterial vector. In other embodiments, the vector is selected from *Salmonella sp.*, *Shigella sp.*, BCG, *L. monocytogenes* and *S. gordonii*. In another embodiment, one or more peptides are delivered by recombinant bacterial vectors modified to escape phagolysosomal fusion and live in the cytoplasm of the cell. In another embodiment, the vector is a viral vector. In other embodiments, the vector is selected from Vaccinia, Avipox, Adenovirus, AAV, Vaccinia virus NYVAC, Modified vaccinia strain Ankara (MVA), Semliki Forest virus, Venezuelan equine encephalitis virus, herpes viruses, and retroviruses. In another embodiment, the vector is a naked DNA vector. In another embodiment, the vector is any other vector known in the

art. Each possibility represents a separate embodiment of the present invention.

[00557] Compositions of this invention may be used in methods of this invention in order to elicit an enhanced anti-tumor T cell response in a subject, in order to inhibit tumor–mediated immunosuppression in a subject, or for increasing the ratio or T effector cells to regulatory T cells (Tregs) in the spleen and tumor of a subject, or any combination thereof.

[00558] In another embodiment, a composition comprising a *Listeria* strain of the present invention further comprises an adjuvant. In one embodiment, a composition of the present invention further comprises an adjuvant. The adjuvant utilized in methods and compositions of the present invention is, in another embodiment, a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein. In another embodiment, the adjuvant comprises a GM-CSF protein. In another embodiment, the adjuvant is a nucleotide molecule encoding GM-CSF. In another embodiment, the adjuvant comprises a nucleotide molecule encoding GM-CSF. In another embodiment, the adjuvant is saponin QS21. In another embodiment, the adjuvant comprises saponin QS21. In another embodiment, the adjuvant is monophosphoryl lipid A. In another embodiment, the adjuvant comprises monophosphoryl lipid A. In another embodiment, the adjuvant is SBAS2. In another embodiment, the adjuvant comprises SBAS2. In another embodiment, the adjuvant is an unmethylated CpG-containing oligonucleotide. In another embodiment, the adjuvant comprises an unmethylated CpG-containing oligonucleotide. In another embodiment, the adjuvant is an immune-stimulating cytokine. In another embodiment, the adjuvant comprises an immune-stimulating cytokine. In another embodiment, the adjuvant is a nucleotide molecule encoding an immune-stimulating cytokine. In another embodiment, the adjuvant comprises a nucleotide molecule encoding an immune-stimulating cytokine. In another embodiment, the adjuvant is or comprises a quill glycoside. In another embodiment, the adjuvant is or comprises a bacterial mitogen. In another embodiment, the adjuvant is or comprises a bacterial toxin. In another embodiment, the adjuvant is or comprises any other adjuvant known in the art.

[00559] In one embodiment, an immunogenic composition of this invention comprises a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to a heterologous antigen or fragment thereof. In another embodiment, an immunogenic composition of this invention comprises a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a

first open reading frame encoding a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

[00560] In one embodiment, an immunogenic composition of this invention comprises a recombinant *Listeria* strain comprising a nucleic acid molecule, said nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide, wherein said fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to a heterologous antigen or fragment thereof, said composition further comprising an antibody or fragment thereof. In another embodiment said antibody or fragment thereof comprises a polyclonal antibody, a monoclonal antibody, an Fab fragment, an F(ab')2 fragment, an Fv fragment, a single chain antibody, or any combination thereof.

[00561] In one embodiment, an immunogenic composition of this invention comprises a recombinant *Listeria* strain provided herein, said composition further comprising an antibody or fragment thereof. In another embodiment said antibody or fragment thereof comprises a polyclonal antibody, a monoclonal antibody, an Fab fragment, an F(ab')2 fragment, an Fv fragment, a single chain antibody, or any combination thereof.

[00562] In another embodiment, an immunogenic composition of this invention comprises a recombinant *Listeria* strain, said composition further comprising an antibody or fragment thereof. In another embodiment said antibody or fragment thereof comprises a polyclonal antibody, a monoclonal antibody, an Fab fragment, an F(ab')2 fragment, an Fv fragment, a single chain antibody, or any combination thereof.

[00563] In some embodiments, the term "antibody" refers to intact molecules as well as functional fragments thereof, also referred to herein as “antigen binding fragments”, such as Fab, F(ab')2, and Fv that are capable of specifically interacting with a desired target as described herein, for example, blocking the binding of a checkpoint inhibitor. In another embodiment, an antibody or functional fragment thereof comprises an immune checkpoint inhibitor antagonist. In another embodiment, an antibody or functional fragment thereof comprises an anti-PD-L1/PD-L2 antibody or fragment thereof. In another embodiment, an antibody or functional fragment thereof comprises an anti-PD-1 antibody or fragment thereof. In another embodiment, an antibody or functional fragment thereof comprises an anti-CTLA-4 antibody or fragment thereof. In another embodiment, an antibody or functional fragment thereof comprises an anti-B7-H4 antibody or fragment thereof.

[00564] In some embodiments, the antibody fragments comprise: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, which can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) $(Fab')_2$, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; or (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Each possibility represents a separate embodiment of the present invention.

[00565] Methods of making these fragments are known in the art. (See for example, Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

[00566] In some embodiments, the antibody fragments may be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment.

[00567] Antibody fragments can, in some embodiments, be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R., Biochem. J., 73: 119-126, 1959. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques

may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

[00568] Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar *et al.*, Proc. Nat'l Acad. Sci. USA 69:2659-62, 1972. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow and Filpula, Methods, 2: 97-105, 1991; Bird *et al.*, Science 242:423-426, 1988; Pack *et al.*, Bio/Technology 11:1271-77, 1993; and Ladner *et al.*, U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

[00569] Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry, Methods, 2: 106-10, 1991.

[00570] In some embodiments, the antibodies or fragments as described herein may comprise "humanized forms" of antibodies. In some embodiments, the term "humanized forms of antibodies" refers to non-human (e.g. murine) antibodies, which are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the

imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

[00571] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature 332:323-327 (1988); Verhoeyen *et al.*, Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[00572] Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks *et al.*, J. Mol. Biol., 222:581 (1991)]. The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)]. Similarly, human can be made by introducing of human immunoglobulin loci into transgenic animals, e.g. mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, Bio/Technology 10, 779-

783 (1992); Lonberg *et al.*, Nature 368 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild *et al.*, Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14, 826 (1996); Lonberg and Huszar, Intern. Rev. Immunol. 13 65-93 (1995).

[00573] In one embodiment, the disease provided herein is a cancer or a tumor. In one embodiment, the cancer treated by a method of the present invention is breast cancer. In another embodiment, the cancer is a cervical cancer. In another embodiment, the cancer is an Her2 containing cancer. In another embodiment, the cancer is a melanoma. In another embodiment, the cancer is pancreatic cancer. In another embodiment, the cancer is ovarian cancer. In another embodiment, the cancer is gastric cancer. In another embodiment, the cancer is a carcinomatous lesion of the pancreas. In another embodiment, the cancer is pulmonary adenocarcinoma. In another embodiment, the cancer is pulmonary adenocarcinoma. In another embodiment, it is a glioblastoma multiforme. In another embodiment, the cancer is colorectal adenocarcinoma. In another embodiment, the cancer is pulmonary squamous adenocarcinoma. In another embodiment, the cancer is gastric adenocarcinoma. In another embodiment, the cancer is an ovarian surface epithelial neoplasm (e.g. a benign, proliferative or malignant variety thereof). In another embodiment, the cancer is an oral squamous cell carcinoma. In another embodiment, the cancer is non-small-cell lung carcinoma. In another embodiment, the cancer is an endometrial carcinoma. In another embodiment, the cancer is a bladder cancer. In another embodiment, the cancer is a head and neck cancer. In another embodiment, the cancer is a prostate carcinoma. In another embodiment, the cancer is oropharyngeal cancer. In another embodiment, the cancer is lung cancer. In another embodiment, the cancer is anal cancer. In another embodiment, the cancer is colorectal cancer. In another embodiment, the cancer is esophageal cancer. In another embodiment, the cancer is mesothelioma.

[00574] In one embodiment, a heterologous antigen provided herein is HPV-E7. In another embodiment, the antigen is HPV-E6. In another embodiment, the HPV-E7 is from HPV strain 16. In another embodiment, the HPV-E7 is from HPV strain 18. In another embodiment, the HPV-E6 is from HPV strain 16. In another embodiment, the HPV-E7 is from HPV strain 18. In another embodiment, fragments of a heterologous antigen provided herein are also encompassed by the present invention.

[00575] In another embodiment, the antigen is Her-2/neu. In another embodiment, the antigen is NY-ESO-1. In another embodiment, the antigen is telomerase (TERT). In another embodiment, the antigen is SCCE. In another embodiment, the antigen is CEA. In another

embodiment, the antigen is LMP-1. In another embodiment, the antigen is p53. In another embodiment, the antigen is carboxic anhydrase IX (CAIX). In another embodiment, the antigen is PSMA. In another embodiment, the antigen is prostate stem cell antigen (PSCA). In another embodiment, the antigen is HMW-MAA. In another embodiment, the antigen is WT-1. In another embodiment, the antigen is HIV-1 Gag. In another embodiment, the antigen is Proteinase 3. In another embodiment, the antigen is Tyrosinase related protein 2. In another embodiment, the antigen is PSA (prostate-specific antigen). In another embodiment, the antigen is a bivalent PSA. In another embodiment, the antigen is an ERG. In another embodiment, the antigen is an ERG construct type III. In another embodiment, the antigen is an ERG construct type VI. In another embodiment, the antigen is an androgen receptor (AR). In another embodiment, the antigen is a PAK6. In another embodiment, the antigen comprises an epitope rich region of PAK6. In another embodiment, the antigen is selected from HPV-E7, HPV-E6, Her-2, NY-ESO-1, telomerase (TERT), SCCE, HMW-MAA, EGFR-III, survivin, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), WT-1, HIV-1 Gag, CEA, LMP-1, p53, PSMA, PSCA, Proteinase 3, Tyrosinase related protein 2, Muc1, PSA (prostate-specific antigen), or a combination thereof. In another embodiment, an antigen comprises the wild-type form of the antigen. In another embodiment, an antigen comprises a mutant form of the antigen.

[00576] In one embodiment, a nucleic acid sequence of PAK6 is set forth in SEQ ID NO: 102. In another embodiment, an amino acid sequence of PAK6 is set for in SEQ ID NO: 103. (See Kwek et al. (2012) *J Immunol* published online 5 September 2012, which is incorporated herein in full.)

[00577] In another embodiment, an “immunogenic fragment” is one that elicits an immune response when administered to a subject alone or in a immunotherapy composition provided herein. Such a fragment contains, in another embodiment, the necessary epitopes in order to elicit either a humoral immune response, and/or an adaptive immune response.

[00578] In one embodiment, compositions of this invention comprise an antibody or a functional fragment thereof. In another embodiment, compositions of this invention comprise at least one antibody or functional fragment thereof. In another embodiment, a composition may comprise 2 antibodies, 3 antibodies, 4 antibodies, or more than 4 antibodies. In another embodiment, a composition of this invention comprises an Lm strain and an antibody or a functional fragment thereof. In another embodiment, a composition of this invention comprises an Lm strain and at least one antibody or a functional fragment thereof. In another

embodiment, a composition of this invention comprises an Lm strain and 2 antibodies, 3 antibodies, 4 antibodies, or more than 4 antibodies. In another embodiment, a composition of this invention comprises an antibody or a functional fragment thereof, wherein the composition does not include a *Listeria* strain provided herein. Different antibodies present in the same or different compositions need not have the same form, for example one antibody may be a monoclonal antibody and another may be a FAb fragment. Each possibility represents a different embodiment.

[00579] In one embodiment, compositions of this invention comprise an antibody or a functional fragment thereof, which specifically binds GITR or a portion thereof. In another embodiment, compositions of this invention comprise an antibody or functional fragment thereof, which specifically binds OX40 or a portion thereof. In another embodiment, a composition may comprise an antibody that specifically bind GITR or a portion thereof, and an antibody that specifically binds OX40. In another embodiment, a composition of this invention comprises an Lm strain and an antibody or a functional fragment thereof that specifically binds GITR. In another embodiment, a composition of this invention comprises an Lm strain and an antibody or a functional fragment thereof that specifically binds OX40. In another embodiment, a composition of this invention comprises an Lm strain and an antibody that specifically binds GITR or a portion thereof, and an antibody that specifically binds OX40 or a portion thereof. In another embodiment, a composition of this invention comprises an antibody or a functional fragment thereof that specifically binds GITR, wherein the composition does not include a *Listeria* strain provided herein. In another embodiment, a composition of this invention comprises an antibody or a functional fragment thereof that specifically binds OX40, wherein the composition does not include a *Listeria* strain provided herein. In another embodiment, a composition of this invention comprises an antibody or a functional fragment thereof that specifically binds GITR, and an antibody that specifically binds GITR, wherein the composition does not include a *Listeria* strain provided herein. Different antibodies present in the same or different compositions need not have the same form, for example one antibody may be a monoclonal antibody and another may be a FAb fragment. Each possibility represents a different embodiment of this invention.

[00580] The term "antibody functional fragment" refers to a portion of an intact antibody that is capable of specifically binding to an antigen to cause the biological effect intended by the present invention. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, and Fv fragments, linear antibodies, scFv

antibodies, and multispecific antibodies formed from antibody fragments.

[00581] An "antibody heavy chain," as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations.

5 [00582] An "antibody light chain," as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring conformations, κ and λ light chains refer to the two major antibody light chain isotypes.

[00583] By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed
10 by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the
15 art.

[00584] In one embodiment, an antibody or functional fragment thereof comprises an antigen binding region. In one embodiment, an antigen binding regions is an antibody or an antigen-binding domain thereof. In one embodiment, the antigen-binding domain thereof is a Fab or a scFv.

20 [00585] It will be appreciated by a skilled artisan that the term “binds” or "specifically binds," with respect to an antibody, encompasses an antibody or functional fragment thereof, which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species, but, such cross-species
25 reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms "specific binding" or "specifically binding," can be used in reference to the interaction of an antibody, a protein, or a peptide with a second
30 chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than a specific amino acid

sequence.

[00586] In one embodiment, a composition of this invention comprises a recombinant *Listeria monocytogenes* (*Lm*) strain. In another embodiment, a composition of this invention comprises an antibody or functional fragment thereof, as described herein.

[00587] In one embodiment, an immunogenic composition comprises an antibody or a functional fragment thereof, provided herein, and a recombinant attenuated *Listeria,* provided herein. In another embodiment, each component of the immunogenic compositions provided herein is administered prior to, concurrently with, or after another component of the immunogenic compositions provided herein. In one embodiment, even when administered concurrently, an Lm composition and an antibody or functional fragment thereof may be administered as two separate compositions. Alternately, in another embodiment, an Lm composition may comprise an antibody or a functional fragment thereof.

[00588] The compositions of this invention, in another embodiment, are administered to a subject by any method known to a person skilled in the art, such as parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intra-dermally, subcutaneously, intra-peritonealy, intra-ventricularly, intra-cranially, intra-vaginally or intra-tumorally.

[00589] In another embodiment, the compositions are administered orally, and are thus formulated in a form suitable for oral administration, i.e. as a solid or a liquid preparation. Suitable solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Suitable liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In another embodiment of the present invention, the active ingredient is formulated in a capsule. In accordance with this embodiment, the compositions of the present invention comprise, in addition to the active compound and the inert carrier or diluent, a hard gelating capsule.

[00590] In another embodiment, compositions are administered by intravenous, intra-arterial, or intra-muscular injection of a liquid preparation. Suitable liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In one embodiment, the pharmaceutical compositions are administered intravenously and are thus formulated in a form suitable for intravenous administration. In another embodiment, the pharmaceutical compositions are administered intra-arterially and are thus formulated in a form suitable for intra-arterial administration. In another embodiment, the pharmaceutical compositions are

administered intra-muscularly and are thus formulated in a form suitable for intra-muscular administration.

[00591] In some embodiments, when the antibody or functional fragment thereof is administered separately from a composition comprising a recombinant Lm strain, the antibody may be injected intravenously, subcutaneously, or directly into the tumor or tumor bed. In one embodiment, a composition comprising an antibody is injected into the space left after a tumor has been surgically removed, e.g., the space in a prostate gland following removal of a prostate tumor.

[00592] In one embodiment, the term "immunogenic composition" may encompass the recombinant *Listeria* provided herein, and an adjuvant, and an antibody or functional fragment thereof, or any combination thereof. In another embodiment, an immunogenic composition comprises a recombinant *Listeria* provided herein. In another embodiment, an immunogenic composition comprises an adjuvant known in the art or as provided herein. It is also to be understood that administration of such compositions enhance an immune response, or increase a T effector cell to regulatory T cell ratio or elicit an anti-tumor immune response, as further provided herein.

[00593] In one embodiment, this invention provides methods of use which comprise administering a composition comprising the described *Listeria* strains, and further comprising an antibody or functional fragment thereof. In another embodiment, methods of use comprise administering more than one antibody provided herein, which may be present in the same or a different composition, and which may be present in the same composition as the *Listeria* or in a separate composition. Each possibility represents a different embodiment of this invention.

[00594] In one embodiment, the term "pharmaceutical composition" encompasses a therapeutically effective amount of the active ingredient or ingredients including the *Listeria* strain, and at least one antibody or functional fragment thereof, together with a pharmaceutically acceptable carrier or diluent. It is to be understood that the term a "therapeutically effective amount" refers to that amount which provides a therapeutic effect for a given condition and administration regimen.

[00595] It will be understood by the skilled artisan that the term "administering" encompasses bringing a subject in contact with a composition of the present invention. In one embodiment, administration can be accomplished *in vitro*, i.e. in a test tube, or *in vivo*, i.e. in cells or tissues of living organisms, for example humans. In one embodiment, the present

invention encompasses administering the *Listeria* strains and compositions thereof of the present invention to a subject.

[00596] The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%. It is to be understood by the skilled artisan that the term "subject" can encompass a mammal including an adult human or a human child, teenager or adolescent in need of therapy for, or susceptible to, a condition or its sequelae, and also may include non-human mammals such as dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. It will also be appreciated that the term may encompass livestock. The term "subject" does not exclude an individual that is normal in all respects.

[00597] Following the administration of the immunogenic compositions provided herein, the methods provided herein induce the expansion of T effector cells in peripheral lymphoid organs leading to an enhanced presence of T effector cells at the tumor site. In another embodiment, the methods provided herein induce the expansion of T effector cells in peripheral lymphoid organs leading to an enhanced presence of T effector cells at the periphery. Such expansion of T effector cells leads to an increased ratio of T effector cells to regulatory T cells in the periphery and at the tumor site without affecting the number of Tregs. It will be appreciated by the skilled artisan that peripheral lymphoid organs include, but are not limited to, the spleen, peyer’s patches, the lymph nodes, the adenoids, etc. In one embodiment, the increased ratio of T effector cells to regulatory T cells occurs in the periphery without affecting the number of Tregs. In another embodiment, the increased ratio of T effector cells to regulatory T cells occurs in the periphery, the lymphoid organs and at the tumor site without affecting the number of Tregs at these sites. In another embodiment, the increased ratio of T effector cells decrease the frequency of Tregs, but not the total number of Tregs at these sites.

Combination Therapies and Methods of Use Thereof

[00598] In one embodiment, this invention provides a method of eliciting an enhanced anti-tumor T cell response in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST

amino acid sequence fused to a heterologous antigen or fragment thereof, wherein said method further comprises a step of administering an effective amount of a composition comprising an immune check-point inhibitor antagonist.

[00599] In one embodiment, an immune check-point inhibitor antagonist is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

[00600] In another embodiment, this invention provides a method of eliciting an enhanced anti-tumor T cell response in a subject, the method comprising the step of administering to the subject an effective amount of an immunogenic composition comprising a recombinant *Listeria* strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence, wherein said method further comprises a step of administering an effective amount of a composition comprising an antibody or fragment thereof to said subject. In another embodiment, the antibody is an agonist antibody or antigen binding fragment thereof. In another embodiment, the antibody is an anti-TNF receptor antibody or antigen binding fragment thereof. In another embodiment, the antibody is an anti-OX40 antibody or antigen binding fragment thereof. In another embodiment, the antibody is an anti-GITR antibody or antigen binding fragment thereof. In another embodiment, said method further comprises administering additional antibodies, which may be comprise in the composition comprising said recombinant *Listeria* strain or may be comprised in a separate composition.

[00601] In one embodiment, any composition comprising a *Listeria* strain described herein may be used in the methods of this invention. In one embodiment, any composition comprising a *Listeria* strain and an antibody or fragment thereof, for example an antibody binding a TNF receptor super family member, or an antibody binding to a T-cell receptor co-stimulatory molecule or an antibody binding to an antigen presenting cell receptor binding a co-stimulatory molecule, as described herein, may be used in the methods of this invention. In one embodiment, any composition comprising an antibody or functional fragment thereof described herein may be used in the methods of this invention. Compositions comprising *Listeria* strains with and without antibodies have been described in detail above. Compositions with antibodies have also been described in detail above. In some embodiment, in a method of this invention a composition comprising an antibody or fragment thereof, for example an antibody binding to a TNF receptor super family member, or an antibody binding

to a T-cell receptor co-stimulatory molecule or an antibody binding to an antigen presenting cell receptor binding a co-stimulatory molecule, may be administered prior to, concurrent with or following administration of a composition comprising a *Listeria* strain.

[00602] In one embodiment, repeat administrations (doses) of compositions of this invention may be undertaken immediately following the first course of treatment or after an interval of days, weeks or months to achieve tumor regression. In another embodiment, repeat doses may be undertaken immediately following the first course of treatment or after an interval of days, weeks or months to achieve suppression of tumor growth. Assessment may be determined by any of the techniques known in the art, including diagnostic methods such as imaging techniques, analysis of serum tumor markers, biopsy, or the presence, absence or amelioration of tumor associated symptoms.

[00603] In one embodiment, provided herein are methods and compositions for preventing, treating and vaccinating against a heterologous antigen-expressing tumor and inducing an immune response against sub-dominant epitopes of the heterologous antigen, while preventing an escape mutation of the tumor.

[00604] In one embodiment, the methods and compositions for preventing, treating and vaccinating against a heterologous antigen-expressing tumor comprise the use of a truncated Listeriolysin (tLLO) protein. In another embodiment, the methods and compositions provided herein comprise a recombinant *Listeria* overexpressing tLLO. In another embodiment, the tLLO is expressed from a plasmid within the *Listeria*.

[00605] In another embodiment, provided herein is a method of preventing or treating a tumor growth or cancer in a subject, the method comprising the step of administering to the subject an immunogenic composition comprising an antibody or functional fragment thereof, as described herein, and a recombinant *Listeria* immunotherapy strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to a heterologous antigen or fragment thereof. In another embodiment, provided herein is a method of preventing or treating a tumor growth or cancer in a subject, the method comprising the step of administering to the subject an immunogenic composition comprising an antibody or functional fragment thereof, as described herein, and a recombinant *Listeria* immunotherapy strain comprising a nucleic acid molecule, the nucleic acid molecule

comprising a first open reading frame encoding a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

[00606] In one embodiment, the term "treating" refers to curing a disease. In another embodiment, "treating" refers to preventing a disease. In another embodiment, "treating" refers to reducing the incidence of a disease. In another embodiment, "treating" refers to ameliorating symptoms of a disease. In another embodiment, "treating" refers to increasing performance free survival or overall survival of a patient. In another embodiment, "treating" refers to stabilizing the progression of a disease. In another embodiment, "treating" refers to inducing remission. In another embodiment, "treating" refers to slowing the progression of a disease. The terms "reducing", "suppressing" and "inhibiting" refer in another embodiment to lessening or decreasing.

[00607] In one embodiment, provided herein is a method of increasing a ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor microenvironments of a subject, comprising administering the immunogenic composition provided herein. In another embodiment, increasing a ratio of T effector cells to regulatory T cells (Tregs) in the spleen and tumor microenvironments in a subject allows for a more profound anti-tumor response in the subject.

[00608] In another embodiment, the T effector cells comprise CD4+FoxP3- T cells. In another embodiment, the T effector cells are CD4+FoxP3- T cells. In another embodiment, the T effector cells comprise CD4+FoxP3- T cells and CD8+ T cells. In another embodiment, the T effector cells are CD4+FoxP3- T cells and CD8+ T cells. In another embodiment, the regulatory T cells is a CD4+FoxP3+ T cell.

[00609] In one embodiment, the present invention provides methods of treating, protecting against, and inducing an immune response against a tumor or a cancer, comprising the step of administering to a subject the immunogenic composition provided herein.

[00610] In one embodiment, the present invention provides a method of preventing or treating a tumor or cancer in a human subject, comprising the step of administering to the subject the immunogenic composition strain provided herein, the recombinant *Listeria* strain comprising a recombinant polypeptide comprising an N-terminal fragment of an LLO protein and tumor-associated antigen, whereby the recombinant *Listeria* strain induces an immune response against the tumor-associated antigen, thereby treating a tumor or cancer in a human subject. In another embodiment, the immune response is a T-cell response. In another

embodiment, the T-cell response is a CD4+FoxP3- T cell response. In another embodiment, the T-cell response is a CD8+ T cell response. In another embodiment, the T-cell response is a CD4+FoxP3- and CD8+ T cell response. In another embodiment, the present invention provides a method of protecting a subject against a tumor or cancer, comprising the step of administering to the subject the immunogenic composition provided herein. In another embodiment, the present invention provides a method of inducing regression of a tumor in a subject, comprising the step of administering to the subject the immunogenic composition provided herein. In another embodiment, the present invention provides a method of reducing the incidence or relapse of a tumor or cancer, comprising the step of administering to the subject the immunogenic composition provided herein. In another embodiment, the present invention provides a method of suppressing the formation of a tumor in a subject, comprising the step of administering to the subject the immunogenic composition provided herein. In another embodiment, the present invention provides a method of inducing a remission of a cancer in a subject, comprising the step of administering to the subject the immunogenic composition provided herein. In one embodiment, the nucleic acid molecule comprising a first open reading frame encoding a fusion polypeptide is integrated into the *Listeria* genome. In another embodiment, the nucleic acid is in a plasmid in the recombinant *Listeria* immunotherapy strain. In another embodiment, the nucleic acid molecule is in a bacterial artificial chromosome in the recombinant *Listeria* immunotherapy strain.

[00611] In one embodiment, the method comprises the step of co-administering the recombinant *Listeria* with an additional therapy. In another embodiment, the additional therapy is surgery, chemotherapy, an immunotherapy, a radiation therapy, antibody based immunotherapy, or a combination thereof. In another embodiment, the additional therapy precedes administration of the recombinant *Listeria*. In another embodiment, the additional therapy follows administration of the recombinant *Listeria*. In another embodiment, the additional therapy is an antibody therapy. In another embodiment, the recombinant *Listeria* is administered in increasing doses in order to increase the T-effector cell to regulatory T cell ration and generate a more potent anti-tumor immune response. It will be appreciated by a skilled artisan that the anti-tumor immune response can be further strengthened by providing the subject having a tumor with cytokines including, but not limited to IFN-γ, TNF-α, and other cytokines known in the art to enhance cellular immune response, some of which can be found in US Patent Serial No. 6,991,785, incorporated by reference herein.

[00612] In one embodiment, the methods provided herein further comprise the step of co-

administering an immunogenic composition provided herein with an antibody or functional fragment thereof that enhances an anti-tumor immune response in said subject.

[00613] In one embodiment, the methods provided herein further comprise the step of co-administering an immunogenic composition provided herein with a indoleamine 2,3-dioxygenase (IDO) pathway inhibitor. IDO pathway inhibitors for use in the present invention include any IDO pathway inhibitor known in the art, including but not limited to, 1-methyltryptophan (1MT), 1-methyltryptophan (1MT), Necrostatin-1, Pyridoxal Isonicotinoyl Hydrazone, Ebselen, 5-Methylindole-3-carboxaldehyde, CAY10581, an anti-IDO antibody or a small molecule IDO inhibitor. In another embodiment, the compositions and methods provided herein are also used in conjunction with, prior to, or following a chemotherapeutic or radiotherapeutic regiment. In another embodiment, IDO inhibition enhances the efficiency of chemotherapeutic agents.

[00614] In another embodiment, provided herein is a method of increasing survival of a subject suffering from cancer or having a tumor, the method comprising the step of administering to the subject an immunogenic composition comprising an antibody or functional fragment thereof, as described herein, and a recombinant *Listeria* immunotherapy strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to a heterologous antigen or fragment thereof.

[00615] In another embodiment, provided herein is a method of increasing antigen-specific T cells in a subject suffering from cancer or having a tumor, the method comprising the step of administering to the subject an immunogenic composition comprising an antibody or functional fragment thereof, as described herein, and a recombinant *Listeria* immunotherapy strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding fusion polypeptide, wherein the fusion polypeptide comprises a truncated listeriolysin O (LLO) protein, a truncated ActA protein, or a PEST amino acid sequence fused to a heterologous antigen or fragment thereof. In another embodiment, provided herein is a method of increasing T cells in a subject suffering from cancer or having a tumor, the method comprising the step of administering to the subject an immunogenic composition comprising an antibody or functional fragment thereof, as described herein, and a recombinant *Listeria* immunotherapy strain comprising a nucleic acid molecule, the nucleic acid molecule comprising a first open reading frame encoding a truncated listeriolysin O

(LLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

[00616] In another embodiment, a method of present invention further comprises the step of boosting the subject with a recombinant *Listeria* strain or an antibody or functional fragment thereof, as provided herein. In another embodiment, the recombinant *Listeria* strain used in the booster inoculation is the same as the strain used in the initial "priming" inoculation. In another embodiment, the booster strain is different from the priming strain. In another embodiment, the antibody used in the booster inoculation binds the same antigen as the antibody used in the initial "priming" inoculation. In another embodiment, the booster antibody is different from the priming antibody. In another embodiment, the same doses are used in the priming and boosting inoculations. In another embodiment, a larger dose is used in the booster. In another embodiment, a smaller dose is used in the booster. In another embodiment, the methods of the present invention further comprise the step of administering to the subject a booster vaccination. In one embodiment, the booster vaccination follows a single priming vaccination. In another embodiment, a single booster vaccination is administered after the priming vaccinations. In another embodiment, two booster vaccinations are administered after the priming vaccinations. In another embodiment, three booster vaccinations are administered after the priming vaccinations. In one embodiment, the period between a prime and a boost strain is experimentally determined by the skilled artisan. In another embodiment, the period between a prime and a boost strain is 1 week, in another embodiment it is 2 weeks, in another embodiment, it is 3 weeks, in another embodiment, it is 4 weeks, in another embodiment, it is 5 weeks, in another embodiment it is 6-8 weeks, in yet another embodiment, the boost strain is administered 8-10 weeks after the prime strain.

[00617] In another embodiment, a method of the present invention further comprises boosting the subject with an immunogenic composition comprising an attenuated *Listeria* strain provided herein. In another embodiment, a method of the present invention comprises the step of administering a booster dose of the immunogenic composition comprising the attenuated *Listeria* strain provided herein. In another embodiment, the booster dose is an alternate form of said immunogenic composition. In another embodiment, the methods of the present invention further comprise the step of administering to the subject a booster immunogenic composition. In one embodiment, the booster dose follows a single priming dose of said immunogenic composition. In another embodiment, a single booster dose is administered after the priming dose. In another embodiment, two booster doses are administered after the priming dose. In another embodiment, three booster doses are

administered after the priming dose. In one embodiment, the period between a prime and a boost dose of an immunogenic composition comprising the attenuated *Listeria* provided herein is experimentally determined by the skilled artisan. In another embodiment, the dose is experimentally determined by a skilled artisan. In another embodiment, the period between a prime and a boost dose is 1 week, in another embodiment it is 2 weeks, in another embodiment, it is 3 weeks, in another embodiment, it is 4 weeks, in another embodiment, it is 5 weeks, in another embodiment it is 6-8 weeks, in yet another embodiment, the boost dose is administered 8-10 weeks after the prime dose of the immunogenic composition.

[00618] Heterologous "prime boost" strategies have been effective for enhancing immune responses and protection against numerous pathogens. Schneider et al., Immunol. Rev. 170:29-38 (1999); Robinson, H. L., Nat. Rev. Immunol. 2:239-50 (2002); Gonzalo, R. M. et al., Strain 20:1226-31 (2002); Tanghe, A., Infect. Immun. 69:3041-7 (2001). Providing antigen in different forms in the prime and the boost injections appears to maximize the immune response to the antigen. DNA strain priming followed by boosting with protein in adjuvant or by viral vector delivery of DNA encoding antigen appears to be the most effective way of improving antigen specific antibody and CD4+ T-cell responses or CD8+ T-cell responses respectively. Shiver J. W. et al., Nature 415: 331-5 (2002); Gilbert, S. C. et al., Strain 20:1039-45 (2002); Billaut-Mulot, O. et al., Strain 19:95-102 (2000); Sin, J. I. et al., DNA Cell Biol. 18:771-9 (1999). Recent data from monkey vaccination studies suggests that adding CRL1005 poloxamer (12 kDa, 5% POE), to DNA encoding the HIV gag antigen enhances T-cell responses when monkeys are vaccinated with an HIV gag DNA prime followed by a boost with an adenoviral vector expressing HIV gag (Ad5-gag). The cellular immune responses for a DNA/poloxamer prime followed by an Ad5-gag boost were greater than the responses induced with a DNA (without poloxamer) prime followed by Ad5-gag boost or for Ad5-gag only. Shiver, J. W. et al. Nature 415:331-5 (2002). U.S. Patent Appl. Publication No. US 2002/0165172 A1 describes simultaneous administration of a vector construct encoding an immunogenic portion of an antigen and a protein comprising the immunogenic portion of an antigen such that an immune response is generated. The document is limited to hepatitis B antigens and HIV antigens. Moreover, U.S. Pat. No. 6,500,432 is directed to methods of enhancing an immune response of nucleic acid vaccination by simultaneous administration of a polynucleotide and polypeptide of interest. According to the patent, simultaneous administration means administration of the polynucleotide and the polypeptide during the same immune response, preferably within 0-10

or 3-7 days of each other. The antigens contemplated by the patent include, among others, those of Hepatitis (all forms), HSV, HIV, CMV, EBV, RSV, VZV, HPV, polio, influenza, parasites (e.g., from the genus Plasmodium), and pathogenic bacteria (including but not limited to M. tuberculosis, M. leprae, Chlamydia, Shigella, B. burgdorferi, enterotoxigenic E. coli, S. typhosa, H. pylori, V. cholerae, B. pertussis, etc.). All of the above references are herein incorporated by reference in their entireties.

[00619] In one embodiment, a treatment protocol of the present invention is therapeutic. In another embodiment, the protocol is prophylactic. In another embodiment, the compositions of the present invention are used to protect people at risk for cancer such as breast cancer or other types of tumors because of familial genetics or other circumstances that predispose them to these types of ailments as will be understood by a skilled artisan. In another embodiment, the immunotherapies are used as a cancer immunotherapy after debulking of tumor growth by surgery, conventional chemotherapy or radiation treatment. Following such treatments, the immunotherapies of the present invention are administered so that the CTL response to the tumor antigen of the immunotherapy destroys remaining metastases and prolongs remission from the cancer. In another embodiment, immunotherapies of the present invention are used to effect the growth of previously established tumors and to kill existing tumor cells.

[00620] In some embodiments, the term “comprise” or grammatical forms thereof, refers to the inclusion of the indicated active agent, such as the *Lm* strains of this invention, as well as inclusion of other active agents, such as an antibody or functional fragment thereof, and pharmaceutically acceptable carriers, excipients, emollients, stabilizers, etc., as are known in the pharmaceutical industry. In some embodiments, the term “consisting essentially of” refers to a composition, whose only active ingredient is the indicated active ingredient, however, other compounds may be included which are for stabilizing, preserving, etc. the formulation, but are not involved directly in the therapeutic effect of the indicated active ingredient. In some embodiments, the term “consisting essentially of” may refer to components, which exert a therapeutic effect via a mechanism distinct from that of the indicated active ingredient. In some embodiments, the term “consisting essentially of” may refer to components, which exert a therapeutic effect and belong to a class of compounds distinct from that of the indicated active ingredient. . In some embodiments, the term “consisting essentially of” may refer to components, which exert a therapeutic effect and may be distinct from that of the indicated active ingredient, by acting via a different mechanism of action, for example. In

some embodiments, the term “consisting essentially of” may refer to components which facilitate the release of the active ingredient. In some embodiments, the term “consisting” refers to a composition, which contains the active ingredient and a pharmaceutically acceptable carrier or excipient.

5 [00621] As used herein, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

[00622] Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is 10 merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub ranges such as from 1 to 3, from 1 to 4, from 1 15 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[00623] Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases “ranging/ranges between” a first indicate number and a second indicate number and “ranging/ranges from” a 20 first indicate number “to” a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals there between.

[00624] As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, 25 means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

[00625] In one embodiment, the term “about”, refers to a deviance of between 0.0001-5% from the indicated number or range of numbers. In one embodiment, the term “about”, refers 30 to a deviance of between 1 -10% from the indicated number or range of numbers. In one embodiment, the term “about”, refers to a deviance of up to 25% from the indicated number or range of numbers.

[00626] The subject matter disclosed herein includes, but is not limited to, the following emdodiments:

1. A personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

5 a. an attenuated Listeria strain delivery vector; and

b. a plasmid vector for transforming said Listeria strain, said plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said

10 disease or condition;

wherein transforming said Listeria strain with said plasmid vector creates a personalized immunotherapy system targeted to said subject's disease or condition.

2. The system of embodiment 1, wherein said disease or condition comprises an infectious disease or a tumor or a cancer.

15 3. The system of embodiment 2, wherein said infectious disease comprises a viral infection.

4. The system of embodiment 2, wherein said infectious disease comprises a bacterial infection.

5. The system of any one of embodiments 1-4, wherein said one or more neo-epitopes

20 comprise a linear neo-epitope(s), or a conformational neo-epitope(s), or any combination thereof.

6. The system of any one of embodiments 1-5, wherein said one or more neo-epitopes comprise a solvent-exposed neo-epitope(s).

7. The system of any one of embodiments 1-6, wherein the immunogenicity of said neo-

25 epitopes was determined using an immunogenic assay analyzing increased secretion of at least one of CD25, CD44, or CD69, or any combination thereof, or an increased secretion of a cytokine selected from the group comprising IFN-γ, TNF-α, IL-1, and IL-2, upon contacting T-cells with said one or more peptides, and wherein said increase identifies said peptide to comprise one or more T-cell neo-epitopes.

30 8. The system of any one of embodiments 1-7, wherein said attenuated Listeria transformed with said plasmid, secretes said one or more immunogenic peptides.

9. The system of any one of embodiments 1-8, wherein said nucleic acid sequence encoding said one or more peptides comprises one or more neo-epitopes each fused to an immunogenic polypeptide or fragment thereof.

10. The system of any one of embodiments 1-9, wherein said nucleic acid sequence encoding said one or more peptides comprises a minigene nucleic acid construct, said construct comprising an open reading frame encoding a chimeric protein, wherein said chimeric protein comprises:

a. a bacterial secretion signal sequence,

b. a ubiquitin (Ub) protein,

c. said one or more peptides comprising one or more neo-epitopes,

wherein said signal sequence, said ubiquitin and said one or more peptides in (a)-(c) are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

11. The system of any one of embodiments 1-10, wherein said plasmid vector is an integrative plasmid.

12. The system of any one of embodiments 1-10, wherein said plasmid vector is an extrachromosomal multicopy plasmid.

13. The system of embodiment 12, wherein following transformation said plasmid is stably maintained in said Listeria strain in the absence of antibiotic selection.

14. The system of embodiment 9, wherein said immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

15. The system of embodiment 14, wherein said tLLO protein is set forth in SEQ ID NO: 3.

16. The system of embodiment 14, wherein said ActA is set forth in SEQ ID NO: 12-13 and 15-18.

17. The system of embodiment 14, wherein said PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

18. The system of embodiment 14, wherein said mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

19. The system of embodiment 18, wherein said mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

20. The system of embodiment 18, wherein said mutation comprises a substitution of 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68with a 1-50 amino acid non-LLO peptide, wherein said non-LLO peptide comprises a peptide comprising a neo-epitope.

21. The system of embodiment 18, wherein said mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

22. The system of any one of embodiments 1-21, wherein said immunogenic one or more neo-epitopes are associated with said disease or condition.

23. The system of any one of embodiments 1-22, wherein said immunogenic peptides comprising one or more neo-epitopes are comprised by a heterologous or a self-antigen or a fragment thereof.

24. The system of embodiment 23, wherein said heterologous or self-antigen is a tumor-associated antigen.

25. The system of any one of embodiments 1-24, wherein said one or more neo-epitopes comprise a cancer-specific or tumor-specific epitope.

26. The system of embodiment 25, wherein said tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

27. The system of any of embodiments 2 or 25, wherein said tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric

adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

5 28. The system of any one of embodiments 1-24, wherein said one or more neo-epitope comprise a infectious disease-associated-specific epitope.

29. The system of embodiment 28, wherein said infections disease is an infectious viral disease.

30. The system of embodiment 28, wherein said infections diseae is an infectious
10 bacterial disease.

31. The system of embodiment 28, wherein said the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus
15 influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral
20 hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola , Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus
25 enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic
30 fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa

(Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis,
5 Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

32. The system of any one of embodiments 1-31, wherein said attenuated Listeria comprises a mutation in one or more endogenous genes.

33. The system of embodiment 32, wherein said endogenous gene mutation is selected
10 from an actA gene mutation, a prfA mutation, an actA and inlB double mutation, a dal/dal gene double mutation, or a dal/dat/actA gene triple mutation, or a combination thereof.

34. The system of embodiment 33, wherein said prfA mutation is complemented by transforming said Listeria with a plasmid comprising a nucleic acid sequence encoding a PrfA comprising a D133V mutation.

15 35. The system of any one of embodiments 32-34, wherein said mutation comprises an inactivation, truncation, deletion, replacement or disruption of the gene or genes.

36. The system of any one of embodiments 1-35, wherein said plasmid further comprises a second nucleic acid sequence comprising an open reading frame encoding a metabolic enzyme.

20 37. The system of embodiment 36, wherein said metabolic enzyme encoded by said open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

38. The system of any one of embodiments 1-37, wherein said Listeria is Listeria monocytogenes.

39. The system of any one of embodiments 1-38, wherein said recombinant Listeria is
25 cultivated or cryopreserved, or any combination thereof, and administered as a form of treatment to that subject either alone or in combination with other potentially beneficial treatments for said disease.

40. The system of any one of embodiments 1-39, wherein administering said attenuated Listeria transformed with said plasmid is accomplished as part of an immunogenic
30 composition comprising said attenuated recombinant Listeria and an adjuvant to said subject.

41. The system of embodiment 40, wherein administering said immunogenic composition further comprises the step of concomitantaly or sequentially administering one or more immunogenic compositions comprising an attenuated Listeria expressing a different peptide comprising one or more neo-epitopes and an adjuvant.

42. The system of any one of embodiments 40-41, wherein said adjuvant comprises wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

43. A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORF) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more neo-epitopres encoded within said one or more ORF from the disease-bearing sample;

b. screening peptides comprising said one or more neo-epitopes for an immunogenic response;

c. transforming an attenuated Listeria strain with a plasmid vector comprising a nucleic acid sequence that encodes a one or more peptides comprising said one or more immunogenic neo-epitopes; and, alternatively storing said attenuated recombinant Listeria for administering to said subject at a pre-determined period or administering said attenuated recombinant Listeria strain to said subject, wherein said attenuated recombinant Listeria strain is administered as part of an immunogenic composition.

44. The process of embodiment 43, wherein said disease or condition is an infectious disease, or a tumor or a cancer.

45. The process of embodiment 44, wherein said infectious disease comprises a viral infection.

46. The process of embodiment 44, wherein said infectiou disease comprise a bacterial infection.

47. The process of any one of embodiments 43-46, wherein said disease-bearing biological sample is obtained from said subject having said diseae or condition.

48. The process of any one of embodiments 43-47, wherein said healthy biological sample is obtained from said subject having said disease or condition, or from a different individual of the same species.

49. The process of any one of embodiments 43-48, wherein said disease-bearing or healthy biological sample comprises a tissue, cells isolated from blood, cells isolated from sputum, cells isolated from saliva, or cells isolated from cerebro spinal fluid.

50. The process of any one of embodiments 43-49, wherein said nucleic acid sequences are determined using exome sequencing.

51. The process of any one of embodiments 43-50, wherein said nucleic acid sequences are determined using transcriptome sequencing.

52. The process of any one of embodiments 43-51, wherein said comparing comprises a use of a screening assay or screening tool and associated digital software for comparing one or more open reading frames (ORF) in nucleic acid sequences extracted from said disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from said healthy biological sample, sample,

i. wherein said associated digital software comprises access to a sequence database that allows screening of mutations within said ORF for identification of immunogenic potential of said neo-epitopes.

53. The process of any one of embodiments 43-51, wherein said screening for an immunogenic response comprises the following steps:

a. contacting a T-cell or cells with said peptide comprising one or more neo-epitopes;

b. analyzing for an immunogenic T-cell response in said cells, wherein presence of an immunogenic T-cell responseidentifies said peptide as an immungenic peptide.

54. The process of any one of embodiments 43-53 wherein said one or more neo-epitopes comprise linear or conformational neo-epitopes.

55. The process of any one of embodiments 43-54, wherein said one or more neo-epitopes comprise a solvent-exposed epitope.

56. The process of any one of embodiments 43-55 wherein said attenuated recombinant Listeria secretes a said one or more immunogenic peptides comprising one or more immunogenic neo-epitopes comprising a T-cell epitope.

57. The process of any one of embodiments 43-56, wherein said transforming is accomplished using a plasmid vector comprising a nucleic acid sequence that encodes said one or more immunogenic peptides comprising one or more immunogenic neo-epitopes each fused to an immunogenic polypeptide or fragment thereof.

5 58. The process of any one of embodiments 43-56, wherein said transforming is accomplished using a plasmid vector comprising a minigene nucleic acid construct, said construct comprising an open reading frame encoding a chimeric protein, wherein said chimeric protein comprises:

a. a bacterial secretion signal sequence,

10 b. a ubiquitin (Ub) protein,

c. said one or more immunogenic peptides comprising said one or more immunogenic neo-epitope,

wherein said signal sequence, said ubiquitin and said peptide in a.-c. are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

15 59. The process of any one of embodiments 43-58, further comprising culturing and characterizing said attenuated recombinant Listeria strain to confirm expression of said one or more immunogenic peptides.

60. The process of any one of embodiments 43-59, wherein said plasmid is an integrative plasmid.

20 61. The process of any one of embodiments 43-59, wherein said plasmid is an extrachromosomal multicopy plasmid.

62. The process of embodiment 61, wherein said plasmid is stably maintained in said Listeria strain in the absence of antibiotic selection.

63. The process of embodiment 57, wherein said immunogenic polypeptide is a mutated
25 Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

64. The process of embodiment 63, wherein said tLLO protein is set forth in SEQ ID NO: 3.

65. The process of embodiment 63, wherein said actA is set forth in SEQ ID NO: 12-13
30 and 15-18.

66. The process of embodiment 63, wherein said PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10

67. The process of embodiment 63, wherein said mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

68. The process of embodiment 67, wherein said mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

69. The process of embodiment 67, wherein said mutation comprises a substitution of 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein said non-LLO peptide comprises a peptide comprising a neo-epitope.

70. The process of embodiment 67, wherein said mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

71. The process of any one of embodiments 43-70, wherein said one or more peptides comprising said one or more neo-epitopes are comprised by a heterologous antigen or a self-antigen associated with said disease.

72. The process of embodiment 71, wherein said heterologous antigen or said self-antigen is a tumor-associated antigen or a fragment thereof.

73. The process of any one of embodiments 43-72, wherein said one or more neo-epitopes comprise a cancer-specific or tumor-specific epitope.

74. The process of embodiment 73, wherein said tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

75. The process of any one of embodiments 44 and 73, wherein said tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non -mall-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

76. The process of any one of embodiments 43-75, wherein said one or more neo-epitopes comprise an infectious disease-associated-specific epitope.

77. The process of embodiment 76, wherein said infections disease is an infectious viral disease.

78. The process of embodiment 76, wherein said infections dieseae is an infectious bacterial disease.

79. The process of embodiment 76, wherein said the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses,

Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV),
5 Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis,
10 Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

80. The process of any one of embodiments 43-79, wherein said attenuated Listeria comprises a mutation in one or more endogenous genes.

81. The process of embodiment 80, wherein said endogenous gene mutation is selected
15 from an actA gene mutation, a prfA mutation, an actA and inlB double mutation, a dal/dal gene double mutation, or a dal/dat/actA gene triple mutation, or a combination thereof.

82. The process of embodiment 81, wherein said prfA mutation is complemented by transforming said Listeria with a plasmid comprising a nucleic acid sequence encoding a PrfA comprising a D133V mutation.

20 83. The process of any one of embodiments 80-82, wherein said mutation comprises an inactivation, truncation, deletion, replacement or disruption of the gene or genes.

84. The process of any one of embodiments 43-83, wherein said plasmid further comprises a second nucleic acid sequence comprising an open reading frame encoding a metabolic enzyme.

25 85. The process of embodiment 84, wherein said metabolic enzyme encoded by said open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

86. The process of any one of embodiments 43-85, wherein said Listeria is Listeria monocytogenes.

87. The process of any one of embodiments 43-86, further comprising administering one
30 or more immunogenic compositions comprising a recombinant Listeria expressing a different peptide comprising one or more different neo-epitopes and an adjuvant to said subject.

88. The process of embodiment 87, wherein administering comprises concomitant administering or sequential administering.

89. The process of any one of embodiments 87-88, wherein said adjuvant comprises wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

90. The process of any one of embodiments 43-89, further comprising administering an immune checkpoint inhibitor antagonist.

91. The process of embodiment 90, wherein said immune checkpoint inhibitor is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

92. The process of any one of embodiments 43-91, wherein said administering generates a personalized enhanced anti-disease, or anti-condition immune response in said subject.

93. The process of embodiment 92, wherein said immune response comprises an anti-cancer or anti-tumor response.

94. The process of embodiment 92, wherein said immune response comprises an anti-infectious disease response.

95. The process of embodiment 94, wherein said infectious disease comprises a viral infection.

96. The process of embodiment 94, wherein said infectious disease comprises a bacterial infection.

97. The process of any one of embodiments 43-96, wherein said process allows personalized treatment or prevention of said disease or condition in said subject.

98. The process of any one of embodiments 43-97, wherein said administration increases survival time in said subject having said disease or condition.

99. A recombinant attenuated Listeria strain produced by the process of any one of embodiments 43-86.

100. A system for providing a personalized immunotherapy system created for a subject having a disease or condition, said system comprising:

a. delivery vector or other vector; and optionally

b. a plasmid vector for transforming said delivery vector, said plasmid vector comprising a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition.

101. The system of embodiment 100, wherein said delivery vector comprises a bacterial delivery vector.

102. The system of embodiment 100, wherein said delivery vector comprises a viral vector delivery vector.

103. The system of embodiment 100, wherein said delivery vector comprises a peptide immunotherapy delivery vector.

104. The system of embodiment 103, wherein said peptide immunotherapy delivery vector comprises a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition

105. The system of embodiment 100, wherein said delivery vector comprises a DNA plasmid immunotherapy vector.

106. The system of embodiment 105, wherein said DNA plasmid immunotherapy vector delivery vector comprises a nucleic acid construct comprising one or more open reading frames encoding one or more peptides comprising one or more neo-epitopes, wherein said neo-epitope(s) comprise immunogenic epitopes present in a disease-bearing tissue or cell of said subject having said disease or condition

107. The system of embodiment 100, wherein said disease or condition comprises an infectious disease, or a tumor or a cancer.

108. The system of embodiment 107, wherein said infectious disease comprises a viral infection.

109. The system of embodiment 107, wherein said infectious disease comprises a bacterial infection.

110. The system of any one of embodiments 100-109, wherein said one or more neo-epitopes comprise a linear neo-epitope(s), or a conformational neo-epitope(s), or any combination thereof.

111. The system of any one of embodiments 100-110, wherein said one or more neo-epitopes comprise a solvent-exposed neo-epitope(s).

112. The system of any one of embodiments 100-111, wherein the immunogenicity of said neo-epitopes was determined using an immunogenic assay analyzing increased secretion of at least one of CD25, CD44, or CD69, or any combination thereof, or an increased secretion of a cytokine selected from the group comprising IFN-γ, TNF-α, IL-1, and IL-2, upon contacting T-cells with said one or more peptides, and wherein said increase identifies said peptide to comprise one or more T-cell neo-epitopes.

113. The system of any one of embodiments 100-112, wherein said delivery vector transformed with said plasmid, secretes said one or more immunogenic peptides.

114. The system of any one of embodiments 100-113, wherein said nucleic acid sequence encoding said one or more peptides comprises one or more neo-epitopes each fused to an immunogenic polypeptide or fragment thereof.

115. The system of any one of embodiments 100-113, wherein said nucleic acid sequence enocding said one or more peptides comprises a minigene nucleic acid construct, said construct comprising an open reading frame encoding a chimeric protein, wherein said chimeric protein comprises:

a. a bacterial secretion signal sequence,

b. a ubiquitin (Ub) protein,

c. said one or more peptides comprising one or more neo-epitopes,

wherein said signal sequence, said ubiquitin and said one or more peptides in (a)-(c) are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

116. The system of any one of embodiments 100-115, wherein said plasmid vector is an integrative plasmid.

117. The system of any one of embodiments 100-115, wherein said plasmid vector is an extrachromosomal multicopy plasmid.

118. The system of embodiment 114, wherein said immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

119. The system of embodiment 118, wherein said tLLO protein is set forth in SEQ ID
5 NO: 3.

120. The system of embodiment 118, wherein said ActA is set forth in SEQ ID NO: 12-13 and 15-18.

121. The system of embodiment 118, wherein said PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

10 122. The system of embodiment 118, wherein said mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

123. The system of embodiment 118, wherein said mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

124. The system of embodiment 118, wherein said mutation comprises a substitution of 1-
15 11 amino acid within the CBD as set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein said non-LLO peptide comprises a peptide comprising a neo-epitope.

125. The system of embodiment 118, wherein said mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

126. The system of any one of embodiments 100-125, wherein said immunogenic one or
20 more neo-epitopes are associated with said disease or condition.

127. The system of any one of embodiments 100-125, wherein said immunogenic peptides comprising one or more neo-epitopes are comprised by a heterologous or a self-antigen or a fragment thereof.

128. The system of embodiment 127, wherein said heterologous or self-antigen is a tumor-
25 associated antigen.

129. The system of any one of embodiments 100-128, wherein said one or more neo-epitopes comprise a cancer-specific or tumor-specific epitope.

130. The system of embodiment 128, wherein said tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-
30 18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA),

ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

131. The system of any of embodiments 107 or 128, wherein said tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

132. The system of any one of embodiments 100-131, wherein said one or more neo-epitope comprise a infectious disease-associated-specific epitope.

133. The system of embodiment 132, wherein said infections disease is an infectious viral disease.

134. The system of embodiment 132, wherein said infections diseae is an infectious bacterial disease.

135. The system of embodiment 132, wherein said the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus

anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola , Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

136. The system of any one of embodiments 100-135, wherein said delivery vector is cultivated, or cryopreserved, or any combination thereof, and administered as a form of treatment to that subject either alone or in combination with other potentially beneficial treatments for said disease.

137. The system of any one of embodiments 100-136, wherein administering said delivery vector optionally transformed with said plasmid is accomplished as part of an immunogenic composition comprising said recombinant delivery vector and an adjuvant to said subject.

138. The system of embodiment 137, wherein administering said immunogenic composition further comprises the step of concomitantly or sequentially administering one or more immunogenic compositions comprising a delivery vector expressing a different peptide comprising one or more neo-epitopes and an adjuvant.

139. The system of any one of embodiments 137-138, wherein said adjuvant comprises wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

5 140. A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORF) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one
10 or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORF from the disease-bearing sample;

b. transforming an attenuated Listeria strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said attenuated recombinant Listeria for administering to said
15 subject at a pre-determined period or administering a composition comprising said attenuated recombinant Listeria strain to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; optionally,

c. Obtaining a second biological sample from said subject comprising a T-cell clone or
20 T-infiltrating cell from said T-cell immune response and characterizing specific peptides comprising one or more neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells , wherein said one or more neo-epitopes are immunogenic;

d. Screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

25 e. Transforming a second attenuated recombinant Listeria strain with a vector comprising a nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes; and, alternatively storing said second attenuated recombinant Listeria for administering to said subject at a pre-determined period or administering a second composition comprising said second attenuated recombinant Listeria
30 strain to said subject,

wherein said process creates a personalized immunotherapy for said subject.

141. The process of embodiment 140, wherein said comparing comprises a use of a screening assay or screening tool and associated digital software for comparing one or more

ORF in nucleic acid sequences extracted from said disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from said healthy biological sample,

i. wherein said associated digital software comprises access to a sequence database that allows screening of mutations within said ORF in said nucleic acid sequences extraced from said disease-bearing biological sample for identification of immunogenic potential of said neo-epitopes.

142. The process of any one of embodiments 140-141, wherein the process of obtaining a second biological sample from said subject comprises obtaining a biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of said second composition comprising said attenuated recombinant Listeria strain.

143. The process of any one of embodiments 140-142, wherein said biological sample is tissue, cells, blood or sera.

144. The process of any one of embodiments 140-143, wherein the process of characterizing comprises the steps of:

i. Identifying, isolating and expanding T cell clones or T-infiltrating cells that respond against said disease;

ii. Screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules to which a T-cell receptor on said T cells binds.

145. The process of embodiment 144, wherein said screening for and identifying comprises T-cell receptor sequencing, multiplex based flow cytometry, or high-performance liquid chromatography.

146. The process of embodiment 145, wherein said sequencing comprises the use of associated digital software and database.

147. The process of any one of embodiments 140-146, wherein said disease or condition is an infectious disease, or a tumor or a cancer.

148. The process of embodiment 147, wherein said infectious disease comprises a viral or bacterial infection.

149. The process of any one of embodiments 140-148, wherein said disease-bearing biological sample is obtained from said subject having said disease or condition.

150. The process of any one of embodiments 140-149, wherein said healthy biological sample is obtained from said subject having said disease or condition.

151. The process of any one of embodiments 140-150, wherein said sequencing of said nucleic acid sequences are determined using exome sequencing or transcriptome sequencing.

5 152. The process of any one of embodiments 140-151, wherein said one or more neo-epitopes comprise linear neo-epitopes.

153. The process of any one of embodiments 140-152, wherein said one or more neo-epitopes comprise a solvent-exposed epitope.

154. The process of any one of embodiments 140-153, wherein said attenuated
10 recombinant Listeria secretes said one or more peptides comprising one or more immunogenic neo-epitopes.

155. The process of any one of claims 140-154, wherein said one or more immunogenic neo-epitopes comprise a T-cell epitope.

156. The process of any one of embodiments 140-155, wherein said transforming is
15 accomplished using a plasmid or phage vector.

157. The process of any one of embodiments 140-156, wherein said one or more peptides comprising one or more immunogenic neo-epitopes are each fused to an immunogenic polypeptide or fragment thereof.

158. The process of any one of embodiments 140-157, wherein said transforming is
20 accomplished using a plasmid vector comprising a minigene nucleic acid construct, said construct comprising one or more open reading frames encoding a chimeric protein, wherein said chimeric protein comprises:

a. a bacterial secretion signal sequence,

b. a ubiquitin (Ub) protein,

25 c. said one or more peptides comprising said one or more neo-epitopes,

wherein said signal sequence, said ubiquitin and said one or more peptides in a.-c. are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

159. The process of any one of embodiments 140-158, further comprising culturing and characterizing said attenuated recombinant Listeria strain to confirm expression and secretion
30 of said one or more peptides.

160. The process of any one of embodiments 156-158, wherein said plasmid is an integrative plasmid.

161. The process of any one of embodiments 156-158, wherein said plasmid is an extrachromosomal multicopy plasmid.

5 162. The process of embodiment 156-158, wherein said plasmid is stably maintained in said Listeria strain in the absence of antibiotic selection.

163. The process of embodiment 157, wherein said immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

10 164. The process of embodiment 163, wherein said tLLO protein is set forth in SEQ ID NO: 3.

165. The process of embodiment 163, wherein said actA is set forth in SEQ ID NO: 12-13 and 15-18.

166. The process of embodiment 163, wherein said PEST amino acid sequence is selected 15 from the sequences set forth in SEQ ID NOs: 5-10.

167. The process of embodiment 163, wherein said mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

168. The process of embodiment 167, wherein said mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

20 169. The process of embodiment 167, wherein said mutation comprises a substitution of 1-11 amino acid within the CBD set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein said non-LLO peptide comprises a peptide comprising a neo-epitope.

170. The process of embodiment 167, wherein said mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

25 171. The process of any one of embodiments 140-170, wherein said one or more peptides are comprised by a heterologous antigen or a self-antigen associated with said disease.

172. The process of embodiment 171, wherein said heterologous antigen or said self-antigen is a tumor-associated antigen or a fragment thereof.

173. The process of any one of embodiments 140-172, wherein said one or more neo-30 epitopes comprise a cancer-specific or tumor-specific epitope.

174. The process of any one of embodiments 171-173, wherein said tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

175. The process of any one of embodiments 147 and 173, wherein said tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non -mall-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

176. The process of any one of embodiments 140-175, wherein said one or more neo-epitopes comprise an infectious disease-associated-specific epitope.

177. The process of embodiment 176, wherein said infections disease is an infectious viral disease.

178. The process of embodiment 176, wherein said infections disease is an infectious bacterial disease.

179. The process of embodiment 176, wherein said the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus

influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

180. The process of any one of embodiments 140-179, wherein said attenuated Listeria comprises a mutation in one or more endogenous genes.

181. The process of embodiment 180, wherein said endogenous gene mutation is selected from an actA gene mutation, a prfA mutation, an actA and inlB double mutation, a dal/dal gene double mutation, or a dal/dat/actA gene triple mutation, or a combination thereof.

182. The process of any one of embodiments 180-181, wherein said mutation comprises an inactivation, truncation, deletion, replacement or disruption of the gene or genes.

183. The process of any one of embodiments 140-182, wherein said vector further comprises an open reading frame or a second nucleic acid sequence comprising an open reading frame encoding a metabolic enzyme.

184. The process of embodiment 183, wherein said metabolic enzyme encoded by said open reading frame is an alanine racemase enzyme or a D-amino acid transferase enzyme.

185. The process of any one of embodiments 140-184, wherein said Listeria is Listeria monocytogenes.

186. The process of any one of embodiments 140-185, further comprising administering an adjuvant to said subject.

187. The process of embodiment 176, wherein said adjuvant comprises wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

188. The process of any one of embodiments 140-187, further comprising administering an immune checkpoint inhibitor antagonist.

189. The process of embodiment 188, wherein said immune checkpoint inhibitor is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

190. The process of any one of embodiments 140-189, wherein said administering generates a personalized enhanced anti-disease, or anti-condition immune response in said subject.

191. The process of embodiment 190, wherein said immune response comprises an anti-cancer or anti-tumor response.

192. The process of embodiment 190, wherein said immune response comprises an anti-infectious disease response.

193. The process of embodiment 192, wherein said infectious disease comprises a viral infection.

194. The process of embodiment 192, wherein said infectious disease comprises a bacterial infection.

195. The process of any one of embodiments 140-194, wherein said process allows personalized treatment or prevention of said disease or condition in said subject.

196. The process of any one of embodiments 140-191, wherein said personalized immunotherapy increases survival time in said subject having said disease or condition.

5 197. A recombinant attenuated Listeria strain produced by the process of any one of embodiments 140-185.

198. A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

a. comparing one or more open reading frames (ORF) in nucleic acid sequences
10 extracted from a disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from a healthy biological sample, wherein said comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within said one or more ORF from the disease-bearing sample;

b. transforming a vector with a nucleic acid sequence encoding one or more peptides
15 comprising said one or more neo-epitopes identified in a., or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more neo-epitopes identified in a.; and, alternatively storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period or administering
20 a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject, and wherein said administering results in the generation of a personalized T-cell immune response against said disease or said condition; and optionally,

c. Obtaining a second biological sample from said subject comprising a T-cell clone or T-infiltrating cell from said T-cell immune response and characterizing specific peptides
25 comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on said T cells;

d. Screening for and selecting a nucleic acid construct encoding one or more peptides comprising one or more immunogenic neo-epitope identified in c.; and,

e. Transforming a second vector with a nucleic acid sequence comprising one or more
30 open reading frames encoding one or more peptides comprising said one or more immunogenic neo-epitopes or generating a DNA immunotherapy vector or a peptide immunotherapy vector using said nucleic acid sequence encoding one or more peptides comprising said one or more immunogenic neo-epitopes identified in c.; and, alternatively

storing said vector or said DNA immunotherapy or said peptide immunotherapy for administering to said subject at a pre-determined period, or administering a composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy to said subject,

5 wherein said process creates a personalized immunotherapy for said subject.

199. The process of embodiment 198, wherein said comparing comprises a use of a screening assay or screening tool and associated digital software for comparing one or more ORF in nucleic acid sequences extracted from said disease-bearing biological sample with one or more ORF in nucleic acid sequences extracted from said healthy biological sample,

10 ii. wherein said associated digital software comprises access to a sequence database that allows screening of mutations within said ORF in said nucleic acid sequences extraced from said disease-bearing biological sample for identification of immunogenic potential of said neo-epitopes.

200. The process of any one of embodiments 198-199, wherein the process of obtaining a

15 second biological sample from said subject comprises obtaining a second biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of said second composition comprising said vector, said DNA immunotherapy or said peptide immunotherapy.

201. The process of any one of embodiments 198-200, wherein said biological sample is

20 tissue, cells, blood or sera.

202. The process of any one of embodiments 198-201, wherein the process of characterizing comprises the steps of:

i. Identifying, isolating and expanding T cell clones or T-infiltrating cells that respond against said disease;

25 ii. Screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules to which a T-cell receptor on said T cells binds.

203. The process of embodiment 202, wherein said screening for and identifying comprises T-cell receptor sequencing, multiplex based flow cytometry, or high-performance liquid

30 chromatography.

204. The process of embodiment 203, wherein said sequencing comprises the use of associated digital software and database.

205. The process of any one of embodiments 198-204 wherein said disease or condition is an infectious disease, or a tumor or a cancer.

206. The process of embodiment 205, wherein said infectious disease comprises a viral or bacterial infection.

5 207. The process of any one of embodiments 198-206, wherein said disease-bearing biological sample is obtained from said subject having said disease or condition.

208. The process of any one of embodiments 198-207, wherein said healthy biological sample is obtained from said subject having said disease or condition.

209. The process of any one of embodiments 198-208, wherein said sequencing of said
10 nucleic acid sequences are determined using exome sequencing or transcriptome sequencing.

210. The process of any one of embodiments 198-209, wherein said one or more neo-epitopes comprise linear neo-epitopes.

211. The process of any one of embodiments 198-210, wherein said one or more neo-epitopes comprise a solvent-exposed epitope.

15 212. The process of any one of embodiments 198-211, wherein said one or more immunogenic neo-epitopes comprise a T-cell epitope.

213. The process of any one of embodiments 198-212, wherein said vector is a vaccinia virus or a virus-like particle.

214. The process of any one of embodiments 198-213, further comprising culturing and
20 characterizing said vaccinia virus or virus-like particle to confirm expression of said one or more peptides.

215. The process of any one of embodiments 198-212, wherein said DNA immunotherapy comprises a nucleic acid sequence comprising one or more ORF encoding one or more peptides comprising one or more immunogenic neo-epitopes.

25 216. The process of embodiment 215, wherein said nucleic acid sequence is in the form of a plasmid.

217. The process of any one of embodiments 216, wherein said plasmid is an integrative or an extrachrosomomal multicopy plasmid.

218. The process of any one of embodiments 59-78, wherein said one or more peptides comprising one or more immunogenic neo-epitopes are each fused to an immunogenic polypeptide or fragment thereof.

219. The process of any one of embodiments 198-212, wherein said peptide immunotherapy comprises one or more peptides comprising one or more immunogenic neo-epitopes, wherein each peptide is fused to or mixed with an immunogenic polypeptide or fragment thereof.

220. The process of any one of embodiments 218-219, wherein said immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

221. The process of embodiment 220, wherein said tLLO protein is set forth in SEQ ID NO: 3.

222. The process of embodiment 220, wherein said actA is set forth in SEQ ID NO: 12-13 and 15-18.

223. The process of embodiment 220, wherein said PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

224. The process of embodiment 220, wherein said mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

225. The process of embodiment 224, wherein said mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

226. The process of embodiment 224, wherein said mutation comprises a substitution of 1-11 amino acid within the CBD set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein said non-LLO peptide comprises a peptide comprising a neo-epitope.

227. The process of embodiment 224, wherein said mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

228. The process of any one of embodiments 198-227, wherein said one or more peptides are comprised by a heterologous antigen or a self-antigen associated with said disease.

229. The process of embodiment 228, wherein said heterologous antigen or said self-antigen is a tumor-associated antigen or a fragment thereof.

230. The process of any one of embodiments 198-229, wherein said one or more neo-epitopes comprise a cancer-specific or tumor-specific epitope.

231. The process of any one of embodiments 229-230, wherein said tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

232. The process of any one of embodiments 205 and 230, wherein said tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, an Her2 expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non -mall-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

233. The process of any one of embodiments 198-232, wherein said one or more neo-epitopes comprise an infectious disease-associated-specific epitope.

234. The process of embodiment 233, wherein said infectious disease is an infectious viral disease or an infectious bacterial disease.

235. The process of embodiment 234, wherein said the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus

influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic E.coli, Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

236. The process of any one of embodiments 198-235, further comprising administering an adjuvant to said subject.

237. The process of embodiment 236, wherein said adjuvant comprises wherein said adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

238. The process of any one of embodiments 198-237, further comprising administering an immune checkpoint inhibitor antagonist.

239. The process of embodiment 238, wherein said immune checkpoint inhibitor is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

240. The process of any one of embodiments 198-239, wherein said administering generates a personalized enhanced anti-disease, or anti-condition immune response in said subject.

241. The process of embodiment 240, wherein said immune response comprises an anti-cancer or anti-tumor response.

242. The process of embodiment 240, wherein said immune response comprises an anti-infectious disease response.

243. The process of embodiment 242, wherein said infectious disease comprises a viral infection or bacterial infection.

244. The process of any one of embodiments 198-243, wherein said process allows personalized treatment or prevention of said disease or condition in said subject.

245. The process of any one of embodiments 198-244, wherein said personalized immunotherapy increases survival time in said subject having said disease or condition.

246. A viral-like particle produced by the process of any one of embodiments 198-214, 218, and 220-235.

247. A vaccinia virus strain produced by the process of any one of embodiments 198-214, 218, and 220-235.

248. A DNA immunotherapy produced by the process of any one of embodiments 198-212, 215-218, and 220-235.

249. A peptide immunotherapy produced by the process of any one of embodiments 198-212, 219, and 220-235.

250. A pharmaceutical composition comprising the Listeria of embodiment 197.

251. A pharmaceutical composition comprising the viral-like particle of embodiment 246.

252. A pharmaceutical composition comprising the vaccinia virus strain of embodiment 247.

253. A pharmaceutical composition comprising the DNA immunotherapy of embodiment 248.

254. A pharmaceutical composition comprising the peptide immunotherapy of embodiment 249.

5 255. A system for creating personalized immunotherapy for a subject, comprising: at least one processor and at least one storage medium containing program instructions for execution by the processor, the program instructions causing the processor to execute steps comprising:

(a) receiving output data containing all neo-epitopes and the human leukocyte antigen (HLA) type of the subject;

10 (b) scoring the hydrophobicity of each neo-epitope and removing epitopes that score above a certain threshold;

(c) numerically rating the remaining neo-epitopes based on their ability to bind to subject HLA and on their predictive MHC binding scores;

(d) inserting the amino acid sequence of each neo-epitope into a plasmid;

15 (e) scoring the hydrophobicity of each construct and removing any constructs that score above a certain threshold;

(f) reverse translating the amino acid sequence of each construct into the corresponding DNA sequence, starting with the highest scored construct;

(g) inserting additional neo-epitopes into the plasmid construct in order of 20 ranking until a predetermined upper limit is reached;

(h) adding a DNA sequence tag to the end of the construct in order to measure the immunotherapeutic response in the subject; and

(i) optimizing the DNA sequence encoding the neo-epitopes and the DNA sequence tag for expression and secretion in *Listeria monocytogenes*.

25 256. The system of embodiment 255, wherein the preferred output data is in FASTA format.

257. The system of any embodiment 255-256, wherein the hydrophobicity is scaled using the Kyte-Doolittle hydropathy plot.

258. The system of any embodiment 255-257, wherein all neo-epitopes scoring above a 1.6 30 on the Kyte-Doolittle plot are removed or de-selected.

259. The system of any embodiment 255-258, wherein each neo-epitope’s ability to bind to

subject HLA is rated using the Immune Epitope Database (IED).

260. The system of any embodiment 255-259, wherein each neo-epitope is 21 amino acids in size (21 mer).

261. The system of any embodiment 255-260, wherein the DNA tag is linked to the neo-epitopes via a linker.

262. The system of any embodiment 255-261, wherein the linker is a 4X glycine linker.

263. The system of any embodiment 255-262, wherein the DNA sequence tag of step (h) is SIINFEKL-6xHis.

264. The system of any embodiment 255-263, wherein neo-epitopes known to have immunosuppressive properties are removed from consideration before step (a).

[00627] In the following examples, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

EXAMPLES

Materials and Experimental Methods (Examples 1-2)

Cell lines

[00628] The C57BL/6 syngeneic TC-1 tumor was immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. TC-1, provided by T. C. Wu (Johns Hopkins University School of Medicine, Baltimore, MD) is a highly tumorigenic lung epithelial cell expressing low levels of with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. TC-1 was grown in RPMI 1640, 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 50 micromolar (mcM) 2-ME, 400 microgram (mcg)/ml G418, and 10% National Collection Type Culture-109 medium at 37° with 10% CO_2. C3 is a mouse embryo cell from C57BL/6 mice immortalized with the complete genome of HPV 16 and transformed with pEJ-ras. EL-4/E7 is the thymoma EL-4 retrovirally transduced with E7.

L. monocytogenes strains and propagation

[00629] ***Listeria*** strains used were Lm-LLO-E7, also referred to herein as ADXS11-001, (hly-E7 fusion gene in an episomal expression system; **Figure 1A**), Lm-E7 (single-copy E7 gene cassette integrated into *Listeria* genome), Lm-LLO-NP ("DP-L2028"; hly-NP fusion gene in an episomal expression system), and Lm-Gag ("ZY-18"; single-copy HIV-1 Gag gene cassette integrated into the chromosome). E7 was amplified by PCR using the primers 5'-GGCTCGAGCATGGAGATACACC-3' (SEQ ID No: 24; XhoI site is underlined) and 5'-GGGGACTAGTTTATGGTTTCTGAGAACA-3' (SEQ ID No: 25; SpeI site is underlined) and ligated into pCR2.1 (Invitrogen, San Diego, CA). E7 was excised from pCR2.1 by XhoI/ SpeI digestion and ligated into pGG-55. The hly-E7 fusion gene and the pluripotential transcription factor prfA were cloned into pAM401, a multicopy shuttle plasmid (Wirth R et al, J Bacteriol, 165: 831, 1986), generating pGG-55. The hly promoter drives the expression of the first 441 AA of the hly gene product, (lacking the hemolytic C-terminus, referred to below as "ΔLLO," and having the sequence set forth in SEQ ID No: 3), which is joined by the XhoI site to the E7 gene, yielding a hly-E7 fusion gene that is transcribed and secreted as LLO-E7. Transformation of a prfA negative strain of *Listeria*, XFL-7 (provided by Dr. Hao Shen, University of Pennsylvania), with pGG-55 selected for the retention of the plasmid *in vivo* (**Figures 1A-B**). The hly promoter and gene fragment were generated using primers 5'-GGGGGCTAGCCCTCCTTTGATTAGTATATTC-3' (SEQ ID No: 26; NheI site is underlined) and 5'-CTCCCTCGAGATCATAATTTACTTCATC-3' (SEQ ID No: 27; XhoI site is underlined). The prfA gene was PCR amplified using primers 5'-GACTACAAGGACGATGACCGACAAGTGATAACCCGGGATCTAAATAAATCCGTT T-3' (SEQ ID No: 28; XbaI site is underlined) and 5'-CCCGTCGACCAGCTCTTCTTGGTGAAG-3' (SEQ ID No: 29; SalI site is underlined). Lm-E7 was generated by introducing an expression cassette containing the hly promoter and signal sequence driving the expression and secretion of E7 into the orfZ domain of the LM genome. E7 was amplified by PCR using the primers 5'-GCGGATCCCATGGAGATACACCTAC-3' (SEQ ID No: 30; BamHI site is underlined) and 5'-GCTCTAGATTATGGTTTCTGAG-3' (SEQ ID No: 31; XbaI site is underlined). E7 was then ligated into the pZY-21 shuttle vector. LM strain 10403S was transformed with the resulting plasmid, pZY-21-E7, which includes an expression cassette inserted in the middle of a 1.6-kb sequence that corresponds to the orfX, Y, Z domain of the LM genome. The homology domain allows for insertion of the E7 gene cassette into the orfZ domain by homologous recombination. Clones were screened for integration of the E7 gene cassette into

the orfZ domain. Bacteria were grown in brain heart infusion medium with (Lm-LLO-E7 and Lm-LLO-NP) or without (Lm-E7 and ZY-18) chloramphenicol (20 μg/ml). Bacteria were frozen in aliquots at -80°C. Expression was verified by Western blotting (**Figure 2**).

Western blotting

5 [00630] *Listeria* strains were grown in Luria-Bertoni medium at 37°C and were harvested at the same optical density measured at 600 nm. The supernatants were TCA precipitated and resuspended in 1x sample buffer supplemented with 0.1 N NaOH. Identical amounts of each cell pellet or each TCA-precipitated supernatant were loaded on 4–20% Tris-glycine SDS-PAGE gels (NOVEX, San Diego, CA). The gels were transferred to polyvinylidene difluoride 10 and probed with an anti-E7 monoclonal antibody (mAb) (Zymed Laboratories, South San Francisco, CA), then incubated with HRP-conjugated anti-mouse secondary Ab (Amersham Pharmacia Biotech, Little Chalfont, U.K.), developed with Amersham ECL detection reagents, and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Measurement of tumor growth

15 [00631] Tumors were measured every other day with calipers spanning the shortest and longest surface diameters. The mean of these two measurements was plotted as the mean tumor diameter in millimeters against various time points. Mice were sacrificed when the tumor diameter reached 20 mm. Tumor measurements for each time point are shown only for surviving mice.

20 ***Effects of Listeria recombinants on established tumor growth***

[00632] Six- to 8-wk-old C57BL/6 mice (Charles River) received 2 x 10^5 TC-1 cells s.c. on the left flank. One week following tumor inoculation, the tumors had reached a palpable size of 4–5 mm in diameter. Groups of eight mice were then treated with 0.1 LD_{50} i.p. Lm-LLO-E7 (10^7 CFU), Lm- E7 (10^6 CFU), Lm-LLO-NP (10^7 CFU), or Lm-Gag (5 x 10^5 CFU) on 25 days 7 and 14.

^{51}Cr release assay

[00633] C57BL/6 mice, 6–8 wk old, were immunized i.p. with 0.1LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Ten days post-immunization, spleens were harvested. Splenocytes were established in culture with irradiated TC-1 cells (100:1, splenocytes:TC-1) 30 as feeder cells; stimulated *in vitro* for 5 days, then used in a standard ^{51}Cr release assay, using the following targets: EL-4, EL-4/E7, or EL-4 pulsed with E7 H-2b peptide (RAHYNIVTF).

E:T cell ratios, performed in triplicate, were 80:1, 40:1, 20:1, 10:1, 5:1, and 2.5:1. Following a 4-h incubation at 37°C, cells were pelleted, and 50 µl supernatant was removed from each well. Samples were assayed with a Wallac 1450 scintillation counter (Gaithersburg, MD). The percent specific lysis was determined as [(experimental counts per minute (cpm)-spontaneous cpm)/(total cpm - spontaneous cpm)] x 100.

TC-1-specific proliferation

[00634] C57BL/6 mice were immunized with 0.1 LD_{50} and boosted by i.p. injection 20 days later with 1 LD_{50} Lm-LLO-E7, Lm-E7, Lm-LLO-NP, or Lm-Gag. Six days after boosting, spleens were harvested from immunized and naive mice. Splenocytes were established in culture at 5 x 10^5/well in flat-bottom 96-well plates with 2.5 x 10^4, 1.25 x 10^4, 6 x 10^3, or 3 x 10^3 irradiated TC-1 cells/well as a source of E7 Ag, or without TC-1 cells or with 10 µg/ml Con A. Cells were pulsed 45 h later with 0.5 µCi [^{3}H]thymidine/well. Plates were harvested 18 h later using a Tomtec harvester 96 (Orange, CT), and proliferation was assessed with a Wallac 1450 scintillation counter. The change in cpm was calculated as experimental cpm - no Ag cpm.

Flow cytometric analysis

[00635] C57BL/6 mice were immunized intravenously (i.v.) with 0.1 LD_{50} Lm-LLO-E7 or Lm-E7 and boosted 30 days later. Three-color flow cytometry for CD8 (53-6.7, PE conjugated), CD62 ligand (CD62L; MEL-14, APC conjugated), and E7 H-2Db tetramer was performed using a FACSCalibur® flow cytometer with CellQuest® software (Becton Dickinson, Mountain View, CA). Splenocytes harvested 5 days after the boost were stained at room temperature (rt) with H-2Db tetramers loaded with the E7 peptide (RAHYNIVTF) or a control (HIV-Gag) peptide. Tetramers were used at a 1/200 dilution and were provided by Dr. Larry R. Pease (Mayo Clinic, Rochester, MN) and by the NIAID Tetramer Core Facility and the NIH AIDS Research and Reference Reagent Program. Tetramer$^+$, CD8$^+$, CD62L^{low} cells were analyzed.

B16F0-Ova experiment

[00636] 24 C57BL/6 mice were inoculated with 5 x 10^5 B16F0-Ova cells. On days 3, 10 and 17, groups of 8 mice were immunized with 0.1 LD_{50} Lm-OVA (10^6 cfu), Lm-LLO-OVA (10^8 cfu) and eight animals were left untreated.

Statistics

[00637] For comparisons of tumor diameters, mean and SD of tumor size for each group were determined, and statistical significance was determined by Student's t test. $p \leq 0.05$ was considered significant.

5 **EXAMPLE 1: LLO-Antigen Fusions Induce Anti-Tumor Immunity**

RESULTS

[00638] Lm-E7 and Lm-LLO-E7 were compared for their abilities to impact on TC-1 growth. Subcutaneous tumors were established on the left flank of C57BL/6 mice. Seven days later tumors had reached a palpable size (4–5 mm). Mice were vaccinated on days 7 and 14
10 with 0.1 LD_{50} Lm-E7, Lm-LLO-E7, or, as controls, Lm-Gag and Lm-LLO-NP. Lm-LLO-E7 induced complete regression of 75% of established TC-1 tumors, while tumor growth was controlled in the other 2 mice in the group (**Fig. 3**). By contrast, immunization with Lm-E7 and Lm-Gag did not induce tumor regression. This experiment was repeated multiple times, always with very similar results. In addition, similar results were achieved for Lm-LLO-E7
15 under different immunization protocols. In another experiment, a single immunization was able to cure mice of established 5 mm TC-1 tumors.

[00639] In other experiments, similar results were obtained with 2 other E7-expressing tumor cell lines: C3 and EL-4/E7. To confirm the efficacy of vaccination with Lm-LLO-E7, animals that had eliminated their tumors were re-challenged with TC-1 or EL-4/E7 tumor
20 cells on day 60 or day 40, respectively. Animals immunized with Lm-LLO-E7 remained tumor free until termination of the experiment (day 124 in the case of TC-1 and day 54 for EL-4/E7).

[00640] Thus, expression of an antigen as a fusion protein with ΔLLO enhances the immunogenicity of the antigen.

25 **EXAMPLE 2: LM-LLO-E7 Treatment Elicits TC-1 Specific Splenocyte Proliferation**

[00641] To measure induction of T cells by Lm-E7 with Lm-LLO-E7, TC-1-specific proliferative responses, a measure of antigen-specific immunocompetence, were measured in immunized mice. Splenocytes from Lm-LLO-E7-immunized mice proliferated when exposed to irradiated TC-1 cells as a source of E7, at splenocyte: TC-1 ratios of 20:1, 40:1, 80:1, and
30 160:1 (**Fig. 4**). Conversely, splenocytes from Lm-E7 and rLm control-immunized mice exhibited only background levels of proliferation.

EXAMPLE 3: ActA-E7 and PEST-E7 Fusions Confer Anti-Tumor Immunity

Materials and Methods

Construction of Lm-ActA-E7

[00642] Lm-ActA-E7 is a recombinant strain of LM, comprising a plasmid that expresses the E7 protein fused to a truncated version of the actA protein. Lm-actA-E7 was generated by introducing a plasmid vector pDD-1, constructed by modifying pDP-2028, into *Listeria*. pDD-1 comprises an expression cassette expressing a copy of the 310 bp hly promoter and the hly signal sequence (ss), which drives the expression and secretion of ActA-E7; 1170 bp of the actA gene that comprises four PEST sequences (SEQ ID NO: 19) (the truncated ActA polypeptide consists of the first 390 AA of the molecule, SEQ ID NO: 11); the 300 bp HPV E7 gene; the 1019 bp prfA gene (controls expression of the virulence genes); and the CAT gene (chloramphenicol resistance gene) for selection of transformed bacteria clones (Sewell et al. (2004), Arch. Otolaryngol. Head Neck Surg., 130: 92-97).

[00643] The hly promoter (pHly) and gene fragment were PCR amplified from pGG55 (Example 1) using primer 5'-GGGGTCTAGACCTCCTTTGATTAGTATATTC-3' (Xba I site is underlined; SEQ ID NO: 32) and primer 5'-ATCTTCGCTATCTGTCGCCGCGGCGCGTGCTTCAGTTTGTTGCGC-'3 (Not I site is underlined. The first 18 nucleotides are the ActA gene overlap; SEQ ID NO: 33). The actA gene was PCR amplified from the LM 10403s wild type genome using primer 5'-GCGCAACAAACTGAAGCAGCGGCCGCGGCGACAGATAGCGAAGAT-3' (NotI site is underlined; SEQ ID NO: 34) and primer 5'-TGTAGGTGTATCTCCATGCTCGAGAGCTAGGCGATCAATTTC-3' (XhoI site is underlined; SEQ ID NO: 35). The E7 gene was PCR amplified from pGG55 (pLLO-E7) using primer 5'-GGAATTGATCGCCTAGCTCTCGAGCATGGAGATACACCTACA-3' (XhoI site is underlined; SEQ ID NO: 36) and primer 5'-AAACGGATTTATTTAGATCCCGGGTTATGGTTTCTGAGAACA-3' (XmaI site is underlined; SEQ ID NO: 37). The prfA gene was PCR amplified from the LM 10403s wild-type genome using primer 5'-TGTTCTCAGAAACCATAACCCGGGATCTAAATAAATCCGTTT-3' (XmaI site is underlined; SEQ ID NO: 38) and primer 5'-GGGGGTCGACCAGCTCTTCTTGGTGAAG-3' (SalI site is underlined; SEQ ID NO: 39). The hly promoter- actA gene fusion (pHly-actA) was PCR generated and amplified from purified pHly DNA and purified actA DNA using the

upstream pHly primer (SEQ ID NO: 32) and downstream actA primer (SEQ ID NO: 35).

[00644] The E7 gene fused to the prfA gene (E7-prfA) was PCR generated and amplified from purified E7 DNA and purified prfA DNA using the upstream E7 primer (SEQ ID NO: 36) and downstream prfA gene primer (SEQ ID NO: 39).

5 [00645] The pHly-actA fusion product fused to the E7-prfA fusion product was PCR generated and amplified from purified fused pHly-actA DNA product and purified fused E7-prfA DNA product using the upstream pHly primer (SEQ ID NO: 32) and downstream prfA gene primer (SEQ ID NO: 39) and ligated into pCRII (Invitrogen, La Jolla, Calif.). Competent *E. coli* (TOP10'F, Invitrogen, La Jolla, Calif.) were transformed with pCRII-
10 ActAE7. After lysis and isolation, the plasmid was screened by restriction analysis using BamHI (expected fragment sizes 770 bp and 6400 bp (or when the insert was reversed into the vector: 2500 bp and 4100 bp)) and BstXI (expected fragment sizes 2800 bp and 3900 bp) and also screened with PCR analysis using the upstream pHly primer (SEQ ID NO: 32) and the downstream prfA gene primer (SEQ ID NO: 39).

15 [00646] The pHly-actA-E7-prfA DNA insert was excised from pCRII by double digestion with Xba I and Sal I and ligated into pDP-2028 also digested with Xba I and Sal I. After transforming TOP10'F competent *E. coli* (Invitrogen, La Jolla, Calif.) with expression system pActAE7, chloramphenicol resistant clones were screened by PCR analysis using the upstream pHly primer (SEQ ID NO: 32) and the downstream PrfA gene primer (SEQ ID NO:
20 39). A clone comprising pActAE7 was grown in brain heart infusion medium (with chloramphenicol (20 mcg (microgram)/ml (milliliter), Difco, Detroit, Mich.) and pActAE7 was isolated from the bacteria cell using a midiprep DNA purification system kit (Promega, Madison, Wis.). A prfA-negative strain of penicillin-treated *Listeria* (strain XFL-7) was transformed with expression system pActAE7, as described in Ikonomidis et al. (1994, J.
25 Exp. Med. 180: 2209-2218) and clones were selected for the retention of the plasmid *in vivo*. Clones were grown in brain heart infusion with chloramphenicol (20 mcg/ml) at 37 °C. Bacteria were frozen in aliquots at -80 °C.

Immunoblot Verification of Antigen Expression

[00647] To verify that Lm-ActA-E7 secretes ActA-E7, (about 64 kD), *Listeria* strains were
30 grown in Luria-Bertoni (LB) medium at 37°C. Protein was precipitated from the culture supernatant with trichloroacetic acid (TCA) and resuspended in 1x sample buffer with 0.1N sodium hydroxide. Identical amounts of each TCA precipitated supernatant were loaded on

4% to 20% Tris-glycine sodium dodecyl sulfate–polyacrylamide gels (NOVEX, San Diego, Calif). Gels were transferred to polyvinylidene difluoride membranes and probed with 1:2500 anti-E7 monoclonal antibody (Zymed Laboratories, South San Francisco, Calif), then with 1:5000 horseradish peroxidase–conjugated anti-mouse IgG (Amersham Pharmacia Biotech, Little Chalfont, England). Blots were developed with Amersham enhanced chemiluminescence detection reagents and exposed to autoradiography film (Amersham) (**Fig. 5A**).

Construction of Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-E7epi (Fig. 6A)

[00648] Lm-PEST-E7 is identical to Lm-LLO-E7, except that it contains only the promoter and PEST sequence of the hly gene, specifically the first 50 AA of LLO. To construct Lm-PEST-E7, the hly promoter and PEST regions were fused to the full-length E7 gene using the SOE (gene splicing by overlap extension) PCR technique. The E7 gene and the hly-PEST gene fragment were amplified from the plasmid pGG-55, which contains the first 441 AA of LLO, and spliced together by conventional PCR techniques. To create a final plasmid, pVS16.5, the hly-PEST-E7 fragment and the prfA gene were subcloned into the plasmid pAM401, which includes a chloramphenicol resistance gene for selection *in vitro*, and the resultant plasmid was used to transform XFL-7.

[00649] Lm-ΔPEST-E7 is a recombinant *Listeria* strain that is identical to Lm- LLO-E7 except that it lacks the PEST sequence. It was made essentially as described for Lm-PEST-E7, except that the episomal expression system was constructed using primers designed to remove the PEST-containing region (bp 333–387) from the hly-E7 fusion gene. Lm-E7epi is a recombinant strain that secretes E7 without the PEST region or LLO. The plasmid used to transform this strain contains a gene fragment of the hly promoter and signal sequence fused to the E7 gene. This construct differs from the original Lm-E7, which expressed a single copy of the E7 gene integrated into the chromosome. Lm-E7epi is completely isogenic to Lm-LLO-E7, Lm-PEST-E7, and Lm-ΔPEST-E7 except for the form of the E7 antigen expressed.

Results

[00650] To compare the anti-tumor immunity induced by Lm-ActA-E7 versus Lm-LLO-E7, 2 x 10^5 TC-1 tumor cells were implanted subcutaneously in mice and allowed to grow to a palpable size (approximately 5 millimeters [mm]). Mice were immunized i.p. with one LD_{50} of either Lm-ActA-E7 (5 x10^8 CFU), (crosses) Lm-LLO-E7 (10^8 CFU) (squares) or Lm-E7 (10^6 CFU) (circles) on days 7 and 14. By day 26, all of the animals in the Lm-LLO-E7 and

Lm-ActA-E7 were tumor free and remained so, whereas all of the naive animals (triangles) and the animals immunized with Lm-E7 grew large tumors (**Fig.** 5B). Thus, vaccination with ActA-E7 fusions causes tumor regression.

[00651] In addition, Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, and Lm-E7epi were compared for their ability to cause regression of E7-expressing tumors. s.c. TC-1 tumors were established on the left flank of 40 C57BL/6 mice. After tumors had reached 4-5 mm, mice were divided into 5 groups of 8 mice. Each groups was treated with 1 of 4 recombinant LM immunotherapies, and 1 group was left untreated. Lm-LLO-E7 and Lm-PEST-E7 induced regression of established tumors in 5/8 and 3/8 cases, respectively. There was no statistical difference between the average tumor size of mice treated with Lm-PEST-E7 or Lm-LLO-E7 at any time point. However, the immunotherapies that expressed E7 without the PEST sequences, Lm-ΔPEST-E7 and Lm-E7epi, failed to cause tumor regression in all mice except one (**Fig. 6B**, top panel). This was representative of 2 experiments, wherein a statistically significant difference in mean tumor sizes at day 28 was observed between tumors treated with Lm-LLO-E7 or Lm-PEST-E7 and those treated with Lm-E7epi or Lm-ΔPEST-E7; $P < 0.001$, Student's t test; **Fig. 6B**, bottom panel). In addition, increased percentages of tetramer-positive splenocytes were seen reproducibly over 3 experiments in the spleens of mice vaccinated with PEST-containing immunotherapies (**Fig. 6C**). Thus, vaccination with PEST-E7 fusions causes tumor regression.

EXAMPLE 4: Fusion of E7 to LLO, Acta, or A Pest-Like Sequence Enhances E7-Specific Immunity and Generates Tumor-Infiltrating E7-Specific CD8$^+$ Cells

Materials and Experimental Methods

[00652] 500 mcl (microliter) of MATRIGEL®, comprising 100 mcl of 2 x 10^5 TC-1 tumor cells in phosphate buffered saline (PBS) plus 400 mcl of MATRIGEL® (BD Biosciences, Franklin Lakes, N.J.) were implanted subcutaneously on the left flank of 12 C57BL/6 mice (n=3). Mice were immunized intraperitoneally on day 7, 14 and 21, and spleens and tumors were harvested on day 28. Tumor MATRIGELs were removed from the mice and incubated at 4 °C overnight in tubes containing 2 milliliters (ml) of RP 10 medium on ice. Tumors were minced with forceps, cut into 2 mm blocks, and incubated at 37 °C for 1 hour with 3 ml of enzyme mixture (0.2 mg/ml collagenase-P, 1 mg/ml DNAse-1 in PBS). The tissue suspension was filtered through nylon mesh and washed with 5% fetal bovine serum + 0.05% of NaN_3 in PBS for tetramer and IFN-gamma staining.

[00653] Splenocytes and tumor cells were incubated with 1 micromole (mcm) E7 peptide for 5 hours in the presence of brefeldin A at 10^7 cells/ml. Cells were washed twice and incubated in 50 mcl of anti-mouse Fc receptor supernatant (2.4 G2) for 1 hour or overnight at 4 °C. Cells were stained for surface molecules CD8 and CD62L, permeabilized, fixed using the permeabilization kit Golgi-stop® or Golgi-Plug® (Pharmingen, San Diego, Calif.), and stained for IFN-gamma. 500,000 events were acquired using two-laser flow cytometer FACSCalibur and analyzed using Cellquest Software (Becton Dickinson, Franklin Lakes, NJ). Percentages of IFN-gamma secreting cells within the activated ($CD62L^{low}$) $CD8^+$ T cells were calculated.

[00654] For tetramer staining, H-$2D^b$ tetramer was loaded with phycoerythrin (PE)-conjugated E7 peptide (RAHYNIVTF, SEQ ID NO: 40), stained at rt for 1 hour, and stained with anti-allophycocyanin (APC) conjugated MEL-14 (CD62L) and FITC-conjugated $CD8^+$ at 4 °C for 30 min. Cells were analyzed comparing $tetramer^+CD8^+$ $CD62L^{low}$ cells in the spleen and in the tumor.

Results

[00655] To analyze the ability of Lm-ActA-E7 to enhance antigen specific immunity, mice were implanted with TC-1 tumor cells and immunized with either Lm-LLO-E7 (1×10^7 CFU), Lm-E7 (1×10^6 CFU), or Lm-ActA-E7 (2×10^8 CFU), or were untreated (naïve). Tumors of mice from the Lm-LLO-E7 and Lm-ActA-E7 groups contained a higher percentage of IFN-gamma-secreting $CD8^+$ T cells (**Fig. 7A**) and tetramer-specific $CD8^+$ cells (**Fig. 7B**) than in Lm-E7 or naive mice.

[00656] In another experiment, tumor-bearing mice were administered Lm-LLO-E7, Lm-PEST-E7, Lm-ΔPEST-E7, or Lm-E7epi, and levels of E7-specific lymphocytes within the tumor were measured. Mice were treated on days 7 and 14 with 0.1 LD_{50} of the 4 immunotherapies. Tumors were harvested on day 21 and stained with antibodies to CD62L, CD8, and with the E7/Db tetramer. An increased percentage of tetramer-positive lymphocytes within the tumor were seen in mice vaccinated with Lm-LLO-E7 and Lm-PEST-E7 (**Fig. 8A**). This result was reproducible over three experiments (**Fig. 8B**).

[00657] Thus, Lm-LLO-E7, Lm-ActA-E7, and Lm-PEST-E7 are each efficacious at induction of tumor-infiltrating $CD8^+$ T cells and tumor regression.

EXAMPLE 5: LLO and ActA Fusions Reduce Autochthonous (Spontaneous) Tumors in E6/E7 Transgenic Mice

[00658] To determine the impact of the Lm-LLO-E7 and Lm-ActA-E7 immunotherapies on autochthonous tumors in the E6/E7 transgenic mouse, 6 to 8 week old mice were immunized with 1 x 10^8 Lm-LLO-E7 or 2.5 x 10^8 Lm-ActA-E7 once per month for 8 months. Mice were sacrificed 20 days after the last immunization and their thyroids removed and weighed. This experiment was performed twice (Table 1).

[00659] Table 1. Thyroid weight (mg) in unvaccinated and vaccinated transgenic mice at 8 months of age (mg)*.

Untreated	± S.D.	Lm-LLO-NP	± S.D.	Lm-LLO-E7	± S.D.	Lm-ActA-E7	± S.D.
Expt. 1 408	123	385	130	225	54	305	92
Expt. 2 588	94	503	86	239	68	275	84

* Statistical analyses performed using Student's t test showed that the difference in thyroid weight between Lm-LLO-NP treated mice and untreated mice was not significant but that the difference between Lm-LLO-E7 and Lm-ActA-E7 treated mice was highly significant ($p<0.001$)

[00660] The difference in thyroid weight between Lm-LLO-E7 treated mice and untreated mice and between Lm-LLO-ActA treated mice and untreated mice was significant ($p<0.001$ and $p<0.05$, respectively) for both experiments, while the difference between Lm-LLO-NP treated mice (irrelevant antigen control) and untreated mice was not significant (Student's t test), showing that Lm-LLO-E7 and Lm-ActA-E7 controlled spontaneous tumor growth. Thus, immunotherapies of the present invention prevent formation of new E7-expressing tumors.

[00661] To summarize the findings in the above Examples, LLO-antigen and ActA-antigen fusions (a) induce tumor-specific immune response that include tumor-infiltrating antigen-specific T cells; and are capable of inducing tumor regression and controlling tumor growth of both normal and particularly aggressive tumors; (b) overcome tolerance to self antigens; and (c) prevent spontaneous tumor growth. These findings are generalizable to a large number of antigens, PEST-like sequences, and tumor types, as evidenced by their successful

implementation with a variety of different antigens, PEST-like sequences, and tumor types.

EXAMPLE 6: LM-LLO-E7 Immunotherapies are Safe and Improve Clinical Indicators in Cervical Cancer Patients

Materials and Experimental Methods

5 [00662] Inclusion criteria. All patients in the trial were diagnosed with "advanced, progressive or recurrent cervical cancer," and an assessment at the time of entry indicated that all were staged as having IVB disease. All patients manifested a positive immune response to an anergy panel containing 3 memory antigens selected from candidin, mumps, tetanus, or Tuberculin Purified Protein Derivative (PPD); were not pregnant or HIV positive, had taken 10 no investigational drugs within 4 weeks, and were not receiving steroids.

[00663] Protocol: Patients were administered 2 vaccinations at a 3-week interval as a 30-minute intravenous (IV) infusion in 250 ml of normal saline to inpatients. After 5 days, patients received a single course of IV ampicillin and were released with an additional 10 days of oral ampicillin. Karnofsky Performance Index, which is a measurement of overall 15 vitality and quality of life such as appetite, ability to complete daily tasks, restful sleep, etc, was used to determine overall well-being. In addition, the following indicators of safety and general wellbeing were determined: alkaline phosphatase; bilirubin, both direct and total; gamma glutamyl transpeptidase (ggt); cholesterol; systole, diastole, and heart rate; Eastern Collaborative Oncology Group's (ECOG)'s criteria for assessing disease progression- a 20 Karnofsky like - quality of life indicator; hematocrit; hemoglobin; platelet levels; lymphocytes levels; AST (aspartate aminotransferase); ALT (alanine aminotransferase); and LDH (lactate dehydrogenase). Patients were followed at 3 weeks and 3 months subsequent to the second dosing, at which time Response Evaluation Criteria in Solid Tumors (RECIST) scores of the patients were determined, scans were performed to determine tumor size, and 25 blood samples were collected for immunological analysis at the end of the trial, which includes the evaluation of IFN-γ, IL-4, $CD4^+$ and $CD8^+$ cell populations.

[00664] *Listeria* strains: The creation of LM-LLO-E7 is described in Example 1.

Results

[00665] Prior to the clinical trial, a preclinical experiment was performed to determine the 30 anti-tumor efficacy of intravenous (i.v.) vs. i.p. administration of LM-LLO-E7. A tumor containing 1 x 10^4 TC-1 cells was established sub-cutaneously. On days 7 and 14, mice were

immunized with either 10^8 LM-LLO-E7 i.p. or LM-LLO-E7 i.v. at doses of 10^8, 10^7, 10^6, or 10^5. At day 35, 5/8 of the mice that received 10^8 LM-LLO-E7 by either route or 10^7 LM-LLO-E7 i.v, and 4/8 of the mice that received 10^6 LM-LLO-E7 i.v, were cured. By contrast, doses of less than 10^7 or in some cases even 10^8 LM-LLO-E7 administered i.p. were
5 ineffective at controlling tumor growth. Thus, i.v. administration of LM-LLO-E7 is more effective than i.p. administration.

Clinical trial

[00666] A phase I/II clinical trial was conducted to assess safety and efficacy of LM-LLO-E7 immunotherapies in patients with advanced, progressive, or recurrent cervical cancer. 5
10 patients each were assigned to cohorts 1-2, which received 1 x 10^9 or 3.3 x 10^9 CFU, respectfully. An additional 5 patients each will be assigned to cohorts 3-4, which will receive 1 x 10^{10} or 3.31 x 10^{10} CFU, respectfully.

Safety data

First cohort

15 [00667] All patients in the first cohort reported onset of mild-to-moderate fever and chills within 1-2 hours after onset of the infusion. Some patients exhibited vomiting, with or without nausea. With 1 exception (described below), a single dose of a non-steroidal agent such as paracetamol was sufficient to resolve these symptoms. Modest, transient cardiovascular effects were observed, consistent with, and sharing the time course of, the
20 fever. No other adverse effects were reported.

[00668] At this late stage of cervical cancer, 1 year survival is typically 10-15% of patients and no tumor therapy has ever been effective. Indeed, Patient 2 was a young patient with very aggressive disease who passed away shortly after completing the trial.

[00669] Quantitative blood cultures were assessed on days 2, 3, and 5 post-administration.
25 Of the 5 evaluable patients in this cohort, 4 exhibited no serum *Listeria* at any time and 1 had a very small amount (35 cfu) of circulating *Listeria* on day 2, with no detectable *Listeria* on day 3 or 5.

[00670] Patient 5 responded to initial vaccination with mild fever over the 48 hours subsequent to administration, and was treated with anti-inflammatory agents. On 1 occasion,
30 the fever rose to moderate severity (at no time above 38.4 °C), after which she was given a course of ampicillin, which resolved the fever. During the antibiotic administration she

experienced mild urticaria, which ended after antibiotic administration. Blood cultures were all sterile, cardiovascular data were within the range observed for other patients, and serum chemistry values were normal, showing that this patient had no *Listeria*l disease. Further, the anergy panel indicated a robust response to 1/3 memory antigens, indicating the presence of functional immunity (similar to the other patients). Patient 5 subsequently evidenced a response similar to all other patients upon receiving the boost.

Second cohort and overall safety observations

[00671] In both cohorts, minor and transient changes in liver function tests were observed following infusion. These changes were determined by the attending physician monitoring the trial to have no clinical significance, and were expected for a short-lived infection of bacteria that are rapidly removed from the systemic circulation to the liver and spleen. In general, all the safety indicators described in the Methods section above displayed little or no net change, indicative of an excellent safety profile. The side effect profile in this cohort was virtually identical to that seen in the in the initial cohort and appeared to be a dose independent series of symptoms related to the consequences of cytokines and similar agents that occur consequent to the induction of an iatrogenic infection. No serum *Listeria* was observed at any time and no dose limiting toxicity was observed in either cohort.

Efficacy- first cohort

[00672] The following indications of efficacy were observed in the 3 patients in the first cohort that finished the trial: (**Fig. 9**).

[00673] Patient 1 entered the trial with 2 tumors of 20 mm each, which shrunk to 18 and 14 mm over the course of the trial, indicating therapeutic efficacy of the immunotherapy. In addition, patient 1 entered the trial with a Karnofsky Performance Index of 70, which rose to 90 after dosing. In the Safety Review Panel meeting, Siniša Radulovic, the chairman of the Department of Oncology, Institute for Oncology and Radiology, Belgrade, Serbia presented the results to a representative of the entity conducting the trials; Michael Kurman, an independent oncologist who works as a consultant for the entity; Kevin Ault, an academic gynecologic oncologist at Emory University who conducted the phase III Gardasil trials for Merck and the Cervarix trials for Glaxo SmithKline; and Tate Thigpen, a founder of the Gynecologic Oncology Group at NCI and professor of gynecologic oncology at the University of Mississippi. In the opinion of Dr. Radulovic, patient 1 exhibited a clinical benefit from treatment with the immunotherapy.

[00674] Before passing away, Patient 2 exhibited a mixed response, with 1/2 tumors shrinking.

[00675] Patient 3 enrolled with paraneoplastic disease, (an epiphenomenon of cancer wherein the overall debilitated state of the patient has other sequelae that are secondary to the cancer), including an elevation of platelet count to 936 x 10^9/ml. The count decreased to 405 x 10^9/ml, approximately a normal level, following the first dose.

[00676] Patient 4 entered the trial with 2 tumors of 20 mm each, which shrunk to 18 and 14 mm over the course of the trial, indicating therapeutic efficacy of the immunotherapy. Patient 4 exhibited a weight gain of 1.6 Kg and an increased hemoglobin count of approximately 10% between the first and second doses.

Efficacy- second cohort and general observations

[00677] In the lowest dose cohort, 2 patients demonstrated the shrinkage of tumors. The timing of this effect was consistent with that observed in immunological responses, in that it followed chronologically development of the immune response. One of the 2 patients in the second cohort evaluated so far for tumor burden exhibited a dramatic tumor load reduction at a post-vaccination time point. At the start of the trial, this patient had 3 tumors of 13, 13, and 14 mm. After the 2 doses of the immunotherapy, 2 of the tumor had shrunk to 9.4 and 12 mm, and the third was no longer detectable.

[00678] Tumors loads for the 2 cohorts are depicted in Fig. 13B. In summary, even relatively low doses of LM-LLO-E7, administered in a therapeutic regimen containing a priming injection and a single boost, achieved 3 objective responses out of 6 patients for whom data has been collected.

Discussion

[00679] At this late stage of cervical cancer, 1 year survival is typically 10-15% of patients and no tumor therapy has ever been effective. No treatment has shown to be effective in reversing stage IVB cervical cancer. Despite the difficulty of treating cervical cancer at this stage, an anti-tumor effect was observed in 2/6 patients. In addition, other indications of efficacy were observed in patients that finished the trial, as described hereinabove.

[00680] Thus, LM-LLO-E7 is safe in human subjects and improves clinical indicators of cervical cancer patients, even when administered at relatively low doses. Additional positive results are likely to be observed when the dose and number of booster vaccinations is

increased; and/or when antibiotics are administered in smaller doses or at a later time point after infusion. Pre-clinical studies have shown that a dose increase of a single order of magnitude can cause dramatic changes in response rate (e.g. a change from 0% response rate to 50-100% complete remission rate. Additional booster doses are also very likely to further enhance the immune responses obtained. Moreover, the positive effects of the therapeutic immune response observed are likely to continue with the passage of additional time, as the immune system continues to attack the cancer.

EXAMPLE 7: Construction of attenuated *Listeria* strain-LmddΔ*actA* and insertion of the human *klk3* gene in frame to the *hly* gene in the *Lmdd* and *Lmdda* strains.

Materials and Methods

[00681] A recombinant *Lm* was developed that secretes PSA fused to tLLO (*Lm*-LLO-PSA), which elicits a potent PSA-specific immune response associated with regression of tumors in a mouse model for prostate cancer, wherein the expression of tLLO-PSA is derived from a plasmid based on pGG55 (Table 2), which confers antibiotic resistance to the vector. We recently developed a new strain for the PSA immunotherapy based on the pADV142 plasmid, which has no antibiotic resistance markers, and referred as *LmddA*-142 (Table 3). This new strain is 10 times more attenuated than *Lm*-LLO-PSA. In addition, *LmddA*-142 was slightly more immunogenic and significantly more efficacious in regressing PSA expressing tumors than the *Lm*-LLO-PSA.

[00682] **Table 2. Plasmids and strains**

Plasmids	**Features**
pGG55	pAM401/pGB354 shuttle plasmid with gram(-) and gram(+) *cm* resistance, LLO-E7 expression cassette and a copy of *Lm prfA* gene
pTV3	Derived from pGG55 by deleting *cm* genes and inserting the *Lm dal* gene
pADV119	Derived from pTV3 by deleting the *prfA* gene
pADV134	Derived from pADV119 by replacing the *Lm dal* gene by the *Bacillus dal* gene
pADV142	Derived from pADV134 by replacing HPV16 *e7* with *klk3*
pADV168	Derived from pADV134 by replacing HPV16 *e7* with $hmw\text{-}maa_{2160\text{-}2258}$
Strains	Genotype
10403S	Wild-type *Listeria monocytogenes*:: *str*
XFL-7	10403S $prfA^{(-)}$
Lmdd	10403S $dal^{(-)}$ $dat^{(-)}$
LmddA	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$
LmddA-134	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV134*
LmddA-142	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV142*
Lmdd-143	10403S $dal^{(-)}$ $dat^{(-)}$ *with klk3 fused to the hly gene in the chromosome*
LmddA-143	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *with klk3 fused to the hly gene in the chromosome*
LmddA-168	10403S $dal^{(-)}$ $dat^{(-)}$ $actA^{(-)}$ *pADV168*
Lmdd-143/134	*Lmdd-143 pADV134*
LmddA-143/134	*LmddA-143 pADV134*
Lmdd-143/168	*Lmdd-143 pADV168*
LmddA-143/168	*LmddA-143 pADV168*

[00683] The sequence of the plasmid pAdv142 (6523 bp) was as follows:

cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgc
gtcagcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatg
gcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttttcc
ataggctccgcccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccagg
cgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattc
cacgcctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgcctt
atccggtaactatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagtta
gtcttgaagtcatgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaagag
ttggtagctcagagaaccttcgaaaaaccgccctgcaaggcggttttttcgttttcagagcaagagattacgcgcagaccaaaacgatct
caagaagatcatcttattaatcagataaaatatttctagccctcctttgattagtatattcctatcttaaagttacttttatgtggaggcattaaca
tttgttaatgacgtcaaaaggatagcaagactagaataaagctataaagcaagcatataatattgcgtttcatctttagaagcgaatttcgc
caatattataattatcaaaagagaggggtggcaaacggtatttggcattattaggttaaaaaatgtagaaggagagtgaaacccatgaaa
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aattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataa
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[00684] The strain Lm *dal dat* (Lmdd) was attenuated by the irreversible deletion of the virulence factor, ActA. An in-frame deletion of *actA* in the Lm*daldat* (Lmdd) background was constructed to avoid any polar effects on the expression of downstream genes. The Lm *dal dat ΔactA* contains the first 19 amino acids at the N-terminal and 28 amino acid residues of the C-terminal with a deletion of 591 amino acids of ActA.

[00685] The actA deletion mutant was produced by amplifying the chromosomal region corresponding to the upstream (657 bp-oligo's Adv 271/272) and downstream (625 bp-oligo's Adv 273/274) portions of *actA* and joining by PCR. The sequence of the primers used for this amplification is given in the **Table 3**. The upstream and downstream DNA regions of *actA* were cloned in the pNEB193 at the EcoRI/PstI restriction site and from this plasmid, the EcoRI/PstI was further cloned in the temperature sensitive plasmid pKSV7, resulting in ΔactA/pKSV7 (pAdv120).

[00686] **Table 3: Sequence of primers that was used for the amplification of DNA sequences upstream and downstream of *actA***

Primer	**Sequence**	**SEQ ID NO:**
Adv271-actAF1	cg GAATTCGGATCCgcgccaaatcattggttgattg	42
Adv272-actAR1	gcgaGTCGACgtcggggttaatcgtaatgcaattggc	43
Adv273-actAF2	gcgaGTCGACccatacgacgttaattcttgcaatg	44
Adv274-actAR2	gataCTGCAGGGATCCttcccttctcggtaatcagtcac	45

[00687] The deletion of the gene from its chromosomal location was verified using primers that bind externally to the *actA* deletion region, which are shown in **Fig. 10A and Fig. 10B** as primer 3 (Adv 305-tgggatggccaagaaattc, SEQ ID NO: 46) and primer 4 (Adv304-ctaccatgtcttccgttgcttg; SEQ ID NO: 47) . The PCR analysis was performed on the chromosomal DNA isolated from Lmdd and Lmdd*ΔactA*. The sizes of the DNA fragments after amplification with two different sets of primer pairs 1/2 and 3/4 in Lmdd chromosomal DNA was expected to be 3.0 Kb and 3.4 Kb. On the other hand, the expected sizes of PCR using the primer pairs 1/2 and 3/4 for the Lmdd*ΔactA* was 1.2 Kb and 1.6 Kb. Thus, PCR analysis in **Fig. 10A and Fig. 10B** confirms that the 1.8 kb region of *actA* was deleted in the Lmdd*ΔactA* strain. DNA sequencing was also performed on PCR products to confirm the deletion of *actA* containing region in the strain, Lmdd*ΔactA*.

EXAMPLE 8: Construction of the antibiotic-independent episomal expression system for antigen delivery by *Lm* vectors.

[00688] The antibiotic-independent episomal expression system for antigen delivery by *Lm* vectors (pAdv142) is the next generation of the antibiotic-free plasmid pTV3 (Verch et al., Infect Immun, 2004. **72**(11):6418-25, incorporated herein by reference). The gene for virulence gene transcription activator, *prfA* was deleted from pTV3 since *Listeria* strain Lmdd contains a copy of *prfA* gene in the chromosome. Additionally, the cassette for p60-*Listeria dal* at the NheI/PacI restriction site was replaced by p60-*Bacillus subtilis dal* resulting in plasmid pAdv134 (**Fig. 11A**). The similarity of the *Listeria* and *Bacillus dal* genes is ~30%, virtually eliminating the chance of recombination between the plasmid and the remaining fragment of the *dal* gene in the *Lmdd* chromosome. The plasmid pAdv134 contained the antigen expression cassette tLLO-E7. The *LmddA* strain was transformed with the pADV134 plasmid and expression of the LLO-E7 protein from selected clones confirmed by Western blot (**Fig. 11B**). The *Lmdd* system derived from the 10403S wild-type strain lacks antibiotic

resistance markers, except for the *Lmdd* streptomycin resistance.

[00689] Further, pAdv134 was restricted with XhoI/XmaI to clone human PSA, *klk3* resulting in the plasmid, pAdv142. The new plasmid, pAdv142 (**Fig. 11C, Table 2**) contains *Bacillus dal* (B-Dal) under the control of *Listeria* p60 promoter. The shuttle plasmid, pAdv142 complemented the growth of both *E. coli ala drx* MB2159 as well as *Listeria monocytogenes* strain Lmdd in the absence of exogenous D-alanine. The antigen expression cassette in the plasmid pAdv142 consists of *hly* promoter and LLO-PSA fusion protein (**Fig. 11C**).

[00690] The plasmid pAdv142 was transformed to the *Listeria* background strains, Lmdd*actA* strain resulting in Lm-ddA-LLO-PSA. The expression and secretion of LLO-PSA fusion protein by the strain, Lm-ddA-LLO-PSA was confirmed by Western Blot using anti-LLO and anti-PSA antibody (**Fig. 11D**). There was stable expression and secretion of LLO-PSA fusion protein by the strain, Lm-ddA-LLO-PSA after two *in vivo* passages.

EXAMPLE 9: *In vitro* and *in vivo* stability of the strain LmddA-LLO-PSA

[00691] The *in vitro* stability of the plasmid was examined by culturing the LmddA-LLO-PSA *Listeria* strain in the presence or absence of selective pressure for eight days. The selective pressure for the strain LmddA-LLO-PSA is D-alanine. Therefore, the strain LmddA-LLO-PSA was passaged in Brain-Heart Infusion (BHI) and BHI+ 100 μg/ml D-alanine. CFUs were determined for each day after plating on selective (BHI) and non-selective (BHI+D-alanine) medium. It was expected that a loss of plasmid will result in higher CFU after plating on non-selective medium (BHI+D-alanine). As depicted in **Fig. 12A**, there was no difference between the number of CFU in selective and non-selective medium. This suggests that the plasmid pAdv142 was stable for at least 50 generations, when the experiment was terminated.

[00692] Plasmid maintenance *in vivo* was determined by intravenous injection of 5×10^7 CFU LmddA-LLO-PSA, in C57BL/6 mice. Viable bacteria were isolated from spleens homogenized in PBS at 24 h and 48 h. CFUs for each sample were determined at each time point on BHI plates and BHI + 100 mg/ml D-alanine. After plating the splenocytes on selective and non-selective medium, the colonies were recovered after 24 h. Since this strain is highly attenuated, the bacterial load is cleared *in vivo* in 24 h. No significant differences of CFUs were detected on selective and non-selective plates, indicating the stable presence of the recombinant plasmid in all isolated bacteria (**Fig. 12B**).

EXAMPLE 10: *In vivo* passaging, virulence and clearance of the strain LmddA-142 (LmddA-LLO-PSA)

[00693] *LmddA*-142 is a recombinant *Listeria* strain that secretes the episomally expressed tLLO-PSA fusion protein. To determine a safe dose, mice were immunized with LmddA-LLO-PSA at various doses and toxic effects were determined. LmddA-LLO-PSA caused minimum toxic effects (data not shown). The results suggested that a dose of 10^8 CFU of LmddA-LLO-PSA was well tolerated by mice. Virulence studies indicate that the strain LmddA-LLO-PSA was highly attenuated.

[00694] The *in vivo* clearance of LmddA-LLO-PSA after administration of the safe dose, 10^8 CFU intraperitoneally in C57BL/6 mice, was determined. There were no detectable colonies in the liver and spleen of mice immunized with LmddA-LLO-PSA after day 2. Since this strain is highly attenuated, it was completely cleared *in vivo* at 48 h (**Fig. 13A**).

[00695] To determine if the attenuation of LmddA-LLO-PSA attenuated the ability of the strain LmddA-LLO-PSA to infect macrophages and grow intracellularly, a cell infection assay was performed. Mouse macrophage-like cell line such as J774A.1, were infected *in vitro* with *Listeria* constructs and intracellular growth was quantified. The positive control strain, wild type *Listeria* strain 10403S grows intracellularly, and the negative control XFL7, a *prfA* mutant, cannot escape the phagolysosome and thus does not grow in J774 cells. The intracytoplasmic growth of LmddA-LLO-PSA was slower than 10403S due to the loss of the ability of this strain to spread from cell to cell (**Fig. 13B**). The results indicate that LmddA-LLO-PSA has the ability to infect macrophages and grow intracytoplasmically.

EXAMPLE 11: Immunogenicity of the strain-LmddA-LLO-PSA in C57BL/6 mice

[00696] The PSA-specific immune responses elicited by the construct LmddA-LLO-PSA in C57BL/6 mice were determined using PSA tetramer staining. Mice were immunized twice with LmddA-LLO-PSA at one week intervals and the splenocytes were stained for PSA tetramer on day 6 after the boost. Staining of splenocytes with the PSA-specific tetramer showed that LmddA-LLO-PSA elicited 23% of PSA tetramer$^+$CD8$^+$CD62L^{low} cells (**Fig. 14A**). The functional ability of the PSA-specific T cells to secrete IFN-γ after stimulation with PSA peptide for 5 h was examined using intracellular cytokine staining. There was a 200-fold increase in the percentage of CD8$^+$CD62L^{low}IFN-γ secreting cells stimulated with PSA peptide in the LmddA-LLO-PSA group compared to the naïve mice (**Fig. 14B**), indicating that the LmddA-LLO-PSA strain is very immunogenic and primes high levels of

functionally active PSA $CD8^+$ T cell responses against PSA in the spleen.

[00697] To determine the functional activity of cytotoxic T cells generated against PSA after immunizing mice with LmddA-LLO-PSA, we tested the ability of PSA-specific CTLs to lyse cells EL4 cells pulsed with H-$2D^b$ peptide in an *in vitro* assay. A FACS-based caspase assay (**Fig. 14C**) and Europium release (**Fig. 14D**) were used to measure cell lysis. Splenocytes of mice immunized with LmddA-LLO-PSA contained CTLs with high cytolytic activity for the cells that display PSA peptide as a target antigen.

[00698] Elispot was performed to determine the functional ability of effector T cells to secrete IFN-γ after 24 h stimulation with antigen. Using ELISpot, a 20-fold increase in the number of spots for IFN-γ in splenocytes from mice immunized with LmddA-LLO-PSA stimulated with specific peptide when compared to the splenocytes of the naïve mice was observed (**Fig. 14E**).

EXAMPLE 12: Immunization with the *LmddA*-142 strains induces regression of a tumor expressing PSA and infiltration of the tumor by PSA-specific CTLs.

[00699] The therapeutic efficacy of the construct *LmddA*-142 (LmddA-LLO-PSA) was determined using a prostrate adenocarcinoma cell line engineered to express PSA (Tramp-C1-PSA (TPSA); Shahabi et al., 2008). Mice were subcutaneously implanted with 2 x 10^6 TPSA cells. When tumors reached the palpable size of 4-6 mm, on day 6 after tumor inoculation, mice were immunized three times at one week intervals with 10^8 CFU LmddA-142, 10^7 CFU Lm-LLO-PSA (positive control) or left untreated. The naïve mice developed tumors gradually (**Fig. 15A**). The mice immunized with LmddA-142 were all tumor-free until day 35 and gradually 3 out of 8 mice developed tumors, which grew at a much slower rate as compared to the naïve mice (**Fig. 15B**). Five out of eight mice remained tumor free through day 70. As expected, Lm-LLO-PSA-vaccinated mice had fewer tumors than naïve controls and tumors developed more slowly than in controls (**Fig. 15C**). Thus, the construct LmddA-LLO-PSA could regress 60 % of the tumors established by TPSA cell line and slow the growth of tumors in other mice. Cured mice that remained tumor free were rechallenged with TPSA tumors on day 68.

[00700] Immunization of mice with the *LmddA*-142 can control the growth and induce regression of 7-day established Tramp-C1 tumors that were engineered to express PSA in more than 60% of the experimental animals (**Fig. 15B**), compared to none in the untreated group (**Fig. 15A**). The *LmddA*-142 was constructed using a highly attenuated vector (*LmddA*)

and the plasmid pADV142 (**Table 2**).

[00701] Further, the ability of PSA-specific CD8 lymphocytes generated by the LmddA-LLO-PSA construct to infiltrate tumors was investigated. Mice were subcutaneously implanted with a mixture of tumors and matrigel followed by two immunizations at seven day intervals with naïve or control (Lm-LLO-E7) *Listeria,* or with LmddA-LLO-PSA. Tumors were excised on day 21 and were analyzed for the population of $CD8^{+}CD62L^{low}$ $PSA^{tetramer+}$ and $CD4^{+}$ $CD25^{+}FoxP3^{+}$ regulatory T cells infiltrating in the tumors.

[00702] A very low number of $CD8^{+}CD62L^{low}$ $PSA^{tetramer+}$ tumor infiltrating lymphocytes (TILs) specific for PSA that were present in the both naïve and Lm-LLO-E7 control immunized mice was observed. However, there was a 10-30-fold increase in the percentage of PSA-specific $CD8^{+}CD62L^{low}$ $PSA^{tetramer+}$ TILs in the mice immunized with LmddA-LLO-PSA (Fig. 7A). Interestingly, the population of $CD8^{+}CD62L^{low}$ $PSA^{tetramer+}$ cells in spleen was 7.5 fold less than in tumor (**Fig. 16A**).

[00703] In addition, the presence of $CD4^{+}/CD25^{+}/Foxp3^{+}$ T regulatory cells (Tregs) in the tumors of untreated mice and *Listeria* immunized mice was determined. Interestingly, immunization with *Listeria* resulted in a considerable decrease in the number of $CD4^{+}$ $CD25^{+}FoxP3^{+}$ T-regs in tumor but not in spleen (**Fig. 16B**). However, the construct LmddA-LLO-PSA had a stronger impact in decreasing the frequency of $CD4^{+}$ $CD25^{+}FoxP3^{+}$ T-regs in tumors when compared to the naïve and Lm-LLO-E7 immunized group (**Fig. 16B**).

[00704] Thus, the *LmddA*-142 immunotherapy can induce PSA-specific $CD8^{+}$ T cells that are able to infiltrate the tumor site (**Fig. 16A**). Interestingly, immunization with *LmddA*-142 was associated with a decreased number of regulatory T cells in the tumor (**Fig. 16B**), probably creating a more favorable environment for an efficient anti-tumor CTL activity.

EXAMPLE 13: *Lmdd*-143 and *LmddA*-143 secretes a functional LLO despite the PSA fusion.

[00705] The *Lmdd*-143 and *LmddA*-143 contain the full-length human *klk3* gene, which encodes the PSA protein, inserted by homologous recombination downstream and in frame with the *hly* gene in the chromosome. These constructs were made by homologous recombination using the pKSV7 plasmid (Smith and Youngman, Biochimie. 1992; 74 (7-8) p705-711), which has a temperature-sensitive replicon, carrying the *hly-klk3-mpl* recombination cassette. Because of the plasmid excision after the second recombination event, the antibiotic resistance marker used for integration selection is lost. Additionally, the

actA gene is deleted in the *LmddA*-143 strain (**Fig. 17A**). The insertion of *klk3* in frame with *hly* into the chromosome was verified by PCR (**Fig. 17B**) and sequencing (data not shown) in both constructs.

[00706] One important aspect of these chromosomal constructs is that the production of LLO-PSA would not completely abolish the function of LLO, which is required for escape of *Listeria* from the phagosome, cytosol invasion and efficient immunity generated by *L. monocytogenes*. Western-blot analysis of secreted proteins from *Lmdd*-143 and *LmddA*-143 culture supernatants revealed an ~81 kDa band corresponding to the LLO-PSA fusion protein and an ~60 kDa band, which is the expected size of LLO (**Fig. 18A**), indicating that LLO is either cleaved from the LLO-PSA fusion or still produced as a single protein by *L. monocytogenes*, despite the fusion gene in the chromosome. The LLO secreted by *Lmdd*-143 and *LmddA*-143 retained 50% of the hemolytic activity, as compared to the wild-type *L. monocytogenes* 10403S (**Fig. 18B**). In agreement with these results, both *Lmdd*-143 and *LmddA*-143 were able to replicate intracellularly in the macrophage-like J774 cell line (**Fig. 18C**).

EXAMPLE 14: Both *Lmdd*-143 and *LmddA*-143 elicit cell-mediated immune responses against the PSA antigen.

[00707] After showing that both *Lmdd*-143 and *LmddA*-143 were able to secrete PSA fused to LLO, the question of if these strains could elicit PSA-specific immune responses *in vivo* was investigated. C57Bl/6 mice were either left untreated or immunized twice with the *Lmdd*-143, *LmddA*-143 or *LmddA*-142. PSA-specific $CD8^+$ T cell responses were measured by stimulating splenocytes with the PSA_{65-74} peptide and intracellular staining for IFN-γ. As shown in **Fig. 19**, the immune response induced by the chromosomal and the plasmid-based vectors is similar.

Materials and Methods (EXAMPLES 15-20)

[00708] Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and DNA sequencing was done by Genewiz Inc., South Plainfield, NJ. Flow cytometry reagents were purchased from Becton Dickinson Biosciences (BD, San Diego, CA). Cell culture media, supplements and all other reagents, unless indicated, were from Sigma (St. Louise, MO). Her2/neu HLA-A2 peptides were synthesized by EZbiolabs (Westfield, IN). Complete RPMI 1640 (C-RPMI) medium contained 2mM glutamine, 0.1 mM non-essential amino acids, and 1mM sodium pyruvate, 10% fetal bovine serum, penicillin/streptomycin, Hepes (25mM). The

polyclonal anti-LLO antibody was described previously and anti-Her2/neu antibody was purchased from Sigma.

Mice and Cell Lines

[00709] All animal experiments were performed according to approved protocols by IACUC at the University of Pennsylvania or Rutgers University. FVB/N mice were purchased from Jackson laboratories (Bar Harbor, ME). The FVB/N Her2/neu transgenic mice, which overexpress the rat Her2/neu onco-protein were housed and bred at the animal core facility at the University of Pennsylvania. The NT-2 tumor cell line expresses high levels of rat Her2/neu protein, was derived from a spontaneous mammary tumor in these mice and grown as described previously. DHFR-G8 (3T3/neu) cells were obtained from ATCC and were grown according to the ATCC recommendations. The EMT6-Luc cell line was a generous gift from Dr. John Ohlfest (University of Minnesota, MN) and was grown in complete C-RPMI medium. Bioluminescent work was conducted under guidance by the Small Animal Imaging Facility (SAIF) at the University of Pennsylvania (Philadelphia, PA).

Listeria constructs and antigen expression

[00710] Her2/neu-pGEM7Z was kindly provided by Dr. Mark Greene at the University of Pennsylvania and contained the full-length human Her2/neu (hHer2) gene cloned into the pGEM7Z plasmid (Promega, Madison WI). This plasmid was used as a template to amplify three segments of hHer-2/neu, namely, EC1, EC2, and IC1, by PCR using pfx DNA polymerase (Invitrogen) and the oligos indicated in **Table 4**.

[00711] **Table 4**: Primers for cloning of Human her-2-Chimera

	DNA sequence	**Base pair region**	**Amino acid region or junctions**
Her-2-Chimera (F)	TGATCTCGAGACCCACCTGGACATGCTC (SEQ ID NO:48)	120-510	40-170
HerEC1-EC2F (Junction)	CTACCAGGACACGATTTTGTGGAAG-AATATCCAGGAGTTTGCTGGCTGC (SEQ ID NO: 49)	510/1077	170/359
HerEC1-EC2R (Junction)	GCAGCCAGCAAACTCCTGGATATT-CTTCCACAAAATCGTGTCCTGGTAG (SEQ ID NO: 50)		
HerEC2-ICIF (Junction)	CTGCCACCAGCTGTGCGCCCGAGGG-CAGCAGAAGATCCGGAAGTACACGA (SEQ ID NO: 51)	1554/2034	518/679
HerEC2-ICIR (Junction)	TCGTGTACTTCCGGATCTTCTGCTGCCCT CGGGC GCACAGCTGGTGGCAG (SEQ ID NO: 84)		
Her-2-Chimera (R)	GTGGCCCGGGTCTAGATTAGTCTAAGAG GCAGCCATAGG (SEQ ID NO:52)	2034-2424	679-808

[00712] The Her-2/neu chimera construct was generated by direct fusion by the SOEing PCR method and each separate hHer-2/neu segment as templates. Primers are shown in **Table 5**.

5 [00713] **Table 5**

	DNA sequence	**Base pair region**	**Amino acid region**
Her-2-EC1(F)	CCGCCTCGAGGCCGCGAGCACCCAAGTG (SEQ ID NO: 53)	58-979	20-326
Her-2-EC1(R)	CGCGACTAGTTTAATCCTCTGCTGTCACCTC (SEQ ID NO: 54)		
Her-2-EC2(F)	CCGCCTCGAGTACCTTTCTACGGACGTG (SEQ ID NO:55)	907-1504	303-501
Her- 2-EC2(R)	CGCGACTAGTTTACTCTGGCCGGTTGGCAG (SEQ ID NO: 56)		
Her-2-Her-2-IC1(F)	CCGCCTCGAGCAGCAGAAGATCCGGAAGTAC (SEQ ID NO: 57)	2034-3243	679-1081
Her-2-IC1(R)	CGCGACTAGTTTAAGCCCCTTCGGAGGGTG (SEQ ID NO: 58)		

[00714] Sequence of primers for amplification of different segments human Her2 regions

[00715] ChHer2 gene was excised from pAdv138 using XhoI and SpeI restriction enzymes,

and cloned in frame with a truncated, non-hemolytic fragment of LLO in the *Lmdd* shuttle vector, pAdv134. The sequences of the insert, LLO and *hly* promoter were confirmed by DNA sequencing analysis. This plasmid was electroporated into electro-competent *actA, dal, dat* mutant *Listeria monocytogenes* strain, *LmddA* and positive clones were selected on Brain Heart infusion (BHI) agar plates containing streptomycin (250μg/ml). In some experiments similar *Listeria* strains expressing hHer2/neu (*Lm*-hHer2) fragments were used for comparative purposes. In all studies, an irrelevant *Listeria* construct (*Lm*-control) was included to account for the antigen independent effects of *Listeria* on the immune system. *Lm*-controls were based on the same *Listeria* platform as ADXS31-164 (*LmddA*-ChHer2), but expressed a different antigen such as HPV16-E7 or NY-ESO-1. Expression and secretion of fusion proteins from *Listeria* were tested. Each construct was passaged twice *in vivo*.

Cytotoxicity assay

[00716] Groups of 3-5 FVB/N mice were immunized three times with one week intervals with 1 x 10^8 colony forming units (CFU) of *Lm*-LLO-ChHer2, ADXS31-164, *Lm*-hHer2 ICI or *Lm*-control (expressing an irrelevant antigen) or were left naïve. NT-2 cells were grown *in vitro*, detached by trypsin and treated with mitomycin C (250 μg/ml in serum free C-RPMI medium) at 37°C for 45 minutes. After 5 washes, they were co-incubated with splenocytes harvested from immunized or naïve animals at a ratio of 1:5 (Stimulator: Responder) for 5 days at 37°C and 5% CO_2. A standard cytotoxicity assay was performed using europium labeled 3T3/neu (DHFR-G8) cells as targets according to the method previously described. Released europium from killed target cells was measured after 4 hour incubation using a spectrophotometer (Perkin Elmer, Victor^2) at 590 nm. Percent specific lysis was defined as (lysis in experimental group-spontaneous lysis)/(Maximum lysis-spontaneous lysis).

Interferon-γ secretion by splenocytes from immunized mice

[00717] Groups of 3-5 FVB/N or HLA-A2 transgenic mice were immunized three times with one week intervals with 1 x 10^8 CFU of ADXS31-164, a negative *Listeria* control (expressing an irrelevant antigen) or were left naïve. Splenocytes from FVB/N mice were isolated one week after the last immunization and co-cultured in 24 well plates at 5 x 10^6 cells/well in the presence of mitomycin C treated NT-2 cells in C-RPMI medium. Splenocytes from the HLA-A2 transgenic mice were incubated in the presence of 1μM of HLA-A2 specific peptides or 1μg/ml of a recombinant His-tagged ChHer2 protein, produced in *E. coli* and purified by a nickel based affinity chromatography system. Samples from supernatants were obtained 24 or

72 hours later and tested for the presence of interferon-γ (IFN-γ) using mouse IFN-γ Enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer's recommendations.

Tumor studies in Her2 transgenic animals

[00718] Six weeks old FVB/N rat Her2/neu transgenic mice (9-14/group) were immunized 6 times with 5 x 10^8 CFU of *Lm*-LLO-ChHer2, ADXS31-164 or *Lm*-control. They were observed twice a week for the emergence of spontaneous mammary tumors, which were measured using an electronic caliper, for up to 52 weeks. Escaped tumors were excised when they reached a size 1cm^2 in average diameter and preserved in RNAlater at -20°C. In order to determine the effect of mutations in the Her2/neu protein on the escape of these tumors, genomic DNA was extracted using a genomic DNA isolation kit, and sequenced.

Effect of ADXS31-164 on regulatory T cells in spleens and tumors

[00719] Mice were implanted subcutaneously (s.c.) with 1 x 10^6 NT-2 cells. On days 7, 14 and 21, they were immunized with 1 x 10^8 CFUs of ADXS31-164, *LmddA*-control or left naïve. Tumors and spleens were extracted on day 28 and tested for the presence of $CD3^+/CD4^+/FoxP3^+$ Tregs by FACS analysis. Briefly, splenocytes were isolated by homogenizing the spleens between two glass slides in C-RPMI medium. Tumors were minced using a sterile razor blade and digested with a buffer containing DNase (12U/ml), and collagenase (2mg/ml) in PBS. After 60 min incubation at RT with agitation, cells were separated by vigorous pipetting. Red blood cells were lysed by RBC lysis buffer followed by several washes with complete RPMI-1640 medium containing 10% FBS. After filtration through a nylon mesh, tumor cells and splenocytes were resuspended in FACS buffer (2% FBS/PBS) and stained with anti-CD3-PerCP-Cy5.5, CD4-FITC, CD25-APC antibodies followed by permeabilization and staining with anti-Foxp3-PE. Flow cytometry analysis was performed using 4-color FACS calibur (BD) and data were analyzed using cell quest software (BD).

Statistical analysis

[00720] The log-rank Chi-Squared test was used for survival data and student's *t*-test for the CTL and ELISA assays, which were done in triplicates. A p-value of less than 0.05 (marked as *) was considered statistically significant in these analyzes. All statistical analysis was done with either Prism software, V.4.0a (2006) or SPSS software, V.15.0 (2006). For all FVB/N rat Her2/neu transgenic studies we used 8-14 mice per group, for all wild-type FVB/N studies we used at least 8 mice per group unless otherwise stated. All studies were repeated at

least once except for the long term tumor study in Her2/neu transgenic mouse model.

EXAMPLE 15: Generation of *L. Monocytogenes* Strains That Secrete LLO Fragments Fused to Her-2 Fragments: Construction of ADXS31-164

[00721] Construction of the chimeric *Her2/neu* gene (ChHer2) was as follows. Briefly, *ChHer2* gene was generated by direct fusion of two extracellular (aa 40-170 and aa 359-433) and one intracellular fragment (aa 678-808) of the Her2/neu protein by SOEing PCR method. The chimeric protein harbors most of the known human MHC class I epitopes of the protein. *ChHer2* gene was excised from the plasmid, pAdv138 (which was used to construct *Lm*-LLO-ChHer2) and cloned into *LmddA* shuttle plasmid, resulting in the plasmid pAdv164 (**Fig. 20A**). There are two major differences between these two plasmid backbones. 1) Whereas pAdv138 uses the chloramphenicol resistance marker (*cat*) for *in vitro* selection of recombinant bacteria, pAdv164 harbors the D-alanine racemase gene (*dal*) from *bacillus subtilis*, which uses a metabolic complementation pathway for *in vitro* selection and *in vivo* plasmid retention in *LmddA* strain which lacks the *dal-dat* genes. This immunotherapy platform was designed and developed to address FDA concerns about the antibiotic resistance of the engineered *Listeria* immunotherapy strains. 2) Unlike pAdv138, pAdv164 does not harbor a copy of the *prfA* gene in the plasmid (see sequence below and **Fig. 20A**), as this is not necessary for *in vivo* complementation of the *Lmdd* strain. The *LmddA* immunotherapy strain also lacks the *actA* gene (responsible for the intracellular movement and cell-to-cell spread of *Listeria*) so the recombinant immunotherapy strains derived from this backbone are 100 times less virulent than those derived from the *Lmdd*, its parent strain. *LmddA*-based immunotherapies are also cleared much faster (in less than 48 hours) than the *Lmdd*-based immunotherapies from the spleens of the immunized mice. The expression and secretion of the fusion protein tLLO-ChHer2 from this strain was comparable to that of the *Lm*-LLO-ChHer2 in TCA precipitated cell culture supernatants after 8 hours of *in vitro* growth (**Fig. 20B**) as a band of ~104 KD was detected by an anti-LLO antibody using Western Blot analysis. The *Listeria* backbone strain expressing only tLLO was used as negative control.

[00722] pAdv164 sequence (7075 base pairs) (see **Figs. 20A and 20B**):

cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgc
gtcagcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatg
gcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttttc
cataggctccgcccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccag
gcgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcatt

ccacgcctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgcct
tatccggtaactatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagtt
agtcttgaagtcatgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaaga
gttggtagctcagagaaccttcgaaaaaccgccctgcaaggcggttttttcgttttcagagcaagagattacgcgcagaccaaaacgat
5 ctcaagaagatcatcttattaatcagataaaatatttctagccctcctttgattagtatattcctatcttaaagttacttttatgtggaggcattaa
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aaaattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcga
10 taagtatatacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaaggtta
caaagatggaaatgaatatattgttgtggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcg
agcctaacctatccaggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaa
cactcagcattgatttgccaggtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagtaa
atacattagtggaaagatggaatgaaaaatatgctcaagcttatccaaatgtaagtgcaaaaaattgattatgatgacgaaatggcttacag
15 tgaatcacaattaattgcgaaatttggtacagcatttaaagctgtaaataatagcttgaatgtaaacttcggcgcaatcagtgaagggaaa
atgcaagaagaagtcattagttttaaacaaatttactataacgtgaatgttaatgaacctacaagaccttccagatttttcggcaaagctgtt
actaaagagcagttgcaagcgcttggagtgaatgcagaaaatcctcctgcatatatctcaagtgtggcgtatggccgtcaagtttatttga
aattatcaactaattcccatagtactaaagtaaaagctgcttttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaaca
aatatcatcaaaaaattcttccttcaaagccgtaatttacggaggttccgcaaaagatgaagttcaaatcatcgacggcaacctcggagac
20 ttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgcttatacaacaaacttcctaaaagacaatg
aattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatggaaaaattaacatcgatcactctggagga
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gaaacggagctggtggagccgctgacacctagcggagcgatgcccaaccaggcgcagatgcggatcctgaaagagacggagctg
aggaaggtgaaggtgcttggatctggcgcttttggcacagtctacaagggcatctggatccctgatggggagaatgtgaaaattccagt

ggccatcaaagtgttgagggaaaacacatcccccaaagccaacaaagaaatcttagacgaagcatacgtgatggctggtgtgggctc
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cattggagttagaaatggaagaagaaaaaagcaatgatttcgtgtgaataatgcacgaaatcattgcttatttttttaaaaagcgatatact
5 agatataacgaaacaacgaactgaataaagaatacaaaaaaagagccacgaccagttaaagcctgagaaactttaactgcgagcctta
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cggcttgttaaagtacaaatttaaaaatagtaaaattcgctcaatcactaccaagccaggtaaaagtaaaggggctatttttgcgtatcgct
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ccaaacgtttatcgttatggtacgtatgcagacgaaaaccgttcatacactaaaggacattctgaaaacaatttaagacaaatcaatacctt
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15 acggacaatgtagaatttttgatcccaattaccgttattctttcaaagaatggcaagattggtctttcaaacaaacagataataagggcttt
actcgttcaagtctaacggttttaagcggtacagaaggcaaaaaacaagtagatgaaccctggtttaatctcttattgcacgaaacgaaat
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aatataatatgtttgagtttaataatcgattagatcaacccttagaagaaaaagaagtaatcaaaattgttagaagtgcctattcagaaaact
atcaaggggctaatagggaatacattaccattctttgcaaagcttgggtatcaagtgatttaaccagtaaagatttatttgtccgtcaaggg
20 tggtttaaattcaagaaaaaaagaagcgaacgtcaacgtgttcatttgtcagaatggaaagaagatttaatggcttatattagcgaaaaaa
gcgatgtatacaagccttatttagcgacgaccaaaaaagagattagagaagtgctaggcattcctgaacggacattagataaattgctg
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actctaaacaaattggcagaacgccccaaaacggacccacaactcgatttgtttagctacgatacaggctgaaaataaaacccgcact
25 atgccattacatttatatctatgatacgtgtttgtttttctttgctggctagcttaattgcttatatttacctgcaataaaggatttcttacttccatta
tactcccattttccaaaaacatacggggaacacgggaacttattgtacaggccacctcatagttaatggtttcgagccttcctgcaatctca
tccatggaaatatattcatccccctgccggcctattaatgtgacttttgtgcccggcggatattcctgatccagctccaccataaattggtcc
atgcaaattcggccggcaattttcaggcgttttcccttcacaaggatgtcggtccctttcaattttcggagccagccgtccgcatagccta
caggcaccgtcccgatccatgtgtctttttccgctgtgtactcggctccgtagctgacgctctcgccttttctgatcagtttgacatgtgaca
30 gtgtcgaatgcagggtaaatgccggacgcagctgaaacggtatctcgtccgacatgtcagcagacgggcgaaggccatacatgccg
atgccgaatctgactgcattaaaaaagcctttttcagccggagtccagcggcgctgttcgcgcagtggaccattagattctttaacggc
agcggagcaatcagctctttaaagcgctcaaactgcattaagaaatagcctctttctttttcatccgctgtcgcaaaatgggtaaatacccc
tttgcactttaaacgagggttgcggtcaagaattgccatcacgttctgaacttcttcctctgtttttacaccaagtctgttcatccccgtatcg
accttcagatgaaaatgaagagaacctttttcgtgtggcgggctgcctcctgaagccattcaacagaataacctgttaaggtcacgtcat

actcagcagcgattgccacatactccgggggaaccgcgccaagcaccaatataggcgccttcaatcccttttgcgcagtgaaatcgc
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ctttcacaactgccatcaagtggacatgttcaccgatatgtttttcatattgctgacattttcctttatcgcggacaagtcaatttccgcccac
gtatctctgtaaaaaggttttgtgctcatggaaaactcctctcttttttcagaaaatcccagtacgtaattaagtatttgagaattaattttatatt
5 gattaatactaagtttacccagttttcacctaaaaaacaaatgatgagataatagctccaaaggctaaagaggactataccaactatttgtt
aattaa (SEQ ID NO: 87)

EXAMPLE 16: ADXS31-164 Is as Immunogenic As Lm-LLO-ChHER2

[00723] Immunogenic properties of ADXS31-164 in generating anti-Her2/neu specific cytotoxic T cells were compared to those of the *Lm*-LLO-ChHer2 immunotherapy in a
10 standard CTL assay. Both immunotherapies elicited strong but comparable cytotoxic T cell responses toward Her2/neu antigen expressed by 3T3/neu target cells. Accordingly, mice immunized with a *Listeria* expressing only an intracellular fragment of Her2-fused to LLO showed lower lytic activity than the chimeras which contain more MHC class I epitopes. No CTL activity was detected in naïve animals or mice injected with the irrelevant *Listeria*
15 immunotherapy (**Fig. 21A**). ADXS31-164 was also able to stimulate the secretion of IFN-γ by the splenocytes from wild type FVB/N mice (**Fig. 21B**). This was detected in the culture supernatants of these cells that were co-cultured with mitomycin C treated NT-2 cells, which express high levels of Her2/neu antigen (**Fig. 21C**).

[00724] Proper processing and presentation of the human MHC class I epitopes after
20 immunizations with ADXS31-164 was tested in HLA-A2 mice. Splenocytes from immunized HLA-A2 transgenics were co-incubated for 72 hours with peptides corresponding to mapped HLA-A2 restricted epitopes located at the extracellular (HLYQGCQVV SEQ ID NO: 59 or KIFGSLAFL SEQ ID NO: 60) or intracellular (RLLQETELV SEQ ID NO: 61) domains of the Her2/neu molecule (**Fig. 21C**). A recombinant ChHer2 protein was used as positive
25 control and an irrelevant peptide or no peptide as negative controls. The data from this experiment show that ADXS31-164 is able to elicit anti-Her2/neu specific immune responses to human epitopes that are located at different domains of the targeted antigen.

EXAMPLE 17: ADXS31-164 was More Efficacious than Lm-LLO-ChHER2 in
30 **Preventing the Onset of Spontaneous Mammary Tumors**

[00725] Anti-tumor effects of ADXS31-164 were compared to those of *Lm*-LLO-ChHer2 in Her2/neu transgenic animals which develop slow growing, spontaneous mammary tumors at

20-25 weeks of age. All animals immunized with the irrelevant *Listeria*-control immunotherapy developed breast tumors within weeks 21-25 and were sacrificed before week 33. In contrast, *Liseria*-Her2/neu recombinant immunotherapies caused a significant delay in the formation of the mammary tumors. On week 45, more than 50% of ADXS31-164 vaccinated mice (5 out of 9) were still tumor free, as compared to 25% of mice immunized with *Lm*-LLO-ChHer2. At week 52, 2 out of 8 mice immunized with ADXS31-164 still remained tumor free, whereas all mice from other experimental groups had already succumbed to their disease (**Fig. 22**). These results indicate that despite being more attenuated, ADXS31-164 is more efficacious than *Lm*-LLO-ChHer2 in preventing the onset of spontaneous mammary tumors in Her2/neu transgenic animals.

EXAMPLE 18: Mutations in HER2/Neu Gene upon Immunization with ADXS31-164

[00726] Mutations in the MHC class I epitopes of Her2/neu have been considered responsible for tumor escape upon immunization with small fragment immunotherapies or trastuzumab (Herceptin), a monoclonal antibody that targets an epitope in the extracellular domain of Her2/neu. To assess this, genomic material was extracted from the escaped tumors in the transgenic animals and sequenced the corresponding fragments of the *neu* gene in tumors immunized with the chimeric or control immunotherapies. Mutations were not observed within the Her-2/neu gene of any vaccinated tumor samples suggesting alternative escape mechanisms (data not shown).

EXAMPLE 19: ADXS31-164 Causes A Significant Decrease in Intra-Tumoral T Regulatory Cells

[00727] To elucidate the effect of ADXS31-164 on the frequency of regulatory T cells in spleens and tumors, mice were implanted with NT-2 tumor cells. Splenocytes and intra-tumoral lymphocytes were isolated after three immunizations and stained for Tregs, which were defined as $CD3^+/CD4^+/CD25^+/FoxP3^+$ cells, although comparable results were obtained with either FoxP3 or CD25 markers when analyzed separately. The results indicated that immunization with ADXS31-164 had no effect on the frequency of Tregs in the spleens, as compared to an irrelevant *Listeria* immunotherapy or the naïve animals (**Fig. 23**). In contrast, immunization with the *Listeria* immunotherapies caused a considerable impact on the presence of Tregs in the tumors (**Fig. 24A**). Whereas in average 19.0% of all $CD3^+$ T cells in untreated tumors were Tregs, this frequency was reduced to 4.2% for the irrelevant immunotherapy and 3.4% for ADXS31-164, a 5-fold reduction in the frequency of intra-

tumoral Tregs (**Fig. 24B**). The decrease in the frequency of intra-tumoral Tregs in mice treated with either of the *LmddA* immunotherapies could not be attributed to differences in the sizes of the tumors. In a representative experiment, the tumors from mice immunized with ADXS31-164 were significantly smaller [mean diameter (mm) ±SD, 6.71±0.43, n=5] than the tumors from untreated mice (8.69±0.98, n=5, p<0.01) or treated with the irrelevant immunotherapy (8.41±1.47, n=5, p=0.04), whereas comparison of these last two groups showed no statistically significant difference in tumor size (p=0.73). The lower frequency of Tregs in tumors treated with *LmddA* immunotherapies resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* immunotherapies. However, only the immunotherapy expressing the target antigen HER2/neu (ADXS31-164) was able to reduce tumor growth, indicating that the decrease in Tregs has an effect only in the presence on antigen-specific responses in the tumor.

EXAMPLE 20: Peripheral Immunization with ADXS31-164 Can Delay the Growth of a Metastatic Breast Cancer Cell Line in the Brain

[00728] Mice were immunized IP with ADXS31-164 or irrelevant *Lm*-control immunotherapies and then implanted intra-cranially with 5,000 EMT6-Luc tumor cells, expressing luciferase and low levels of Her2/neu (**Fig. 25A**). Tumors were monitored at different times post-inoculation by *ex vivo* imaging of anesthetized mice. On day 8 post-tumor inoculation tumors were detected in all control animals, but none of the mice in ADXS31-164 group showed any detectable tumors (**Fig. 25A and 25B**). ADXS31-164 could clearly delay the onset of these tumors, as on day 11 post-tumor inoculation all mice in negative control group had already succumbed to their tumors, but all mice in ADXS31-164 group were still alive and only showed small signs of tumor growth. These results strongly suggest that the immune responses obtained with the peripheral administration of ADXS31-164 could possibly reach the central nervous system and that *LmddA*-based immunotherapies might have a potential use for treatment of CNS tumors.

EXAMPLE 21: Peptide "Minigene" Expression System

Materials and Methods

[00729] This expression system is designed to facilitate cloning of panels of recombinant proteins containing distinct peptide moieties at the carboxy-terminus. This is accomplished by a simple PCR reaction utilizing a sequence encoding one of the SS-Ub-Peptide constructs as a

template. By using a primer that extends into the carboxy-terminal region of the Ub sequence and introducing codons for the desired peptide sequence at the 3' end of the primer, a new SS-Ub-Peptide sequence can be generated in a single PCR reaction. The 5' primer encoding the bacterial promoter and first few nucleotides of the ActA signal sequence is the same for all constructs. The constructs generated using this strategy are represented schematically in **Figs. 26A-26C.** In this example, two constructs are described. One contains a model peptide antigen presented on mouse MHC class I and the second construct indicates where a therapeutically relevant peptide, such as one derived from a human glioblastoma (GBM) TAA, would be substituted. For clarity, we have designated the constructs diagramed in **Figs. 26A-C** as containing an $ActA_{1-100}$ secretion signal. However, an LLO based secretion signal could be substituted with equal effect.

[00730] One of the advantages of the proposed system is that it will be possible to load cells with multiple peptides using a single *Listeria* vector construct. Multiple peptides will be introduce into recombinant attenuated *Listeria* (e.g. prfA mutant *Listeria* or a dal/dat/actA mutant *Listeria*) using a modification of the single peptide expression system described above. A chimeric protein encoding multiple distinct peptides from sequential SS-Ub-Peptide sequences encoded in one insert. Shine-Dalgarno ribosome binding sites are introduced before each SS-Ub-Peptide coding sequence to enable separate translation of each of the peptide constructs. **Fig. 26C** demonstrates a schematic representation of a construct designed to express 4 separate peptide antigens from one strain of recombinant *Listeria*. Since this is strictly a representation of the general expression strategy, we have included 4 distinct MHC class I binding peptides derived from known mouse or human tumor associated- or infectious disease antigens.

MATERIALS & METHODS (EXAMPLES 22-24)

[00731] Plasmid pAdv142 and strain LmddA142 have been described above at Example 7. Additional details are provided below.

Construction of plasmid pAdv142 and strain LmddA142

[00732] This plasmid is next generation of the antibiotic free plasmid, pTV3 that was previously constructed by Verch et al. The unnecessary copy of the virulence gene transcription activator, *prfA* was deleted from plasmid pTV3 since Lm-ddA contains a copy of *prfA* gene in the chromosome. Therefore, the presence of *prfA* gene in the *dal* containing plasmid was not essential. Additionally, the cassette for p60-*Listeria dal* at the NheI/PacI

restriction site was replaced by p60-*Bacillus subtilis dal* (*dal*$_{Bs}$) resulting in the plasmid pAdv134. Further, pAdv134 was restricted with XhoI/XmaI to clone human PSA, *klk3* resulting in the plasmid, pAdv142. The new plasmid pAdv 142 (**Fig. 11C**) contains *dal*$_{Bs}$ and its expression was under the control of Lm p60 promoter. The shuttle plasmid pAdv142 could
5 complement the growth of both *E. coli ala drx* MB2159 as well as Lmdd in the absence of exogenous addition of D-alanine. The antigen expression cassette in the plasmid pAdv 142 consists of *hly* promoter and tLLO-PSA fusion protein (**Fig. 27**).

[00733] The plasmid pAdv142 was transformed to the *Listeria* background strain, LmddA resulting in LmddA142 or ADXS31-142. The expression and secretion of LLO-PSA fusion
10 protein by the strain, ADXS31-142 was confirmed by western analysis using anti-LLO and anti-PSA antibody and is shown in **Fig. 11D**. There was stable expression and secretion of LLO-PSA fusion protein by the strain, ADXS31-142 after two *in vivo* passages in C57BL/6 mice.

Construction of LmddA211, LmddA223 and LmddA224 strains

15 [00734] The different ActA/PEST regions were cloned in the plasmid pAdv142 to create the three different plasmids pAdv211, pAdv223 and pAdv224 containing different truncated fragments of ActA protein.

LLO signal sequence (LLOss)-ActAPEST2 (pAdv211)/ LmddA211

[00735] First two fragments PsiI-LLOss-XbaI (817 bp in size) and LLOss-XbaI-ActA-
20 PEST2 (602 bp in size) were amplified and then fused together by using SOEing PCR method with an overlap of 25 bases. This PCR product now contains PsiI-LLOss- Xbal- ActAPEST2-XhoI a fragment of 762 bp in size. The new PsiI-LLOss- Xbal- ActAPEST2-XhoI PCR product and pAdv142 (LmddA-PSA) plasmid were digested with PsiI/XhoI restriction enzymes and purified. Ligation was set up and transformed into MB2159 electro competent
25 cells and plated onto LB agar plates. The PsiI-LLOss- Xbal- ActAPEST2 / pAdv 142 (PSA) clones were selected and screened by insert-specific PCR reaction PsiI-LLOss- Xbal-ActAPEST2 / pAdv 142 (PSA) clones #9, 10 were positive and the plasmid purified by mini preparation. Following screening of the clones by PCR screen, the inserts from positive clones were sequenced. The plasmid PsiI-LLOss- Xbal- ActAPEST2 / pAdv 142 (PSA)
30 referred as pAdv211.10 was transformed into *Listeria* LmddA mutant electro competent cells and plated onto BHI/strep agar plates. The resulting LmddA211 strain was screened by colony PCR. Several *Listeria* colonies were selected and screened for the expression and

secretion of endogenous LLO and ActAPEST2-PSA (LA229-PSA) proteins. There was stable expression of ActAPEST2-PSA fusion proteins after two in vivo passages in mice.

LLOss-ActAPEST3 and PEST4:

[00736] ActAPEST3 and ActAPEST4 fragments were created by PCR method. PCR products containing LLOss-XbaI- ActAPEST3-XhoI (839 bp in size) and LLOss-XbaI-ActAPEST4-XhoI a fragments (1146 bp in size) were cloned in pAdv142. The resulting plasmid pAdv223 (PsiI-LLOss- Xbal- ActAPEST3-XhoI / pAdv 142) and pAdv224 (PsiI-LLOss- Xbal- ActAPEST4 / pAdv 142) clones were selected and screened by insert-specific PCR reaction. The plasmids pAdv223 and pAdv224 were transformed to the LmddA backbone resulting in LmddA223 and LmddA224, respectively. Several *Listeria* colonies were selected and screened for the expression and secretion of endogenous LLO, ActAPEST3-PSA (LmddA223) or ActAPEST4-PSA (LmddA224) proteins. There was stable expression and secretion of the fusion protein ActAPEST3-PSA (LmddA223) or ActAPEST4-PSA (LmddA224) after two in vivo passages in mice.

Experimental plan 1

[00737] The therapeutic efficacy of the ActA-PEST-PSA (PEST3, PEST2 and PEST4 sequences) and tLLO-PSA using TPSA23 (PSA expressing tumor model) were evaluated and compared. Untreated mice were used as control group. In parallel evaluated the immune responses were also using intracellular cytokine staining for interferon–gamma and PSA tetramer staining.

For the tumor regression study.

[00738] Ten groups of eight C57BL/6 mice (7 weeks old males) were implanted subcutaneously with 1 x 10^6 of TPSA23 cells on day 0. On Day 6 they received immunization which was followed by 2 booster doses which were 1 week apart. Tumor growth was monitored every week until they reached a size of 1.2 cm in average diameter.

Immunogenicity study.

[00739] 2 groups of C57BL/6 mice (7 weeks old males) were immunized 3 times with one week interval with the immunotherapies listed in the table below. Six days after the last boost injection, mice were sacrificed, and the spleens will be harvested and the immune responses were tested for tetramer staining and IFN-γ secretion by intracellular cytokine staining.

Experimental plan 2

[00740] This experiment was a repeat of Experimental plan 1, however, the Naïve, tLLO, ActA/PEST2-PSA and tLLO-PSA groups were only included. Similar to Experimental plan 1, the therapeutic efficacy was evaluated using TPSA23 (PSA expressing tumor model). Five C57BL/6 mice per group were implanted subcutaneously with $1x10^6$ of TPSA23 cells on day 0. On Day 6 they received immunization ($1x10^8$ CFU/mL) which was followed by booster 1 week later. Spleen and tumor was collected on day 6 post last treatment. The immune response was monitored using PSA pentamer staining in both spleen and tumor.

Materials & Methods:

[00741] TPSA23 cells are cultured in complete medium. Two days prior to implanting tumor cells in mice, TPSA23 cells were sub-cultured in complete media. On the day of the experiment (Day 0), cells were trypsinized and washed twice with PBS. Cells were counted and re-suspended at a concentration of $1x10^6$ cells/200ul in PBS/mouse for injection. Tumor cells were injected subcutaneously in the flank of each mouse.

Complete Medium for TPSA23 cells

[00742] Complete medium for TPSA23 cells was prepared by mixing 430ml of DMEM with Glucose, 45ml of fetal calf serum (FCS), 25ml of Nu-Serum IV, 5ml 100X L-Glutamine, 5ml of 100mM Na-Pyruvate, 5ml of 10,000U/mL Penicillin/Streptomycin. 0.005mg/ml of Bovine Insulin and 10nM of Dehydroisoandrosterone was added to the flask while splitting cells.

Complete Medium for splenocytes (c-RPMI)

[00743] Complete medium was prepared by mixing 450ml of RPMI 1640, 50ml of fetal calf serum (FCS), 5ml of 1M HEPES, 5ml of 100X Non-essential amino acids (NEAA), 5ml of 100X L-Glutamine, 5ml of 100mM Na-Pyruvate, 5ml of 10,000U/mL Penicillin/Streptomycin and 129ul of 14.6M 2-Mercaptoethanol.

Preparing isolated splenocytes

[00744] Work was performed in biohazard hood. Spleens were harvested from experimental and control mice groups using sterile forceps and scissors. They were transport in 15 ml tubes containing 10 ml PBS to the lab. Spleen from each mouse was processed separately. Spleen was taken in a sterile Petri dish and mashed using the back of plunger from a 3 mL syringe. Spleen cells were transferred to a 15 ml tube containing 10 ml of RPMI 1640. Cells were pelleted by centrifugation at 1,000 RPM for 5 min at 4°C. The supernatant was discarded in

10% bleach. Cell pellet was gently broken by tapping. RBC was lysed by adding 2 ml of RBC lysis buffer per spleen to the cell pellet. RBC lysis was allowed for 2 min. Immediately, 10 ml of c-RPMI medium was added to the cell suspension to deactivate RBC lysis buffer. Cells were pelleted by centrifugation at 1,000 RPM for 5 min at 4°C. The supernatant was discarded and cell pellet was re-suspended in 10 ml of c-RPMI and passed through a cell strainer. Cells were counted using hemocytometer and the viability was checked by mixing 10 ul of cell suspension with 90 ul of Trypan blue stain. About 2 X 10^6 cells were used for pentamer staining. (Note: each spleen should yield 1-2 x 10^8 cells).

Preparing single cell suspension from tumors using Miltenyi mouse tumor dissociation kit

[00745] Enzyme mix was prepared by adding 2.35 mL of RPMI 1640, 100 µL of Enzyme D, 50 µL of Enzyme R, and 12.5 µL of Enzyme A into a gentleMACS C Tube. Tumor (0.04–1 g) was cut into small pieces of 2–4 mm and transferred into the gentleMACS C Tube containing the enzyme mix. The tube was attached upside down onto the sleeve of the gentle MACS Dissociator and the Program **m_impTumor_02** was run. After termination of the program, C Tube was detached from the gentle MACS Dissociator. The sample was incubated for 40 minutes at 37°C with continuous rotation using the MACSmix Tube Rotator. After completion of incubation the C tube was again attached upside down onto the sleeve of the gentle MACS Dissociator and the program **m_impTumor_03** was run twice. The cell suspension was filtered through 70 µm filter placed on a 15 mL tube. The filter was also washed with 10 mL of RPMI 1640. The cells were centrifuged at 300×g for 7 minutes. The supernatant was discarded and the cells were re-suspended in 10 ml of RPMI 1640. At this point one can divide the cells for pentamer staining.

Pentamer staining of splenocytes

[00746] The PSA-specific T cells were detected using commercially available PSA-H-$2D^b$ pentamer from ProImmune using manufacturers recommended protocol. Splenocytes were stained for CD8, CD62L, CD3 and Pentamer. While tumor cells were stained for CD8, CD62L, CD45 and Pentamer. The $CD3^+CD8^+$ $CD62L^{low}$ cells were gated to determine the frequency of $CD3^+CD8^+$ $CD62L^{low}$ PSA pentamer$^+$ cells. The stained cells were acquired and analyzed on FACS Calibur using Cell quest software.

Materials needed for Pentamer staining

[00747] Splenocytes (preparation described above), Pro5® Recombinant MHC PSA Pentamer conjugated to PE. (Note: Ensure that the stock Pentamer is stored consistently at

4°C in the dark, with the lid tightly closed), anti-CD3 antibody conjugated to PerCP Cy5.5, anti-CD8 antibody conjugated to FITC and anti-CD62L antibody conjugated to APC, wash buffer (0.1% BSA in PBS) and fix solution (1% heat inactivated fetal calf serum (HI-FCBS), 2.5% formaldehyde in PBS)

5 Standard Staining Protocol

[00748] Pro5® PSA Pentamer was centrifuged in a chilled microcentrifuge at 14,000×*g* for 5-10 minutes to remove any protein aggregates present in the solution. These aggregates may contribute to non-specific staining if included in test volume. 2×10^6 splenocytes were allocated per staining condition and 1 ml of wash buffer was added per tube. Cells were 10 centrifuged at 500 x *g* for 5 min in a chilled centrifuge at 4 °C. The cell pellet was re-suspended in the residual volume (~ 50µl). All tubes were chilled on ice for all subsequent steps, except where otherwise indicated. 10µl of labeled Pentamer was added to the cells and mixed by pipetting. The cells were incubated at room temperature (22 °C) for 10 minutes, shielded from light. Cells were washed with 2 ml of wash buffer per tube and re-suspend in 15 residual liquid (~ 50 µl). An optimal amount of anti-CD3, anti-CD8 and anti-CD62L antibodies were added (1:100 dilution) and mixed by pipetting. Single stain control samples were also made at this point. Samples were incubated on ice for 20 minutes, shielded from light. Cells were washed twice with 2 ml wash buffer per tube. The cell pellet was re-suspended in the residual volume (~ 50 µl). 200 µl of fix solution was added to each tube and 20 vortexed. The tubes were stored in dark in the refrigerator until ready for data acquisition. (Note: the morphology of the cell changes after fixing, so it is advisable to leave the samples for 3 hours before proceeding with data acquisition. Samples can be stored for up to 2 days).

Intracellular Cytokine Staining (IFN-γ) protocol:

[00749] $2x10^7$ cells/ml splenocytes were taken in FACS tubes and 100µl of Brefeldin A (BD 25 Golgi Plug) was added to the tube. For stimulation, 2µM Peptide was added to the tube and the cells were incubated at room temperature for 10-15 minutes. For positive control samples, PMA (10ng/ml) (2x) and ionomycin (1µg/ml) (2x) was added to corresponding tubes. 100µl of medium from each treatment was added to the corresponding wells in a U-bottom 96-well plate. 100µl of cells were added to the corresponding wells (200µl final volume – medium + 30 cells). The plate was centrifuged at 600rpm for 2 minutes and incubated at 37°C 5%CO_2 for 5 hours. Contents from the plate was transferred to FACS tubes. 1ml of FACS buffer was added to each tube and centrifuged at 1200 rpm for 5 min. The supernatant was discarded. 200µl of

2.4G2 supernatant and 10μl of rabbit serum was added to the cells and incubated for 10 minutes at room temperature. The cells were washed with 1 mL of FACS buffer. The cells were collected by centrifugation at 1200rpm for 5 minutes. Cells were suspended in 50μl of FACS buffer containing the fluorochrome-conjugated monoclonal antibodies (CD8 FITC, CD3 PerCP-Cy5.5, CD62L APC) and incubated at 4°C for 30 minutes in the dark. Cells were washed twice with 1 mL FACS buffer and re-suspended in 200μl of 4% formalin solution and incubated at 4°C for 20 min. The cells were washed twice with 1 mL FACS buffer and re-suspended in BD Perm/Wash (0.25ml/tube) for 15 minutes. Cells were collected by centrifugation and re-suspended in 50μl of BD Perm/Wash solution containing the fluorochrome-conjugated monoclonal antibody for the cytokine of interest (IFNg- PE). The cells were incubated at 4°C for 30 minutes in the dark. Cells were washed twice using BD Perm/Wash (1ml per tube) and re-suspended in 200 μl FACS buffer prior to analysis.

RESULTS

EXAMPLE 22: VACCINATION WITH RECOMBINANT *LISTERIA* CONSTRUCTS LEADS TO TUMOR REGRESSION

[00750] The data showed that by week 1, all groups had developed tumor with the average size of 2-3mm. On week 3 (Day 20) mice immunized with ActA/PEST2 (also known as "LA229")-PSA, ActA/PEST3-PSA and ActA/PEST3-PSA and *Lm*ddA-142 (ADXS31-142), which expresses a tLLO fused to PSA showed, tumor regression and slow down of the tumor growth. By week 6, all mice in naïve and most in ActAPEST4-PSA treated group had big tumors and had to be euthanized (**Fig. 28A**). However, LmddA-142, ActA-PEST2 and ActA-PEST3 mice groups showed better tumor regression and survival rate (**Figs. 28A and 28B**).

EXAMPLE 23: VACCINATION WITH RECOMBINANT *LISTERIA* GENERATES HIGH LEVELS OF ANTIGEN-SPECIFIC T CELLS

[00751] *Lm*ddA-ActAPEST2-PSA immunotherapy generated high levels of PSA-specific T cells response compared to LmddA-ActAPEST (3 or 4) - PSA, or *Lm*ddA-142 (**Fig. 29A**). The magnitude of PSA tetramer specific T cells in PSA-specific immunotherapies was 30 fold higher than naïve mice. Similarly, higher levels of IFN-γ secretion was observed for *Lm*ddA-ActAPEST2-PSA immunotherapy in response to stimulation with PSA-specific antigen (**Fig. 29B**).

EXAMPLE 24: VACCINATION WITH ACTA/PEST2 (LA229) GENERATES A HIGH NUMBER OF ANTIGEN-SPECIFIC CD8+ T CELLS IN SPLEEN

[00752] *Lm* expressing ActA/PEST2 fused PSA was able to generate higher numbers of PSA specific CD8+ T cells in spleen compared to *Lm* expressing tLLO fused PSA or tLLO treated group. The number of PSA specific CD8+ T cells infiltrating tumors were similar for both *Lm*-tLLO-PSA and *Lm*-ActA/PEST2-PSA immunized mice (**Figs. 30B and 30C**). Also, tumor regression ability of *Lm* expressing ActA/PEST2-PSA was similar to that seen for *Lm*ddA-142 which expresses tLLO-PSA (**Fig. 30A**).

EXAMPLE 25: SITE-DIRECTED MUTAGENESIS OF THE LLO CHOLESTEROL-BINDING DOMAIN

[00753] Site-directed mutagenesis was performed on LLO to introduce inactivating point mutations in the CBD, using the following strategy. The resulting protein is termed "mutLLO":

Subcloning of LLO into pET29b

[00754] The amino acid sequence of wild-type LLO is:

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKKHADEIDKYIQGLDYN
KNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQVVNAISSLTYPGALVKANSELV
ENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNATKSNVNNAVNTLVERWNEKYAQAYSNVSAKID
YDDEMAYSESQLIAKFGTAFKAVNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAV
TKEQLQALGVNAENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF
KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIKNNSEYIETTSKA
YTDGKINIDHSGGYVAQFNISWDEVNYDPEGNEIVQHKNWSENNKSKLAHFTSSIYLPGNARNINVYA
KE***C***TGLAWE***WW***RTVIDDRNLPLVKNRNISIWGTTLYPKYSNKVDNPIE (SEQ ID NO: 2). The signal peptide and the cholesterol-binding domain (CBD) are underlined, with 3 critical residues in the CBD (C484, W491, and W492) in bold-italics.

[00755] A 6xHis tag (HHHHHH) was added to the C-terminal region of LLO. The amino acid sequence of His-tagged LLO is:

MKKIMLVFITLILVSLPIAQQTEAKDASAFNKENSISSVAPPASPPASPKTPIEKKHADE
IDKYIQGLDYNKNNVLVYHGDAVTNVPPRKGYKDGNEYIVVEKKKKSINQNNADIQ
VVNAISSLTYPGALVKANSELVENQPDVLPVKRDSLTLSIDLPGMTNQDNKIVVKNA
TKSNVNNAVNTLVERWNEKYAQAYSNVSAKIDYDDEMAYSESQLIAKFGTAFKAV
NNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTRPSRFFGKAVTKEQLQALGVN
AENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSGKSVSGDVELTNIIKNSSF
KAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPIAYTTNFLKDNELAVIK

NNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEVNYDPEGNEIVQHKNWSENNK SKLAHFTSSIYLPGNARNINVYAKE*C*TGLAWE***WW***RTVIDDRNLPLVKNRNISIWGTT LYPKYSNKVDNPIEHHHHHH (SEQ ID NO: 62).

[00756] A gene encoding a His-tagged LLO protein was digested with NdeI/BamHI, and the NdeI/BamHI was subcloned into the expression vector pET29b, between the NdeI and BamHI sites. The sequence of the gene encoding the LLO protein is:

catatgaaggatgcatctgcattcaataaagaaaattcaatttcatccgtggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgc
ggatgaaatcgataagtatatacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaaggttacaaag
atggaaatgaatatattgttgtggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcgagcctaacctatccaggtgctct
cgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaacactcagcattgatttgccaggtatgactaatcaagacaata
aaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagtaaatacattagtggaaagatggaatgaaaaatatgctcaagcttattcaaatgtaagtgcaaa
aattgattatgatgacgaaatggcttacagtgaatcacaattaattgcgaaatttggtacagcatttaaagctgtaaataatagcttgaatgtaaacttcggcgcaatcag
tgaagggaaaatgcaagaagaagtcattagttttaaacaaatttactataacgtgaatgttaatgaacctacaagaccttccagatttttcggcaaagctgttactaaag
agcagttgcaagcgcttggagtgaatgcagaaaatcctcctgcatatatctcaagtgtggcgtatggccgtcaagtttatttgaaattatcaactaattcccatagtacta
aagtaaaagctgcttttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaacaaatatcatcaaaaattcttccttcaaagccgtaatttacggaggt
tccgcaaaagatgaagttcaaatcatcgacggcaacctcggagacttacgcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgct
tatacaacaaacttcctaaaagacaatgaattagctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatggaaaaattaacatcgatca
ctctggaggatacgttgctcaattcaacatttcttgggatgaagtaaattatgatcctgaaggtaacgaaattgttcaacataaaaactggagcgaaaacaataaaagc
aagctagctcatttcacatcgtccatctatttgcctggtaacgcgagaaatattaatgtttacgctaaagaa***tgc***actggtttagcttgggaa***tggtgg***agaacggtaatt
gatgaccggaacttaccacttgtgaaaaatagaaatatctccatctggggcaccacgctttatccgaaatatagtaataaagtagataatccaatcgaacaccaccac
caccaccactaataaggatcc (SEQ ID NO: 63). The underlined sequences are, starting from the beginning of the sequence, the NdeI site, the NheI site, the CBG-encoding region, the 6x His tag, and the BamHI site. The CBD resides to be mutated in the next step are in bold-italics.

Splicing by Overlap Extension (SOE) PCR

[00757] Step 1: PCR reactions #1 and #2 were performed on the pET29b-LLO template. PCR reaction #1, utilizing primers #1 and #2, amplified the fragment between the NheI site and the CBD, inclusive, introducing a mutation into the CBD. PCR reaction #2, utilizing primers #3 and #4, amplified the fragment between the CBD and the BamHI site, inclusive, introducing the same mutation into the CBD (**Fig. 31A**).

[00758] PCR reaction #1 cycle: A) 94°C 2min30sec, B) 94°C 30sec, C) 55°C 30sec, D) 72°C 1min, Repeat steps B to D 29 times (30 cycles total), E) 72°C 10min.

[00759] PCR reaction #2 cycle: A) 94°C 2min30sec, B) 94°C 30sec, C) 60°C 30sec, D) 72°C 1min, Repeat steps B to D 29 times (30 cycles total), E) 72°C 10min.

[00760] Step 2: The products of PCR reactions #1 and #2 were mixed, allowed to anneal (at

the mutated CBD-encoding region), and PCR was performed with primers #1 and #4 for 25 more cycles (**Fig. 31B**). PCR reaction cycle: A) 94°C 2min30sec, B) 94°C 30sec, C) 72°C 1min, Repeat steps B to C 9 times (10 cycles total), Add primers #1 and #4, D) 94°C 30sec, E) 55°C 30sec, F) 72°C 1min, Repeat steps D to F 24 times (25 cycles total), G) 72°C 10min.

5 **Primer Sequences:**

[00761] Primer 1: GCTAGCTCATTTCACATCGT (SEQ ID NO: 64; NheI sequence is underlined).

[00762] Primer 2:
TCT***TGCAGC***TTCCCAAGCTAAACCAGT***CGC***TTCTTTAGCGTAAACATTAATATT
10 (SEQ ID NO: 65; CBD-encoding sequence is underlined; mutated codons are in bold-italics).

[00763] Primer 3:
GAA***GCG***ACTGGTTTAGCTTGGGAA***GCTGCA***AGAACGGTAATTGATGACCGGAAC (SEQ ID NO: 66; CBD-encoding sequence is underlined; mutated codons are in bold-italics).

[00764] Primer 4: GGATCCTTATTAGTGGTGGTGGTGGTGGTGTTCGATTGG (SEQ
15 ID NO: 67; BamHI sequence is underlined).

[00765] The wild-type CBD sequence is ECTGLAWEWWR (SEQ ID NO: 68).

[00766] The mutated CBD sequence is EATGLAWEAAR (SEQ ID NO: 69).

[00767] The sequence of the mutated NheI-BamHI fragment is

GCTAGCTCATTTCACATCGTCCATCTATTTGCCTGGTAACGCGAGAAATATTAATGTTTACGCTAAA
20 GAA*GCG*ACTGGTTTAGCTTGGGAA*GCTGCA*AGAACGGTAATTGATGACCGGAACTTACCACTTGT
GAAAAATAGAAATATCTCCATCTGGGGCACCACGCTTTATCCGAAATATAGTAATAAAGTAGATA
ATCCAATCGAACACCACCACCACCACCACTAATAAGGATCC (SEQ ID NO: 70).

EXAMPLE 26: REPLACEMENT OF PART OF THE LLO CBD WITH A CTL EPITOPE

25 [00768] Site-directed mutagenesis was performed on LLO to replace 9 amino acids (AA) of the CBD with a CTL epitope from the antigen NY-ESO-1. The sequence of the CBD (SEQ ID NO: 68) was replaced with the sequence ESLLMWITQCR (SEQ ID NO: 71; mutated residues underlined), which contains the HLA-A2 restricted epitope 157-165 from NY-ESO-1, termed "ctLLO."

30 [00769] The subcloning strategy used was similar to the previous Example.

[00770] The primers used were as follows:

[00771] Primer 1: GCTAGCTCATTTCACATCGT (SEQ ID NO: 64; NheI sequence is underlined).

[00772] Primer 2:
TCT***GCACTGGGTGATCCACATCAGCAGGCT***TTCTTTAGCGTAAACATTAATATT (SEQ ID NO: 72; CBD-encoding sequence is underlined; mutated (NY-ESO-1) codons are in bold-italics).

[00773] Primer 3:
GAA***AGCCTGCTGATGTGGATCACCCAGTGC***AGAACGGTAATTGATGACCGGAAC (SEQ ID NO: 73; CBD-encoding sequence is underlined; mutated (NY-ESO-1) codons are in bold-italics).

[00774] Primer 4: GGATCCTTATTAGTGGTGGTGGTGGTGGTGTTCGATTGG (SEQ ID NO: 67; BamHI sequence is underlined).

[00775] The sequence of the resulting NheI/BamHI fragment is as follows:
GCTAGCTCATTTCACATCGTCCATCTATTTGCCTGGTAACGCGAGAAATATTAAT GTTTACGCTAAAGAA***AGCCTGCTGATGTGGATCACCCAGTGC***AGAACGGTAATTG ATGACCGGAACTTACCACTTGTGAAAAATAGAAATATCTCCATCTGGGGCACCAC GCTTTATCCGAAATATAGTAATAAAGTAGATAATCCAATCGAACACCACCACCAC CACCACTAATAAGGATCC (SEQ ID NO: 74).

EXAMPLE 27: mutLLO AND ctLLO ARE ABLE TO BE EXPRESSED AND PURIFIED IN *E. coli* EXPRESSION SYSTEMS

[00776] To show that mutLLO and ctLLO could be expressed in *E. coli*, *E. coli* were transformed with pET29b and induced with 0.5 mM IPTG, then cell lysates were harvested 4 hours later and the total proteins were separated in a SDS-PAGE gel and subject to Coomassie staining (**Fig. 32A**) and anti-LLO Western blot, using monoclonal antibody B3-19 (**Fig. 32B**). Thus, LLO proteins containing point mutations or substitutions in the CBD can be expressed and purified in *E. coli* expression systems.

EXAMPLE 28: mutLLO AND ctLLO EXHIBIT SIGNIFICANT REDUCTION IN HEMOLYTIC ACTIVITY

MATERIALS AND EXPERIMENTAL METHODS

Hemolysis assay

[00777] 1. Wild-type and mutated LLO were diluted to the dilutions indicated in **Figs. 33A-B** in 900μl of 1x PBS-cysteine (PBS adjusted to pH 5.5 with 0.5 M Cysteine hydrochloride or was adjusted to 7.4). 2. LLO was activated by incubating at 37°C for 30 minutes. 3. Sheep red blood cells (200 μl/sample) were washed twice in PBS-cysteine and 3 to 5 times in 1x PBS until the supernatant was relatively clear. 4. The final pellet of sheep red blood cells was resuspended in PBS-cysteine and 100 μl of the cell suspension was added to the 900 μl of the LLO solution (10% final solution). 5. 50 μl of sheep red blood cells was added to 950 μl of water + 10% Tween 20 (Positive control for lysis, will contain 50% the amount of lysed cells as the total amount of cells add to the other tubes; "50% control.") 6. All tubes were mixed gently and incubated at 37°C for 45 minutes. 7. Red blood cells were centrifuged in a microcentrifuge for 10 minutes at 1500 rpm. 8. A 200 μl aliquot of the supernatant was transferred to 96-well ELISA plate and read at 570 nm to measure the concentration of released hemoglobin after hemolysis, and samples were titered according to the 50% control.

RESULTS

[00778] The hemolytic activity of mutLLO and ctLLO was determined using a sheep red blood cell assay. mutLLO exhibited significantly reduced (between 100-fold and 1000-fold) hemolytic titer at pH 5.5, and undetectable hemolytic activity at pH 7.4. ctLLO exhibited undetectable hemolytic activity at either pH (**Figs. 33A-B**).

[00779] Thus, point (mutLLO) or substitution (ctLLO) mutation of LLO CBD residues, including C484, W491, and W492, abolishes or severely reduces hemolytic activity. Further, replacement of the CBD with a heterologous antigenic peptide is an effective means of creating an immunogenic carrier of a heterologous epitope, with significantly reduced hemolytic activity relative to wild-type LLO.

EXAMPLE 29: FULLY ENCLOSED SINGLE USE CELL GROWTH SYSTEM

[00780] The innovative system leverages readily available bioprocessing components and technologies arranged in a unique configuration to grow the engineered *Lm* bacteria, concentrate the fermentation broth, wash and purify the cells, exchange the fermentation

media for formulation buffer, and dispense the patient-specific doses into ready-to-use IV bags using a single fully enclosed system. This type of system provides a complete segregation and control of each patient's immunotherapy. This system is particularly well suited for integration in the overall work stream of identification and clinical use of 5 personalized neo-epitope targeting immunotherapeutics (**Fig. 37 A-B**).

[00781] The custom designed system is assembled using single use bioprocessing bags, patient IV bags, sampling bags, tubing, filters, quick connectors, and sensors. Its small footprint allows manufacture for an individual patient but can be replicated to manufacture product for multiple patients in parallel (**Fig. 38**). The entire assembly is comprised of 4 10 sections: 1) Inoculation and Fermentation, 2) Concentration, 3) Diafiltration, and 4) Drug Product Fill. Since the system has a fully enclosed fluid flow path and is sterilized prior to use, final formulated immunotherapies are dispensed directly into IV bags, frozen and shipped to the healthcare center. Therefore, this eliminates the need for the typical fill/finish and packaging involved when dispensing into vials or pre-filled syringes. This addresses the 15 expectation for rapid turnaround and delivery to the patient.

[00782] The Inoculation and Fermentation section of the assembly (**Fig. 39**) is filled with growth media and warmed to the specified temperature. The cell bank is then inoculated into either a single use rocking style bag fermentor or into a single use agitated bioreactor vessel. Once the bacteria grows to a specific density, the Concentration section of the assembly (**Fig.** 20 **40**) is used to remove the fermentation media and concentrate the batch using a hollow fiber filter. A wash/formulation buffer bag is connected to the Diafiltration section of the assembly (**Fig. 41**) and the bacterial cells are washed/purified, the remaining media is exchanged with formulation buffer via a cross flow filtration in the hollow fiber filter, and the product is diluted to the final concentration. Finally, the batch is aliquoted into sterile single use IV bags 25 and sampling bags for QC testing using the Drug Product Fill section of the assembly (**Fig. 42**). The patient-specific immunotherapy is supplied frozen in a small volume parenteral IV bag containing a pure culture strain of the live attenuated engineered Lm bacteria at a specified concentration. Prior to patient administration, the IV bag is thawed, cells re-suspended, and the required dose withdrawn with a syringe and added to the larger infusion 30 IV bag.

[00783] Several fully enclosed assemblies are used in parallel to manufacture personalized immunotherapeutic compositions either for several patients or for a single patient (**Fig. 43**). In order to increase throughput, additional rockers or agitated vessel bioreactors systems are

added to the processing train, as required (*see e.g.* **Fig. 38**).

[00784] The fully enclosed design of the growth system allow complete quality control of immunotherapeutic compositions while in the process of manufacture, resulting in additional time savings. A full analytical control strategy is implemented in parallel with growing *Listeria* delivery vector (**Table 6**). Thus the dispensed product is ready for immediate delivery to the patient with no additional testing required.

[00785] **Table 6.** Analytical Control Strategy

Parameter	Quality Attribute	Test Method	Test Duration	Comment
Identity	Plasmid ID	PCR	5 days	3 days + 2VCC
Safety	Attenuation	Macrophage or THP1	5 days	3 days + 2VCC
General	Solution Appearance		1 day	
General	pH		1 day	
General	Osmolality		1 day	
Content	Fill Weight	In Process Test	0 day	
Content	Viable Cell Count	Plate	2 days	
Content	Plasmid Copy Number	PCR	5 days	3 days + 2VCC
Potency	In vitro Potency	J774 Infectivity Intracellular Express	5 - 10 days	3-7 days + 2VCC
Purity	Plasmid Stability		5 days	
Purity	Microbial Purity	Plate Method	21 days	Need Rapid ID method
Purity	Percent of Live and Dead Cells		5 days	
Safety	Endotoxin		5 days	

EXAMPLE 30: Construction of a Neo-Epitope Expression vector

[00786] Constructing the Lm vector comprising one or more neo-epitopes is performed using the steps detailed below.

Whole Genome Sequencing

[00787] First, comparative whole genome sequencing including locating non-synonymous mutations present in approximately >20% of tumor cells is performed and the results are provided in FASTA format. Matched normal/tumor samples from whole exomes are sequenced by an outside vendor, and output data is given in the preferred FASTA format listing all neo-antigens as 21 amino acid sequence peptides, for example a peptide having 10 non-mutant amino acids on either side of a mutant amino acid. Also included are patient HLA

types.

[00788] DNA and RNA from a biological sample obtained from human tissue (or any non-human animal) are extracted in triplicates. Another source of neo-antigens could be from sequencing metastases or circulating tumor cells. They may contain additional mutations that are not resident in the initial biopsy but could be included in the vector to specifically target cytotoxic T cells (CTC's) or metastases that have mutated differently than the primary biopsy that was sequenced. Triplicates of each sample are sequenced by DNA exome sequencing. In brief, 3 μg purified genomic DNA (gDNA) are fragmented to about 150-200 bp using an ultrasound device. Fragments are end repaired, 5' phosphorylated, 3' adenylated, and then Illumina paired end adapters are ligated to the gDNA fragments according to the manufacturer's instructions. Enriched pre capture and flow cell specific sequences are added using Illumina PE PCR primers. About 500 ng of adapter ligated, PCR enriched gDNA fragments are hybridized to biotinylated exome (human exome or any other non-human animal exome e.g. mouse, guinea pig, rat, dog, sheep). RNA library baits for 24 hrs at 65 °C. Hybridized gDNA/RNA bait complexes are then removed using streptavidin coated magnetic beads, washed and the RNA baits cleaved off. These eluted gDNA fragments are PCR amplified and then sequenced on an Illumina sequencing apparatus.

RNA gene expression profiling (RNA-Seq)

[00789] Barcoded mRNA-seq cDNA libraries are prepared in triplicates from a total of about 5 μg of total RNA, then, in brief, mRNA are isolated and fragmented. Following, mRNA fragments are converted to cDNA and connected to specific Illumina adaptors, clustered and sequenced according to standard illumine protocol. The output sequence reads are aligned to a referenced sequence (RefSeq). Genome alignments and transcriptome alignments are made. Reads are also aligned to exon-exon junctions. Expression values are determined by intersecting read coordinates with those of RefSeq transcripts, counting overlapping exon and exon junction reads, and normalized to standard normalizing units such as RPKM expression units (Reads which map per Kilobase of transcript per Million mapped reads).

Detecting mutations

[00790] Fragments of isolated gDNA from a disease or condition bearing tissue sample are aligned to referenced matched gDNA of a healthy tissue, by vendor available software, e.g. Samtools, GATK, and Somatic Sniper.

[00791] About 10 flanking amino acids on each side of the detected mutation are

incorporated to accommodate class1 MHC-1 presentation, in order to provide at least some of the different HLA TCR reading frames.

[00792] Table 7 shows a sample list of 50 neo-epitope peptides wherein each mutation is indicated by a Bolded amino acid letter and is flanked by 10 amino acids on each side providing a 21 amino acid peptide neo-epitope.

[00793] **Table 7.**

Name	Sequence1	Sequence ID NO:
MUT1	FMVAVAHVA**A**FLLEDRAVCV	88
MUT2	AENVEQVLVT**S**IQGAVDYPDP	89
MUT3	SFKKKFEECQ**H**NIIKLQNGHT	90
MUT4	SALIESLNQK**T**QSTGDHPQPT	91
MUT5	KAYLPVNESF**A**FTADLRSNTG	92
MUT6	HTLLEITEES**G**AVLVDKSDSD	93
MUT7	SVMCTYSPPL**D**KLFCQLAKTC	94
MUT8	ESGKHKYRQT**A**MFTATMPPAV	95
MUT9	AAPSAASSPA**D**VQSLKKAMSS	96
MUT10	SQLFSLNPRG**R**SLVTAGRIDR	97
MUT11	SLARGPLSEA**G**LALFDPYSKE	98
MUT12	QKKLCHLSST**G**LPRETIASLP	99
MUT13	LTASNMEGKS**W**PSEVLVCTTS	100
MUT14	YAAQQHETFL**T**NGDRAGFLIG	101
MUT15	QAKVPFSEET**Q**NLILPYISDM	102
MUT16	CNRAGEKHCF**S**SNEAARDFGG	103
MUT17	RNPQFLDPVL**A**YLMKGLCEKP	104
MUT18	LECERGKQEA**K**LLAERSRFED	105
MUT19	APLEWLRYFD**K**KELELMLCGM	106
MUT20	KAFLHWYTGE**A**MDEMEFTEAE	107
MUT21	DEVALVEGVQ**S**LGFTYLRLKD	108
MUT22	DFSQLQRNIL**P**SNPRVTRFHI	109
MUT23	ISTNGSFIRL**L**DAFKGVVMHT	110
MUT24	ITPPTTTTKK**A**RVSTPKPATP	111
MUT25	NYNTSHLNND**V**WQIFENPVDW	112
MUT26	QKTLHNLLRK**V**VPSFSAEIER	113
MUT27	VELCPGNKYE**M**RRHGTTHSLV	114
MUT28	GIDKLTQLKK**P**FLVNNKINKI	115
MUT29	GTTILNCFHD**V**LSGKLSGGS	116
MUT30	PSFQEFVDWE**N**VSPELNSTDQ	117
MUT31	PALVEEYLER**G**NFVANDLDWL	118
MUT32	ELKACKPNGK**R**NPYCEVSMGS	119
MUT33	SPFPAAVILR**D**ALHMARGLKY	120
MUT34	QQLDTYILKN**V**VAFSRTDKYR	121
MUT35	SFVGQTRVLM**I**NGEEVEETEL	122
MUT36	AFFINFIAIY**H**HASRAIPFGT	123

Name	Sequence[1]	Sequence ID NO:
MUT37	GLALPNNYCD**V**CLGDSKINKK	124
MUT38	EGQISIAKYE**N**CPKDNPMYYC	125
MUT39	NFKRKRVAAF**Q**KNLIEMSELE	126
MUT40	KMKGELGMML**I**LQNVIQKTTT	127
MUT41	SIECKGIDKE**I**NESKNTHLDI	128
MUT42	ELEAAIETVV**C**TFFTFAGREG	129
MUT43	SLSHREREQM**K**ATLNYEDHCF	130
MUT44	HIKAFDRTFA**N**NPGPMVVFAT	131
MUT45	ITSNFVIPSE**Y**WVEEKEEKQK	132
MUT46	GLVTFQAFID**V**MSRETTDTDT	133
MUT47	HLLGRLAAIV**G**KQVLLGRKVV	134
MUT48	HWNDLAVIPA**G**VVHNWDFEPR	135
MUT49	SMDHKTGTIA**M**QNTTQLRSRY	136
MUT50	QPLRRLVLHV**V**SAAQAERLAR	137

1 Bolded letter indicates mutated amino acid

[00794] Output FASTA file is used to design patient-specific constructs, either manually or by programmed script according to one or more of criteria detailed below. The programmed script automates the creation of the personalized plasma construct containing one or more neo-epitopes for each subject using a series of protocols (**Fig. 45**). The output FASTA file is inputted and after running the protocols, the DNA sequence of a LM vector including one or more neo-epitopes is outputted. The software program is useful for creating personalized immunotherapy for each subject.

Prioritization of neo-epitopes for incorporation into constructs.

[00795] Neo-epitopes are scored by Kyte and Doolittle hydropathy index 21 amino acid window, all scoring above cutoff (around 1.6) are excluded as they are unlikely to be secretable by *Listeria monocytogenes*. The remaining 21 amino acid long peptides are then scored for their ability to bind patient HLA (for example by using IEDB, Immune epitope database and analysis source,www.iedb.org/) and ranked by best MHC binding score from each 21 amino acid sequence peptide. Cut-offs may be different for different expression vectors such as Salmonella.

[00796] Determination of the number of constructs vs. mutational burden, are performed to determine efficiency of expression and secretion of neo-epitopes. Ranges of linear neo-epitopes are tested, starting with about 50 epitopes per vector. In certain cases constructs will include at least one neo-epitope per vector. The number of vectors to be used is determined considering for example the efficiency of translation and secretion of multiple epitopes from a single vector, and the MOI needed for each Lm vector harboring specific neo-epitopes, or in

reference to the number of neo-epitopes. Another consideration can be by predefining groups of known tumor-associated mutations/mutations found in circulating tumor cells/known cancer "driver" mutations/known chemotherapy resistance mutations and giving them priority in the 21 amino acid sequence peptide selection. This can be accomplished by screening identified mutated genes against the COSMIC (Catalogue of somatic mutations in cancer, cancer.Sanger.ac.uk) or Cancer Genome Analysis or other similar cancer-associated gene database. Further, screening for immunosuppressive epitopes (T-reg epitopes, IL-10 inducing T helper epitopes, etc.) is utilized to de-selected or to avoid immunosuppressive influences on the vector. Selected codons are codon optimized to efficient translation and secretion according to specific *Listeria* strain. Example for codons optimized for *L. monocytogenes* as known in the art is presented in table 8.

[00797] **Table 8. Preliminary *Listeria monocytogenes* preferred (most common) codon table**

A = GCA
C = TGT
D = GAT
E = GAA
F = TTC
G = GGT
H = CAT
I = ATT
K = AAA
L = TTA
M = ATG
N = AAC
P = CCA
Q = CAA
R = CGT
S = TCT
T = ACA
V = GTT
W = TGG
Y = TAT
STOP = TAA

[00798] The remaining 21amino acid peptide neo-epitopes are assembled into a pAdv134-MCS (SEQ ID NO: 138) plasmid, or optionally into pAdv134, exchanging the LLO-E7 cassette as shown in Example 8 above, to create the tLLO-neo-epitope-tag fusion

polypeptide. The compatible insert as an amino acid sequence and the whole insert are rechecked by Kyte and Doolittle test to confirm no hydropathy problems across the whole construct. If needed, the insert order is rearranged or the problem 21 amino acid sequence peptides is removed from construct.

5 [00799] The construct amino acid sequence is reverse translated into the corresponding DNA sequence for DNA synthesis/cloning into pAdv134-MCS SEQ ID NO: 138:

cggagtgtatactggcttactatgttggcactgatgagggtgtcagtgaagtgcttcatgtggcaggagaaaaaaggctgcaccggtgc
gtcagcagaatatgtgatacaggatatattccgcttcctcgctcactgactcgctacgctcggtcgttcgactgcggcgagcggaaatg
gcttacgaacggggcggagatttcctggaagatgccaggaagatacttaacagggaagtgagagggccgcggcaaagccgtttttcc
10 ataggctccgcccccctgacaagcatcacgaaatctgacgctcaaatcagtggtggcgaaacccgacaggactataaagataccagg
cgtttccccctggcggctccctcgtgcgctctcctgttcctgcctttcggtttaccggtgtcattccgctgttatggccgcgtttgtctcattc
cacgcctgacactcagttccgggtaggcagttcgctccaagctggactgtatgcacgaaccccccgttcagtccgaccgctgcgcctt
atccggtaactatcgtcttgagtccaacccggaaagacatgcaaaagcaccactggcagcagccactggtaattgatttagaggagtta
gtcttgaagtcatgcgccggttaaggctaaactgaaaggacaagttttggtgactgcgctcctccaagccagttacctcggttcaaagag
15 ttggtagctcagagaaccttcgaaaaaccgccctgcaaggcggttttttcgttttcagagcaagagattacgcgcagaccaaaacgatct
caagaagatcatcttattaatcagataaaatatttctagccctcctttgattagtatattcctatcttaaagttacttttatgtggaggcattaaca
tttgttaatgacgtcaaaaggatagcaagactagaataaagctataaagcaagcatataatattgcgtttcatctttagaagcgaatttcgc
caatattataattatcaaaagagaggggtggcaaacggtatttggcattattaggttaaaaaatgtagaaggagagtgaaacccatgaaa
aaaataatgctagtttttattacacttatattagttagtctaccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaataaagaa
20 aattcaatttcatccatggcaccaccagcatctccgcctgcaagtcctaagacgccaatcgaaaagaaacacgcggatgaaatcgataa
gtatatacaaggattggattacaataaaaacaatgtattagtataccacggagatgcagtgacaaatgtgccgccaagaaaaggttaca
aagatggaaatgaatatattgttgtggagaaaaagaagaaatccatcaatcaaaataatgcagacattcaagttgtgaatgcaatttcgag
cctaacctatccaggtgctctcgtaaaagcgaattcggaattagtagaaaatcaaccagatgttctccctgtaaaacgtgattcattaaca
ctcagcattgatttgccaggtatgactaatcaagacaataaaatagttgtaaaaaatgccactaaatcaaacgttaacaacgcagtaaata
25 cattagtggaaagatggaatgaaaaatatgctcaagcttatccaaatgtaagtgcaaaaattgattatgatgacgaaatggcttacagtga
atcacaattaattgcgaaatttggtacagcatttaaagctgtaaataatagcttgaatgtaaacttcggcgcaatcagtgaagggaaaatg
caagaagaagtcattagttttaaacaaatttactataacgtgaatgttaatgaacctacaagaccttccagatttttcggcaaagctgttact
aaagagcagttgcaagcgcttggagtgaatgcagaaaatcctcctgcatatatctcaagtgtggcgtatggccgtcaagtttatttgaaat
tatcaactaattcccatagtactaaagtaaaagctgcttttgatgctgccgtaagcggaaaatctgtctcaggtgatgtagaactaacaaat
30 atcatcaaaaattcttccttcaaagccgtaatttacggaggttccgcaaaagatgaagttcaaatcatcgacggcaacctcggagacttac
gcgatattttgaaaaaaggcgctacttttaatcgagaaacaccaggagttcccattgcttatacaacaaacttcctaaaagacaatgaatta
gctgttattaaaaacaactcagaatatattgaaacaacttcaaaagcttatacagatggaaaaattaacatcgatcactctggaggatacgt
tgctcaattcaacatttcttgggatgaagtaaattatgatCTCGAGGAGCTCCTGCAGTCTAGAGTCGACAC

TAGTGGATCCAGATCTCCCGGGccactaactcaacgctagtagtggatttaatcccaaatgagccaacagaacca
gaaccagaaacagaacaagtaacattggagttagaaatggaagaagaaaaaagcaatgatttcgtgtgaataatgcacgaaatcattg
cttatttttttaaaaagcgatatactagatataacgaaacaacgaactgaataaagaatacaaaaaaagagccacgaccagttaaagcct
gagaaactttaactgcgagccttaattgattaccaccaatcaattaaagaagtcgagacccaaaatttggtaaagtatttaattactttattaa
5 tcagatacttaaatatctgtaaacccattatatcgggtttttgaggggatttcaagtctttaagaagataccaggcaatcaattaagaaaaac
ttagttgattgcctttttgttgtgattcaactttgatcgtagcttctaactaattaattttcgtaagaaaggagaacagctgaatgaatatccctt
ttgttgtagaaactgtgcttcatgacggcttgttaaagtacaaatttaaaaatagtaaaattcgctcaatcactaccaagccaggtaaaagt
aaaggggctatttttgcgtatcgctcaaaaaaaagcatgattggcggacgtggcgttgttctgacttccgaagaagcgattcacgaaaat
caagatacatttacgcattggacaccaaacgtttatcgttatggtacgtatgcagacgaaaaccgttcatacactaaaggacattctgaaa
10 acaatttaagacaaatcaataccttctttattgattttgatattcacacggaaaaagaaactatttcagcaagcgatattttaacaacagctatt
gatttaggttttatgcctacgttaattatcaaatctgataaaggttatcaagcatattttgttttagaaacgccagtctatgtgacttcaaaatca
gaatttaaatctgtcaaagcagccaaaataatctcgcaaaatatccgagaatattttggaaagtctttgccagttgatctaacgtgcaatcat
tttgggattgctcgtataccaagaacggacaatgtagaatttttgatcccaattaccgttattctttcaaagaatggcaagattggtctttca
aacaaacagataataagggctttactcgttcaagtctaacggttttaagcggtacagaaggcaaaaaacaagtagatgaaccctggttta
15 atctcttattgcacgaaacgaaattttcaggagaaaagggtttagtagggcgcaatagcgttatgtttaccctctctttagcctactttagttc
aggctattcaatcgaaacgtgcgaatataatatgtttgagtttaataatcgattagatcaacccttagaagaaaaagaagtaatcaaaattg
ttagaagtgcctattcagaaaactatcaaggggctaatagggaatacattaccattctttgcaaagcttgggtatcaagtgatttaaccagt
aaagatttatttgtccgtcaagggtggtttaaattcaagaaaaaaagaagcgaacgtcaacgtgttcatttgtcagaatggaaagaagatt
taatggcttatattagcgaaaaaagcgatgtatacaagccttatttagcgacgaccaaaaaagagattagagaagtgctaggcattcctg
20 aacggacattagataaattgctgaaggtactgaaggcgaatcaggaaattttctttaagattaaaccaggaagaaatggtggcattcaac
ttgctagtgttaaatcattgttgctatcgatcattaaattaaaaaaagaagaacgagaaagctatataaaggcgctgacagcttcgtttaatt
tagaacgtacatttattcaagaaactctaaacaaattggcagaacgccccaaaacggacccacaactcgatttgtttagctacgatacag
gctgaaaataaaacccgcactatgccattacatttatatctatgatacgtgtttgtttttctttgctggctagcttaattgcttatatttacctgca
ataaaggatttcttacttccattatactcccattttccaaaaacatacggggaacacgggaacttattgtacaggccacctcatagttaatgg
25 tttcgagccttcctgcaatctcatccatggaaatatattcatccccctgccggcctattaatgtgacttttgtgcccggcggatattcctgatc
cagctccaccataaattggtccatgcaaattcggccggcaattttcaggcgttttcccttcacaaggatgtcggtccctttcaattttcgga
gccagccgtccgcatagcctacaggcaccgtcccgatccatgtgtctttttccgctgtgtactcggctccgtagctgacgctctcgccttt
tctgatcagtttgacatgtgacagtgtcgaatgcagggtaaatgccggacgcagctgaaacggtatctcgtccgacatgtcagcagacg
ggcgaaggccatacatgccgatgccgaatctgactgcattaaaaaagcctttttcagccggagtccagcggcgctgttcgcgcagtg
30 gaccattagattctttaacggcagcggagcaatcagctctttaaagcgctcaaactgcattaagaaatagcctctttctttttcatccgctgt
cgcaaaatgggtaaataccccttgcactttaaacgagggttgcggtcaagaattgccatcacgttctgaacttcttcctctgtttttacacc
aagtctgttcatccccgtatcgaccttcagatgaaaatgaagagaacctttttcgtgtggcgggctgcctcctgaagccattcaacagaa
taacctgttaaggtcacgtcatactcagcagcgattgccacatactccgggggaaccgcgccaagcaccaatataggcgccttcaatc
ccttttgcgcagtgaaatcgcttcatccaaaatggccacggccaagcatgaagcacctgcgtcaagagcagcctttgctgtttctgcat

caccatgcccgtaggcgtttgctttcacaactgccatcaagtggacatgttcaccgatatgtttttcatattgctgacattttcctttatcacg gacaagtcaatttccgcccacgtatctctgtaaaaaggttttgtgctcatggaaaactcctctctttttcagaaaatcccagtacgtaattaa gtatttgagaattaattttatattgattaatactaagtttacccagttttcacctaaaaaacaaatgatgagataatagctccaaaggctaaag aggactataccaactatttgttaattaa; Capital letters refer to multi-cloning site by outside vendor.

5 Individual 21 amino acid peptides sequences and the SIINFEKL-6xHis tag DNA sequences (for example SEQ ID NO: 87) are optimized for expression and secretion in *L. monocytogenes* while the 4x glycine linker sequences are one of eleven preset DNA sequences (G1-G11, SEQ ID NO: 76-86). Linker sequence codons are varied to avoid excess repetition to better enable DNA synthesis. Examples of the different sequence codons (G1-

10 G11, SEQ ID NO: 76-86) for 4Xglycine linkers are presented in Table 9.

[00800] **Table 9**. 4x glycine linker DNA sequences and terminal tag sequence

Name	Sequence	Sequence ID NO:
G1	GGTGGTGGAGGA	76
G2	GGTGGAGGTGGA	77
G3	GGTGGAGGAGGT	78
G4	GGAGGTGGTGGA	79
G5	GGAGGAGGTGGT	80
G6	GGAGGTGGAGGT	81
G7	GGAGGAGGAGGT	82
G8	GGAGGAGGTGGA	83
G9	GGAGGTGGAGGA	84
G10	GGTGGAGGAGGA	85
G11	GGAGGAGGAGGA	86
C-terminal SIINFEKL and 6xHis AA sequence	ARSIINFEKLSHHHHHH	87

[00801] Each neo-epitope is connected with a linker sequence to the following neo-epitope encoded on the same vector. The final neo-epitope in an insert is fused to a TAG sequence

15 followed by a stop codon. The TAG fused is set forth in SEQ ID NO: 87, a C-terminal SIINFEKL and 6xHis amino acid sequence. The TAG allows for easy detection of the tLLO-neo-epitope during for example secretion from the Lm vector or when testing construct for affinity to specific T-cells, or presentation by antigen presenting cells. The linker is 4Xglycine DNA sequence, selected from a group comprising G1-G11 (SEQ ID NO: 76-86)

20 accordingly, or any combination thereof.

[00802] If there are more usable 21 amino acid peptides than can fit into a single plasmid (maximum payload currently being tested), the different 21 amino acid peptides are designated into 1st, 2nd, etc. construct by priority rank as needed/desired. The priority of

assignment to one of multiple vectors composing the entire set of desired neo-epitopes is determined based on factors like relative size, priority of transcription, and overall hydrophobicity of the translated polypeptide.

[00803] In one embodiment, the construct structure disclosed herein comprises a nucleic acid sequence encoding a N terminal truncated LLO fused to one or more 21 mer neo-epitope(s) amino acid sequence flanked by a linker sequence and followed by at least one second neo epitope flanked by another linker and terminated by a SIINFEKL-6xHis tag-and 2 stop codons closing the open reading frame: p*Hly*-tLLO-21mer #1-4x glycine linker G1-21mer #2-4x glycine linker G2-…-SIINFEKL-6xHis tag-2x stop codon. In another embodiment, the above construct's expression is driven by an *hly* gene promoter sequence or other suitable promoter sequence known in the art and further disclosed herein. It will be appreciated by a skilled artisan that each 21 mer neo-epitope sequence may also be fused to an immunogenic polypeptide such as a tLLO, truncated ActA or PEST amino acid sequence disclosed herein.

[00804] Different linker sequences are distributed between the neo-epitopes for minimizing repeats. This reduces possible secondary structures thereby allowing efficient transcription, translation, secretion, maintenance, or stabilization of the plasmid including the insert within the Lm recombinant vector strain population.

[00805] DNA synthesis is achieved by ordering nucleotide sequence from a vendor comprising the construct including the open reading frame comprising tLLO or tActA or ActA or PEST amino acid sequence fused to at least one neo-epitope. Additionally or alternatively multiple neo-epitopes are separated by one or more linker 4xglycine sequences. Additionally or alternatively inserts are constructed to comprise the desired sequence by molecular biology technics for example: by sewing PCR with specific over lapping primers and specific primers, or ligating different nucleotide sequences by an appropriate enzyme (e.g., Ligase), optionally following dissection by restriction enzymes, and any combination thereof.

[00806] In an embodiment different linker sequences are distributed between the neo-epitopes for minimizing repeats. This reduces possible secondary structures thereby allowing efficient transcription, translation, secretion, maintenance, or stabilization of the plasmid including the insert within the Lm recombinant vector strain population.

[00807] Selected DNA inserts are synthesized by techniques standard in the art (e.g., PCR, DNA replication – bio-replication, oligonucleotide chemical synthesis) and cloned to a

plasmid, for example as presented in Example 8. The plasmid is then transfected or conjugated into Lm vector. Additionally or alternatively, the insert is integrated into a phage vector and inserted into Lm vector by phage infection. Confirmation of the construct is performed utilizing techniques known in the art, for example bacterial colony PCR with insert specific primers, or purifying said plasmid and sequencing at least a portion comprising the insert.

EXAMPLE 31: Expression of Neo-Epitopes from Neo-Epitope Expression Vector

[00808] As cancer is driven by mutations, the capability of providing a comprehensive map of somatic mutations in individual tumors provides a powerful tool to better understand and intervene against cancer. Human cancers carry 10s to 100s of non-synonymous mutations. *See, e.g.,* Castle *et al.* (2012) *Cancer Res.* 72(5):1081-1091, herein incorporated by reference in its entirety for all purposes. However, shared mutations among patients are rare, and the great majority of mutations are patient-specific, which has hindered exploitation of the mutanome for the development of broadly applicable drugs.

[00809] In this example, neo-epitope expression vectors were constructed as in Example 30 based on approximately 200 non-synonymous mutations identified in non-small-cell lung cancer tissue that are not present in healthy lung tissue. Tissues came from UMassMed cancer center of excellence tissue bank (http://www.umassmed.edu/ccoe/core-services/tissue-and-tumor-bank/banked-tumor-by-organ-of-origin). Others typically screen based on predictive algorithms for immunogenicity of the epitopes. These algorithms are at best 20% accurate in predicting which peptide will generate a T cell response. This is done because they cannot include all 200 mutations. Here, all mutations could be included, so no screening/predictive algorithms were used. Screening was performed for hydrophobicity, to determine what is likely to be secretable by the Lm strain (i.e. not too hydrophobic). The non-synonymous mutations (neo-epitopes) are provided in the table below.

<table>
<tr><th></th><th>Name</th><th>Hydropathy Score</th><th>SEQ ID NO for Amino Acid Sequence</th><th>SEQ ID NO for Nucleotide Sequence</th></tr>
<tr><td>1</td><td>>RERE|p.R523P|nonsynonymous SNV [mutant]</td><td>-0.852</td><td>140</td><td>141</td></tr>
<tr><td>2</td><td>>CA6|p.M68V|nonsynonymous SNV [mutant]</td><td>-0.814</td><td>142</td><td>143</td></tr>
<tr><td>3</td><td>>AKR7A2|p.R19S|nonsynonymous SNV [mutant]</td><td>0.305</td><td>144</td><td>145</td></tr>
<tr><td>4</td><td>>KIAA0319L|p.E927Q|nonsynonymous SNV [mutant]</td><td>0.314</td><td>146</td><td>147</td></tr>
<tr><td>5</td><td>>EFCAB14|p.S166F|nonsynonymous SNV [mutant]</td><td>-0.062</td><td>148</td><td>149</td></tr>
<tr><td>6</td><td>>FOXD2|p.S180C|nonsynonymous SNV [mutant]</td><td>-0.31</td><td>150</td><td>151</td></tr>
<tr><td>7</td><td>>COL11A1|p.L377M|nonsynonymous SNV [mutant]</td><td>-0.295</td><td>152</td><td>153</td></tr>
</table>

	Name	Hydropathy Score	SEQ ID NO for Amino Acid Sequence	SEQ ID NO for Nucleotide Sequence
8	>PHGDH|p.T213A|nonsynonymous SNV [mutant]	0.448	154	155
9	>HIST2H2BE|p.N85S|nonsynonymous SNV [mutant]	-0.748	156	157
10	>LYSMD1|p.T97P|nonsynonymous SNV [mutant]	-0.519	158	159
11	>NES|p.R113Q|nonsynonymous SNV [mutant]	-1.414	160	161
12	>SLAMF1|p.I306T|nonsynonymous SNV [mutant]	-0.295	162	163
13	>ILDR2|p.S270C|nonsynonymous SNV [mutant]	-0.148	164	165
14	>TIPRL|p.S177C|nonsynonymous SNV [mutant]	1.457	166	167
15	>PRRC2C|p.I444M|nonsynonymous SNV [mutant]	-2.424	168	169
16	>SEC16B|p.R975S|nonsynonymous SNV [mutant]	-0.657	170	171
17	>SMG7|p.E479K|nonsynonymous SNV [mutant]	-0.076	172	173
18	>PRG4|p.P99Q|nonsynonymous SNV [mutant]	-2.114	174	175
19	>PTPRC|p.Y1120S|nonsynonymous SNV [mutant]	-1.567	176	177
20	>PLXNA2|p.R1042W|nonsynonymous SNV [mutant]	-0.729	178	179
21	>VASH2|p.T120I|nonsynonymous SNV [mutant]	0.095	180	181
22	>USH2A|p.S1231I|nonsynonymous SNV [mutant]	0.714	182	183
23	>DISC1|p.E726Q|nonsynonymous SNV [mutant]	-0.881	184	185
24	>KIF26B|p.H264D|nonsynonymous SNV [mutant]	-0.724	186	187
25	>ARMC4|p.Q321K|nonsynonymous SNV [mutant]	-1.324	188	189
26	>FRMPD2|p.Q91K|nonsynonymous SNV [mutant]	-0.986	190	191
27	>TET1|p.E995K|nonsynonymous SNV [mutant]	-0.829	192	193
28	>AIFM2|p.E219Q|nonsynonymous SNV [mutant]	-0.848	194	195
29	>PLCE1|p.S61L|nonsynonymous SNV [mutant]	1.443	196	197
30	>GPAM|p.G444V|nonsynonymous SNV [mutant]	-1.562	198	199
31	>TECTB|p.A132S|nonsynonymous SNV [mutant]	-0.4	200	201
32	>HABP2|p.W356R|nonsynonymous SNV [mutant]	0.871	202	203
33	>ADRB1|p.E250K|nonsynonymous SNV [mutant]	-0.248	204	205
34	>DOCK1|p.W103C|nonsynonymous SNV [mutant]	-1.024	206	207
35	>KRTAP5-3|p.S166C|nonsynonymous SNV [mutant]	0.143	208	209
36	>OR51S1|p.A58V|nonsynonymous SNV [mutant]	0.624	210	211
37	>DCDC5|p.R345K|nonsynonymous SNV [mutant]	0.905	212	213
38	>OR5M8|p.E82Q|nonsynonymous SNV [mutant]	-0.01	214	215
39	>OR1S1|p.G154V|nonsynonymous SNV [mutant]	1.243	216	217
40	>MS4A6A|p.S22C|nonsynonymous SNV [mutant]	-0.305	218	219
41	>MS4A4A|p.W32L|nonsynonymous SNV [mutant]	-0.01	220	221
42	>MAP3K11|p.S300C|nonsynonymous SNV [mutant]	0.362	222	223
43	>PCNXL3|p.V1269L|nonsynonymous SNV [mutant]	0.871	224	225
44	>RCE1|p.E22Q|nonsynonymous SNV [mutant]	-0.343	226	227
45	>CARNS1|p.L351V|nonsynonymous SNV [mutant]	1.014	228	229
46	>PDE2A|p.D232H|nonsynonymous SNV [mutant]	0.357	230	231
47	>MYO7A|p.D2029Y|nonsynonymous SNV [mutant]	-0.481	232	233
48	>FAT3|p.I3772M|nonsynonymous SNV [mutant]	-0.186	234	235
49	>FUT4|p.E298Q|nonsynonymous SNV [mutant]	-0.981	236	237
50	>B3GAT1|p.D122N|nonsynonymous SNV [mutant]	-0.319	238	239
51	>CACNA1C|p.P1820T|nonsynonymous SNV [mutant]	-1.352	240	241
52	>SLC2A3|p.G109E|nonsynonymous SNV [mutant]	1.052	242	243
53	>SLCO1C1|p.G396A|nonsynonymous SNV [mutant]	0.957	244	245
54	>NELL2|p.S596I|nonsynonymous SNV [mutant]	-0.7	246	247

<table>
<tr><th></th><th>Name</th><th>Hydropathy Score</th><th>SEQ ID NO for Amino Acid Sequence</th><th>SEQ ID NO for Nucleotide Sequence</th></tr>
<tr><td>55</td><td>>KMT2D|p.E2866K|nonsynonymous SNV [mutant]</td><td>-0.424</td><td>248</td><td>249</td></tr>
<tr><td>56</td><td>>PAN2|p.E630Q|nonsynonymous SNV [mutant]</td><td>-0.49</td><td>250</td><td>251</td></tr>
<tr><td>57</td><td>>LRIG3|p.I341V|nonsynonymous SNV [mutant]</td><td>0.033</td><td>252</td><td>253</td></tr>
<tr><td>58</td><td>>ZDHHC17|p.D154N|nonsynonymous SNV [mutant]</td><td>0.181</td><td>254</td><td>255</td></tr>
<tr><td>59</td><td>>OTOGL|p.L43V|nonsynonymous SNV [mutant]</td><td>-0.79</td><td>256</td><td>257</td></tr>
<tr><td>60</td><td>>PPFIA2|p.S16R|nonsynonymous SNV [mutant]</td><td>-1.443</td><td>258</td><td>259</td></tr>
<tr><td>61</td><td>>ALDH1L2|p.K754N|nonsynonymous SNV [mutant]</td><td>-0.367</td><td>260</td><td>261</td></tr>
<tr><td>62</td><td>>ATP8A2|p.E680Q|nonsynonymous SNV [mutant]</td><td>0.348</td><td>262</td><td>263</td></tr>
<tr><td>63</td><td>>MTUS2|p.W145R|nonsynonymous SNV [mutant]</td><td>-0.681</td><td>264</td><td>265</td></tr>
<tr><td>64</td><td>>MTUS2|p.T550K|nonsynonymous SNV [mutant]</td><td>-0.552</td><td>266</td><td>267</td></tr>
<tr><td>65</td><td>>BRCA2|p.K2750N|nonsynonymous SNV [mutant]</td><td>0.31</td><td>268</td><td>269</td></tr>
<tr><td>66</td><td>>NBEA|p.E2100Q|nonsynonymous SNV [mutant]</td><td>-0.19</td><td>270</td><td>271</td></tr>
<tr><td>67</td><td>>RAB20|p.S52C|nonsynonymous SNV [mutant]</td><td>-0.8</td><td>272</td><td>273</td></tr>
<tr><td>68</td><td>>F7|p.A429T|nonsynonymous SNV [mutant]</td><td>0.162</td><td>274</td><td>275</td></tr>
<tr><td>69</td><td>>NPAS3|p.G35R|nonsynonymous SNV [mutant]</td><td>-1.71</td><td>276</td><td>277</td></tr>
<tr><td>70</td><td>>DDX24|p.T554K|nonsynonymous SNV [mutant]</td><td>-0.695</td><td>278</td><td>279</td></tr>
<tr><td>71</td><td>>DYNC1H1|p.V2568I|nonsynonymous SNV [mutant]</td><td>-0.257</td><td>280</td><td>281</td></tr>
<tr><td>72</td><td>>KIF26A|p.A254S|nonsynonymous SNV [mutant]</td><td>0.795</td><td>282</td><td>283</td></tr>
<tr><td>73</td><td>>HERC2|p.S319C|nonsynonymous SNV [mutant]</td><td>-0.105</td><td>284</td><td>285</td></tr>
<tr><td>74</td><td>>MTMR10|p.E387K|nonsynonymous SNV [mutant]</td><td>0.076</td><td>286</td><td>287</td></tr>
<tr><td>75</td><td>>ARHGAP11A|p.E36D|nonsynonymous SNV [mutant]</td><td>-1.067</td><td>288</td><td>289</td></tr>
<tr><td>76</td><td>>SLC27A2|p.Y500N|nonsynonymous SNV [mutant]</td><td>-0.652</td><td>290</td><td>291</td></tr>
<tr><td>77</td><td>>PRTG|p.N908S|nonsynonymous SNV [mutant]</td><td>-0.181</td><td>292</td><td>293</td></tr>
<tr><td>78</td><td>>ALDH1A2|p.S22L|nonsynonymous SNV [mutant]</td><td>0.49</td><td>294</td><td>295</td></tr>
<tr><td>79</td><td>>CHTF18|p.A858T|nonsynonymous SNV [mutant]</td><td>-0.938</td><td>296</td><td>297</td></tr>
<tr><td>80</td><td>>IFT140|p.R1404W|nonsynonymous SNV [mutant]</td><td>-0.49</td><td>298</td><td>299</td></tr>
<tr><td>81</td><td>>SNX29|p.D644E|nonsynonymous SNV [mutant]</td><td>-0.743</td><td>300</td><td>301</td></tr>
<tr><td>82</td><td>>EEF2K|p.D425G|nonsynonymous SNV [mutant]</td><td>-1.514</td><td>302</td><td>303</td></tr>
<tr><td>83</td><td>>QPRT|p.R102W|nonsynonymous SNV [mutant]</td><td>0.671</td><td>304</td><td>305</td></tr>
<tr><td>84</td><td>>CD2BP2|p.S49G|nonsynonymous SNV [mutant]</td><td>-2.076</td><td>306</td><td>307</td></tr>
<tr><td>85</td><td>>PMFBP1|p.L835P|nonsynonymous SNV [mutant]</td><td>-1.405</td><td>308</td><td>309</td></tr>
<tr><td>86</td><td>>PLCG2|p.S1192C|nonsynonymous SNV [mutant]</td><td>-0.3</td><td>310</td><td>311</td></tr>
<tr><td>87</td><td>>ADAD2|p.G44A|nonsynonymous SNV [mutant]</td><td>-0.238</td><td>312</td><td>313</td></tr>
<tr><td>88</td><td>>ZNF469|p.G680D|nonsynonymous SNV [mutant]</td><td>-0.162</td><td>314</td><td>135</td></tr>
<tr><td>89</td><td>>MYH13|p.M80I|nonsynonymous SNV [mutant]</td><td>-0.71</td><td>316</td><td>317</td></tr>
<tr><td>90</td><td>>TRPV2|p.Q199H|nonsynonymous SNV [mutant]</td><td>-0.114</td><td>318</td><td>319</td></tr>
<tr><td>91</td><td>>LRRC75A|p.Q199E|nonsynonymous SNV [mutant]</td><td>-1.243</td><td>320</td><td>321</td></tr>
<tr><td>92</td><td>>ATXN7L3|p.L249V|nonsynonymous SNV [mutant]</td><td>-0.2</td><td>322</td><td>323</td></tr>
<tr><td>93</td><td>>HOXB2|p.P91Q|nonsynonymous SNV [mutant]</td><td>-0.938</td><td>324</td><td>325</td></tr>
<tr><td>94</td><td>>MPO|p.F508L|nonsynonymous SNV [mutant]</td><td>-0.676</td><td>326</td><td>327</td></tr>
<tr><td>95</td><td>>TRIM37|p.R192W|nonsynonymous SNV [mutant]</td><td>0.243</td><td>328</td><td>329</td></tr>
<tr><td>96</td><td>>CHMP1B|p.Q146H|nonsynonymous SNV [mutant]</td><td>-0.29</td><td>330</td><td>331</td></tr>
<tr><td>97</td><td>>SEH1L|p.D118N|nonsynonymous SNV [mutant]</td><td>-0.071</td><td>332</td><td>333</td></tr>
<tr><td>98</td><td>>KCTD1|p.S137L|nonsynonymous SNV [mutant]</td><td>-0.548</td><td>334</td><td>335</td></tr>
<tr><td>99</td><td>>EVI5L|p.P715S|nonsynonymous SNV [mutant]</td><td>0.1</td><td>336</td><td>337</td></tr>
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<tr><th></th><th>Name</th><th>Hydropathy Score</th><th>SEQ ID NO for Amino Acid Sequence</th><th>SEQ ID NO for Nucleotide Sequence</th></tr>
<tr><td>100</td><td>>KEAP1|p.E218K|nonsynonymous SNV [mutant]</td><td>-0.371</td><td>338</td><td>339</td></tr>
<tr><td>101</td><td>>MRI1|p.G69E|nonsynonymous SNV [mutant]</td><td>1.338</td><td>340</td><td>341</td></tr>
<tr><td>102</td><td>>ZNF257|p.T304N|nonsynonymous SNV [mutant]</td><td>-1.019</td><td>342</td><td>343</td></tr>
<tr><td>103</td><td>>VSTM2B|p.E161Q|nonsynonymous SNV [mutant]</td><td>-0.414</td><td>344</td><td>345</td></tr>
<tr><td>104</td><td>>DMKN|p.D93H|nonsynonymous SNV [mutant]</td><td>0.224</td><td>346</td><td>347</td></tr>
<tr><td>105</td><td>>BCKDHA|p.E238K|nonsynonymous SNV [mutant]</td><td>0.086</td><td>348</td><td>349</td></tr>
<tr><td>106</td><td>>CEACAM16|p.S155R|nonsynonymous SNV [mutant]</td><td>-0.752</td><td>350</td><td>351</td></tr>
<tr><td>107</td><td>>NKPD1|p.Q125E|nonsynonymous SNV [mutant]</td><td>-0.338</td><td>352</td><td>353</td></tr>
<tr><td>108</td><td>>EXOC3L2|p.R39L|nonsynonymous SNV [mutant]</td><td>-0.267</td><td>354</td><td>355</td></tr>
<tr><td>109</td><td>>CA11|p.Q282H|nonsynonymous SNV [mutant]</td><td>-0.748</td><td>356</td><td>357</td></tr>
<tr><td>110</td><td>>NLRP8|p.R781S|nonsynonymous SNV [mutant]</td><td>0.11</td><td>358</td><td>3.59</td></tr>
<tr><td>111</td><td>>ZNF470|p.F462L|nonsynonymous SNV [mutant]</td><td>-0.557</td><td>360</td><td>361</td></tr>
<tr><td>112</td><td>>ZNF586|p.R56T|nonsynonymous SNV [mutant]</td><td>-0.314</td><td>362</td><td>363</td></tr>
<tr><td>113</td><td>>ZSCAN1|p.Q134P|nonsynonymous SNV [mutant]</td><td>-0.419</td><td>364</td><td>365</td></tr>
<tr><td>114</td><td>>TPO|p.A90E|nonsynonymous SNV [mutant]</td><td>-0.171</td><td>366</td><td>367</td></tr>
<tr><td>115</td><td>>LTBP1|p.V937L|nonsynonymous SNV [mutant]</td><td>0.748</td><td>368</td><td>369</td></tr>
<tr><td>116</td><td>>AFF3|p.E31Q|nonsynonymous SNV [mutant]</td><td>-2.981</td><td>370</td><td>371</td></tr>
<tr><td>117</td><td>>CKAP2L|p.K30N|nonsynonymous SNV [mutant]</td><td>-1.19</td><td>372</td><td>373</td></tr>
<tr><td>118</td><td>>MYO7B|p.A1791V|nonsynonymous SNV [mutant]</td><td>-0.843</td><td>374</td><td>375</td></tr>
<tr><td>119</td><td>>TANC1|p.K906M|nonsynonymous SNV [mutant]</td><td>0.648</td><td>376</td><td>377</td></tr>
<tr><td>120</td><td>>SLC4A10|p.G309V|nonsynonymous SNV [mutant]</td><td>0.186</td><td>378</td><td>379</td></tr>
<tr><td>121</td><td>>SCN2A|p.S661C|nonsynonymous SNV [mutant]</td><td>0.824</td><td>380</td><td>381</td></tr>
<tr><td>122</td><td>>SP9|p.T14K|nonsynonymous SNV [mutant]</td><td>0.329</td><td>382</td><td>383</td></tr>
<tr><td>123</td><td>>TTN|p.C8217Y|nonsynonymous SNV [mutant]</td><td>-0.695</td><td>384</td><td>385</td></tr>
<tr><td>124</td><td>>HECW2|p.D1350H|nonsynonymous SNV [mutant]</td><td>-0.405</td><td>386</td><td>387</td></tr>
<tr><td>125</td><td>>PARD3B|p.Y789C|nonsynonymous SNV [mutant]</td><td>-0.819</td><td>388</td><td>389</td></tr>
<tr><td>126</td><td>>DIS3L2|p.P77S|nonsynonymous SNV [mutant]</td><td>0.114</td><td>390</td><td>391</td></tr>
<tr><td>127</td><td>>LZTS3|p.E465K|nonsynonymous SNV [mutant]</td><td>0.081</td><td>392</td><td>393</td></tr>
<tr><td>128</td><td>>KCNG1|p.R205H|nonsynonymous SNV [mutant]</td><td>-1.376</td><td>394</td><td>395</td></tr>
<tr><td>129</td><td>>COL20A1|p.N255D|nonsynonymous SNV [mutant]</td><td>-0.119</td><td>396</td><td>397</td></tr>
<tr><td>130</td><td>>BRWD1|p.A2213V|nonsynonymous SNV [mutant]</td><td>-0.586</td><td>398</td><td>399</td></tr>
<tr><td>131</td><td>>DSCAM|p.F271L|nonsynonymous SNV [mutant]</td><td>-0.043</td><td>400</td><td>401</td></tr>
<tr><td>132</td><td>>KRTAP10-4|p.S221T|nonsynonymous SNV [mutant]</td><td>0.048</td><td>402</td><td>403</td></tr>
<tr><td>133</td><td>>NEFH|p.V446A|nonsynonymous SNV [mutant]</td><td>-0.729</td><td>404</td><td>405</td></tr>
<tr><td>134</td><td>>SFI1|p.T128A|nonsynonymous SNV [mutant]</td><td>-0.21</td><td>406</td><td>407</td></tr>
<tr><td>135</td><td>>POLR3H|p.R149C|nonsynonymous SNV [mutant]</td><td>-0.062</td><td>408</td><td>409</td></tr>
<tr><td>136</td><td>>STAB1|p.S681R|nonsynonymous SNV [mutant]</td><td>-0.352</td><td>410</td><td>411</td></tr>
<tr><td>137</td><td>>SLC25A26|p.S82L|nonsynonymous SNV [mutant]</td><td>-0.219</td><td>412</td><td>413</td></tr>
<tr><td>138</td><td>>EPHA6|p.V196L|nonsynonymous SNV [mutant]</td><td>-0.462</td><td>414</td><td>415</td></tr>
<tr><td>139</td><td>>PLXNA1|p.E607K|nonsynonymous SNV [mutant]</td><td>-0.619</td><td>416</td><td>417</td></tr>
<tr><td>140</td><td>>DNAJC13|p.K514I|nonsynonymous SNV [mutant]</td><td>-0.433</td><td>418</td><td>419</td></tr>
<tr><td>141</td><td>>ESYT3|p.K496N|nonsynonymous SNV [mutant]</td><td>-0.905</td><td>420</td><td>421</td></tr>
<tr><td>142</td><td>>GPR149|p.R145G|nonsynonymous SNV [mutant]</td><td>0.09</td><td>422</td><td>423</td></tr>
</table>

	Name	Hydropathy Score	SEQ ID NO for Amino Acid Sequence	SEQ ID NO for Nucleotide Sequence
143	>PDCD10|p.E68Q|nonsynonymous SNV [mutant]	-0.033	424	425
144	>MECOM|p.A78T|nonsynonymous SNV [mutant]	-0.39	426	427
145	>KIAA0226|p.R150K|nonsynonymous SNV [mutant]	-0.176	428	429
146	>MSX1|p.S92L|nonsynonymous SNV [mutant]	0.043	430	431
147	>LIMCH1|p.Q60H|nonsynonymous SNV [mutant]	-0.648	432	433
148	>PTPN13|p.Q2276H|nonsynonymous SNV [mutant]	0	434	435
149	>PDHA2|p.C179Y|nonsynonymous SNV [mutant]	0.2	436	437
150	>EXOSC9|p.L266F|nonsynonymous SNV [mutant]	-0.11	438	439
151	>TBC1D9|p.E837Q|nonsynonymous SNV [mutant]	-0.005	440	441
152	>FGG|p.G294E|nonsynonymous SNV [mutant]	-0.757	442	443
153	>SLC9A3|p.E821Q|nonsynonymous SNV [mutant]	-0.662	444	445
154	>NSUN2|p.F48L|nonsynonymous SNV [mutant]	-0.6	446	447
155	>SPEF2|p.F1436S|nonsynonymous SNV [mutant]	-0.495	448	449
156	>ITGA2|p.P43T|nonsynonymous SNV [mutant]	-0.162	450	451
157	>IPO11|p.N30S|nonsynonymous SNV [mutant]	0.076	452	453
158	>NR2F1|p.V380M|nonsynonymous SNV [mutant]	0.833	454	455
159	>SLCO4C1|p.K663N|nonsynonymous SNV [mutant]	0.781	456	457
160	>WDR55|p.F237L|nonsynonymous SNV [mutant]	-0.114	458	459
161	>PCDHA9|p.T662S|nonsynonymous SNV [mutant]	0.286	460	461
162	>PCDHGA12|p.K590M|nonsynonymous SNV [mutant]RSAEPGYLVTMVVAVDRDSGQ	-0.138	462	463
163	>SLC6A7|p.D151H|nonsynonymous SNV [mutant]	0.162	464	465
164	>TCOF1|p.P566S|nonsynonymous SNV [mutant]	-0.048	466	467
165	>LCP2|p.P138H|nonsynonymous SNV [mutant]	-2.005	468	469
166	>KCNIP1|p.I19M|nonsynonymous SNV [mutant]	0.448	470	471
167	>BTN3A1|p.A186S|nonsynonymous SNV [mutant]	-0.39	472	473
168	>ZBTB12|p.C266S|nonsynonymous SNV [mutant]	-0.348	474	475
169	>CYP21A2|p.E295K|nonsynonymous SNV [mutant]	-1.167	476	477
170	>TREM2|p.E202V|nonsynonymous SNV [mutant]	0.633	478	479
171	>TTK|p.L309F|nonsynonymous SNV [mutant]	-1.219	480	481
172	>SYNCRIP|p.D284H|nonsynonymous SNV [mutant]	-0.667	482	483
173	>HS3ST5|p.R82G|nonsynonymous SNV [mutant]	-1.048	484	485
174	>RFX6|p.Y802C|nonsynonymous SNV [mutant]	-1.086	486	487
175	>SYNE1|p.T5594A|nonsynonymous SNV [mutant]	-0.438	488	489
176	>CYP2W1|p.R328H|nonsynonymous SNV [mutant]	-0.729	490	491
177	>GNAT3|p.E216Q|nonsynonymous SNV [mutant]	0.433	492	493
178	>SEMA3C|p.Y141C|nonsynonymous SNV [mutant]	-0.752	494	495
179	>PCLO|p.E1590D|nonsynonymous SNV [mutant]	-1.938	496	497
180	>SAMD9|p.I635V|nonsynonymous SNV [mutant]	-0.114	498	499
181	>COG5|p.A54G|nonsynonymous SNV [mutant]	0.11	500	501
182	>CBLL1|p.S471F|nonsynonymous SNV [mutant]	-1.19	502	503
183	>FOXP2|p.S139C|nonsynonymous SNV [mutant]	-0.129	504	505
184	>MDFIC|p.L78F|nonsynonymous SNV [mutant]	-0.786	506	507
185	>IMPDH1|p.P138L|nonsynonymous SNV [mutant]	-0.148	508	509
186	>TRIM24|p.T772S|nonsynonymous SNV [mutant]	-0.376	510	511
187	>CASP2|p.A338S|nonsynonymous SNV [mutant]	-1.533	512	513
188	>ASIC3|p.L531Q|nonsynonymous SNV [mutant]	-0.41	514	515
189	>INSIG1|p.L126F|nonsynonymous SNV [mutant]	-0.09	516	517

<table>
<tr><th></th><th>Name</th><th>Hydropathy Score</th><th>SEQ ID NO for Amino Acid Sequence</th><th>SEQ ID NO for Nucleotide Sequence</th></tr>
<tr><td>190</td><td>>TNKS|p.I1189F|nonsynonymous SNV [mutant]</td><td>-0.205</td><td>518</td><td>519</td></tr>
<tr><td>191</td><td>>CHD7|p.Q1704E|nonsynonymous SNV [mutant]</td><td>-0.748</td><td>520</td><td>521</td></tr>
<tr><td>192</td><td>>ZFHX4|p.T3413A|nonsynonymous SNV [mutant]</td><td>-0.381</td><td>522</td><td>523</td></tr>
<tr><td>193</td><td>>CNGB3|p.L227H|nonsynonymous SNV [mutant]</td><td>1.152</td><td>524</td><td>525</td></tr>
<tr><td>194</td><td>>HAS2|p.M118V|nonsynonymous SNV [mutant]</td><td>0.11</td><td>526</td><td>527</td></tr>
<tr><td>195</td><td>>LRRC6|p.D453N|nonsynonymous SNV [mutant]</td><td>-1.286</td><td>528</td><td>529</td></tr>
<tr><td>196</td><td>>PLEC|p.E1404D|nonsynonymous SNV [mutant]</td><td>-1.4</td><td>530</td><td>531</td></tr>
<tr><td>197</td><td>>RORB|p.R372G|nonsynonymous SNV [mutant]</td><td>0.267</td><td>532</td><td>533</td></tr>
<tr><td>198</td><td>>SPATA31C2|p.A1072E|nonsynonymous SNV [mutant]</td><td>-1.09</td><td>534</td><td>535</td></tr>
<tr><td>199</td><td>>SVEP1|p.R146S|nonsynonymous SNV [mutant]</td><td>-0.571</td><td>536</td><td>537</td></tr>
<tr><td>200</td><td>>REXO4|p.T241S|nonsynonymous SNV [mutant]</td><td>0.581</td><td>538</td><td>539</td></tr>
<tr><td>201</td><td>>RPS6KA3|p.P617L|nonsynonymous SNV [mutant]</td><td>-0.081</td><td>540</td><td>541</td></tr>
<tr><td>202</td><td>>SMC1A|p.K402Q|nonsynonymous SNV [mutant]</td><td>-1.81</td><td>542</td><td>543</td></tr>
<tr><td>203</td><td>>MSN|p.K211N|nonsynonymous SNV [mutant]</td><td>-0.395</td><td>544</td><td>545</td></tr>
<tr><td>204</td><td>>TEX11|p.S163C|nonsynonymous SNV [mutant]</td><td>-0.148</td><td>546</td><td>547</td></tr>
<tr><td>205</td><td>>SERPINA7|p.K290R|nonsynonymous SNV [mutant]</td><td>-0.695</td><td>548</td><td>549</td></tr>
<tr><td>206</td><td>>IL9R|p.V287E|nonsynonymous SNV [mutant]</td><td>-1.505</td><td>550</td><td>551</td></tr>
</table>

Reagents

[00810] The following reagents were used to test the lung permutation constructs:

- Bacteria: *Lmdda* constructs tagged grown overnight in BHI
- Cell lines: DC2.4
- 2% trypsin in HBSS
- RPMI 10%FBS glutamax
- FACS Buffer (PBS 2% FBS)
- Cell counting solution
- Gentamicin antibiotic
- 25D-APC conjugated antibody 100X

Harvesting Antigen Presenting Cells

[00811] In some experiments, murine dendritic DC2.4 cells were stimulated with 20 ng/mL recombinant mouse IFN gamma for 48 hours. Media was removed and collected into two 50 mL conical tubes per flask. A volume of 10ml 2% trypsin HBSS solution is added to the flask to remove residual FBS and was decanted into the two 50 mL collection tubes equally (5 mL: each). A volume of 10 mL 2% trypsin HBSS solution was added to the flask to coat, and adherence was checked under a microscope, and a 5 min incubation followed at 37°C. The suspension was collected into the two 50-mL collection tubes (5 mL each) and spun for 5

minutes at 1200 rpm. The supernatant was discarded, and the pellet was resuspended in tube 1 with 25 mL RPMI 10%FBS glutamax solution. This was then decanted into the second collection tube to combine the two tubes into one.

[00812] Tubes (1.5 mL) were then labeled for counting. A volume of 135 uL counting solution and a volume of 15 uL cells was added, and the cells were incubated for 2 minutes at room temperature. The DC2.4 cells were then set up for infection in 24-well plates. The 24-well plates were incubated overnight at 37°C (5% CO_2). The plates were then spun for 5 seconds at 2000 rpm, supernatant was removed, and 1 mL fresh c-RPMI was added to each well.

Infection

[00813] The cells were then infected with *Lmdda*-PSA-Survivin-tag expressing vectors (grown overnight dry 37). Total *Lmdda*-neo construct cfu were $1x10^9$/mL The *Lmdda* was spun down in 1.5 mL and resuspended in 1 mL room temperature RPMI-10% FBS media. A volume of the *Lmdda* was added to the DC2.4 wells to reach the correct MOI for $2x10^6$ cells (MOI: 10 = 20 uL *Lmdda*). The plate was then spun at 1200 rpm for 15 minutes and placed in an incubator at 37°C with 5% CO_2 for a four hour infection. To stop *Lmdda* killing of the cells, 10 ug/mL gentamicin was added after 1 hour of the incubation.

Staining with 25D-APC (SIINFEKL) and Flow Cytometry

[00814] After four hours of infection, the plate was spun for 30 seconds at 2000 rpm, and the supernatant was discarded. To block the cells were resuspended in 200 uL 2.4G2 and transferred to a 96-well plate for 10 minute on ice. The cells were washed with FACS buffer (PBS + 2% FBS). Staining master mix was then added, and the cells were vortexed and placed on ice for 20 minutes. The cells were then washed with FACS buffer and resuspended in approximately 300 uL FACS buffer (depending on size of pellet/cell number). The samples were then run on the flow cytometer for detection of 25D-APC.

Experiment 1

[00815] **Table 10.** Samples Tested for Detection of 25D-APC.

Sample #	Lung Construct	Order of Neo-Epitopes
1	SVN-tag	
2	SVN-no	
3	PSMA	
4	R1 (SEQ ID NOS: 552, 553) (DNA sequence, peptide sequence)	160, 171, 19, 129, 96, 127, 115, 42, 131, 196, 118, 36, 113, 56, 30, 84, 80, 32, 200, 21
5	R2 (SEQ ID NOS: 554, 555)	178, 182, 148, 3, 106, 173, 187, 52, 168, 160, 65, 119, 181, 16, 23, 156, 31, 122, 42, 54
6	R5 (SEQ ID NOS: 556, 557)	48, 178, 65, 165, 50, 185, 55, 119, 84, 180, 98, 35, 166, 110, 176, 49, 41, 163, 20, 152
7	R6 (SEQ ID NOS: 558, 559)	105, 66, 65, 69, 152, 3, 46, 91, 79, 114, 58, 73, 125, 8, 163, 156, 31, 44, 7, 103
8	R7 opt (SEQ ID NOS: 560, 561)	158, 176, 36, 150, 56, 15, 120, 40, 167, 29, 187, 5, 102, 1, 91, 44, 22, 108, 193, 68
9	R8 (SEQ ID NOS: 562, 563)	38, 31, 89, 22, 152, 169, 82, 144, 184, 49, 30, 5, 187, 94, 95, 167, 194, 118, 136, 9
10	R9 (SEQ ID NOS: 564, 565)	65, 144, 127, 194, 94, 186, 67, 32, 117, 7, 92, 165, 179, 8, 10, 129, 145, 130, 104, 60
11	R10 opt (SEQ ID NOS: 566, 567)	8, 116, 29, 185, 194, 47, 95, 101, 51, 21, 195, 162, 123, 10, 20, 63, 117, 2, 184, 130
12	R11 (SEQ ID NOS: 568, 569)	184, 129, 92, 154, 159, 167, 40, 67, 113, 189, 77, 18, 150, 87, 196, 31, 13, 100, 15, 22
13	R 14 opt (SEQ ID NOS: 570, 571)	69, 155, 100, 1, 81, 139, 78, 154, 49, 38, 52, 5, 133, 39, 42, 161, 83, 166, 163, 157
14	R18 (SEQ ID NOS: 572, 573)	155, 75, 35, 135, 112, 19, 59, 160, 14, 16, 103, 120, 127, 31, 157, 167, 12, 52, 70, 177
15	R19 (SEQ ID NOS: 574, 575)	94, 57, 174, 149, 117, 24, 170, 20, 12, 176, 146, 127, 108, 53, 46, 27, 157, 29, 59, 130
16	R20 (SEQ ID NOS: 576, 577)	128, 97, 122, 170, 18, 106, 37, 100, 49, 132, 185, 19, 59, 167, 188, 115, 127, 64, 169, 117
17	rM 2 (SEQ ID NOS: 578, 579)	LCMV, 14, 169, 28, VSV, 144, 195, 189, PSA, 90,101, 155, VV, 102, 43, 26, IAV, 82, 31, 95
18	1-20 (SEQ ID NOS: 580, 581)	1-20
19	21-40 opt (SEQ ID NOS: 582, 583)	21-40 except #39 moved to 4th position
20	41-60 (SEQ ID NOS: 584, 585)	41-60
21	81-100 (SEQ ID NOS: 586, 587)	81-100
22	101-120 (SEQ ID NOS: 588, 589)	101-120
23	121-140 (SEQ ID NOS: 590, 591)	121-140
24	141-160 (SEQ ID NOS: 592, 593)	141-160
25	61-80 H (SEQ ID NO: 618) (peptide sequence only)	61-80
26	81-100 H (SEQ ID NO: 587) (peptide sequence only)	81-100
27	101-120 H (SEQ ID NO: 589) (peptide sequence only)	101-120

Sample #	Lung Construct	Order of Neo-Epitopes
28	121-140 H (SEQ ID NO: 591) (peptide sequence only)	121-140
29	141-160 H (SEQ ID NO: 593) (peptide sequence only)	141-160
30	No Infection	
31	Unstained	

[00816] An "H" at the end of the construct (such as "121-140 H") indicates that the peptide sequence is identical to the construct lacking the H, but the underlying nucleotide sequence which resulted in the same peptide sequence was modified.

5 [00817] **Table 11.** Detection of 25D-APC.

Sample #	Lung Construct	live | Mean (R 670-20)	Percent Positive	MFI Ratio (Exp-Background)/ (SVN-background)	Percent Ratio (Exp-Background)/ (SVN-background)
1	SVN-tag	605 Positive Ctrl	16.7	-	-
2	SVN-no	115 Background	.30	-	-
3	PSMA	253 PSMA Ctrl	5.13	0.281633	0.294512
4	R1	350	8.61	0.479592	0.506707
5	R2	309	6.81	0.395918	0.396951
6	R5	162	1.13	0.095918	0.05061
7	R6	286	6.59	0.34898	0.383537
8	R7 opt	152	.39	0.07551	0.005488
9	R8	155	.60	0.081633	0.018293
10	R9	788	18.9	1.373469	1.134146
11	R10 opt	182	.99	0.136735	0.042073
12	R11	222	2.40	0.218367	0.128049
13	R 14 opt	112	.09	-0.00612	-0.0128
14	R18	117	.26	0.004082	-0.00244
15	R19	109	.1	-0.01224	-0.0122
16	R20	203	3.3	0.179592	0.182927
17	rM 2	116	.15	0.002041	-0.00915
18	1-20	134	.27	0.038776	-0.00183
19	21-40 opt	129	.26	0.028571	-0.00244
20	41-60	299	6.52	0.37551	0.379268
21	81-100	187	1.51	0.146939	0.07378
22	101-120	428	10	0.638776	0.591463
23	121-140	368	9.26	0.516327	0.546341
24	141-160	144	.61	0.059184	0.018902
25	61-80 H	312	7.68	0.402041	0.45
26	81-100 H	151	.59	0.073469	0.017683
27	101-120 H	363	8.05	0.506122	0.472561
28	121-140 H	281	5.33	0.338776	0.306707
29	141-160 H	204	2.04	0.181633	0.106098
30	No Infection	102	.07	-0.02653	-0.01402
31	Unstained	63.8	.04	-0.10449	-0.01585

[00818] Detection of the C-terminal SIINFEKL tag with the 25D-APC conjugated antibody is shown in **Table 11**. As indicated in **Table 11**, the SVN-tag and PSMA-tag positive controls showed high levels of positive staining, whereas the SVN-no tag, the no infection,

and the unstained negative controls were below the limit of detection. Similarly, samples 4-7, 10, 12, 16, 20-23, 25, and 27-29 showed high levels of positive staining. This demonstrates confirmation that the neo-antigens express and secrete in antigen-presenting cells upon infection.

5 ***Experiment 2***

[00819] The above was repeated in a second experiment with additional lung neo-epitope constructs, as indicated in **Table 12**. In this experiment, the tag was moved to different locations within the lung constructs.

[00820] **Table 12.** Samples Tested for Detection of 25D-APC.

Sample #	Lung Construct	Explanation of Sample
1	SVN	
2	No Tag	
3	21416 (SEQ ID NOS: 594, 595) (DNA sequence, peptide sequence)	121-135-SIINFEKL-136-140-6xHIS
4	21417 (SEQ ID NOS: 596, 597)	SIINFEKL-random8 1-20-6xHIS
5	21419 (SEQ ID NOS: 598, 599)	random8 1-10-SIINFEKL-11-20-6xHIS
6	20724 (SEQ ID NO: 581) (peptide sequence only)	2712 1-20-tags H
7	21412 (SEQ ID NOS: 600, 601)	41-55-SIINFEKL-56-60-6xHIS
8	20726 (SEQ ID NO: 585) (peptide sequence only)	2712 41-60-tags H
9	20725 (SEQ ID NO: 583) (peptide sequence only)	2712 21-40opt-tags H
10	21411 (SEQ ID NOS: 602, 603)	41-50-SIINFEKL-51-60-6xHIS
11	21409 (SEQ ID NOS: 604, 605)	SIINFEKL-41-60-6xHIS
12	21420 (SEQ ID NOS: 606, 607)	random8 1-15-SIINFEKL-16-20-6xHIS
13	21418 (SEQ ID NOS: 619, 620)	2712 random8 1-5-SIINFEKL-6-20-HIS
14	19411 (SEQ ID NOS: 621, 622)	2712 random16opt-tags
15	41-60 (SEQ ID NOS: 584, 585)	41-60
16	no infection	
17	unstained	

[00821] **Table 13.** Detection of 25D-APC.

Sample #	Lung Construct	live | Mean (R 670-20)	Percent Positive	MFI Ratio (Exp-Background)/ (SVN-background)	Percent Ratio (Exp-Background)/ (SVN-background)
1	SVN	325	2.46	-	-
2	No Tag	239	0.29	-	-
3	21416	285	1.16	0.534884	0.400922
4	21417	248	0.4	0.104651	0.050691
5	21419	223	0.23	-0.18605	-0.02765
6	20724	236	0.3	-0.03488	0.004608
7	21412	255	0.79	0.186047	0.230415
8	20726	265	1.12	0.302326	0.382488
9	20725	218	0.24	-0.24419	-0.02304
10	21411	269	0.45	0.348837	0.073733
11	21409	240	0.32	0.011628	0.013825
12	21420	231	0.16	-0.09302	-0.05991
13	21918	222	0.31	-0.19767	0.009217
14	19411	228	0.25	-0.12791	-0.01843
15	41-60	232	0.46	-0.0814	0.078341
16	no infection	263	0.81	0.27907	0.239631
17	unstained	78.4	0.02	-1.86744	-0.12442

[00822] Detection of the C-terminal SIINFEKL tag with the 25D-APC conjugated antibody is shown in **Table 13**. As indicated in **Table 13**, the SVN-tag positive control showed high levels of positive staining, whereas the no tag, the no infection, and the unstained negative controls were below the limit of detection. Similarly, samples 3, 7, and 8 showed high levels of positive staining. This demonstrates confirmation that the neo-antigens express and secrete in antigen-presenting cells upon infection.

Experiment 3

[00823] The above was repeated in a third experiment with additional lung constructs, as indicated in **Table 14**. In these lung constructs, the tag was moved to different locations in the construct.

[00824] **Table 14.** Samples Tested for Detection of 25D-APC.

Sample #	Lung Construct	Order of Neo-Epitopes and Tags
1	PSA Survivin	
2	Minigene	
3	No Tag	
4	21409 (SEQ ID NOS:604, 605) (DNA sequence, peptide sequence)	SIINFEKL-41-60-6xHIS
5	21410 (SEQ ID NOS:608, 609)	41-45-SIINFEKL-46-60-6xHIS
6	21411 (SEQ ID NOS:602, 603)	41-50-SIINFEKL-51-60-6xHIS
7	21412 (SEQ ID NOS:600, 601)	41-55-SIINFEKL-56-60-6xHIS
8	21414 (SEQ ID NOS:610, 611)	121-125-SIINFEKL-126-140-6xHIS
9	21415 (SEQ ID NOS:612, 613)	121-130-SIINFEKL-131-140-6xHIS
10	21416 (SEQ ID NOS:595, 595)	121-135-SIINFEKL-136-140-6xHIS
11	41-60 (SEQ ID NOS:614, 615)	41-60-SIINFEKL-6xHIS
12	121-140 (SEQ ID NOS:616, 618)	121-140-SIINFEKL-6xHIS
13	no inf	

[00825] **Table 15.** Detection of 25D-APC.

Sample #	Lung Construct	Percent Positive	Percent Ratio (Exp-Background)/ (SVN-background)	MFI	MFI Ratio (Exp-Background)/ (SVN-background)
1	PSA Survivin	30.9		1392	
2	Minigene	37.4		2350	
3	No Tag	1.62		424	
4	21409	6.85	0.17862	553	0.133264
5	21410	1.58	-0.00137	422	-0.00207
6	21411	5.92	0.146858	507	0.085744
7	21412	11.2	0.327186	692	0.27686
8	21414	4.95	0.11373	536	0.115702
9	21415	13.5	0.405738	741	0.327479
10	21416	4.96	0.114071	538	0.117769
11	41-60	11	0.320355	678	0.262397
12	121-140	12.2	0.361339	709	0.294421
13	no inf	0.01	-0.05499	140	-0.29339

[00826] Detection of the C-terminal SIINFEKL tag with the 25D-APC conjugated antibody is shown in **Table 15**. As indicated in **Table 15**, the PSA Survivin and the Minigene positive controls showed high levels of positive staining, whereas the no tag and the no infection negative controls were below the limit of detection. Similarly, samples 4 and 5-12 showed high levels of positive staining. This demonstrates confirmation that the neo-antigens express and secrete in antigen-presenting cells upon infection.

[00827] Figure 45 shows surface K^b-SIINFEKL on DC2.4 cells infected with *Lm* constructs with SIINFEKL at various positions. The graph depicts a summary of the raw 25D data, depicting that the SIINFEKL tag identifies a secreted neo-epitope whether SIINFEKL is located at the C-terminus, the N-temrinus, or in between. The last five bars correspond with the following constructs: 2712 SIINFEKL-121-140-6xHIS; 2712 121-125-SIINFEKL-126-140-6xHIS; 2712 121-130-SIINFEKL-131-140-6xHIS; 2712 121-135-SIINFEKL-136-140-6xHIS; and 2712 121-140-SIINFEKL-6xHIS, respectively.

Experiment 4

[00828] The above was repeated in a fourth experiment with additional *Lmdda* constructs, as indicated in **Table 16**.

[00829] **Table 16.** Samples Tested for Detection of 25D-APC.

Sample #	Lung Construct	Strain Name	Neo-Epitopes from N-term to C-term
1	SVN		
2	No Tag		
3	599	1-5 (SEQ ID NOS: 623, 624) (DNA sequence, peptide sequence)	1-5
4	600	6-10 (SEQ ID NOS: 625, 626)	6-10
5	601	11-15 (SEQ ID NOS: 627, 628)	11-15
6	605	1-20r1 11-20 (SEQ ID NOS: 629, 630)	13, 11, 9, 7, 20, 5, 2, 4, 3, 12
7	606	1-20r1 1-5 (SEQ ID NOS: 631, 632)	10, 19, 16, 17, 15
8	607	1-20r1 6-10 (SEQ ID NOS: 633, 634)	8, 6, 18, 14, 1
9	608	1-20r1 11-15 (SEQ ID NOS:635, 636)	13, 11, 9, 7, 20
10	609	1-20r1 16-20 (SEQ ID NOS: 637, 638)	5, 2, 4, 3, 12
11	614	1-20r2 6-10 (SEQ ID NOS: 639, 640)	18, 7, 16, 17, 6
12	615	1-20r2 11-15 (SEQ ID NOS: 641, 642)	20, 12, 4, 8, 2
13	619	21-40 1-5 (SEQ ID NOS: 643, 644)	21-25
14	620	21-40 6-10 (SEQ ID NOS: 645, 646)	26-30
15	622	21-40 16-20 (SEQ ID NOS: 647, 648)	36-40
16	623	21-40r1 (SEQ ID NOS: 649, 650)	38, 22, 31, 36, 35, 24, 32, 33, 29, 39, 30, 28, 34, 37, 21, 27, 25, 40, 26, 23
17	624	21-40r1 1-10 (SEQ ID NOS: 651, 652)	38, 22, 31, 36, 35, 24, 32, 33, 29, 39
18	625	21-40r1 11-20 (SEQ ID NOS: 653, 654)	30, 28, 34, 37, 21, 27, 25, 40, 26, 23
19	626	21-40r1 1-5 (SEQ ID NOS: 655, 656)	38, 22, 31, 36, 35
20	627	21-40r1 6-10 (SEQ ID NOS: 657, 658)	24, 32, 33, 29, 39
21	628	21-40r1 11-15 (SEQ ID NOS: 659, 660)	30, 28, 34, 37, 21
22	629	21-40r1 16-20 (SEQ ID NOS: 661, 662)	5, 2, 4, 3, 12
23	632	21-40r2 11-20 (SEQ ID NOS: 663, 664)	38, 22, 25, 27, 33, 26, 31, 24, 40, 29
24	634	21-40r2 6-10 (SEQ ID NOS: 665, 666)	35, 21, 28, 34, 36
25	398	2712 random3 (SEQ ID NOS: 667, 668)	71, 45, 89, 122, 31, 199, 95, 131, 35, 192, 154, 136, 185, 124, 194, 73, 150, 159, 93, 190
26	412	2712 random17 (SEQ ID NOS: 669, 670)	64, 156, 93, 179, 187, 119, 90, 55, 9, 14, 153, 59, 78, 151, 107, 170, 134, 148, 97, 29
27	413	SIINFEKL 121-140-6xHIS (SEQ ID NOS: 671, 672)	121-140

Sample #	Lung Construct	Strain Name	Neo-Epitopes from N-term to C-term
28	602	16-20 (SEQ ID NOS: 673, 674)	16-20
29	603	1-20r1 (SEQ ID NOS: 675, 676)	10, 19, 16, 17, 15, 8, 6, 18, 14, 1, 13, 11, 9, 7, 20, 5, 2, 4 ,3, 12
30	604	1-20r1 1-10 (SEQ ID NOS: 677, 678)	10, 19, 16, 17, 15, 8, 6, 18, 14, 1
31	ISG15		

[00830] **Table 17.** Detection of 25D-APC.

Sample #	Lung Construct	Strain Name	Percent Positive	Percent Ratio (Exp-Background)/ (SVN-background)
1	SVN		14.0 %	
2	No Tag		1.43 %	
3	599	1-5	9.56 %	0.646778
4	600	6-10	12.7 %	0.896579
5	601	11-15	4.67 %	0.257757
6	605	1-20r1 11-20	15.5 %	1.119332
7	606	1-20r1 1-5	15.1 %	1.08751
8	607	1-20r1 6-10	6.50 %	0.403341
9	608	1-20r1 11-15	6.51 %	0.404137
10	609	1-20r1 16-20	15.0 %	1.079554
11	614	1-20r2 6-10	7.49 %	0.4821
12	615	1-20r2 11-15	8.88 %	0.592681
13	619	21-40 1-5	0.51 %	-0.07319
14	620	21-40 6-10	0.47 %	-0.07637
15	622	21-40 16-20	5.54 %	0.326969
16	623	21-40r1	0.88 %	-0.04375
17	624	21-40r1 1-10	1.62 %	0.015115
18	625	21-40r1 11-20	11.5 %	0.801114
19	626	21-40r1 1-5	3.48 %	0.163087
20	627	21-40r1 6-10	0.64 %	-0.06285
21	628	21-40r1 11-15	9.81 %	0.666667
22	629	21-40r1 16-20	17.0 %	1.238663
23	632	21-40r2 11-20	0.84 %	-0.04694
24	634	21-40r2 6-10	4.19 %	0.21957
25	398	2712 random3	1.61 %	0.01432
26	412	2712 random17	0.86 %	-0.04535
27	413	SIINFEKL 121-140-6xHIS	3.13 %	0.135243
28	602	16-20	2.33 %	0.071599
29	603	1-20r1	2.46 %	0.081941
30	604	1-20r1 1-10	4.04 %	0.207637
31	ISG15		11.0 %	0.761337

[00831] Detection of the C-terminal SIINFEKL tag with the 25D-APC conjugated antibody is shown in **Table 17**. As indicated in **Table 17**, the SVN positive control showed high levels of positive staining, whereas the no tag negative control showed a low level of staining. Similarly, samples 43-12, 15, 18, 19, 21, 22, 24, and 27-30 showed high levels of positive staining. This demonstrates confirmation that the neo-antigens express and secrete in

antigen-presenting cells upon infection. Fig. 50 shows the effects of randomization of the order of neo-epitopes on presentation and secretion of the neo-epitopes. Ordering 1 thru 20 sequentially does not secrete. However, randomizing the entire order, or breaking down individual pieces, or randomizing those pieces results in successful secretion. Likewise, ordering 21-40 sequentially does not secrete. Individual regions of that 20'mer (1-5, 6-10) do not work, and other regions work (16-20). However, randomizing individual regions results in the successful secretion of each individual region.

EXAMPLE 32: Therapeutic Effects of *Lm* Neo-Antigen Constructs in B16F10 Murine Melanoma Model

[00832] After non-synonymous mutations are identified in cancer cells that are not present in corresponding healthy cells, major efforts are typically invested to determine the mutational functional impact, such as cancer driver versus passenger status, to form a basis for selecting therapeutic targets. However, little attention has been devoted to either define the immunogenicity of these mutations or characterize the immune responses they elicit. From the immunologic perspective, mutations may be particularly potent vaccination targets, as they can create neo-antigens that are not subject to central immune tolerance. When attention has been devoted to define the immunogenicity of these mutations or characterize the immune responses they elicit, efforts are typically directed to narrowing down the non-synonymous mutations to a single mutation to be included in a peptide for immunization. For example, in Castle *et al.*, 962 non-synonymous point mutations were identified in B16F10 murine melanoma cells, with 563 of those mutations in expressed genes. Fifty of these mutations were selected based on selection criteria including low false discovery rate (FDR) confident value, location in an expressed gene, and predicted immunogenicity. Out of these 50, only 16 were found to elicit immune responses in immunized mice, and only 11 of the 16 induced an immune response preferentially recognizing the mutated epitope. Two of the mutations were then found to induce tumor growth inhibition. *See, e.g.,* Castle *et al.* (2012) *Cancer Res.* 72(5):1081-1091, herein incorporated by reference in its entirety for all purposes. In the constructs described in the following experiments, however, our data suggest that Neo 20 and Neo 30 are better at controlling tumor growth. In our constructs, Neo-12 contains the 12 most immunogenic epitopes. Neo-12 contains both tumor controlling epitopes (Mut30 and Mut44, as disclosed above in **Table** 7). Neo 20 contains Mut30-Mut2-Mut3-Mut3-Mut4…Mut19). Neo 30 contains Mut30-Mut2-Mut3…Mut-29). Neo 20 and Neo 30 only contain one of the tumor controlling epitopes identified by Castle (Mut30), and then

they contain both immunogenic and non-immunogenic eptiopes. Despite not having multiple tumor controlling epitopes, and containing many non-tumor controlling and even non-immunogenic eptiopes,

Experiment 1

5 [00833] To determine therapeutic response generated by *Lm* neo-antigen constructs, a tumor regression study was designed to examine the therapeutic effects of such constructs on tumor growth in the B16F10 C57Bl/6 murine melanoma model. Specifically, *Lm* neo-antigen vectors were designed with 12 neo-antigens (*Lm*-Castle 12, containing Mut30, Mut5, Mut17, Mut20, Mut22, Mut24, Mut25, Mut44, Mut46, Mut48, and Mut50) or 20 neo-antigens (*Lm*-
10 Castle 20, containing Mut30, Mut2, Mut3, Mut4, Mut5, Mut6, Mut7, Mut8, Mut9, Mut10, Mut11, Mut12, Mut13, Mut14, Mut15, Mut16, Mut17, Mut18, Mut19, and Mut20) identified by Castle et al. *See, e.g.*, Castle *et al.* (2012) *Cancer Res.* 72(5):1081-1091, herein incorporated by reference in its entirety for all purposes.

[00834] ***Tumor Cell Line Expansion.*** The B16F10 melanoma cell line was cultured in c-
15 RPMI containing 10% FBS (50 mL) and 1X Glutamax (5 mL). The c-RPMI media includes the following components:

RPMI 1640	450 mL
FCS	50 mL
HEPES	5 mL
NEAA	5 ml
L-Glutamine	5 mL
Na-Pyruvate	5 mL
Pen/step	5 mL
2-ME (14.6M)	129 μL

[00835] ***Tumor Inoculation.*** On Day 0, B16F10 cells were trypsinized and washed twice with media. Cells were counted and re-suspended at a concentration of 1 x 10^5 cells/200 uL
20 of PBS for injection. B16F10 cells were then implanted subcutaneously in the right flank of each mouse. Mice were vaccinated on Day 3 of the study. Tumors were measured and recorded twice per week until reaching a size of 12 mm in diameter. Once tumors met sacrifice criteria, mice were euthanized, and tumors were excised and measured.

[00836] ***Immunotherapy Treatment.*** On Day 3, immunotherapies and treatments began.
25 Groups were treated with *Lm* (IP), and boosted twice. Details are listed in Table 18.

[00837] **Table 18.** Treatment Schedule.

Groups (10 mice/group)	**B16F10 Tumor Inoculation 1 x 10^5 cells/200uL/mouse**	**Dose 1: Treatments at 1 week intervals 21JAN16**	**Dose 2: 28FEB16**	**Dose 3: 10FEB16**
1-PBS ONLY (neg control)	18JAN16	200 uL/mouse	200uL/mouse	NA
2-Poly (I:C) ONLY (50 ug in 200 uL PBS) (neg control)	18JAN16	(50 ug in 200 uL PBS-SQ)	(50ug in 200uL PBS- SQ)	NA
3- *LmddA*-274 ONLY (neg control)	18JAN16	1x10^8 IP	1x10^8 IP	NA
4-*Lm*-Castle 12 (SEQ ID NO: 679)	18JAN16	1x10^8 IP	1x10^8 IP	1x10^8 IP
5-*Lm* Castle 20 (SEQ ID NO: 680)	18JAN16	1x10^8 IP	1x10^8 IP	1x10^8 IP

[00838] ***Immunotherapy Treatment Preparation.***

1. PBS ONLY – 200 uL/mouse IP.
2. *LmddA*-274 (Titer: 1.5 x 10^9 CFU/mL)
    a. Thaw 1 vial from -80°C in 37°C water bath.
    b. Spin at 14, 000 rpm for 2 min and discard supernatant.
    c. Wash 2 times with 1 mL PBS and discard PBS.
    d. Re-suspend in PBS to a final concentration of 5x10^8 CFU/mL.
3. *Lm*-Castle 12 (Titer: 1.59 x 10^9 CFU/mL and *Lm*-Castle 20 (Titer: 1.6 x 10^9 CFU/mL)
    a. Thaw 1 vial from -80°C in 37°C water bath.
    b. Spin at 14, 000 rpm for 2min and discard supernatant.
    c. Wash 2 times with 1 mL PBS and discard PBS.
    d. Re-suspend in PBS to a final concentration of 5x10^8 CFU/mL.

[00839] As shown in Fig. 46B, growth of tumors was inhibited by *Lm*-Neo 12 and *Lm*-Neo 20 as compared with the control groups (PBS and *Lmdd*A274). LmddA274 is the listeria control, and is an empty vector. It includes the truncated LLO (tLLO), however no neo-epitopes are attached. In addition, *Lm*-Neo 20, which contained 20 neo-antigens, inhibited tumor growth to a greater extent than *Lm*-Neo 12, which contained 12 neo-antigens. Likewise, *Lm*-Neo 20 and *Lm*-Neo 12 each result in increased survival time when compared with the control groups, with *Lm*-Neo 20 providing the greatest protective effect (Fig. 46C). These data show that vaccination with *Lm* carrying neo-epitopes is able to confer antitumoral effects, and increasing the number of neo-epitopes increases the antitumoral effects.

Experiment 2

[00840] To further compare therapeutic responses generated by different *Lm* neo-antigen constructs, a tumor regression study was designed to examine the therapeutic effects of such constructs on tumor growth in the B16F10 C57Bl/6 murine melanoma model. Specifically, *Lm* neo-antigen vectors were designed with 12 neo-antigens (*Lm*-Castle 12), 20 neo-antigens (*Lm*-Castle 20), or 39 neo-antigens (*Lm*-Castle 39; no linker, no 20-29 (*Lm*-Castle 30)) identified by Castle et al. *See, e.g.,* Castle *et al.* (2012) *Cancer Res.* 72(5):1081-1091, herein incorporated by reference in its entirety for all purposes.

[00841] ***Tumor Cell Line Expansion.*** The B16F10 melanoma cell line was cultured in c-RPMI containing 10% FBS (50 mL) and 1X Glutamax (5 mL).

[00842] ***Tumor Inoculation.*** On Day 0, B16F10 cells were trypsinized and washed twice with media. Cells were counted and re-suspended at a concentration of 1 x 10^5 cells/200 uL of PBS for injection. B16F10 cells were then implanted subcutaneously in the right flank of each mouse. Mice were vaccinated on Day 4 of the study. Tumors were measured and recorded twice per week until reaching a size of 1500 mm^3 in volume. Once tumors met sacrifice criteria, mice were euthanized, and tumors were excised and measured.

[00843] ***Immunotherapy Treatment.*** On Day 4, immunotherapies and treatments began. Animals were treated once every 7 days until the end of the study. Groups were treated with either PBS, *Lmdd*A274, *Lm*-Castle 12, *Lm*-Castle 20, *Lm*-Castle 39 no linker no 20-29, detailed in Table 19.

[00844] **Table 19.** Treatment Schedule.

Groups (10 =N/ group)	**B16F10 Tumor Inoculation 1 x 10^5 cells/200uL/ mouse**	**Dose 1: 01MAR16**	**Dose 2: 08MAR16**	**Dose 3: 15MAR16**	**Dose 4: 22MAR16**	**Dose 5: 29MAR16**
1-PBS ONLY (neg control)	26FEB16	200 uL/ Mouse IP	200 uL/ Mouse IP	200 uL/ Mouse IP	200 uL/ Mouse IP	200 uL/ Mouse IP
2-LmddA-274 ONLY (neg control)	26FEB16	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP
3- *Lm* Castle 12 (SEQ ID NO: 679)	26FEB16	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP
4- *Lm* Castle 20 (SEQ ID NO: 680)	26FEB16	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP
5- *Lm* Castle 39 (no link no 20-29) (also called *Lm* Castle 30) (SEQ ID NO: 681)	26FEB16	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP	1x10^8 IP

[00845] ***Immunotherapy Treatment Preparation.***

1. PBS ONLY – 200 uL/mouse IP.
2. *LmddA*-274 (Titer: 1.7 x 10^9 CFU/mL)
    a. Thaw 1 vial from -80°C in 37°C water bath.
    b. Spin at 14,000 rpm for 2 min and discard supernatant.
    c. Wash 2 times with 1 mL PBS and discard PBS.
    d. Re-suspend in PBS to a final concentration of 5x10^8 CFU/mL.
3. *Lm*-Castle 12 (Titer: 1.59 x 10^9 CFU/mL and *Lm*-Castle 20 (Titer: 1.6 x 10^9 CFU/mL) and *Lm*-Castle 39)Titer: 1 x 10^9 CFU/mL)
    a. Thaw 1 vial from -80°C in 37°C water bath.
    b. Spin at 14,000 rpm for 2min and discard supernatant.
    c. Wash 2 times with 1 mL PBS and discard PBS.

d. Re-suspend in PBS to a final concentration of $5x10^8$ CFU/mL.

[00846] ***Harvesting Details.*** The spleen from each mouse was collected in an individual tube containing 5 mL of c-RPMI medium. Detailed steps are described below. All tumors were excised and measured at termination of the study.

1. Harvest spleens using sterile forceps and scissors.
2. Mash each spleen in wash medium (RPMI only) using two glass slides or the back of plunger from a 3 mL syringe.
3. Transfer cells in the medium to a 15 mL tube.
4. Pellet cells at 1,000 RPM for 5 min at room temperature.
5. Discard supernatant, re-suspend cells in the remaining wash buffer gently, and add 2 mL RBC lysis buffer per spleen to the cell pellet. Mix cells gently with lysis buffer by tapping the tube and wait for 1 min.
6. Immediately add 10 mL of c-RPMI medium to the cell suspension to deactivate the lysis buffer.
7. Spin cells at 1,000 for 5 min at room temperature.
8. Pass the cells through a cell strainer and wash them one more time with 10 mL c-RPMI.
9. Count cells using hemocytometer/moxi flow and check the viability by Trypan blue staining. Each spleen should yield ~1-2 x 10^8 cells.
10. Divide the cells for staining.
11. Follow immudex dextramer staining protocol: with the one exception of adding the cell surface antibodies (CD8, CD62L) in 2.4G2 instead of staining buffer (www.immudex.com/media/12135/tf1003.03_general_staining_procedure_mhc_dextramer.pdf).

[00847] ***CD8+ T Cell Response.*** 25D assays were done as explained above to measure expression and secretion of the *Lm*-Neo 20 construct in antigen presenting cells. Fig. 47A is a positive control (PSA-Survivin-SIINFEKL), Fig. 47B is a negative control (PSA-Survivin without SIINFEKL), and Fig. 47C is the *Lm*-Neo 20 (with SIINFEKL tag at C-terminus). As indicated in Fig. 47, the *Lm*-Neo 20 expresses and is secreted, but only at low levels compared to the positive control. However, despite these low secretion levels, a specific CD8+ T cell response to SIINFEKL was observed. Fig. 48 shows the SIINFEKL-specific CD8+ T cell response to the "low secretion" *Lm*-Neo 20 construct. As shown in Fig. 48,

approximately 20% of the CD8+ T cells are specific for antigens in the *Lm* Neo 20 construct.

[00848] ***Antitumor Effects.*** As shown in Fig. 49A, growth of tumors was inhibited by *Lm*-Neo 12, *Lm*-Neo 20, and *Lm*-Neo 30 as compared with the control groups (PBS and *Lmdd*A274). In addition, *Lm*-Neo 30, which contained 30 neo-antigens, inhibited tumor growth to a greater extent than *Lm*-Neo 20, which contained 20 neo-antigens, which inhibited tumor growth to a greater extent than *Lm*-Neo 12, which contained 12 neo-antigens. Likewise, *Lm*-Neo 30, *Lm*-Neo 20, and *Lm*-Neo 12 each result in increased survival time when compared with the control groups, with *Lm*-Neo 30 providing the greatest protective effect and *Lm*-Neo 20 providing the next greatest protective effect (Fig. 46C). These data show that vaccination with *Lm* carrying neo-epitopes is able to confer antitumoral effects, and increasing the number of neo-epitopes increases the antitumoral effects.

EXAMPLE 33: Neo-Epitope-Specific Immunity in Mice Immunized with *Lm* Neo-Antigen Constructs

[00849] An experiment was designed to evaluate the generation of neo-epitope and signal peptide-specific responses in C57BL/6 mice after immunization with r M2, 1-20, 81-100, 101-120, 121-140, *Lm* 2712#1 & *Lm* 2712#3 neo-epitope constructs. The neo-epitope and signal peptide-specific immune response will be detected by pentamer staining using the known T cell H-2 D^b PSA_{65-73} (HCIRNKSVI**)**, VvB8R(TSYKFESV), IAV PA(SSLENFRAYV) as well as neo-epitope peptide-specific responses as evaluated by intracellular cytokine staining for IFN-γ. The details of immunization schedule and strains are given in Table 20.

[00850] **Table 20.** Immunization Schedule.

Immunotherapy/Group	Titer-CFU/mL	Mice/Group	Dose 1	Dose 2	Dose 3	Spleen harvest
1) r M 2 (VV B8R)	1.04×10^9	5	22MAR16	05APR16	19APR16	26APR16
2) 1-20	1.16×10^9	5	22MAR16	05APR16	19APR16	26APR16
3) 81-100	1.12×10^9	5	22MAR16	05APR16	19APR16	26APR16
4) 101-120	1.09×10^9	5	22MAR16	05APR16	19APR16	26APR16
5) 121-140	0.96×10^9	5	22MAR16	05APR16	19APR16	26APR16
6) 2712#1 (PSA)	0.95×10^9	5	22MAR16	05APR16	19APR16	26APR16
7) 2712#3 (IAV PA	1×10^9	5	22MAR16	05APR16	19APR16	26APR16

[00851] Each of these constructs target mutations from the same 2712 Lung sample used in all of the lung construct experiments. rM2 is a random order of 20 21'mers from the 200 non-synonomous mutation pool . 1-20 includes the first 20 non-synonomous mutations, in

that order. The same applies for 81-100, 101-120, and 121-140. 2712#1 includes 50 21-mers (1-50). 2712 #3 includes 50 21-mers (101-150).

[00852] ***Immunotherapy Preparation.***

1. Thaw 1 vial from -80°C in 37°C water bath.
2. Spin at 14,000 rpm for 2 min and discard supernatant.
3. Wash 2 times with 1 mL PBS and discard PBS.
4. Re-suspend in PBS to appropriate final concentration.

[00853] ***Preparing Isolated Splenocytes.***

1. Harvest spleens using sterile forceps and scissors.
2. Mash each spleen in wash medium (RPMI only) using two glass slides or the back of plunger from a 3 mL syringe.
3. Transfer cells in the medium to a 15 mL tube.
4. Pellet cells at 1,000 RPM for 5 min at room temperature.
5. Discard supernatant, re-suspend cells in the remaining wash buffer gently, and add 2 mL RBC lysis buffer per spleen to the cell pellet. Mix cells gently with lysis buffer by tapping the tube and wait for 1 min.
6. Immediately add 10 mL of c-RPMI medium to the cell suspension to deactivate the lysis buffer.
7. Spin cells at 1,000 for 5 min at room temperature.
8. Pass the cells through a cell strainer and wash them one more time with 10 mL c-RPMI.
9. Count cells using hemocytometer/moxi flow and check the viability by Trypan blue staining. Each spleen should yield ~1-2 x 10^8 cells.
10. Divide the cells for pentamer staining and ELISpot.

ELISPOT for IFN Gamma

[00854] **Day 1.** ***Tubes to prepare:*** PMA (dilute 1:1000 in complete medium). Cells were prepared as mentioned below (5 x 10^6/ml) for each mouse).

1) Prepare complete medium (with BME) **100 mL**
2) Grp 1/ mouse 1 cells (5X10^6/mL of complete medium with BME) **2 mL**
3) Grp 2/ mouse 1 cells (5X10^6/mL of complete medium with BME) **2 mL**

4) Grp 3/ mouse 1 cells ($5X10^6$/mL of complete medium with BME) **2 mL**
5) Grp 1/ mouse 1 cells ($5X10^4$/mL of complete medium with BME) **2 mL**
6) Grp 2/ mouse 1 cells ($5X10^4$/mL of complete medium with BME) **2 mL**
7) Grp 3/ mouse 1 cells ($5X10^4$/mL of complete medium with BME) **2 mL**
8) No peptide (Medium from tube 1) **3 mL**
9) E7 peptide (add 2 ul of 1mM peptide/mL of medium from tube 1) – **16 mL**
10) PMA (add 10 ul of 1 ug/mL stock/mL of medium from tube 1) + Ionomycin (add 1 µL of 1 mg/mL stock/mL of medium from tube 1) **5 mL**

[00855] ***Stimulation with Peptide:***

a) Wash plate 4 times with sterile PBS (200 uL/well).
b) Add 200 uL/well of complete medium. Incubate for at least 30 min at RT.
c) Remove the medium and add the cell suspension (100 uL/well) + stimulants (100 uL/well) as planned in the table.
d) Wrap the plate in aluminum foil and incubate the plate at 37°C, 5% CO_2 for 24h.

[00856] **Day 2.** ***Detection of Spots.***

a) Remove cells by emptying the plate and wash 5 times with PBS (200 uL/well).
b) Add 100 uL/well of diluted R4-6A2-biotin and incubate for 2 h at room temperature.
c) Wash 5 times with PBS (200 uL/well).
d) Add 100 uL/well of diluted Streptavidin-ALP and incubate for 1 h at room temperature.
e) Wash 5 times with PBS (200 uL/well).
f) Add 100 uL/well of filtered (0.45µm filter) ready to use substrate solution (BCIP/NBT) and develop until distinct spots emerge.
g) Stop color development by washing extensively in tap water.
h) Leave the plate to dry and count spots the next day.

Antibody	Dilutions (uL)
R4-6A2	40 uL + 39960 PBS with 0.5 % FCS
Streptavidin-ALP	40 uL + 39960 PBS with 0.5 % FCS

[00857] **Results**.

[00858] Table 21 details whether we were able to detect secretion of the 21'mers via 25D

assay.

[00859] **Table 21** – Lung immunogenicity summary

Strain	Secretion/presentation by 25D Assay
1) r M 2 (VV B8R)	No
2) 1-20	No
3) 81-100	Minimal but positive
4) 101-120	Yes
5) 121-140	Yes
6) 2712 #1	No
7) 2712 #3	No

[00860] Fig. 51. summarizes the SIINFEKL-specific CD8 T cell response in mice
5 immunized with the various constructs. An immune response against SIINFEKL (e.g. surrogate tag for the neo-epitope 21-mer amino acid chain being secreted into the host and the host generating an immune response against the 21-mer amino acid chain), was detected in all constructs, except the 2712 #1 50-21-mer construct.

[00861] Of importance, 3 constructs (r M 2, 1-20 & 2712 #3) that did not screen as a positive
10 screener via 25D assay did in fact generate an in vivo immune response (although the response is less pronounced than constructs that screened positive by 25D). Additionally, an immune response to constructs up to 50 21-mers was able to be generated.

[00862] **EXAMPLE 34:** Testing of 27-mers

[00863] A 25D assay was performed, as described above, using Neo-eptiope constructs
15 comprised of 27 amino acid “27-mers.” The results are shown below in Table 22, and show that the constructs may be composed of oligomers other than 21-mers.

[00864] **Table 22:** Detection of 25D-APC (Assay using 27-mers)

Name	%25D Positive	Pass	Sequence: Neo-eptiope constructs comprised of 27 amino acid: “27’mers.”
Svn	14.6 %	YES	Same PSA-Survivin-SIINFEKL-tag sequence in all other 25D assays
No Tag	2.66 %	NO	Same PSA-Survivin-no tag sequence in all other 25D assays
B16_6	16.6 %	YES	**SEQ ID NO: 682**
B16_8	17.2 %	YES	**SEQ ID NO: 683**
B16_1-13	9.61 %	YES	**SEQ ID NO: 684**
B16_14-27	2.71 %	NO	**SEQ ID NO: 685**
B16_M30	20.4 %	YES	**SEQ ID NO: 686**
B16_M20	17.4 %	YES	**SEQ ID NO: 687**
B16_M50	2.23 %	YES	**SEQ ID NO: 688**

[00865] All patent filings, websites, other publications, accession numbers and the like cited above or below are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant unless otherwise indicated. Any feature, step, element, embodiment, or aspect of the invention can be used in combination with any other unless specifically indicated otherwise. While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

CLAIMS

What is claimed is:

1. A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

(a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample from the subject with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein the comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within the one or more ORFs from the disease-bearing sample;

(b) transforming an attenuated *Listeria* strain with a vector comprising a nucleic acid sequence encoding the one or more peptides comprising the one or more neo-epitopes identified in step (a); and

(c) alternatively (i) storing the attenuated recombinant *Listeria* strain for administering to the subject at a pre-determined period, or (ii) administering a composition comprising the attenuated recombinant *Listeria* strain to the subject, wherein the administering results in the generation of a personalized T-cell immune response against the disease or condition.

2. The process of claim 1, further comprising:

(d) obtaining a second biological sample from the subject comprising a T-cell clone or T-infiltrating cell from the T-cell immune response in step (c) and characterizing specific peptides comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on the T cells;

(e) screening for and selecting a nucleic acid construct encoding the one or more peptides comprising the one or more immunogenic neo-epitopes identified in step (d);

(f) transforming a second attenuated recombinant *Listeria* strain with a vector comprising a nucleic acid sequence encoding the one or more peptides comprising the one or more immunogenic neo-epitopes; and

(g) alternatively (i) storing the second attenuated recombinant *Listeria* for administering to the subject at a pre-determined period, or (ii) administering a second composition comprising the second attenuated recombinant *Listeria* strain to the subject.

3. The process of any preceding claim, wherein each of the one or more peptides

comprising the one or more neo-epitopes is about 5-50 amino acids in length.

4. The process of claim 3, wherein each of the one or more peptides comprising the one or more neo-epitopes is about 8-27 amino acids in length.

5. The process of any preceding claim, wherein the one or more neo-epitopes comprise 5-100 neo-epitopes.

6. The process of claim 5, wherein the one or more neo-epitopes comprise 15-35 neo-epitopes, 8-11 neo-epitopes or 11-16 neo-epiopes.

7. The process of any preceding claim, wherein the one or more neo-epitopes comprise a plurality of neo-epitopes, wherein step (b) further comprises one or more iterations of randomizing the order of the one or more peptides comprising the plurality of neo-epitopes within the nucleic acid sequence of step (b).

8. The process of any preceding claim, wherein the process is repeated to create a plurality of attenuated recombinant *Listeria* strains, each comprising a different set of one or more neo-epitopes.

9. The process of claim 8, wherein the plurality of attenuated recombinant *Listeria* strains comprises 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 attenuated recombinant *Listeria* strains.

10. The process of claim 8 or 9, wherein the combination of the plurality of attenuated recombinant *Listeria* strains comprises about 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes.

11. The process of any preceding claim, wherein the comparing in step (a) comprises use of a screening assay or screening tool and associated digital software for comparing the one or more ORFs in the nucleic acid sequences extracted from the disease-bearing biological sample with the one or more ORFs in the nucleic acid sequences extracted from the healthy biological sample,

wherein the associated digital software comprises access to a sequence database that allows screening of mutations within the ORFs in the nucleic acid sequences extracted from the disease-bearing biological sample for identification of immunogenic potential of the neo-epitopes.

12. The process of any preceding claim, wherein the process of obtaining a second biological sample from the subject in step (d) comprises obtaining a biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of the composition comprising the attenuated recombinant *Listeria* strain.

13. The process of claim 12, wherein the disease-bearing biological sample is tissue, cells, blood, or sera.

14. The process of any preceding claim, wherein the process of characterizing in step (d) comprises the steps of:

(i) identifying, isolating, and expanding T cell clones or T-infiltrating cells that respond against the disease; and

(ii) screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules to which a T-cell receptor on the T cells binds.

15. The process of claim 14, wherein the screening for and identifying in step (ii) comprises T-cell receptor sequencing, multiplex based flow cytometry, or high-performance liquid chromatography.

16. The process of claim 15, wherein the sequencing comprises the use of associated digital software and database.

17. The process of any preceding claim, wherein the disease or condition is an infectious disease, a tumor, or a cancer.

18. The process of any preceding claim, wherein the infectious disease comprises a viral or bacterial infection.

19. The process of any preceding claim, wherein the healthy biological sample is obtained from the subject having the disease or condition.

20. The process of any preceding claim, wherein the nucleic acid sequences extracted from the disease-bearing biological sample and the nucleic acid sequences extracted from the healthy biological sample are determined using exome sequencing or transcriptome sequencing.

21. The process of any preceding claim, wherein the one or more neo-epitopes comprise linear neo-epitopes.

22. The process of any preceding claim, wherein the one or more neo-epitopes comprise a solvent-exposed epitope.

23. The process of any preceding claim, wherein the attenuated recombinant *Listeria* secretes the one or more peptides comprising the one or more neo-epitopes.

24. The process of any preceding claim, wherein the one or more neo-epitopes comprise a T-cell epitope.

25. The process of any preceding claim, wherein the transforming in step (b) is accomplished using a plasmid or phage vector.

26. The process of any preceding claim, wherein the one or more peptides comprising the one or more neo-epitopes are each fused to an immunogenic polypeptide or fragment thereof.

27. The process of any preceding claim, wherein the transforming in step (b) is accomplished using a plasmid vector comprising a minigene nucleic acid construct, the construct comprising one or more open reading frames encoding a chimeric protein, wherein the chimeric protein comprises:

a. a bacterial secretion signal sequence;

b. a ubiquitin (Ub) protein; and

c. the one or more peptides comprising the one or more neo-epitopes,

wherein the bacterial secretion signal sequence, the ubiquitin protein, and the one or more peptides are operatively linked or arranged in tandem from the amino-terminus to the carboxy-terminus.

28. The process of any preceding claim, wherein step (b) further comprises culturing and characterizing the attenuated recombinant *Listeria* strain to confirm expression and secretion of the one or more peptides.

29. The process of any preceding claim 25, wherein the plasmid is an integrative plasmid.

30. The process of claim 29, wherein the plasmid is an extrachromosomal multicopy plasmid.

31. The process of claim 29 or 30, wherein the plasmid is stably maintained in the *Listeria* strain in the absence of antibiotic selection.

32. The process of claim 26, wherein the immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

33. The process of claim 32, wherein the tLLO protein is set forth in SEQ ID NO: 3.

34. The process of claim 32, wherein the actA is set forth in SEQ ID NO: 12-13 and 15-18.

35. The process of claim 32, wherein the PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

36. The process of claim 32, wherein the mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

37. The process of claim 36, wherein the mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

38. The process of claim 36, wherein the mutation comprises a substitution of 1-11 amino acid within the CBD set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO peptide, wherein the non-LLO peptide comprises a peptide comprising a neo-epitope.

39. The process of claim 36, wherein the mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

40. The process of any preceding claim, wherein the one or more peptides comprise a heterologous antigen or a self-antigen associated with the disease.

41. The process of any preceding claim, wherein the heterologous antigen or the self-antigen is a tumor-associated antigen or a fragment thereof.

42. The process of any preceding claim, wherein the one or more neo-epitopes

comprise a cancer-specific or tumor-specific epitope.

43. The process of any preceding claim, wherein the tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

44. The process of any preceding claim, wherein the tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, a Her2-expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or tumor, a blood cancer, or a brain cancer or tumor.

45. The process of any preceding claim, wherein the one or more neo-epitopes comprise an infectious-disease-associated epitope.

46. The process of any preceding claim, wherein the infectious disease is an infectious viral disease.

47. The process of any preceding claim, wherein the infectious disease is an infectious bacterial disease.

48. The process of claim 47, wherein the infectious disease is caused by one of the

following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic *E.coli,* Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea, Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

49. The process of any preceding claim, wherein the attenuated *Listeria* comprises a mutation in one or more endogenous genes.

50. The process of any preceding claim, wherein the mutation is selected from an *actA* gene mutation, a *prfA* mutation, an *actA* and *inlB* double mutation, a *dal/dal* gene double mutation, a *dal/dat/actA* gene triple mutation, or a combination thereof.

51. The process of any preceding claim, wherein the mutation comprises an inactivation, truncation, deletion, replacement, or disruption of the one or more endogenous genes.

52. The process of any preceding claim, wherein the vector further comprises an open reading frame encoding a metabolic enzyme.

53. The process of claim 52, wherein the metabolic enzyme is an alanine racemase enzyme or a D-amino acid transferase enzyme.

54. The process of any preceding claim, wherein the *Listeria* is *Listeria monocytogenes*.

55. The process of any preceding claim, wherein step (c)(ii) further comprises administering an adjuvant to the subject.

56. The process of claim 55, wherein the adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

57. The process of any preceding claim, wherein step (c)(ii) further comprises administering an immune checkpoint inhibitor antagonist.

58. The process of claim 57, wherein the immune checkpoint inhibitor is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

60. The process of any preceding claim, wherein the administering in step (c)(ii) generates a personalized enhanced anti-disease or anti-condition immune response in the subject.

61. The process of claim 60, wherein the immune response comprises an anti-cancer or anti-tumor response.

62. The process of claim 60, wherein the immune response comprises an anti-infectious disease response.

63. The process of claim 62, wherein the infectious disease comprises a viral infection.

64. The process of claim 62, wherein the infectious disease comprises a bacterial infection.

65. The process of any preceding claim, wherein the process allows personalized treatment or prevention of the disease or condition in the subject.

66. The process of any preceding claim, wherein the personalized immunotherapy increases survival time in the subject having the disease or condition.

67. A recombinant attenuated *Listeria* strain produced by the process of any one of claims 1-66.

68. A process for creating a personalized immunotherapy for a subject having a disease or condition, the process comprising the steps of:

(a) comparing one or more open reading frames (ORFs) in nucleic acid sequences extracted from a disease-bearing biological sample with one or more ORFs in nucleic acid sequences extracted from a healthy biological sample, wherein the comparing identifies one or more nucleic acid sequences encoding one or more peptides comprising one or more neo-epitopes encoded within the one or more ORFs from the disease-bearing sample;

(b) transforming a vector with a nucleic acid sequence encoding the one or more peptides comprising the one or more neo-epitopes identified in step (a), or generating a DNA immunotherapy vector or a peptide immunotherapy vector using the nucleic acid sequence encoding the one or more peptides comprising the one or more neo-epitopes identified in step (a); and

(c) alternatively (i) storing the vector or the DNA immunotherapy or the peptide immunotherapy for administering to the subject at a pre-determined period, or (ii) administering a composition comprising the vector, the DNA immunotherapy, or the peptide immunotherapy to the subject, and wherein the administering results in the generation of a personalized T-cell immune response against the disease or condition.

69. The process of claim 68, further comprising:

(d) obtaining a second biological sample from the subject comprising a T-cell clone or T-infiltrating cell from the T-cell immune response in step (c) and characterizing

specific peptides comprising one or more immunogenic neo-epitopes bound by MHC Class I or MHC Class II molecules on the T cells;

(e) screening for and selecting a nucleic acid construct encoding the one or more peptides comprising the one or more immunogenic neo-epitopes identified in step (d);

(f) transforming a second vector with a nucleic acid sequence comprising the one or more open reading frames encoding the one or more peptides comprising the one or more immunogenic neo-epitopes or generating a second DNA immunotherapy vector or a second peptide immunotherapy vector using the nucleic acid sequence encoding the one or more peptides comprising the one or more immunogenic neo-epitopes identified in step (d); and

(g) alternatively (i) storing the second vector or the second DNA immunotherapy or the second peptide immunotherapy for administering to the subject at a pre-determined period, or administering a composition comprising the second vector, the second DNA immunotherapy, or the second peptide immunotherapy to the subject.

70. The process of any one of claims 68 or 69, wherein each of the one or more peptides comprising the one or more neo-epitopes is about 5-50 amino acids in length.

71. The process of claim 70, wherein each of the one or more peptides comprising the one or more neo-epitopes is about 8-27 amino acids in length.

72. The process of any one of claims 68 or 69, wherein the one or more neo-epitopes comprise 5-100 neo-epitopes.

73. The process of claim 72, wherein the one or more neo-epitopes comprise 15-35 neo-epitopes, 8-11 neo-epitopes or 11-16 neo-epitopes.

74. The process of any one of claims 68-73, wherein the one or more neo-epitopes comprise a plurality of neo-epitopes, wherein step (b) further comprises one or more iterations of randomizing the order of the one or more peptides comprising the plurality of neo-epitopes within the nucleic acid sequence of step (b).

75. The process of any one of claims 68-74, wherein the method is repeated to create a plurality of vectors, DNA immunotherapies, or peptide immunotherapies, each comprising a different set of one or more neo-epitopes.

76. The process of claim 75, wherein the plurality of vectors, DNA immunotherapies, or peptide immunotherapies comprises 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 attenuated recombinant *Listeria* strains.

77. The process of claim 75 or 76, wherein the combination of the plurality of vectors, DNA immunotherapies, or peptide immunotherapies comprises about 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes.

78. The process of claim 77, wherein the comparing in step (a) comprises use of a screening assay or screening tool and associated digital software for comparing the one or more ORFs in the nucleic acid sequences extracted from the disease-bearing biological sample with the one or more ORFs in the nucleic acid sequences extracted from the healthy biological sample,

wherein the associated digital software comprises access to a sequence database that allows screening of mutations within the ORFs in the nucleic acid sequences extracted from the disease-bearing biological sample for identification of immunogenic potential of the neo-epitopes.

79. The process of any one of claims 69-78, wherein the process of obtaining a second biological sample from the subject in step (d) comprises obtaining a second biological sample comprising T-cell clones or T-infiltrating cells that expand following administration of the composition comprising the vector, the DNA immunotherapy, or the peptide immunotherapy.

80. The process of any one of claims 68-79, wherein the disease-bearing biological sample is tissue, cells, blood, or sera.

81. The process of any one of claims 69-80, wherein the process of characterizing in step (d) comprises the steps of:

(i) identifying, isolating, and expanding T cell clones or T-infiltrating cells that respond against the disease; and

(ii) screening for and identifying one or more peptides comprising one or more immunogenic neo-epitopes loaded on specific MHC Class I or MHC Class II molecules to which a T-cell receptor on the T cells binds.

82. The process of claim 81, wherein the screening for and identifying in step (ii) comprises T-cell receptor sequencing, multiplex based flow cytometry, or high-performance liquid chromatography.

83. The process of claim 82, wherein the sequencing comprises the use of associated digital software and database.

84. The process of any one of claims 68-83 wherein the disease or condition is an infectious disease, a tumor, or a cancer.

85. The process of claim 84, wherein the infectious disease comprises a viral or bacterial infection.

86. The process of any one of claims 68-86, wherein the healthy biological sample is obtained from the subject having the disease or condition.

87. The process of any one of claims 68-87, wherein the nucleic acid sequences extracted from the disease-bearing biological sample and the nucleic acid sequence extracted from the healthy biological sample are determined using exome sequencing or transcriptome sequencing.

89. The process of any one of claims 68-88, wherein the one or more neo-epitopes comprise linear neo-epitopes.

90. The process of any one of claims 68-89, wherein the one or more neo-epitopes comprise a solvent-exposed epitope.

91. The process of any one of claims 68-90, wherein the one or more neo-epitopes comprise a T-cell epitope.

92. The process of any one of claims 68-91, wherein the vector is a vaccinia virus or a virus-like particle.

93. The process of claim 92, wherein step (b) further comprises culturing and characterizing the vaccinia virus or virus-like particle to confirm expression of the one or more peptides.

94. The process of any one of claims 68-93, wherein the DNA immunotherapy

comprises the nucleic acid sequence comprising the one or more peptides comprising the one or more immunogenic neo-epitopes.

95. The process of claim 94, wherein the nucleic acid sequence is in the form of a plasmid.

96. The process of any one of claims 68-95, wherein the plasmid is an integrative or an extrachrosomomal multicopy plasmid.

97. The process of any one of claims 68-96, wherein the one or more peptides comprising the one or more neo-epitopes are each fused to an immunogenic polypeptide or fragment thereof.

98. The process of any one of claims 68-96, wherein the peptide immunotherapy comprises the one or more peptides comprising the one or more neo-epitopes, wherein each peptide is fused to or mixed with an immunogenic polypeptide or fragment thereof.

99. The process of any one of claims 97-98, wherein the immunogenic polypeptide is a mutated Listeriolysin O (LLO) protein, a truncated LLO (tLLO) protein, a truncated ActA protein, or a PEST amino acid sequence.

100. The process of claim 99, wherein the tLLO protein is set forth in SEQ ID NO: 3.

101. The process of claim 99, wherein the actA is set forth in SEQ ID NO: 12-13 and 15-18.

102. The process of claim 99, wherein the PEST amino acid sequence is selected from the sequences set forth in SEQ ID NOs: 5-10.

103. The process of claim 99, wherein the mutated LLO comprises a mutation in a cholesterol-binding domain (CBD).

104. The process of claim 103, wherein the mutation comprises a substitution of residue C484, W491, or W492 of SEQ ID NO: 2, or any combination thereof.

105. The process of claim 103, wherein the mutation comprises a substitution of 1-11 amino acid within the CBD set forth in SEQ ID NO: 68 with a 1-50 amino acid non-LLO

peptide, wherein the non-LLO peptide comprises a peptide comprising a neo-epitope.

106. The process of claim 103, wherein the mutation comprises a deletion of a 1-11 amino acid within the CBD as set forth in SEQ ID NO: 68.

107. The process of any one of claims 68-106, wherein the one or more peptides comprise a heterologous antigen or a self-antigen associated with the disease.

108. The process of claim 107, wherein the heterologous antigen or the self-antigen is a tumor-associated antigen or a fragment thereof.

109. The process of any one of claims 68-108, wherein the one or more neo-epitopes comprise a cancer-specific or tumor-specific epitope.

110. The process of any one of claims 108-109, wherein the tumor-associated antigen or fragment thereof comprises a Human Papilloma Virus (HPV)-16-E6, HPV-16-E7, HPV-18-E6, HPV-18-E7, a Her/2-neu antigen, a chimeric Her2 antigen, a Prostate Specific Antigen (PSA), bivalent PSA, ERG, Androgen receptor (AR), PAK6, Prostate Stem Cell Antigen (PSCA), NY-ESO-1, a Stratum Corneum Chymotryptic Enzyme (SCCE) antigen, Wilms tumor antigen 1 (WT-1), HIV-1 Gag, human telomerase reverse transcriptase (hTERT), Proteinase 3, Tyrosinase Related Protein 2 (TRP2), High Molecular Weight Melanoma Associated Antigen (HMW-MAA), synovial sarcoma, X (SSX)-2, carcinoembryonic antigen (CEA), Melanoma-Associated Antigen E (MAGE-A, MAGE 1, MAGE2, MAGE3, MAGE4), interleukin-13 Receptor alpha (IL13-R alpha), Carbonic anhydrase IX (CAIX), survivin, GP100, an angiogenic antigen, a ras protein, a p53 protein, a p97 melanoma antigen, KLH antigen, carcinoembryonic antigen (CEA), gp100, MART1 antigen, TRP-2, HSP-70, beta-HCG, or Testisin.

111. The process of claim 84, wherein the tumor or cancer comprises a breast cancer or tumor, a cervical cancer or tumor, a Her2-expressing cancer or tumor, a melanoma, a pancreatic cancer or tumor, an ovarian cancer or tumor, a gastric cancer or tumor, a carcinomatous lesion of the pancreas, a pulmonary adenocarcinoma, a glioblastoma multiforme, a colorectal adenocarcinoma, a pulmonary squamous adenocarcinoma, a gastric adenocarcinoma, an ovarian surface epithelial neoplasm, an oral squamous cell carcinoma, non-small-cell lung carcinoma, an endometrial carcinoma, a bladder cancer or tumor, a head and neck cancer or tumor, a prostate carcinoma, a renal cancer or tumor, a bone cancer or

tumor, a blood cancer, or a brain cancer or tumor.

112. The process of any one of claims 68-111, wherein the one or more neo-epitopes comprise an infectious-disease-associated epitope.

113. The process of claim 112, wherein the infectious disease is an infectious viral disease or an infectious bacterial disease.

114. The process of claim 113, wherein the infectious disease is caused by one of the following pathogens: leishmania, Entamoeba histolytica (which causes amebiasis), trichuris, BCG/Tuberculosis, Malaria, Plasmodium falciparum, plasmodium malariae, plasmodium vivax, Rotavirus, Cholera, Diptheria-Tetanus, Pertussis, Haemophilus influenzae, Hepatitis B, Human papilloma virus, Influenza seasonal), Influenza A (H1N1) Pandemic, Measles and Rubella, Mumps, Meningococcus A+C, Oral Polio Immunotherapies, mono, bi and trivalent, Pneumococcal, Rabies, Tetanus Toxoid, Yellow Fever, Bacillus anthracis (anthrax), Clostridium botulinum toxin (botulism), Yersinia pestis (plague), Variola major (smallpox) and other related pox viruses, Francisella tularensis (tularemia), Viral hemorrhagic fevers, Arenaviruses (LCM, Junin virus, Machupo virus, Guanarito virus, Lassa Fever), Bunyaviruses (Hantaviruses, Rift Valley Fever), Flaviruses (Dengue), Filoviruses (Ebola, Marburg), Burkholderia pseudomallei, Coxiella burnetii (Q fever), Brucella species (brucellosis), Burkholderia mallei (glanders), Chlamydia psittaci (Psittacosis), Ricin toxin (from Ricinus communis), Epsilon toxin of Clostridium perfringens, Staphylococcus enterotoxin B, Typhus fever (Rickettsia prowazekii), other Rickettsias, Food- and Waterborne Pathogens, Bacteria (Diarrheagenic *E.coli,* Pathogenic Vibrios, Shigella species, Salmonella BCG/, Campylobacter jejuni, Yersinia enterocolitica), Viruses (Caliciviruses, Hepatitis A, West Nile Virus, LaCrosse, California encephalitis, VEE, EEE, WEE, Japanese Encephalitis Virus, Kyasanur Forest Virus, Nipah virus, hantaviruses, Tickborne hemorrhagic fever viruses, Chikungunya virus, Crimean-Congo Hemorrhagic fever virus, Tickborne encephalitis viruses, Hepatitis B virus, Hepatitis C virus, Herpes Simplex virus (HSV), Human immunodeficiency virus (HIV), Human papillomavirus (HPV)), Protozoa (Cryptosporidium parvum, Cyclospora cayatanensis, Giardia lamblia, Entamoeba histolytica, Toxoplasma), Fungi (Microsporidia), Yellow fever, Tuberculosis, including drug-resistant TB, Rabies, Prions, Severe acute respiratory syndrome associated coronavirus (SARS-CoV), Coccidioides posadasii, Coccidioides immitis, Bacterial vaginosis, Chlamydia trachomatis, Cytomegalovirus, Granuloma inguinale, Hemophilus ducreyi, Neisseria gonorrhea,

Treponema pallidum, Streptococcus mutans, or Trichomonas vaginalis.

115. The process of any one of claims 68-114, wherein step (c)(ii) further comprises administering an adjuvant to the subject.

116. The process of claim 115, wherein the adjuvant comprises a granulocyte/macrophage colony-stimulating factor (GM-CSF) protein, a nucleotide molecule encoding a GM-CSF protein, saponin QS21, monophosphoryl lipid A, or an unmethylated CpG-containing oligonucleotide.

117. The process of any one of claims 68-116, wherein step (c)(ii) further comprises administering an immune checkpoint inhibitor antagonist.

118. The process of claim 117, wherein the immune checkpoint inhibitor is an anti-PD-L1/PD-L2 antibody or fragment thereof, an anti-PD-1 antibody or fragment thereof, an anti-CTLA-4 antibody or fragment thereof, or an anti-B7-H4 antibody or fragment thereof.

119. The process of any one of claims 68-118, wherein the administering in step (c)(ii) generates a personalized enhanced anti-disease, or anti-condition immune response in said subject.

120. The process of claim 119, wherein the immune response comprises an anti-cancer or anti-tumor response.

121. The process of claim 119, wherein the immune response comprises an anti-infectious disease response.

122. The process of claim 121, wherein the infectious disease comprises a viral infection or bacterial infection.

123. The process of any one of claims 68-122, wherein the process allows personalized treatment or prevention of the disease or condition in the subject.

124. An immunogenic mixture of compositions comprising one or more attenuated recombinant *Listeria* strains produced by the process of any one of claims 68-123.

125. An immunogenic mixture of compositions comprising one or more attenuated recombinant *Listeria* strains, wherein each attenuated recombinant *Listeria* strain comprises a

nucleic acid sequence encoding one or more peptides comprising one or more neo-epitopes present in a disease-bearing biological sample from a subject having a disease or condition.

126. The immunogenic mixture of claim 125, wherein the one or more attenuated recombinant *Listeria* strains comprise a plurality of attenuated recombinant *Listeria* strains, where the nucleic acid sequence in each attenuated recombinant *Listeria* strain encodes a different set of one or more neo-epitopes.

127. The immunogenic mixture of any one of claims 125-126, wherein the plurality of attenuated recombinant *Listeria* strains comprises 5-10, 10-15, 15-20, 10-20, 20-30, 30-40, or 40-50 attenuated recombinant *Listeria* strains.

128. The immunogenic mixture of any one of claims 125-127, wherein the combination of the plurality of attenuated recombinant *Listeria* strains comprises about 5-10, 10-15, 15-20, 10-20, 20-30, 30-40,40-50, 50-60, 60-70, 70-80, 80-90, 90-100, or 100-200 neo-epitopes.

129. The immunogenic mixture of claim 128, wherein each of the attenuated recombinant *Listeria* strains in the mixture comprises a nucleic acid molecule encoding a fusion polypeptide or chimeric protein comprising one or more neo-epitopes.

130. The immunogenic mixture of compositions of claim 129, wherein each of the recombinant *Listeria* strains in the mixture expresses 1-20 neo-epitopes.

131. A method of eliciting a personalized anti-tumor response in a subject, the method comprising the step of concomitantly or sequentially administering to the subject the immunogenic mixture of any one of claims 128-131.

132. A method of preventing or treating a tumor in a subject, the method comprising concomitantly or sequentially administering to the subject the immunogenic mixture of any one of claims 125-130.

132. A nucleic acid construct encoding a chimeric protein comprising the following elements: an immunogenic polypeptide fused to a first neo-epitope amino acid sequence, wherein the first neo-epitope amino acid sequence is operatively linked to a second neo-epitope amino acid sequence via a first linker sequence, wherein the second neo-epitope amino acid sequence is operatively linked to at least one additional neo-epitope amino acid

sequence via a second linker sequence.

133. A nucleic acid construct encoding a chimeric protein comprising the following elements: an N-terminal truncated LLO (tLLO) fused to a first neo-epitope amino acid sequence, wherein the first neo-epitope amino acid sequence is operatively linked to a second neo-epitope amino acid sequence via a first linker sequence, wherein the second neo-epitope amino acid sequence is operatively linked to at least one additional neo-epitope amino acid sequence via a second linker sequence, and wherein a last neo-epitope is operatively linked to a histidine tag at the C-terminus via a third linker sequence.

134. The nucleic acid construct of claim 133, wherein the first, the second, and the at least one addition neo-epitope amino acid sequences each about 5-50 amino acids.

135. The nucleic acid construct of claim 134, wherein the first, the second, and the at least one addition neo-epitope amino acid sequences each about 8-27 amino acids, 8-11 amino acids or 11-16 amino acids.

136. The nucleic acid construct of claim 135, wherein the first, the second, and the at least one addition neo-epitope amino acid sequences each comprises 21 amino acids.

137. The nucleic acid construct of any one of claims 132-136, wherein the nucleic acid construct encodes 5-100 neo-epitopes.

138. The nucleic acid construct of claim 137, wherein the nucleic acid construct encodes 15-35 neo-epitopes.

139. The nucleic acid construct of any one of claims 132-138, wherein the elements are arranged or are operatively linked from N-terminus to C-terminus.

140. The nucleic acid construct of any one of claims 137-139, wherein the tLLO is operatively linked to a promoter sequence.

141. The nucleic acid construct of claim 140, wherein the promoter sequence is an *hly* promoter sequence.

142. The nucleic acid construct of any one of claims 132-141, wherein the nucleic acid construct comprises 2 stop codons following the sequence encoding the histidine tag.

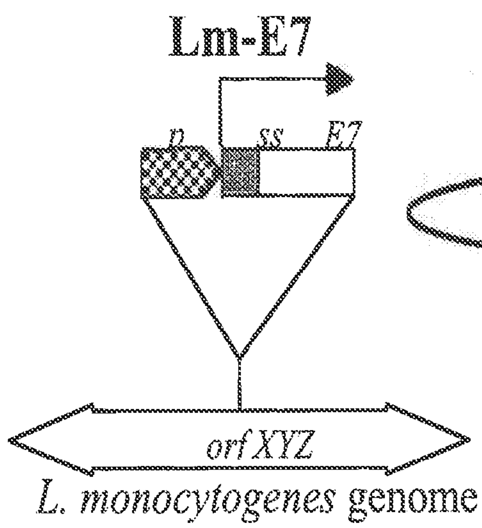
143. The nucleic acid construct of any one of claims 132-142, wherein the histidine tag is a 6X histidine tag that is operatively linked at the N-terminus to a SIINFEKL peptide.

144. The nucleic acid construct of any one of claims 132-143, wherein one or more of the first, second, and third linker sequences is a 4X glycine linker.

145. The nucleic acid construct of any one of claims 132-144, wherein the construct comprises the following components: p*Hly*-tLLO-21mer #1-4x glycine linker G1-21mer #2-4x glycine linker G2-…-SIINFEKL-6xHis tag-2x stop codon.

146. A chimeric protein encoded by the nucleic acid construct of any one of claims 132-145.

147. A recombinant *Listeria* strain comprising the nucleic acid construct of any one of claims 132-145 or expressing the chimeric protein of claim 146.



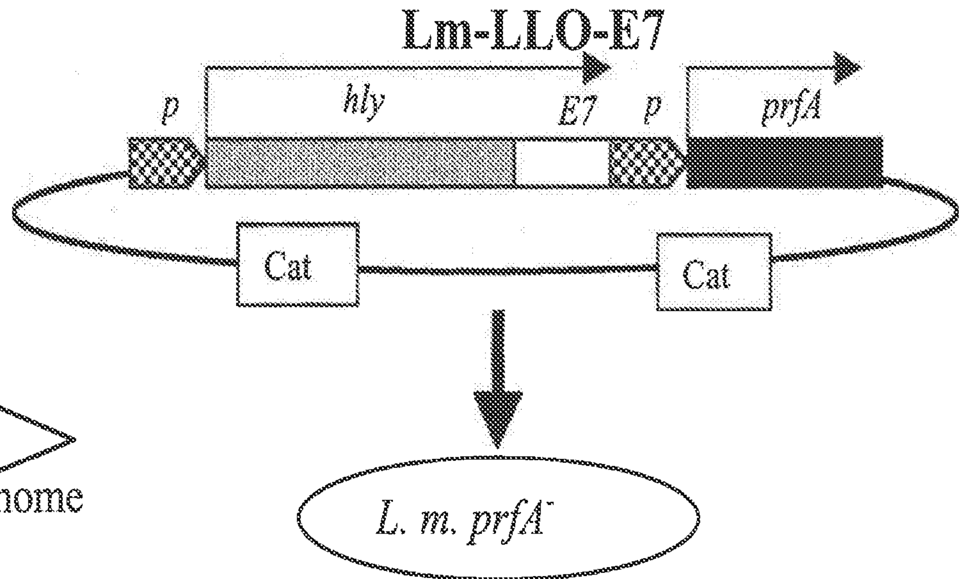


Figure 1A

Figure 1B

FIGURE 1

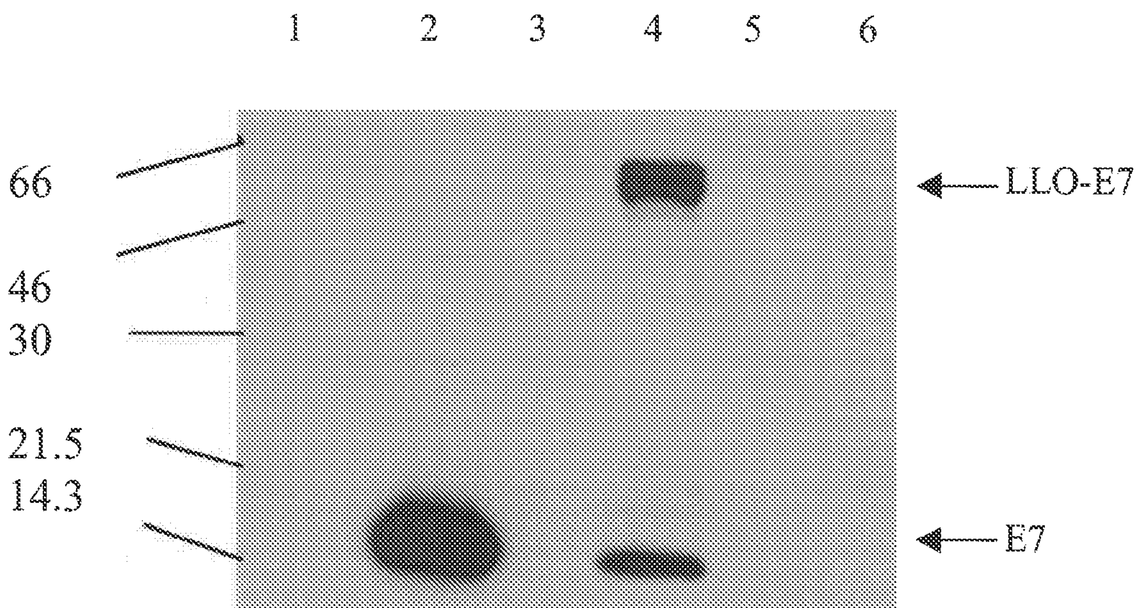


FIGURE 2

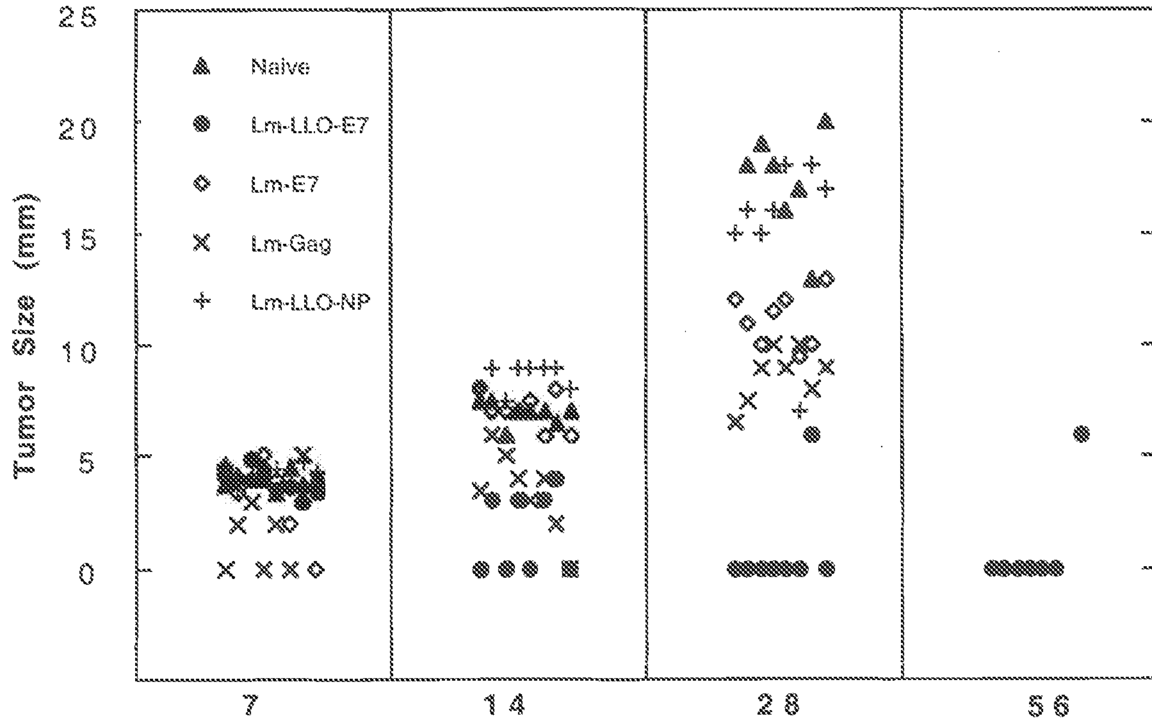


FIGURE 3

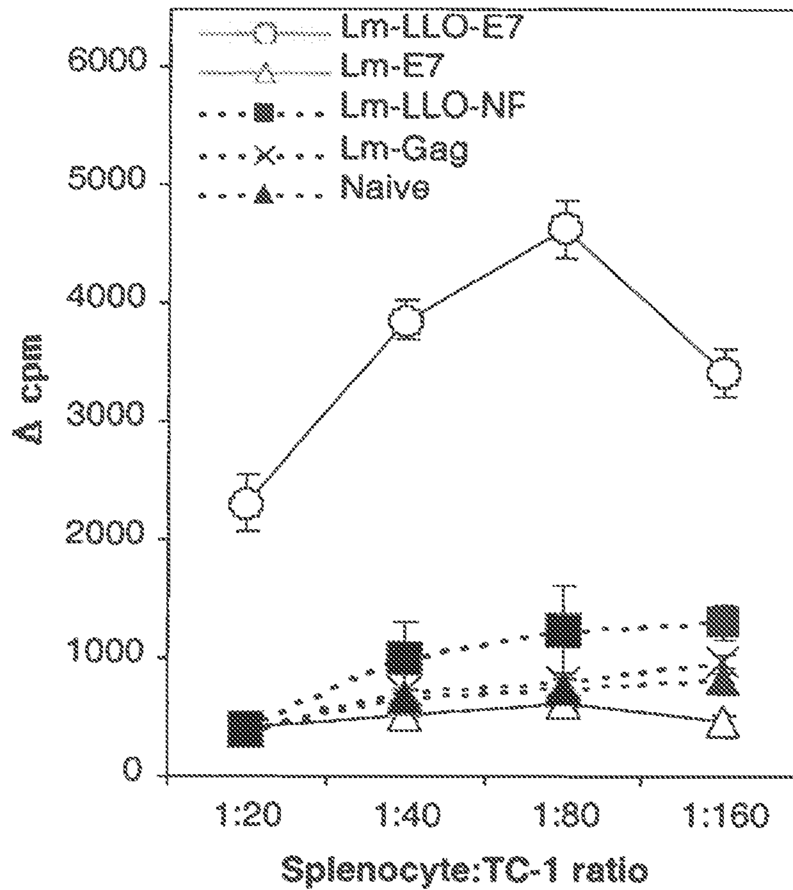


FIGURE 4

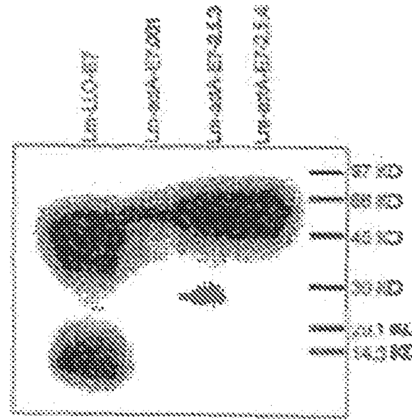


Figure 5A

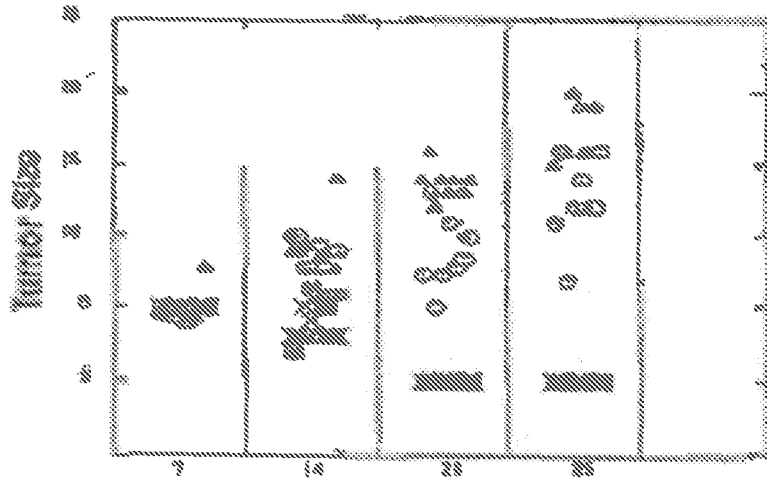


Figure 5B

FIGURE 5

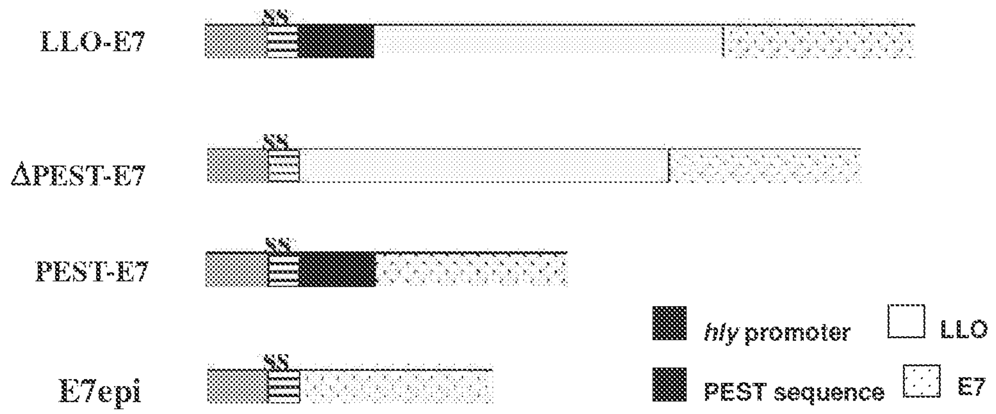
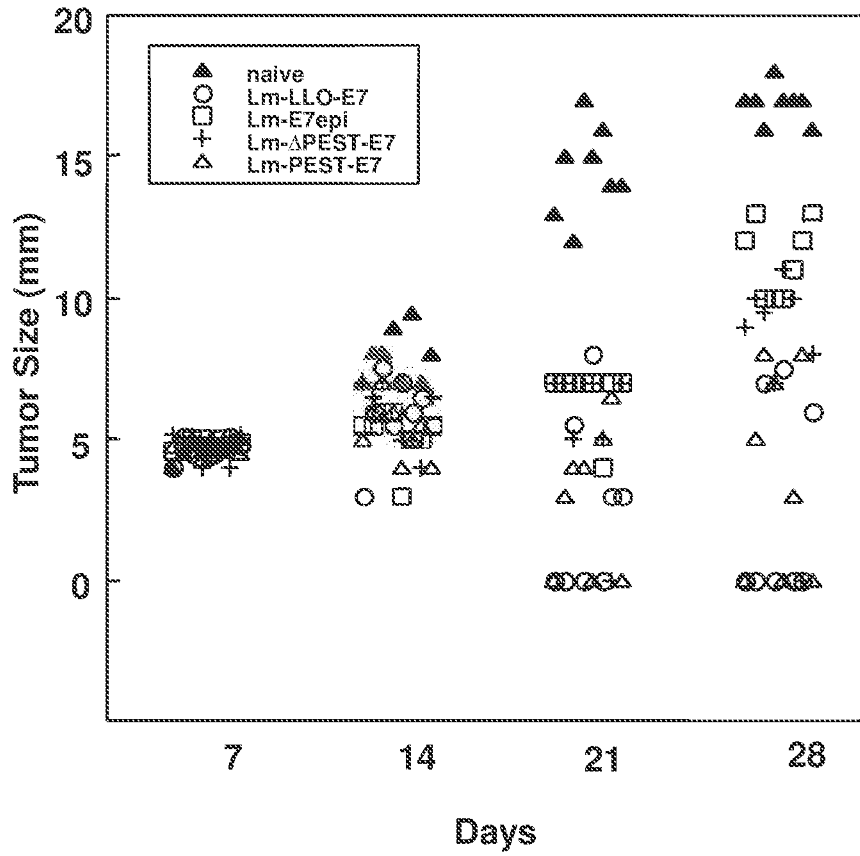


Figure 6A



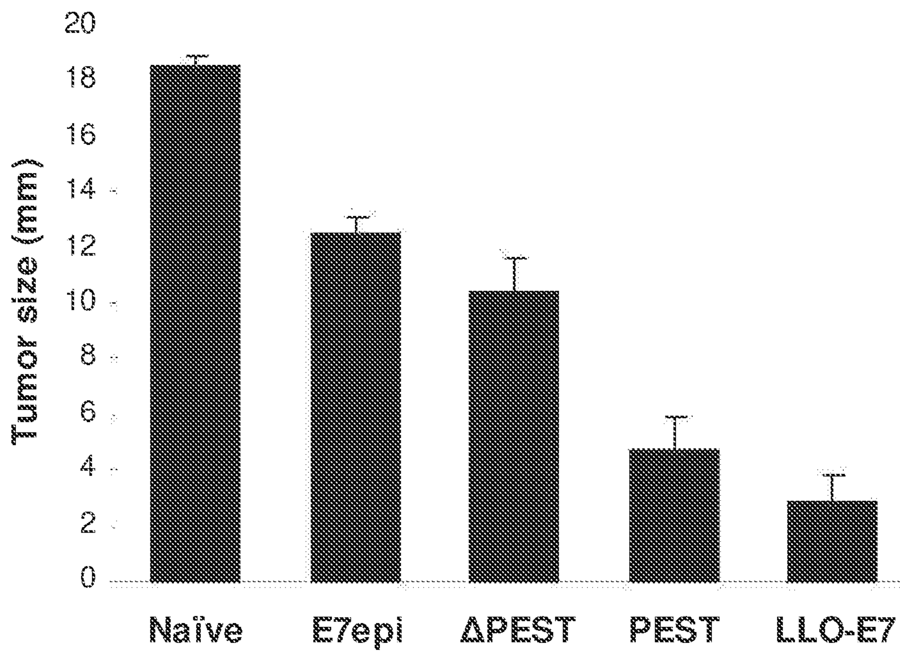


Figure 6B

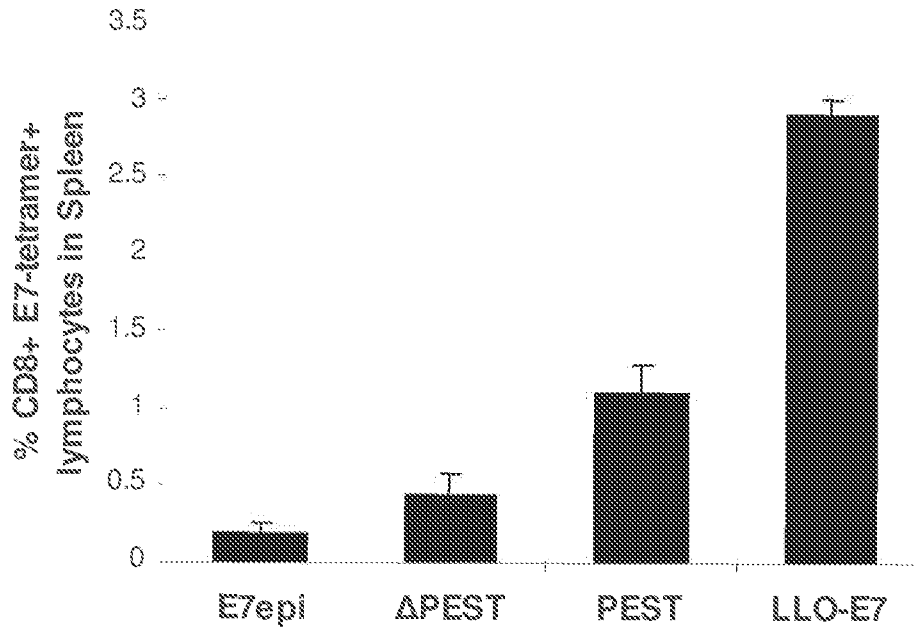


Figure 6C

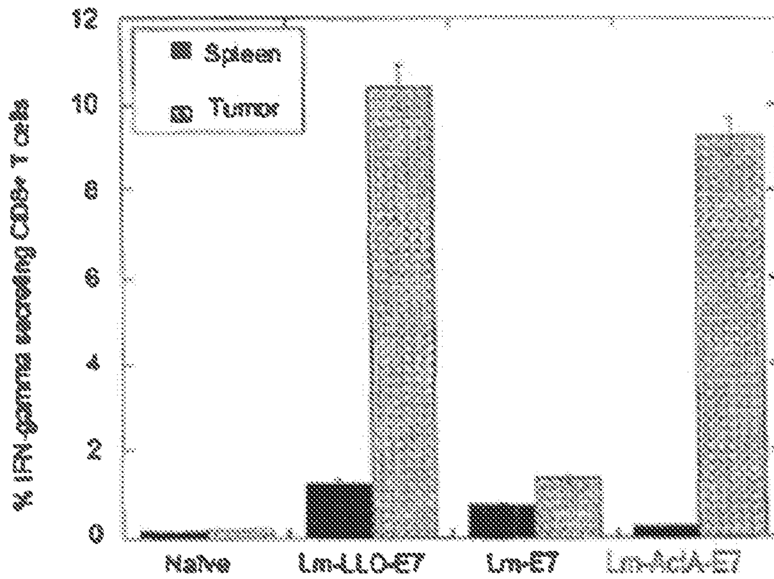


Figure 7A

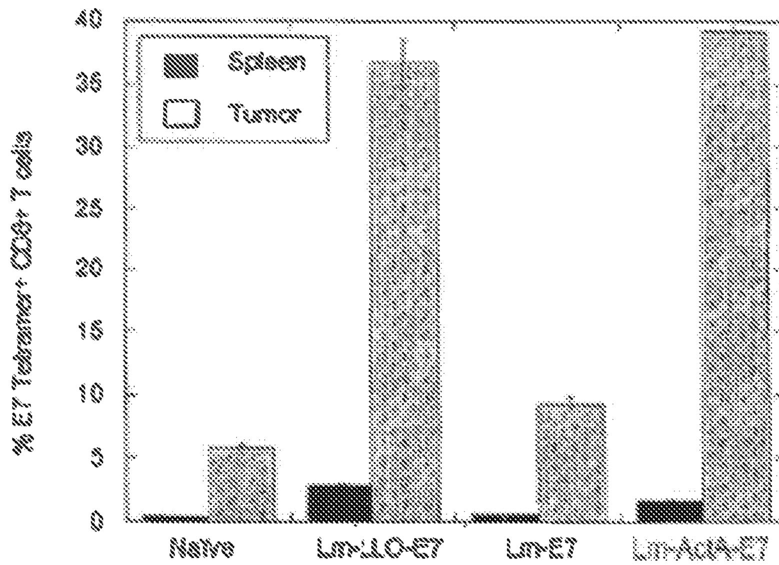


FIGURE 7B

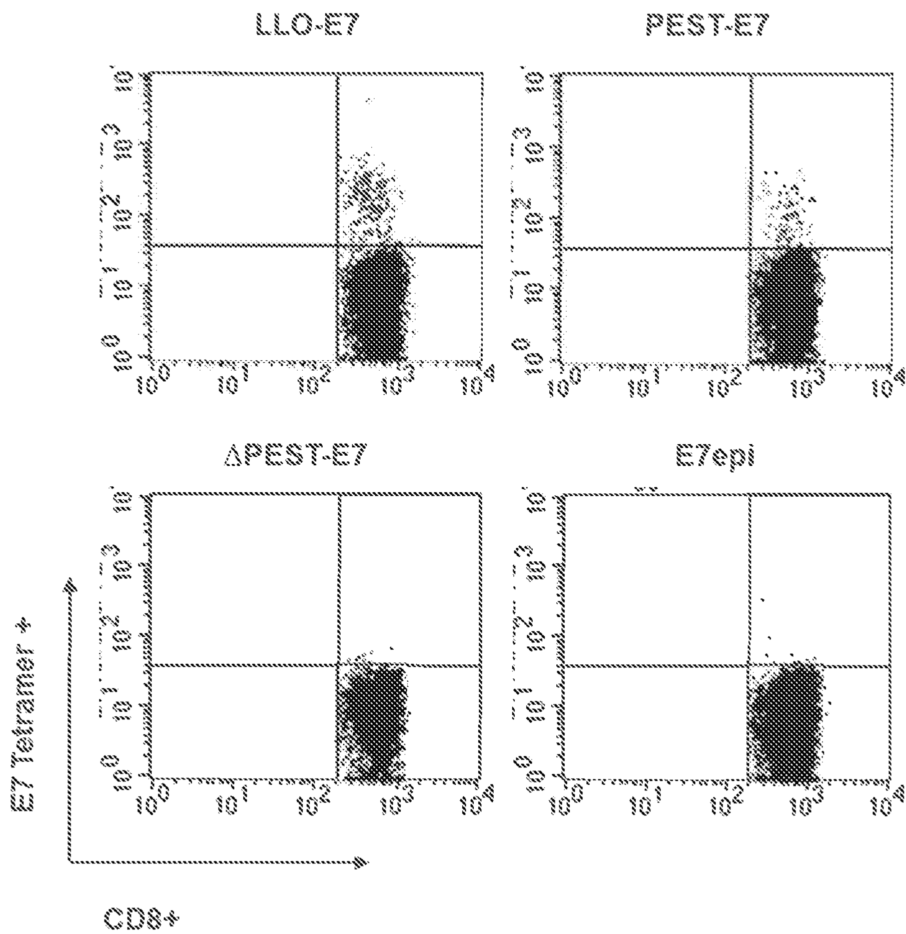


Figure 8A

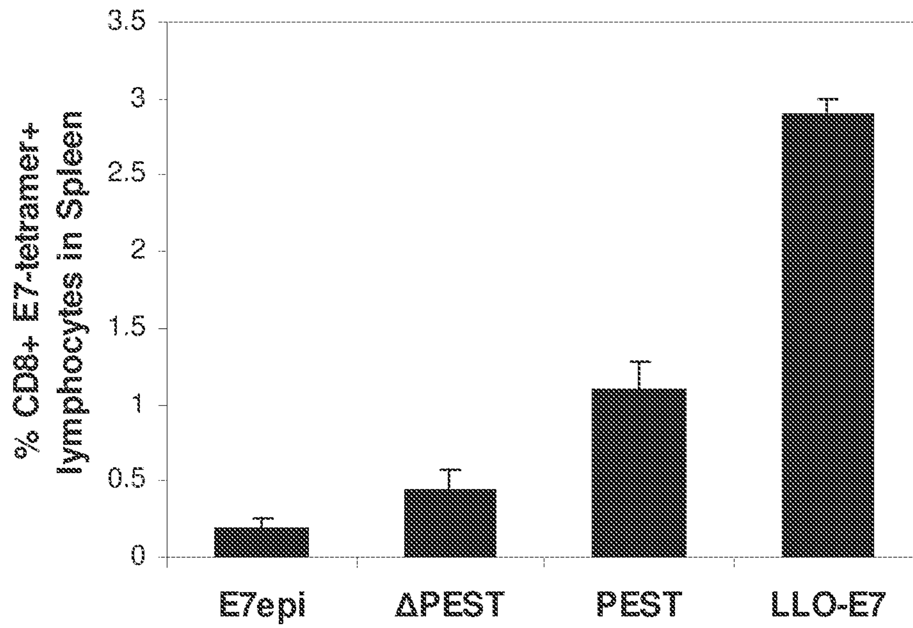


FIGURE 8B

COHORT 1				Tumor Site 1- Size				Tumor Site 2 - Size			
Subj #	Site 1	Site 2	Site 3	Screening S1	d43 S1	d79 S1	d111 S1	Screening S2 Size	d43 S2 Size	d79 S2 Size	d111 S2 Size
1	Paraortal lymph nodes			40	40	44	47				
2	Supraclavicular sin	Parahilar right		30	45	70		10	35	27	
3	Pelvis (soft tissue)			50	60	60	65				
4	Right, upper lobe	Right, lower lobe		20	20	18	18	20	20	14	14
5*	Cervix	Paraortic node	paracaval node *	65	72			13	15		
mean				41	47.4	48	43.33	14.33	23.3	20.5	14
SE				7.81	8.87	11.34	13.69	2.96	6.01	6.5	

* Patient 5 in cohort 1 had a third tumor site, which changed size from 13 to 14 mm between screening and d43.

COHORT 2											
Subj #	Site 1	Site 2	Site 3	Screening S1	d43 S1	d79 S1	d111 S1	Screening S2 Size	d43 S2 Size	d79 S2 Size	d111 S2 Size
03-001-T	Retroperitoneal nodes	Liver tumor		90				34			
04-002-T	Vagina	Vagina		20	23			15			
04-003-T	Uterus	Retrocrural	Iliac Right Region	13	11	9.4		14	14	12	
04-004-T	Para aortic node			32							
04-005-T	Uterus	Ovary and salping	Iliac node left	65				38			
Mean				44	17	9.4		25.25	14	12	

Subj #	SITE 3 DATA- COHORT 2	Screening S1	d43 S1	d79 S1	d111 S1
3		13	13	0	
5		21			
mean		17	13	0	

FIGURE 9

Figure 10A

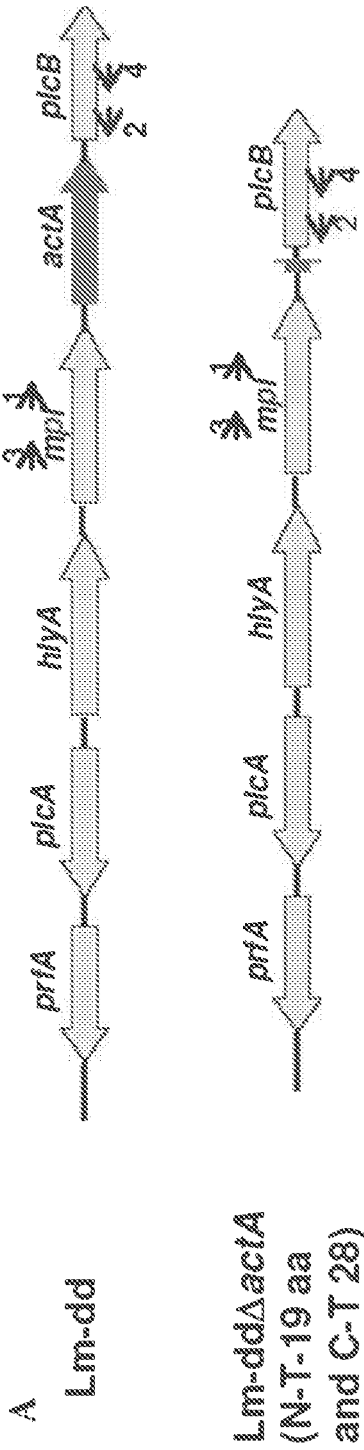


Figure 10B

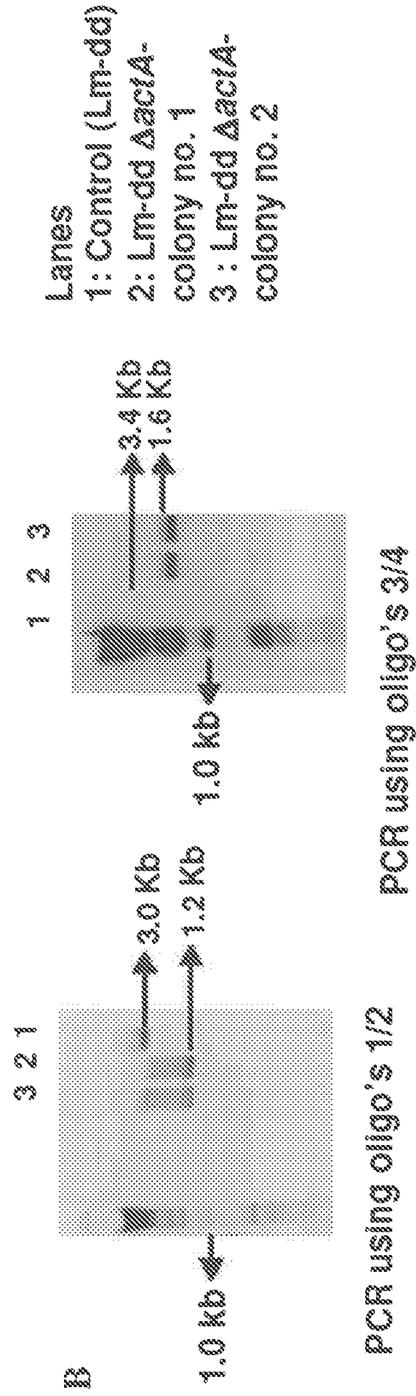


FIGURE 10

Figure 11A

A

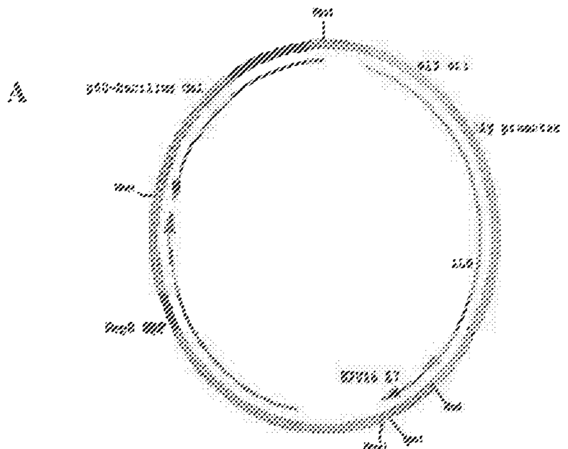


Figure 11B

B

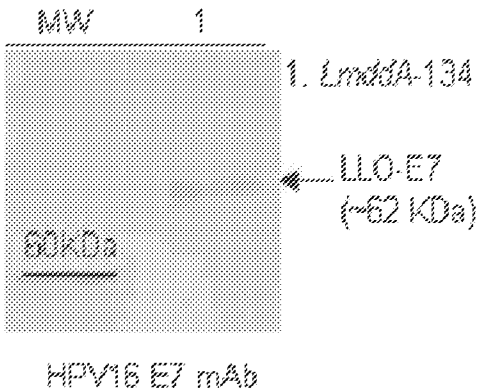


Figure 11C

C

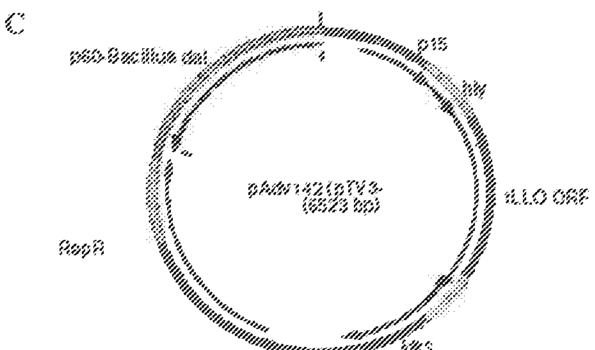


Figure 11D

D

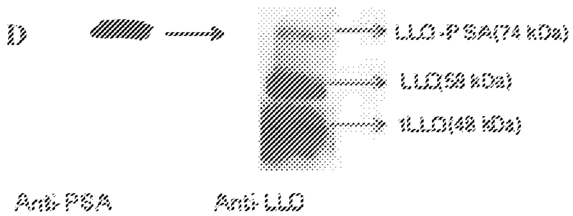


FIGURE 11

Figure 12A

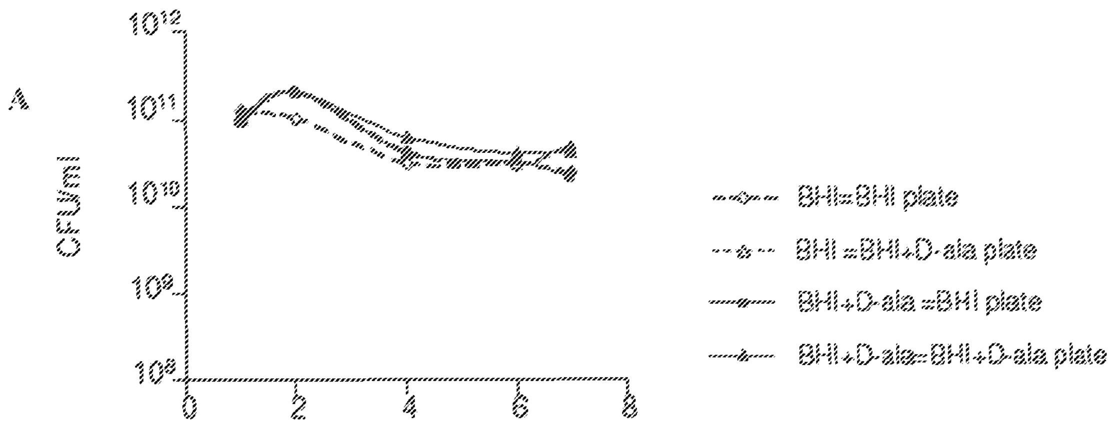


Figure 12B

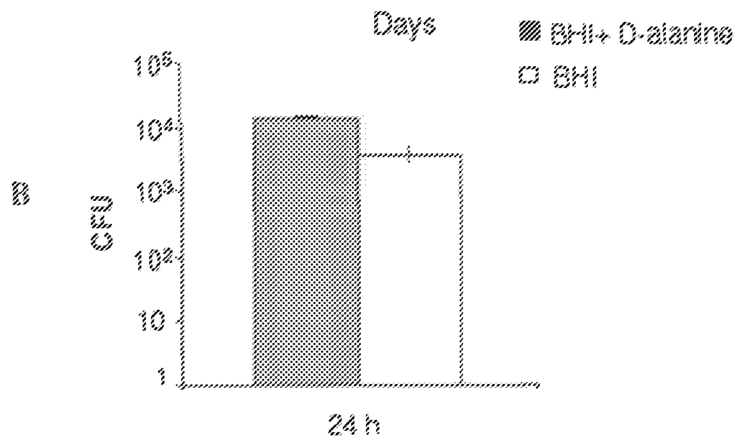


FIGURE 12

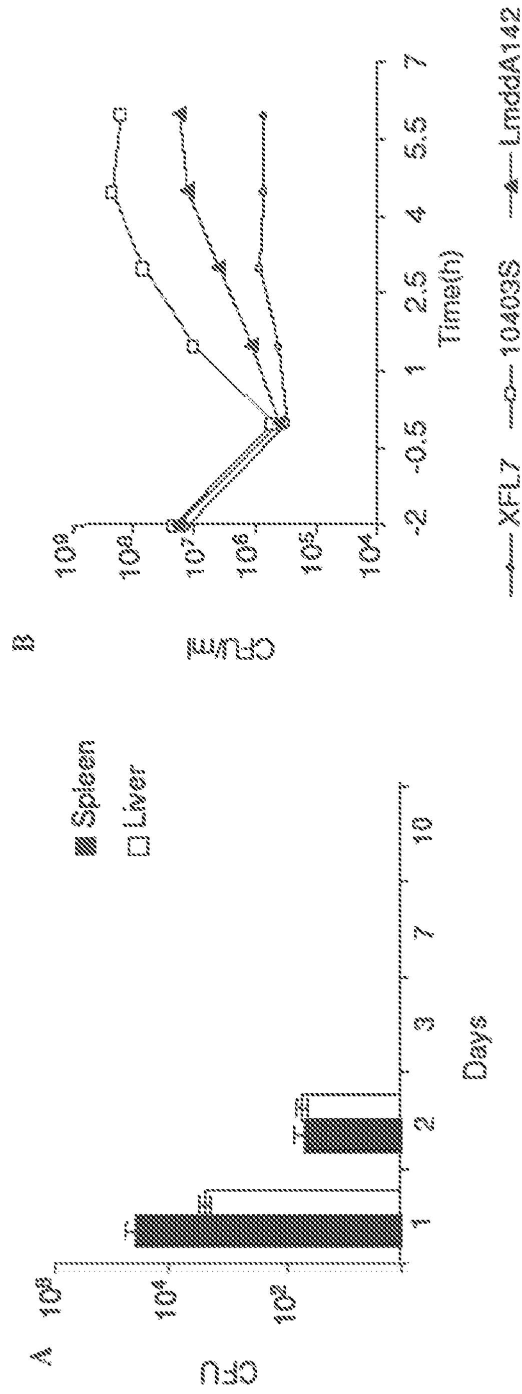


Figure 13A

Figure 13B

FIGURE 13

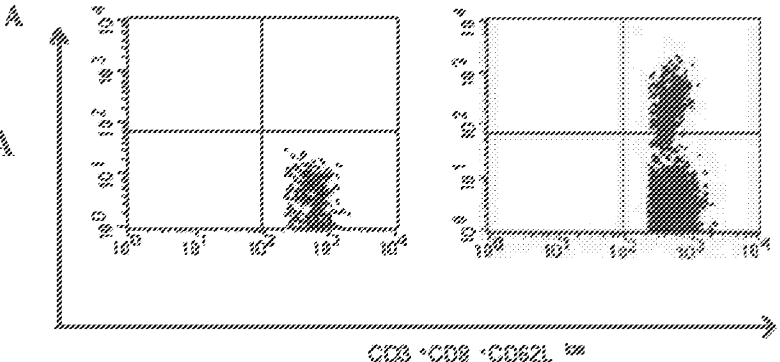


Figure 14A

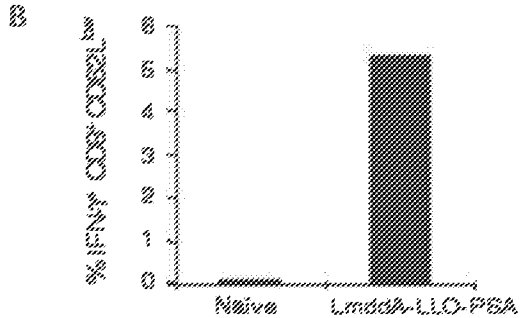


Figure 14B

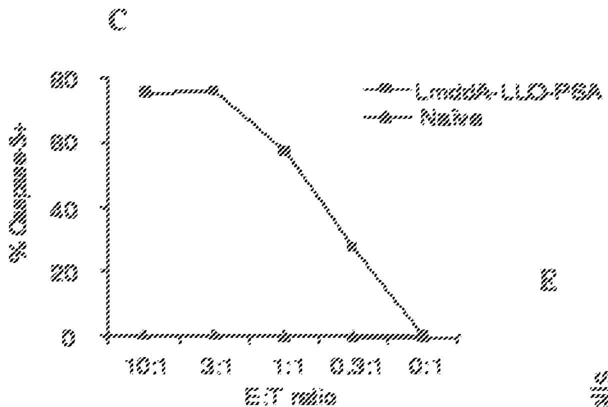


Figure 14C

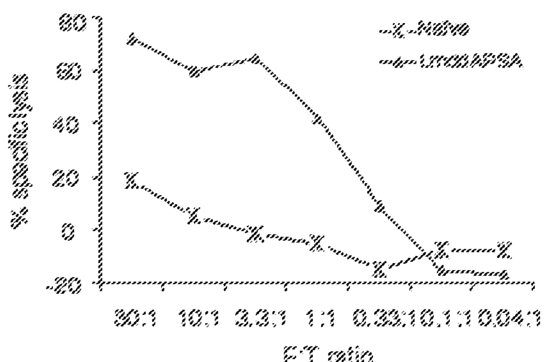


Figure 14D

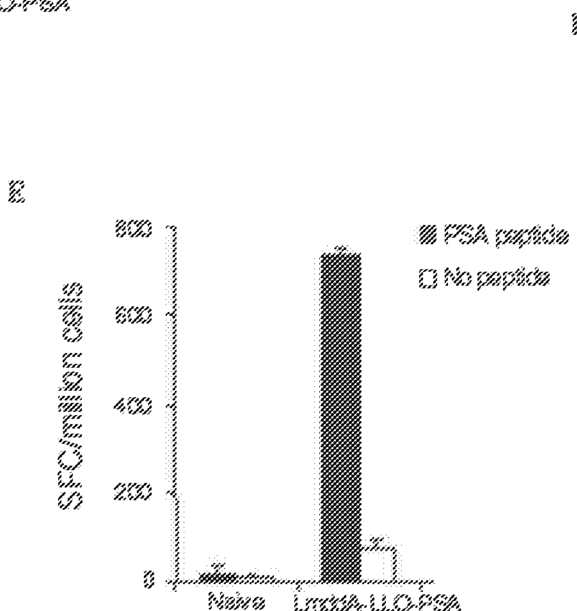


Figure 14E

FIGURE 14

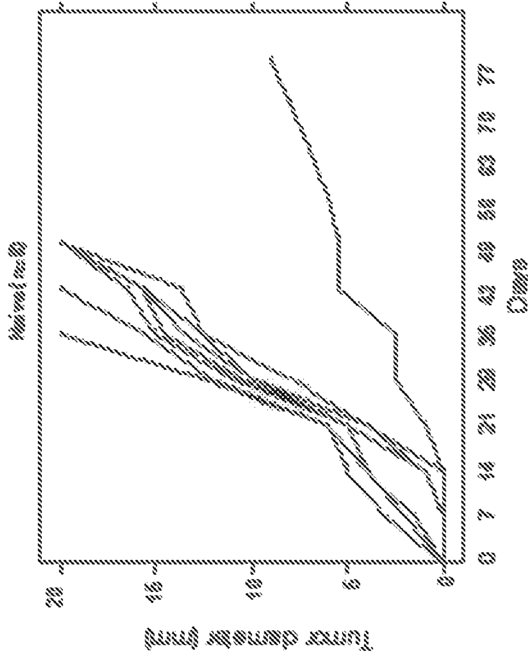


Figure 15A

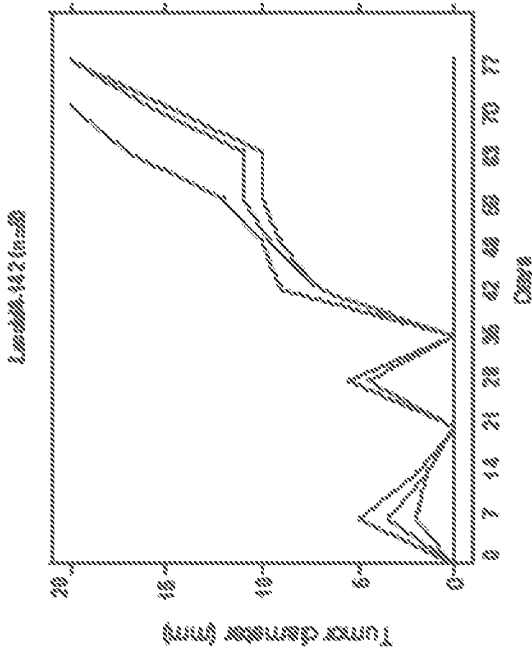


Figure 15B

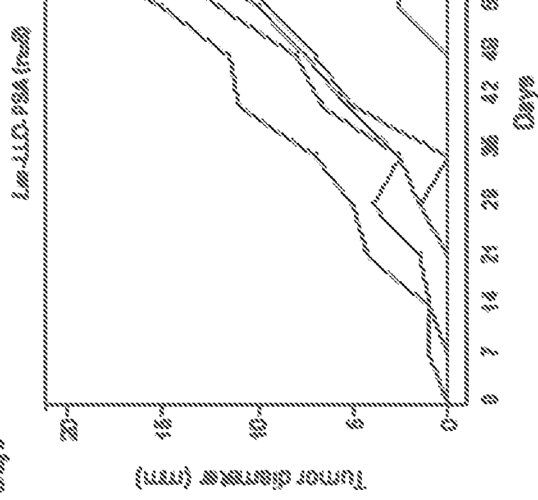


Figure 15C

B

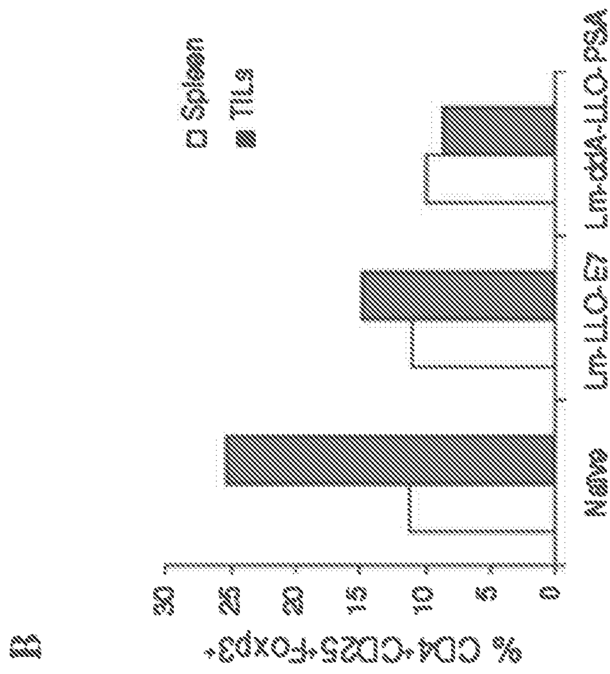


Figure 16B

A

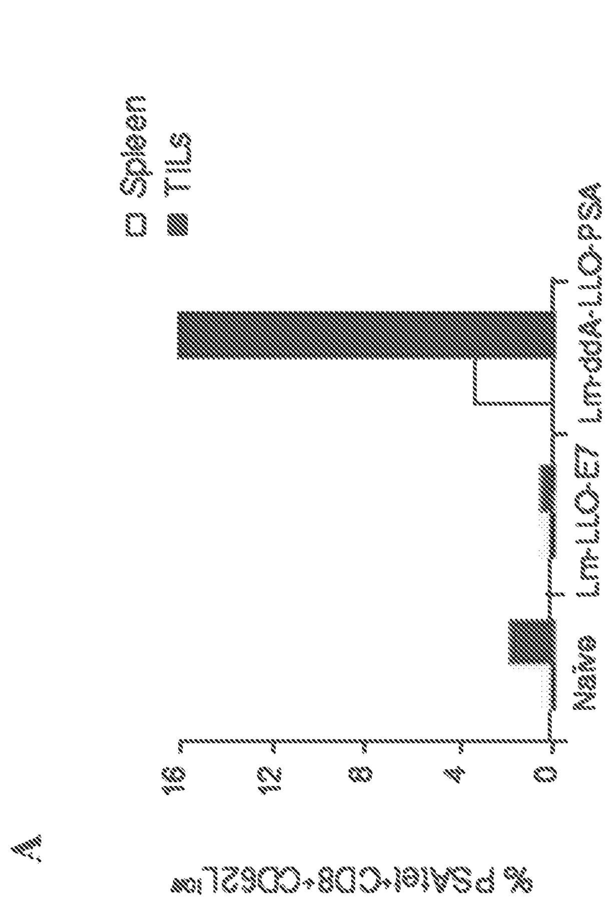


Figure 16A

FIGURE 16

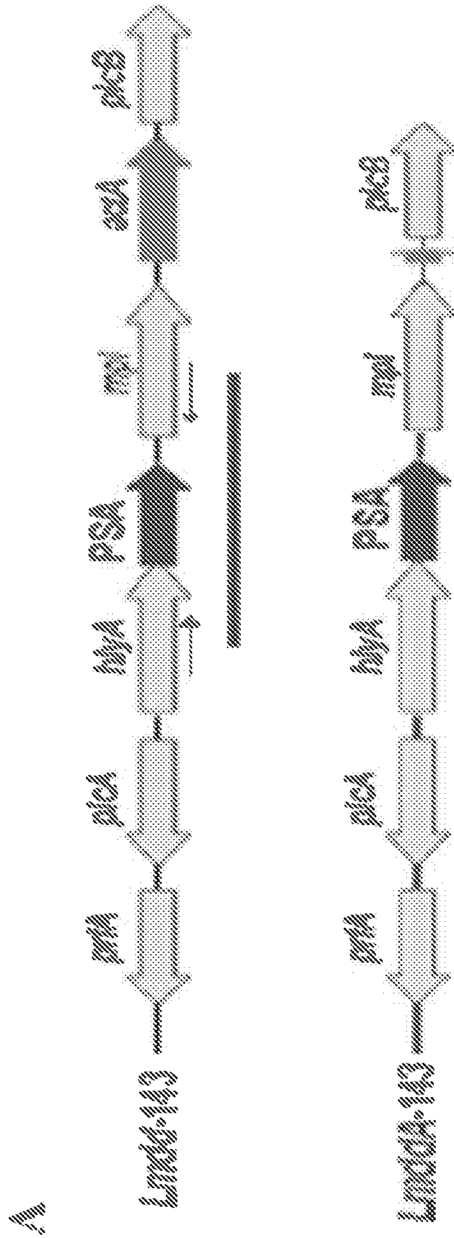


Figure 17A

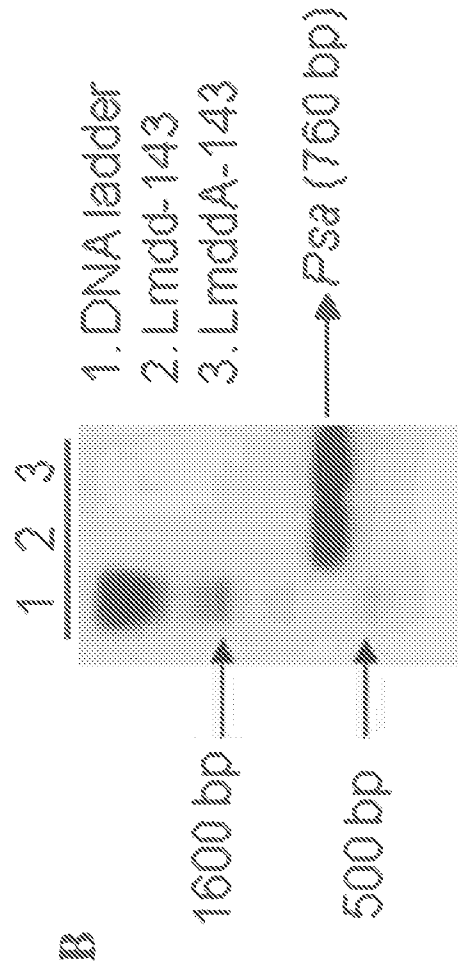
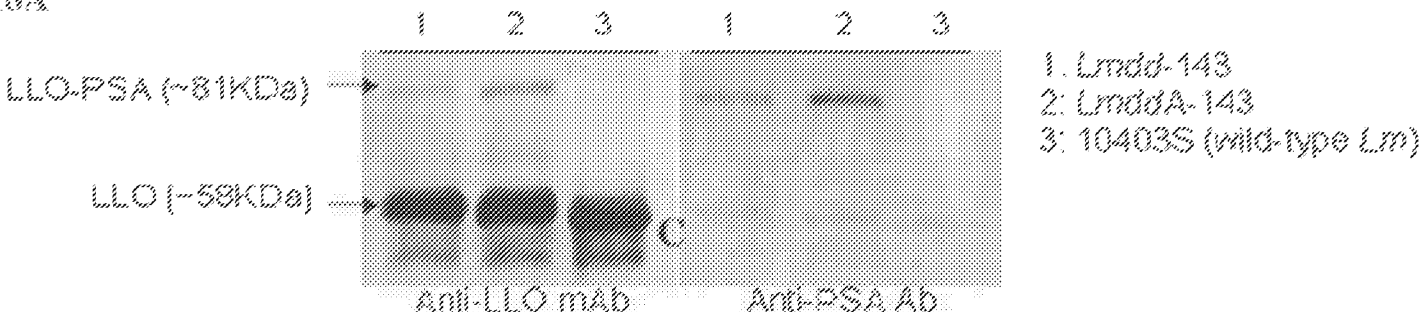


Figure 17B

Figure 18A



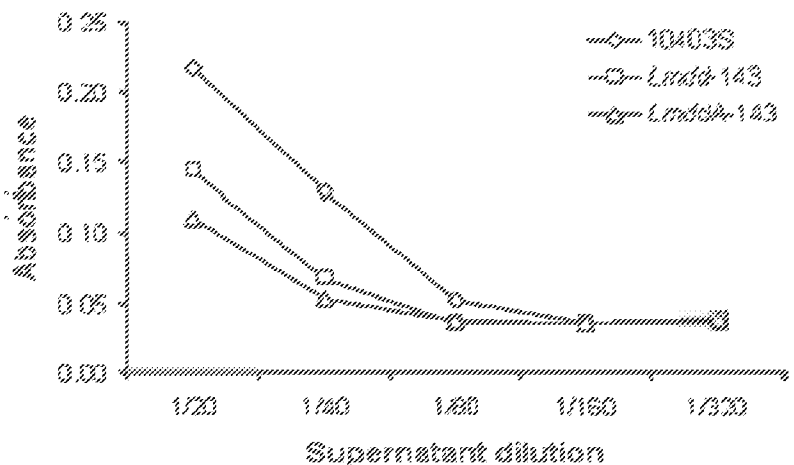


Figure 18B

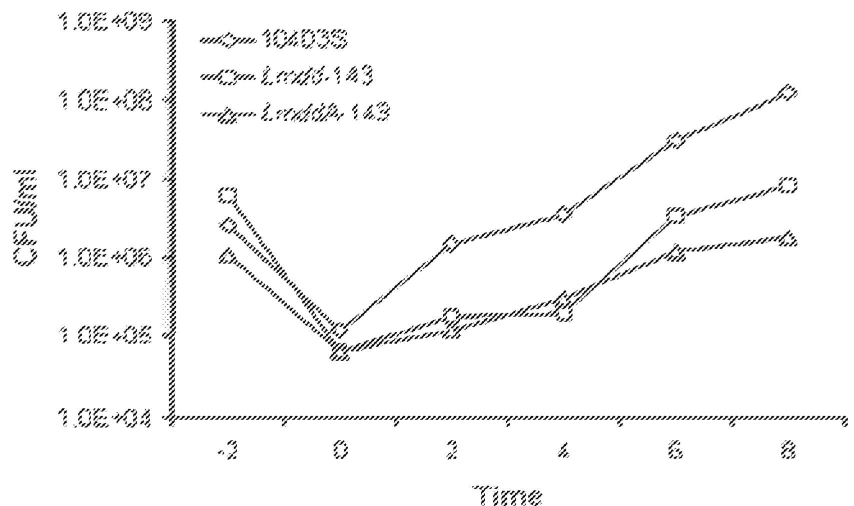


Figure 18C

FIGURE 18

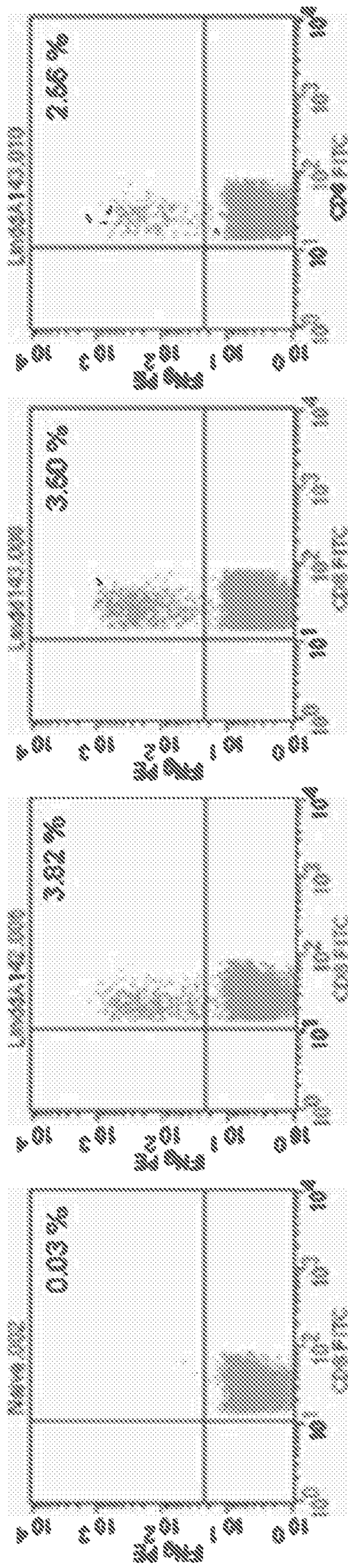


FIGURE 19

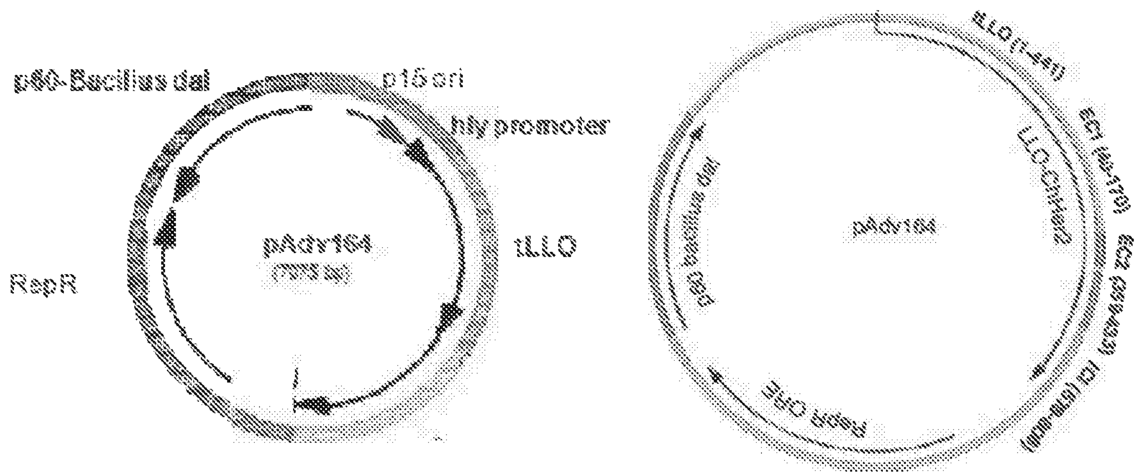


Figure 20A

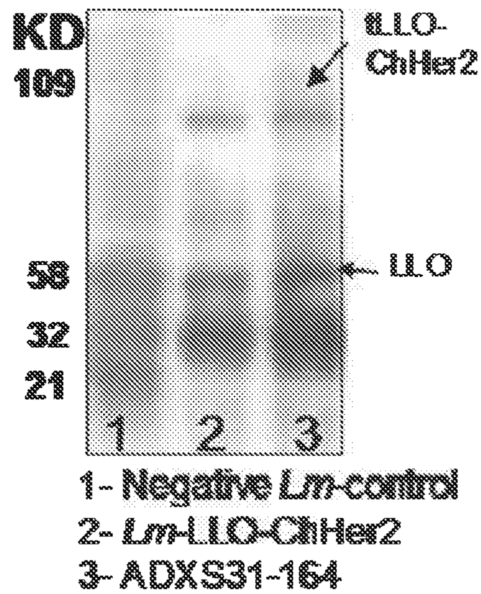


Figure 20B

Figure 21A

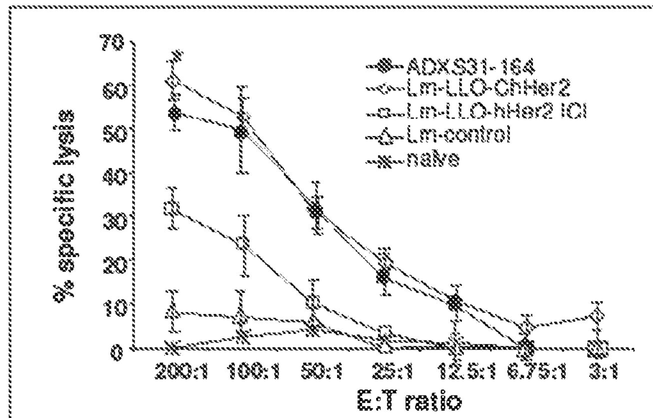


Figure 21B

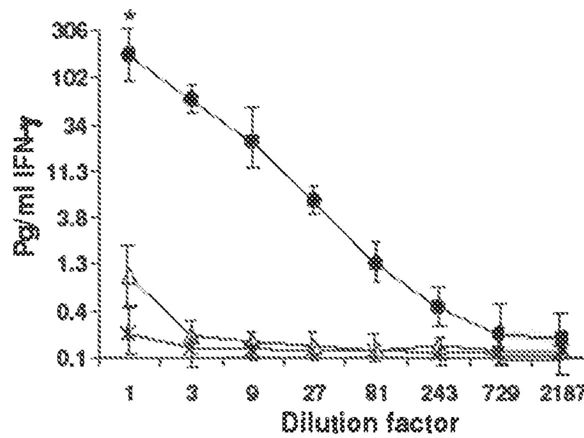


Figure 21C

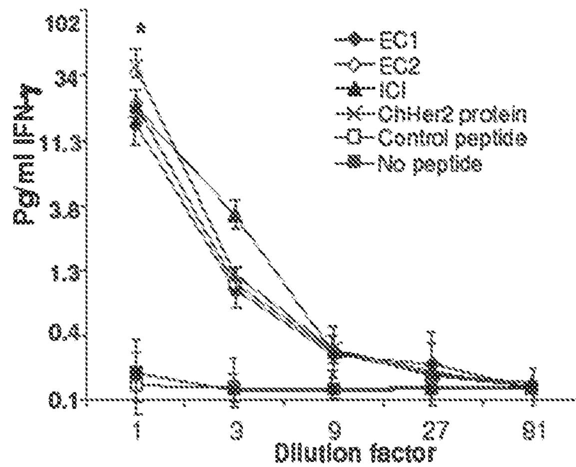


FIGURE 21

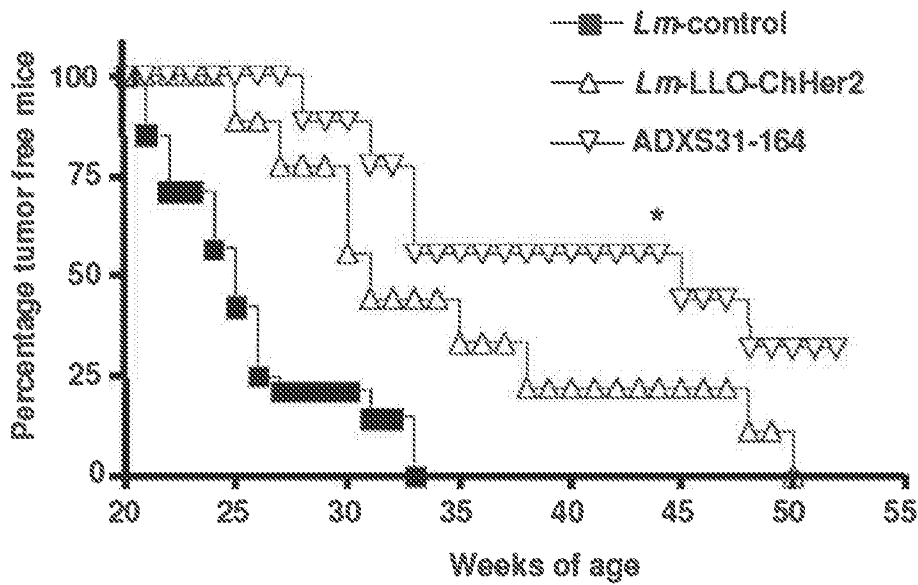


FIGURE 22

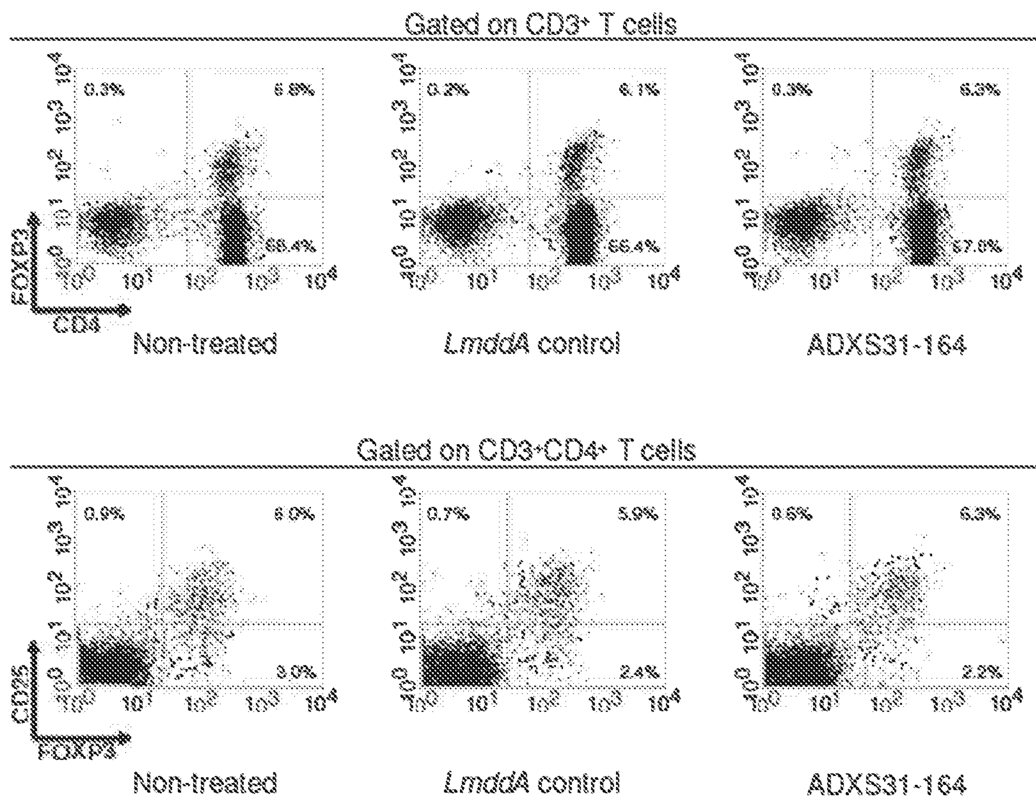


FIGURE 23

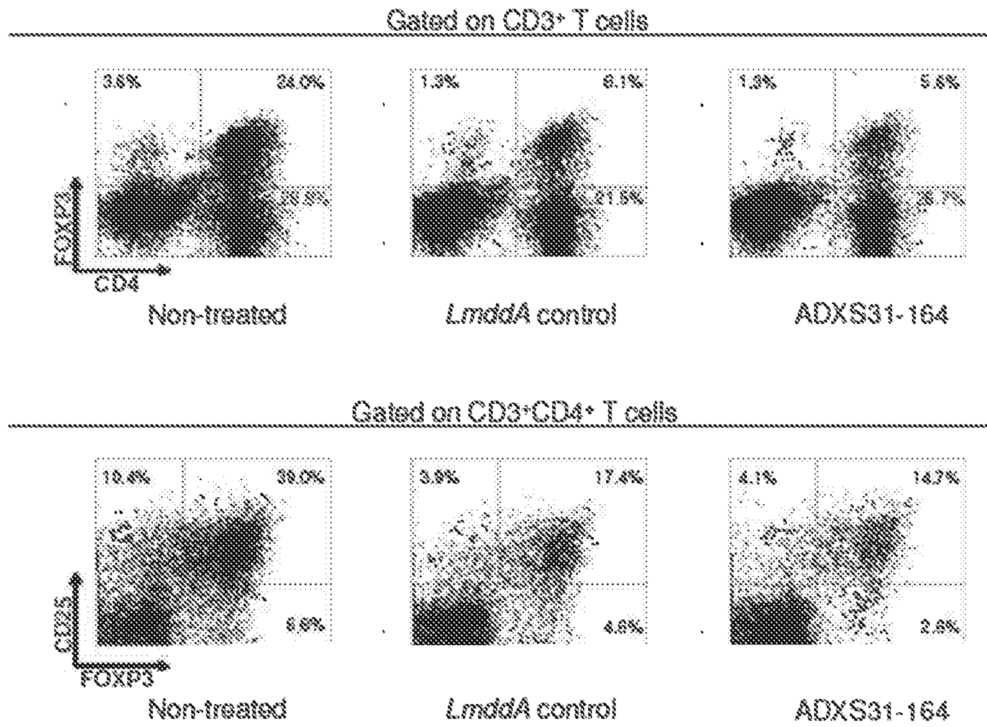


Figure 24A

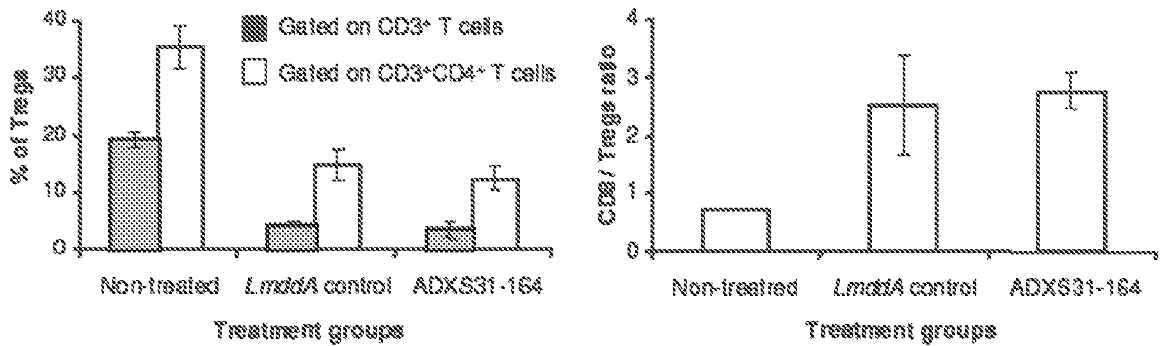


Figure 24B

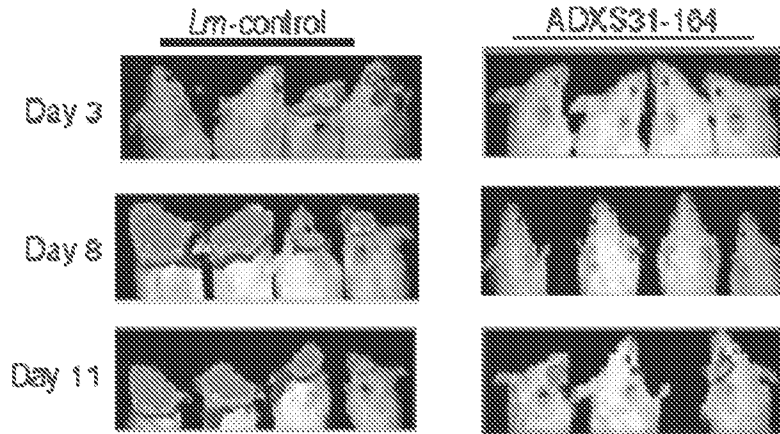


Figure 25A

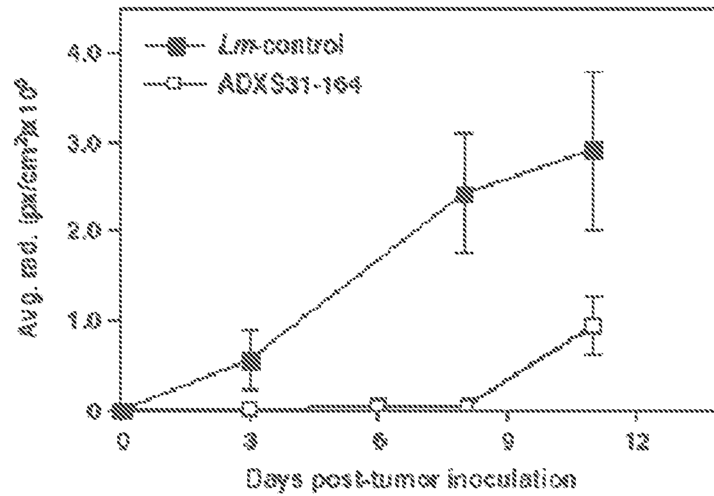


Figure 25B

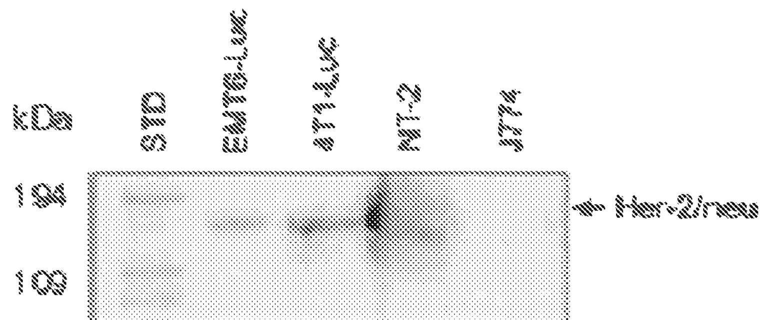


Figure 25C

Figure 26A

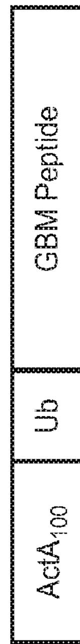


Figure 26B

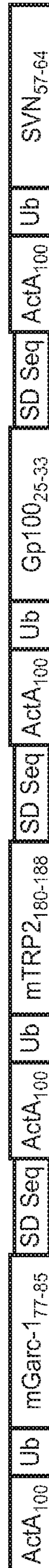


Figure 26C

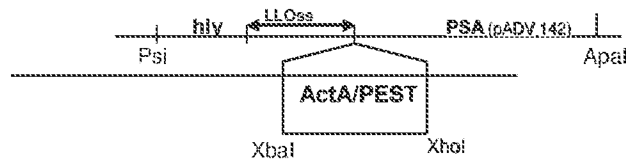


FIGURE 27

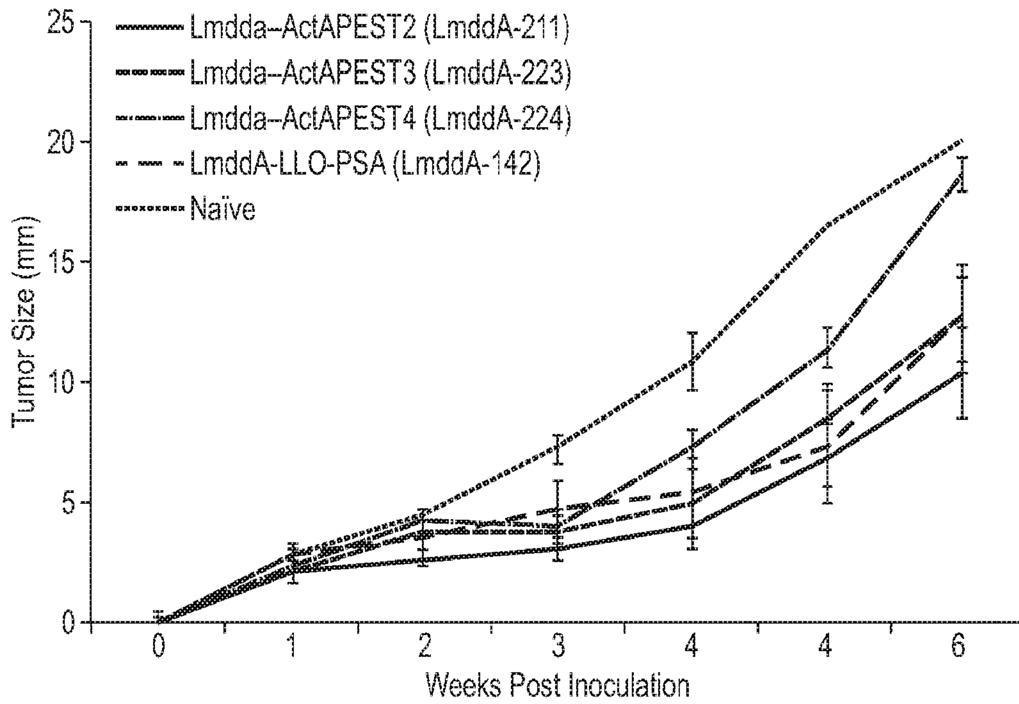


Figure 28A

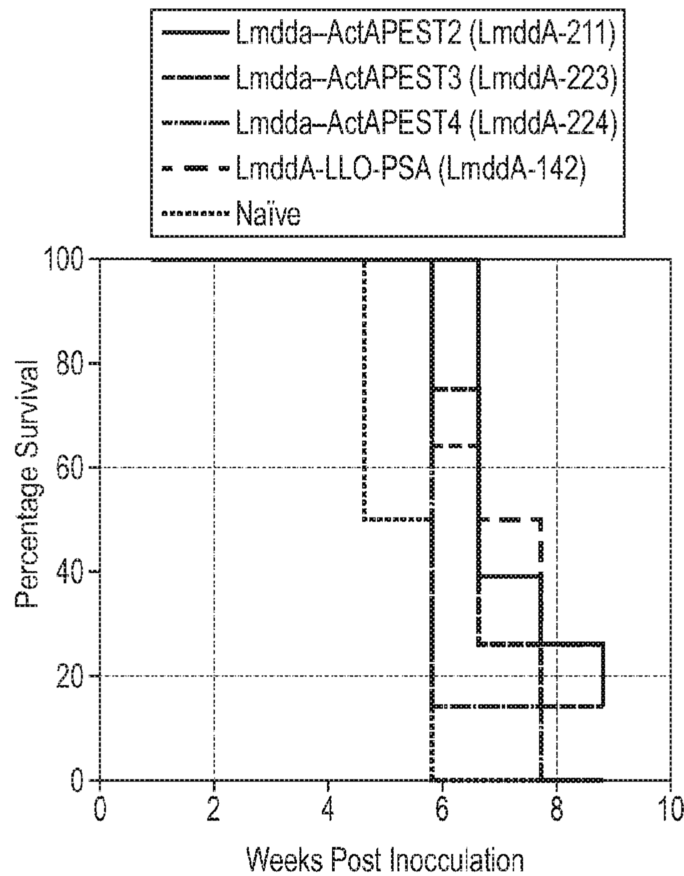


Figure 28B

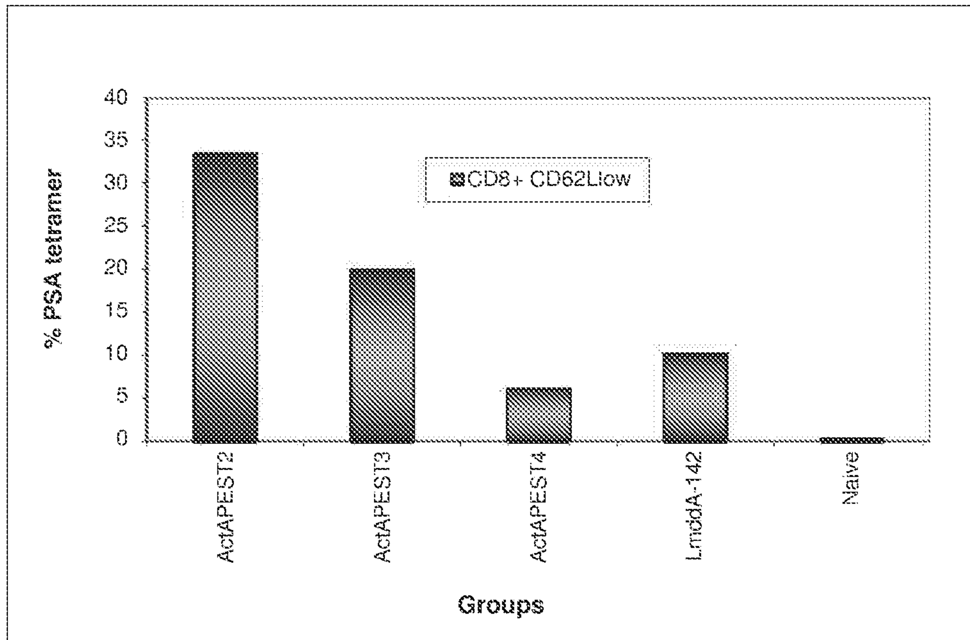


Figure 29A

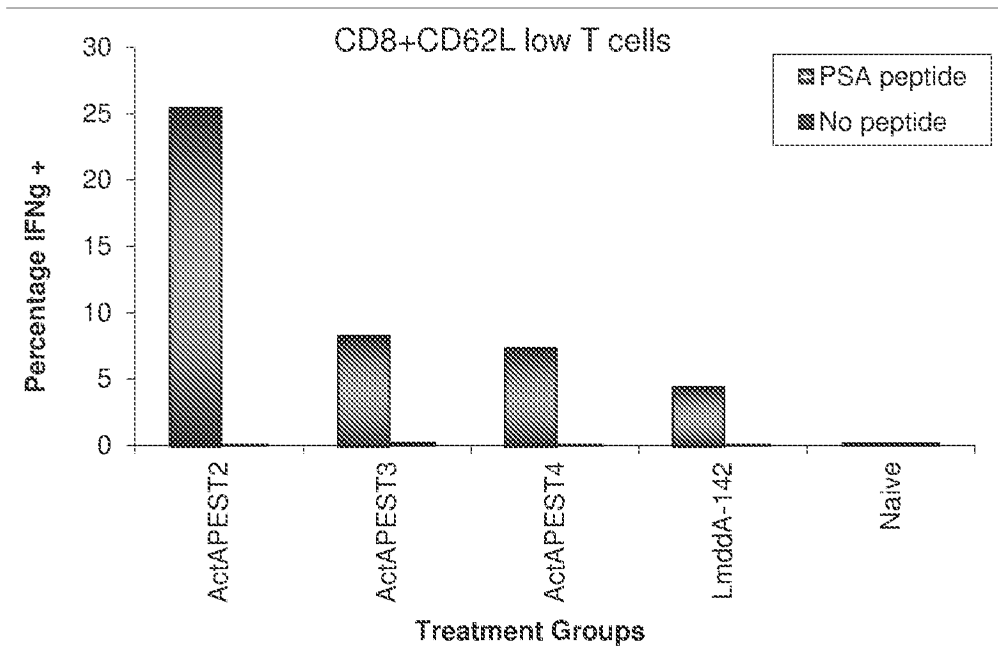


Figure 29B

Treatment Groups	Tumor volume on Day 13 post treatment (mm3)
Naïve	126.1084
LmddA274 (tLLO)	28.3242
ADXS31-142 (tLLO-PSA)	26.2576
LmddA211 (ActA/PEST2-PSA)	36.5254

Figure 30A

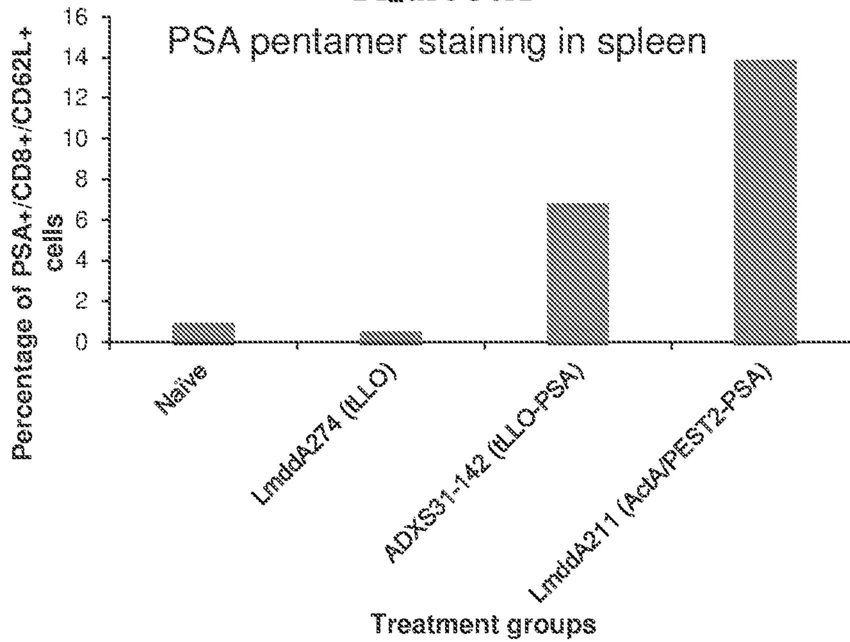


Figure 30B

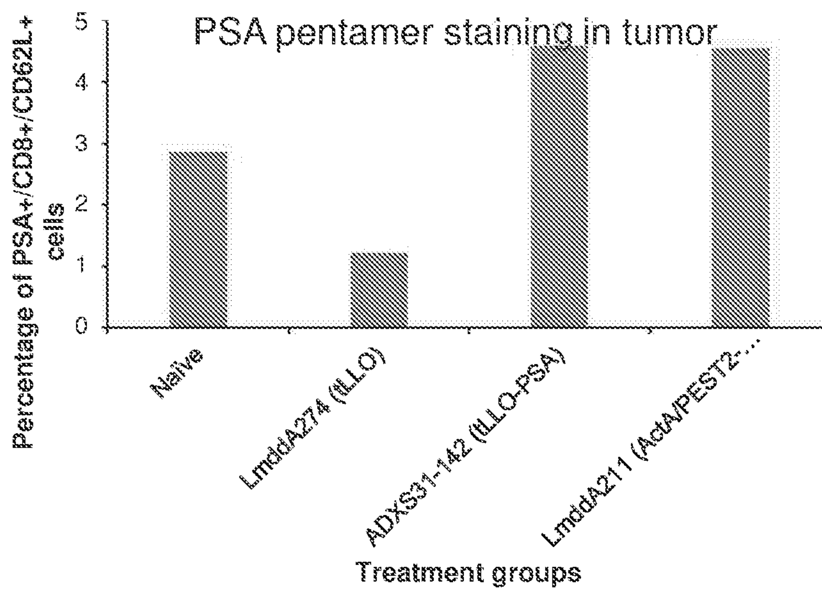


Figure 30C

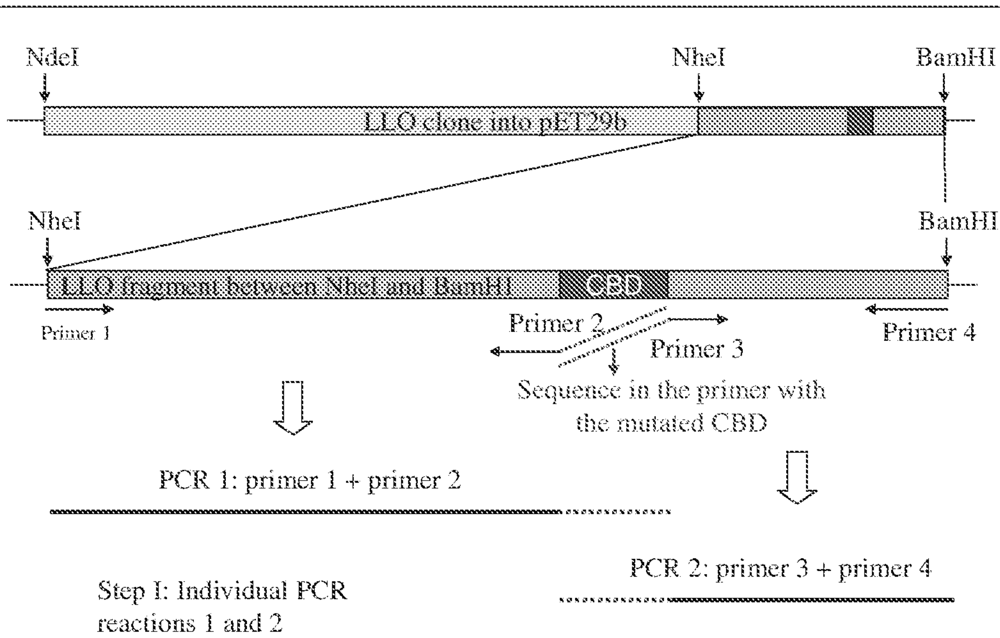
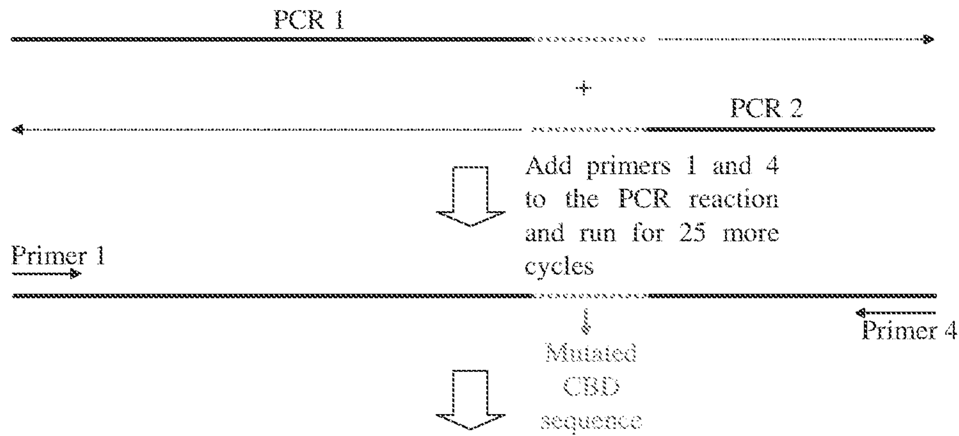


Figure 31A

Step II: mix the products of PCR 1 and PCR 2 and allow the annealing of the complementary region (mutated CBD) between the products from PCR 1 and 2



Clone PCR product into the pCR2.1 vector (Invitrogen)
Verify the sequence of LLO and the mutated CBD

Figure 31B

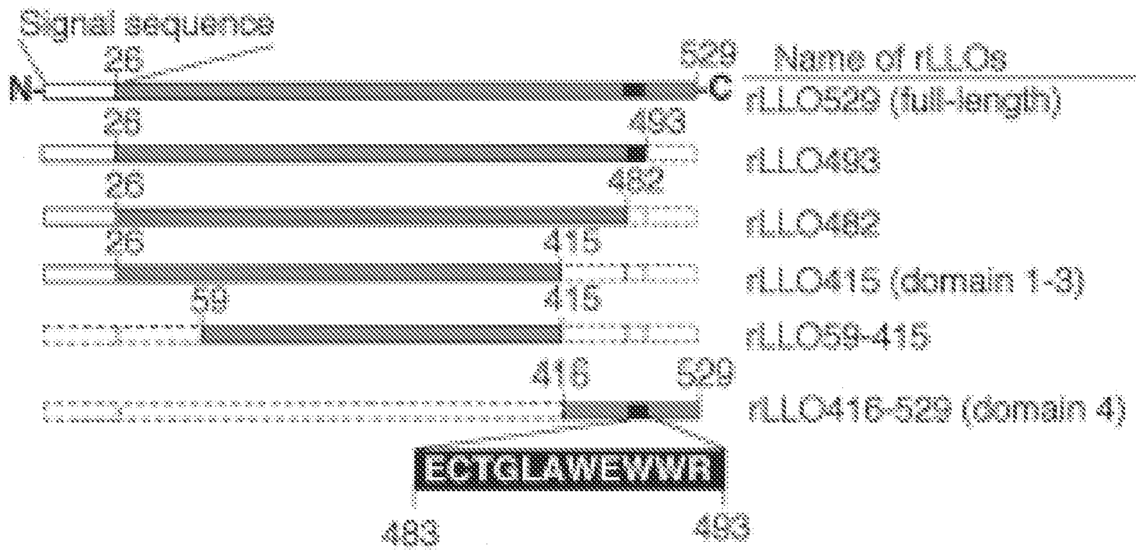


Figure 31C

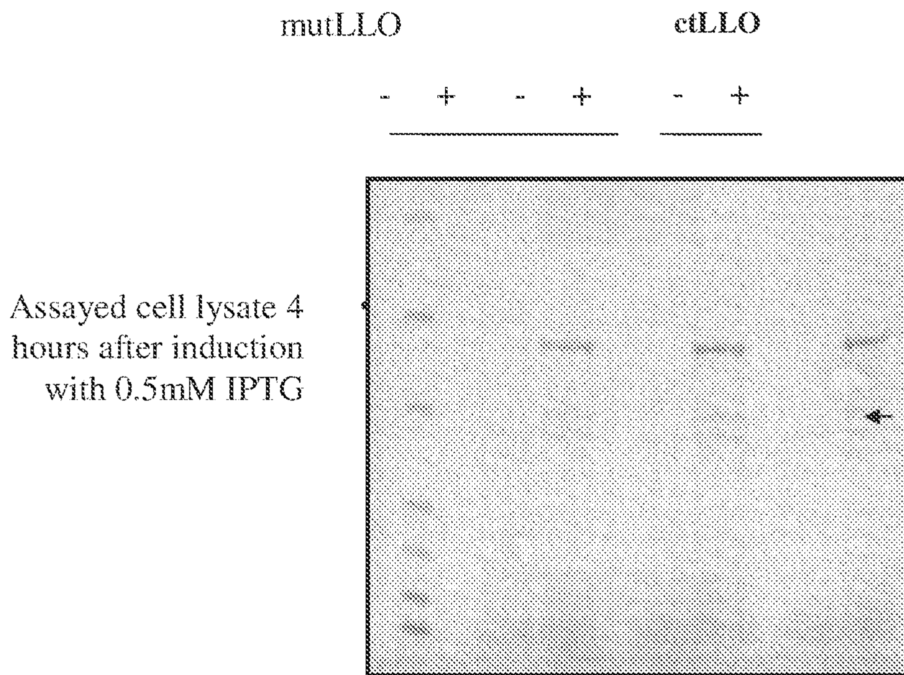


Figure 32A

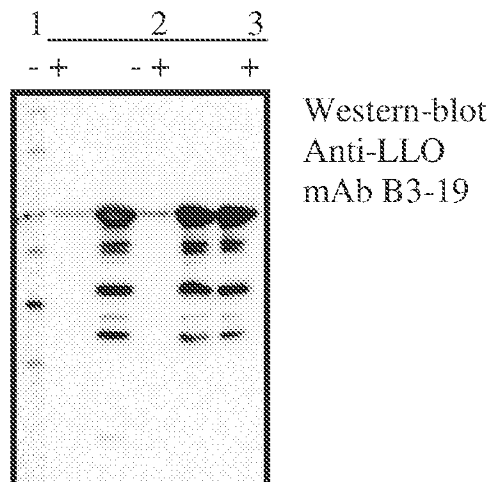
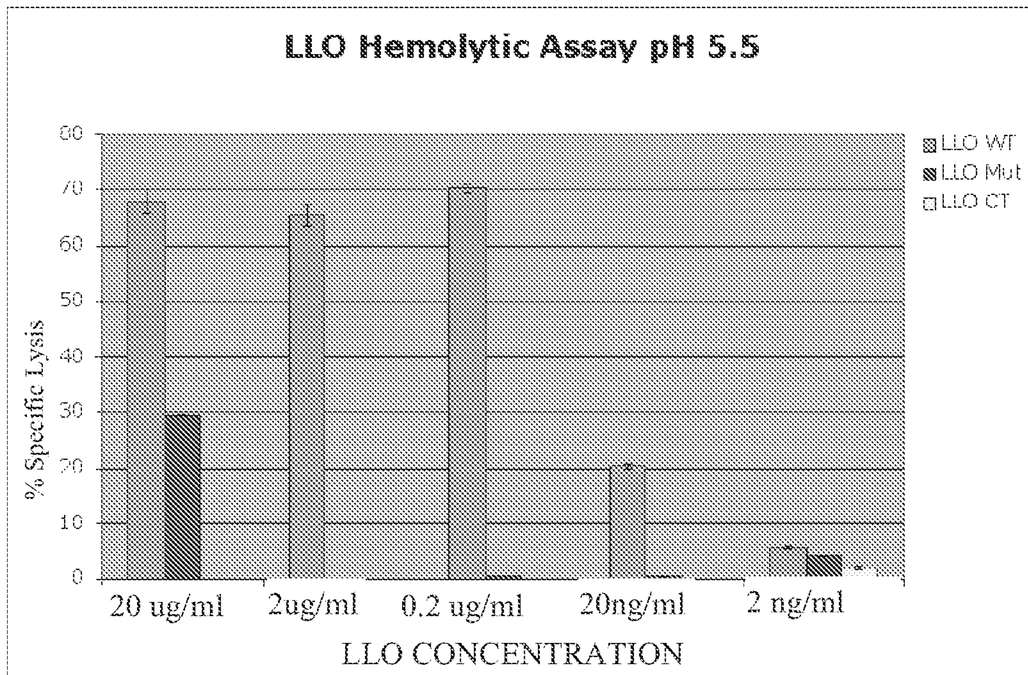
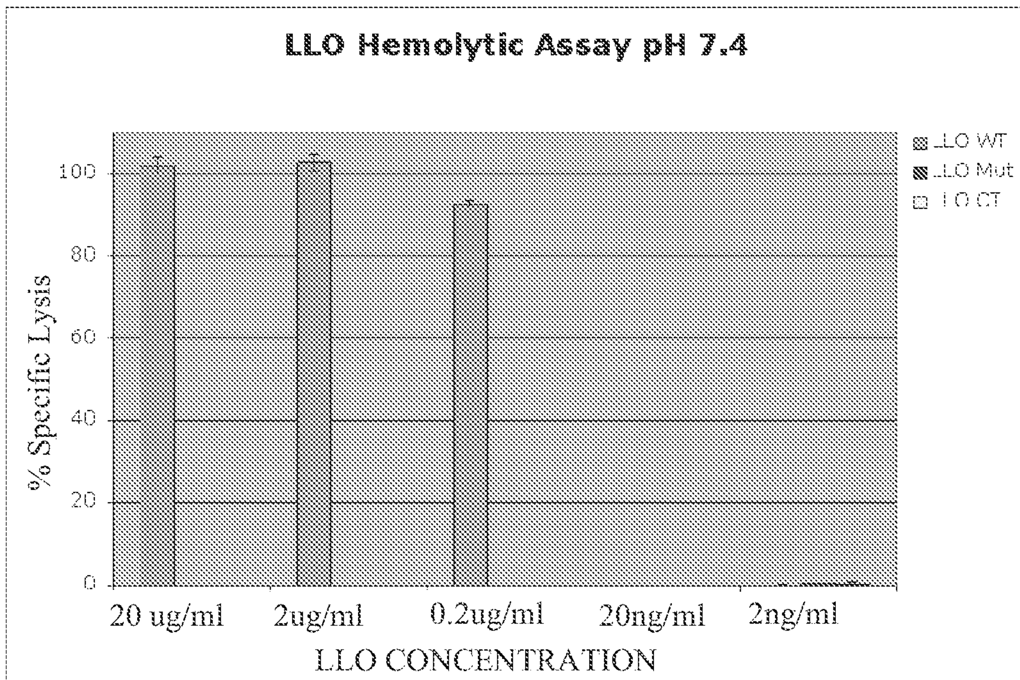


Figure 32B



Figures 33A



Figures 33B

Figure 34

gggctgctcaacgacatccagaagttgtcagtcatcagctccaacaccctgcgtggccgcagccccaccagccggcggcgggcacagtccctggggctgctgggggatgagcactgggccaccgacccagacatgtacctccag
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agggctacctgagtgcctggtgcccctg

Figure 35

G L L N D I Q K L S V I S S N T L R G R S P T S R R R A Q S L G L L G D E H W A T D P D M
Y L Q S P Q S E R T D P H G L Y L S C N G G T P A G H K Q M P W P E P Q S P R V L P N G L
A A K A Q S L G P A E F Q G A S Q R C L Q L G A C L Q S S P P G A S P P T G T N R H G M K
A A K H G S E E A R P Q S C L V G S A T G R P G G E G S P S P K T R E S S L K R R L F R S M
F L S T A A T A P P S S S K P G P P P Q S K P N S S F R P P Q K D N P P S L V A K A Q S L P
S D Q P V G T F S P L T T S D T S S P Q K S L R T A P A T G Q L P G R S S P A G S P R T W
H A Q I S T S N L Y L P Q D P T V A K G A L A G E D T G V V T H E Q F K A A L R M V V D Q
G D P R L L L D S Y V K I G E G S T G I V C L A R E K H S G R Q V A V K M M D L R K Q Q R
R E L L F N E V V I M R D Y Q H F N V V E M Y K S Y L V G E E L W V L M E F L Q G G A L T
D I V S Q V R L N E E Q I A T V C E A V L Q A L A Y L H A Q G V I H R D I K S D S I L L T L D
G R V K L S D F G F C A Q I S K D V P K R K S L V G T P Y W M A P E V I S R S L Y A T E V D
I W S L G I M V I E M V D G E P P Y F S D S P V Q A M K R L R D S P P P K L K N S H K V S
P V L R D F L E R M L V R D P Q E R A T A Q E L L D H P F L L Q T G L P E C L V P L

Figure 36

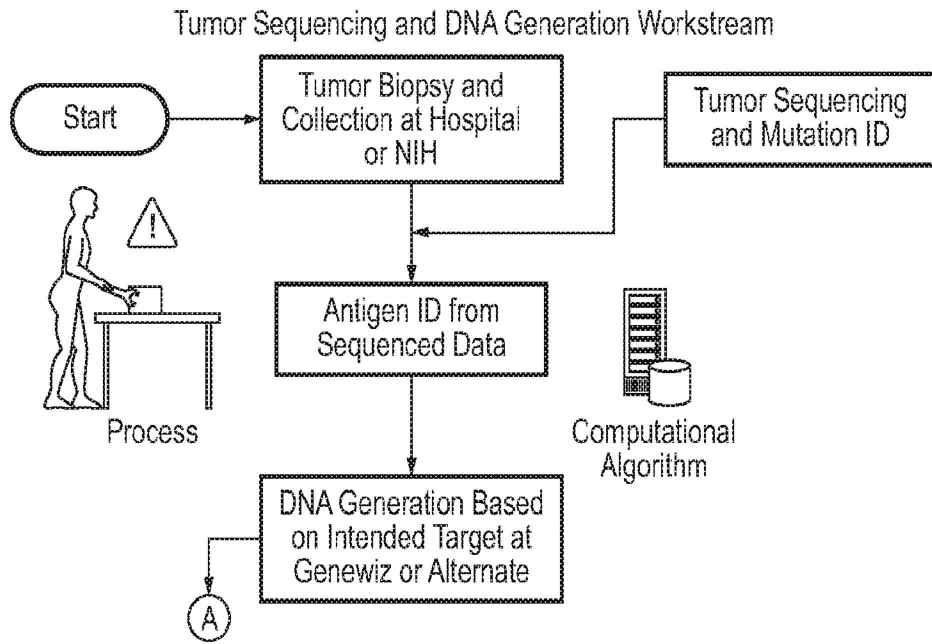


Figure 37A

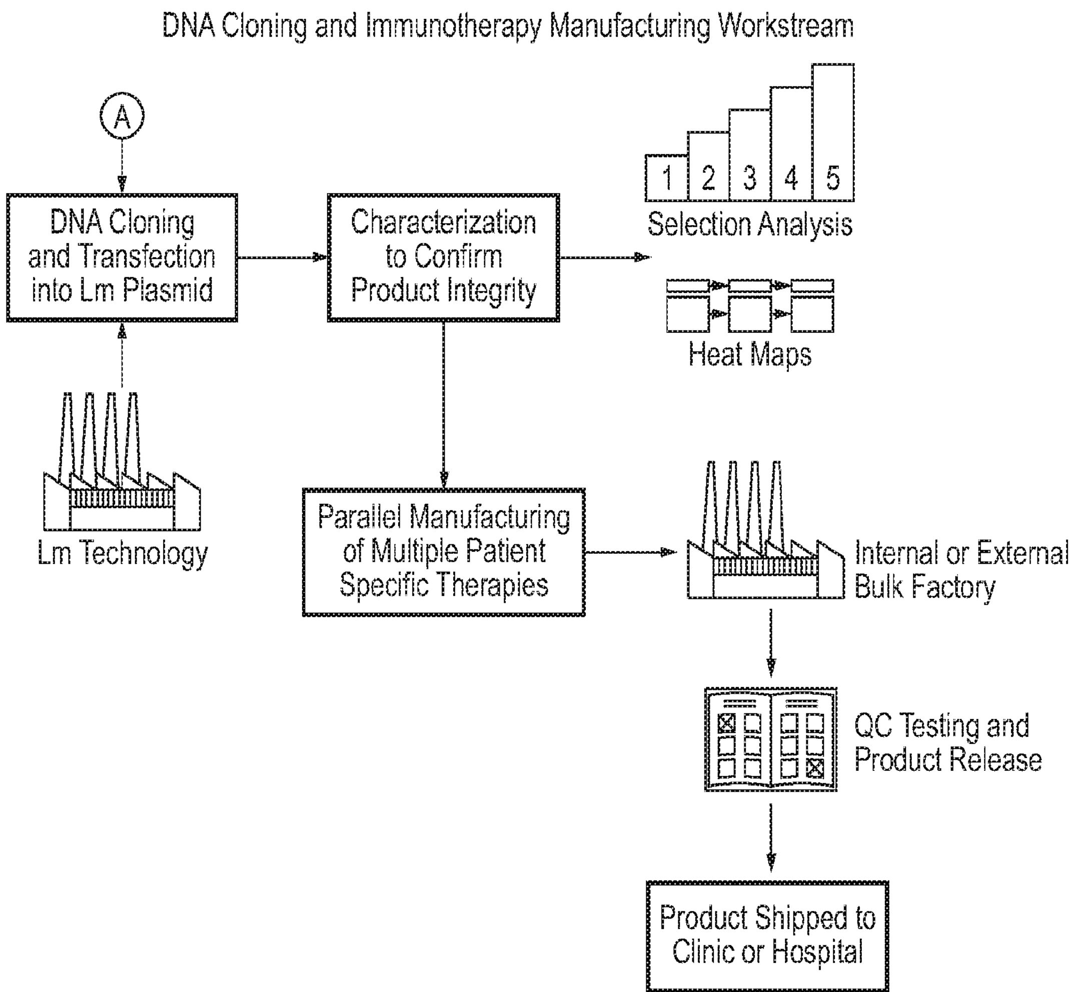


Figure 37B

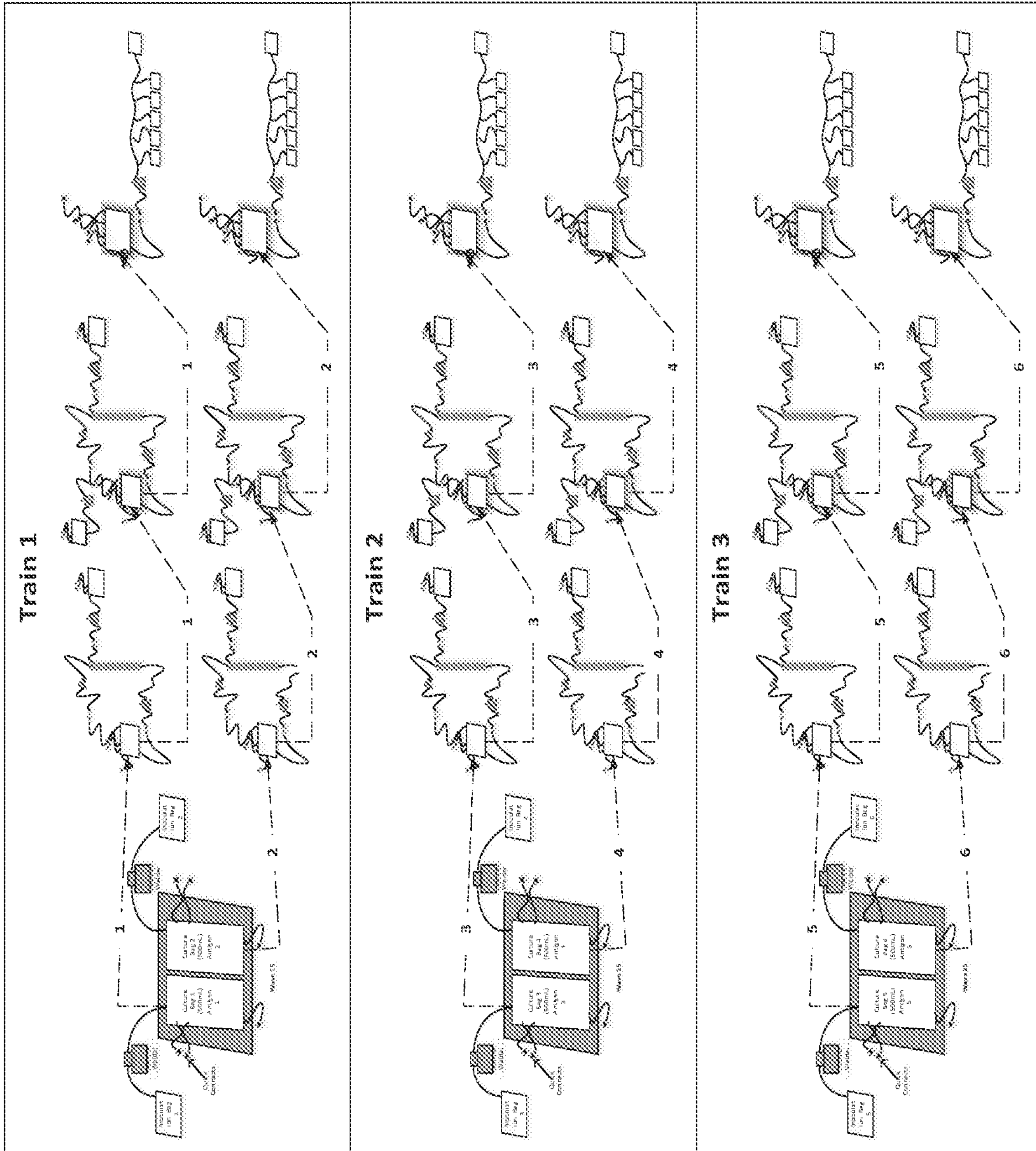


Figure 38

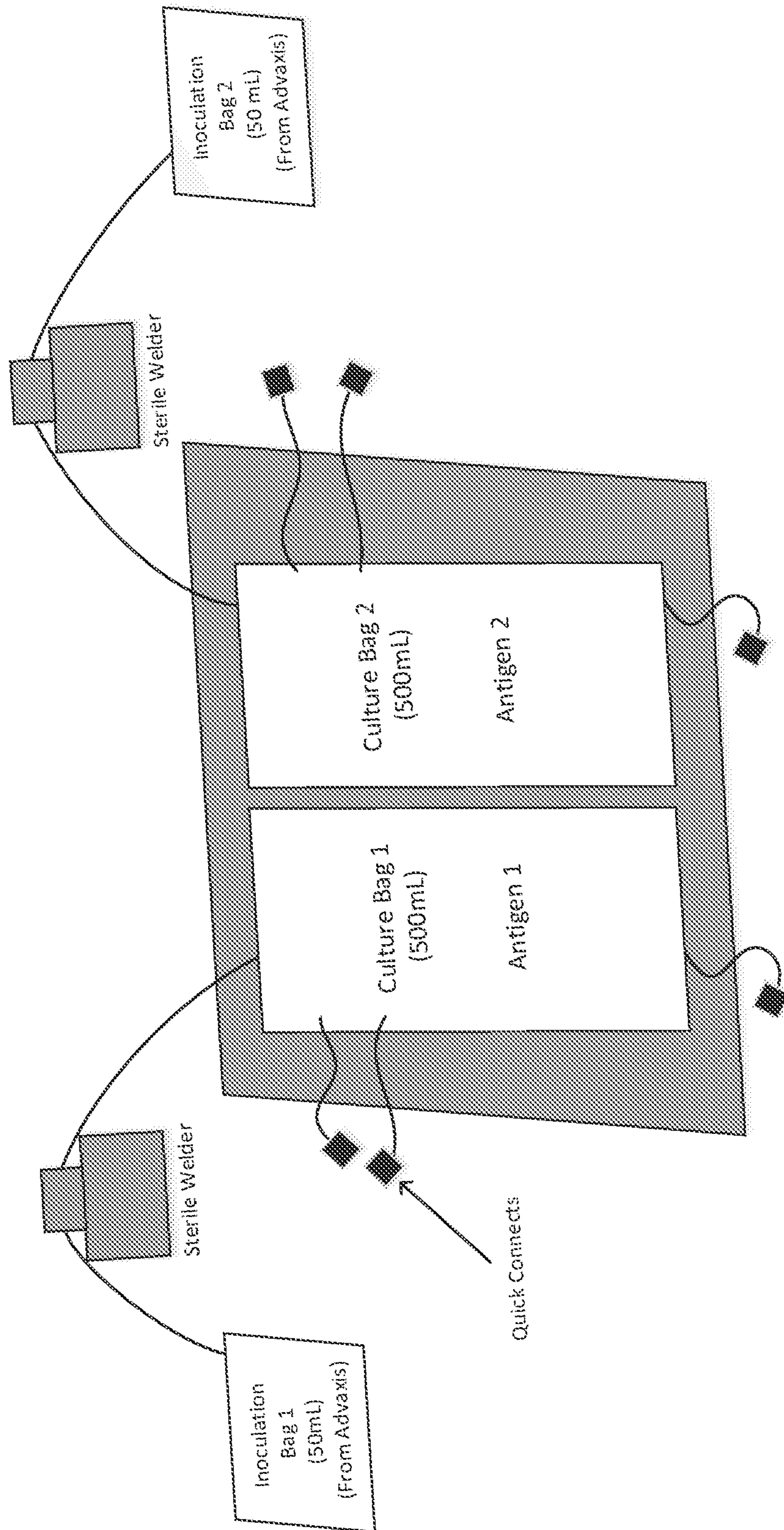


Figure 39

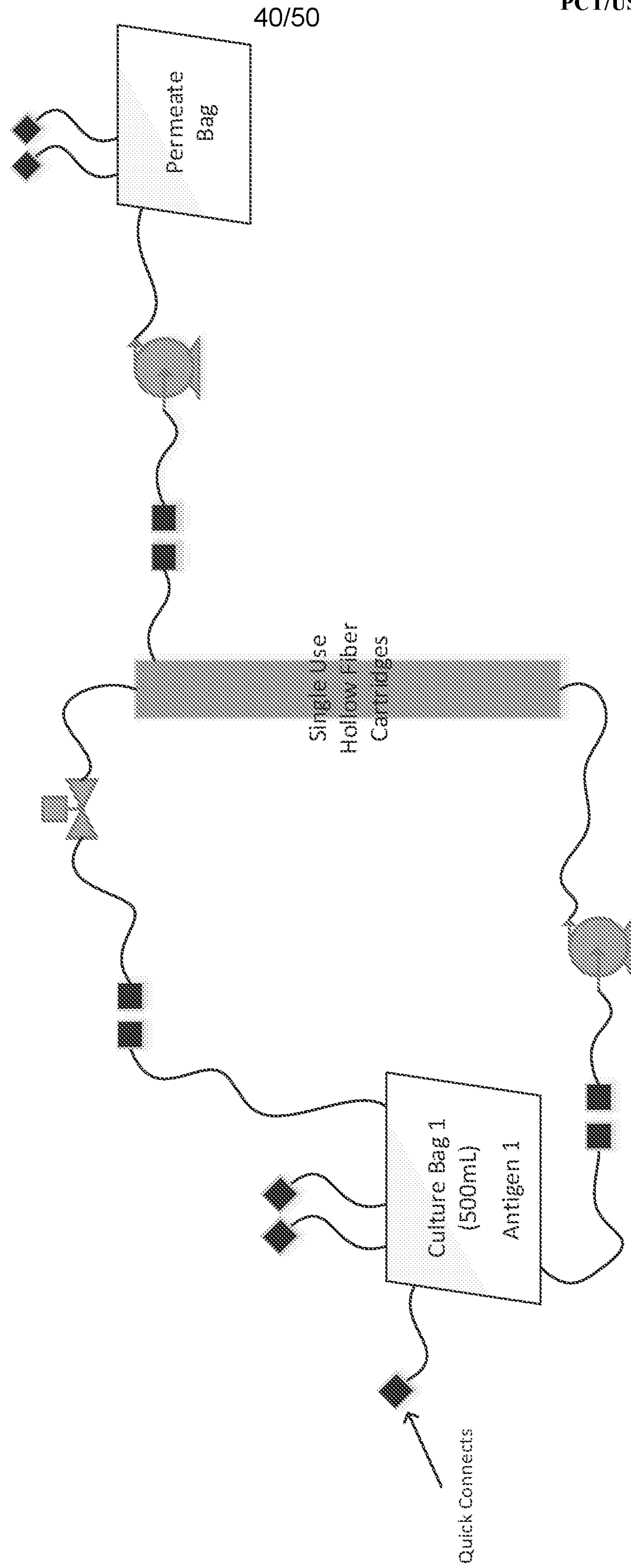


Figure 40

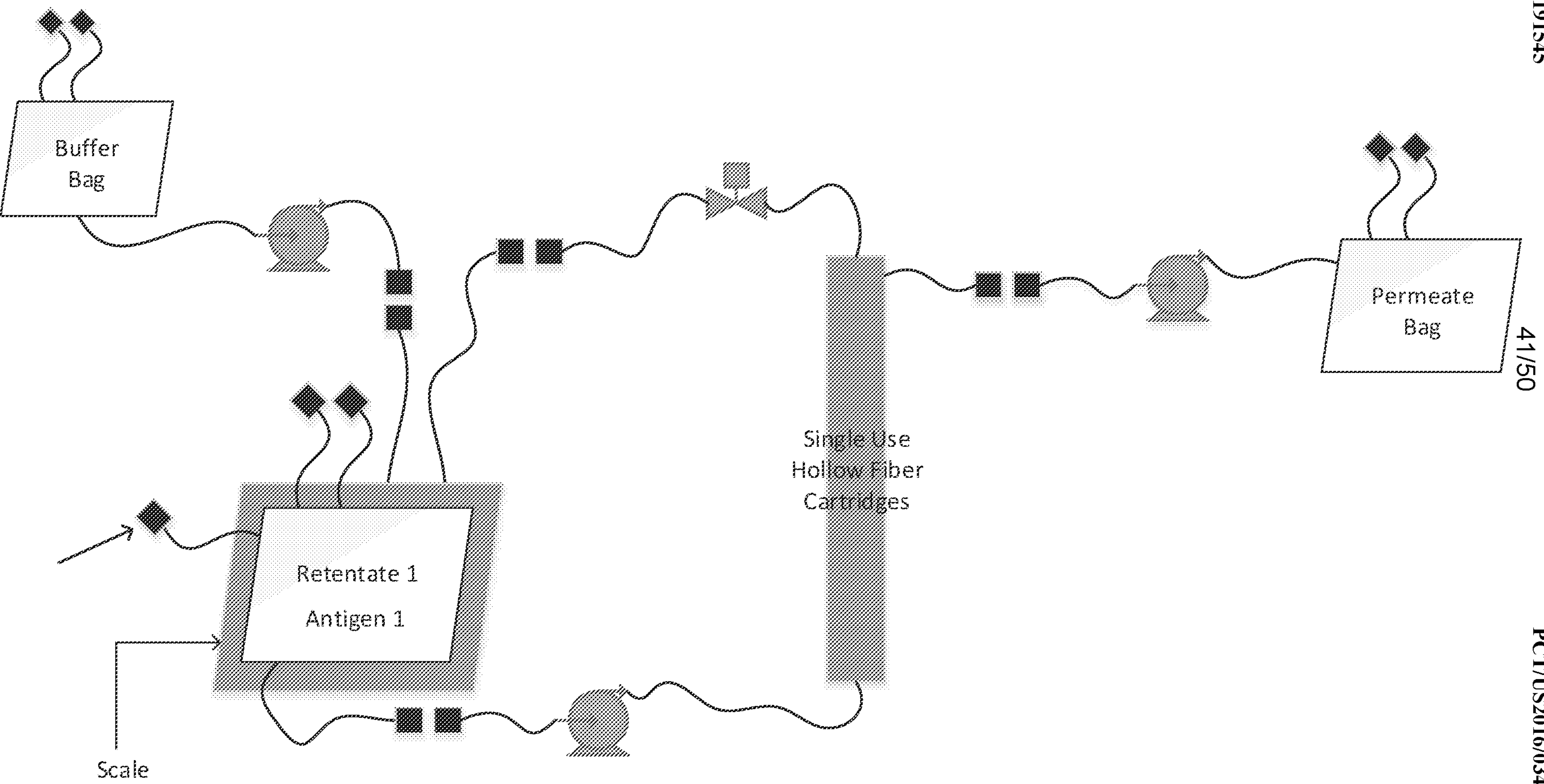


Figure 41

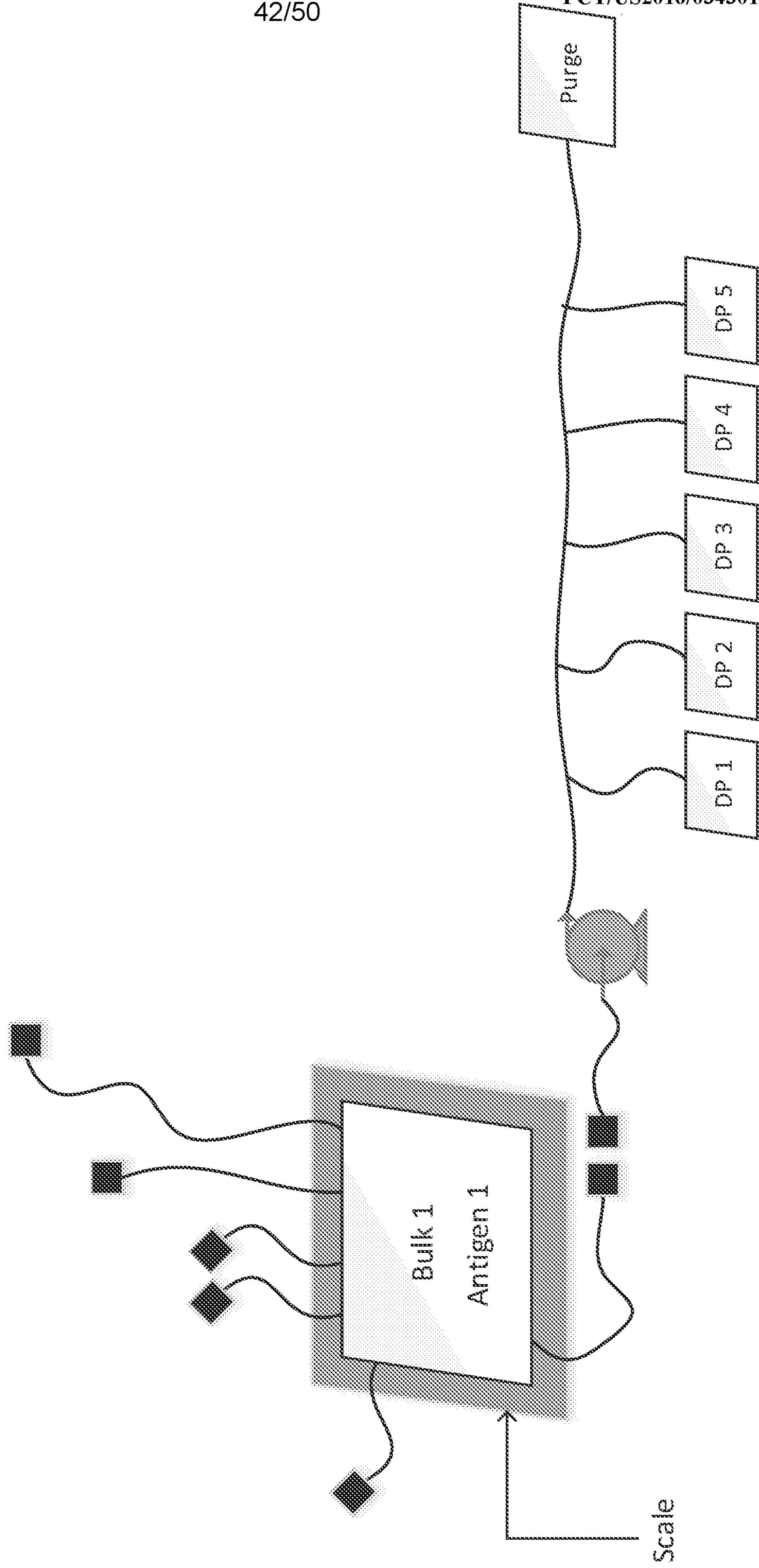
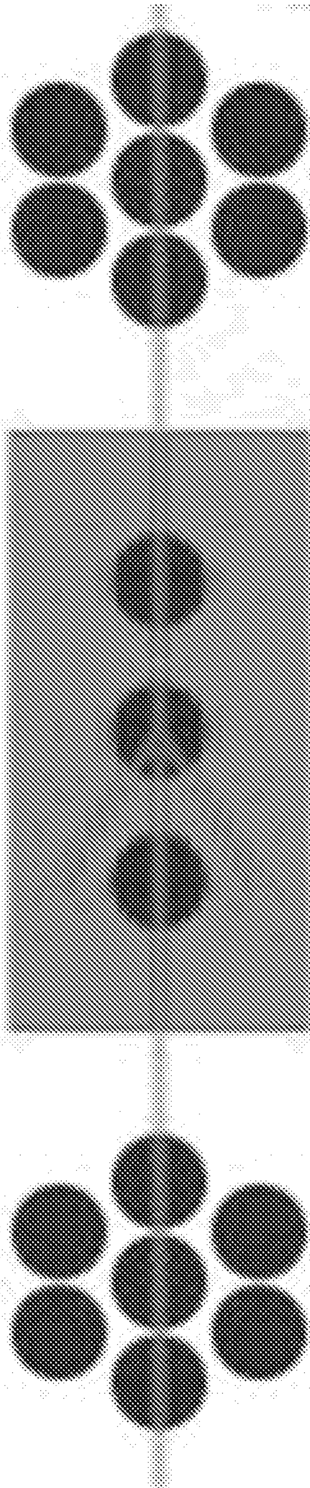


Figure 42



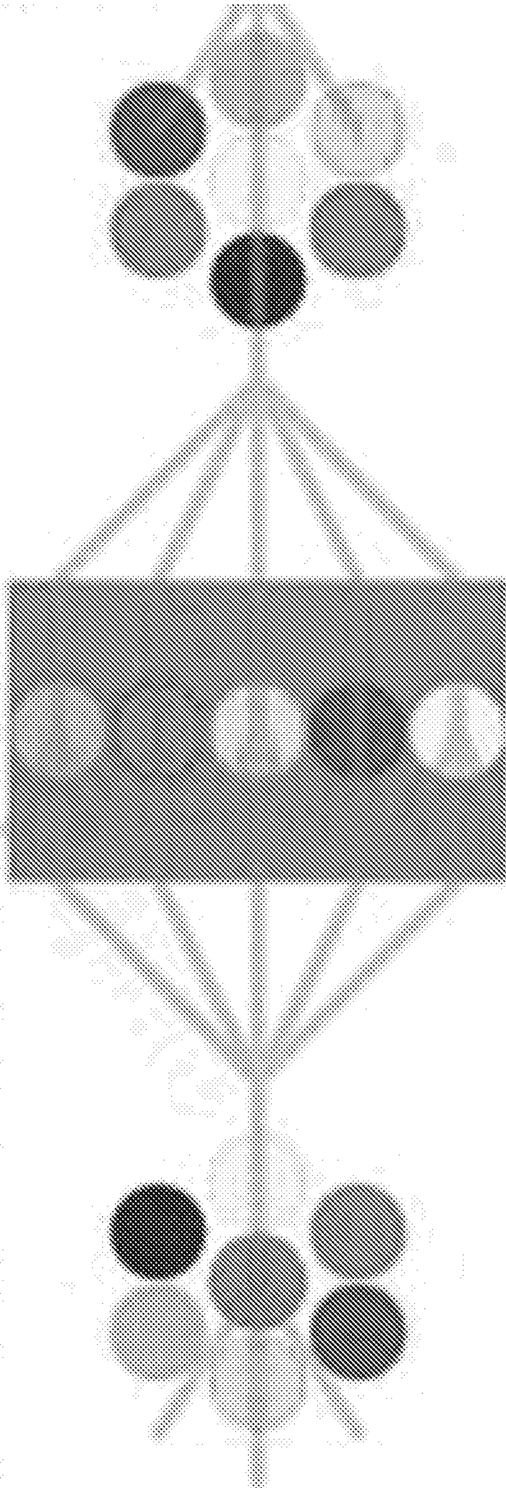


FIGURE 43

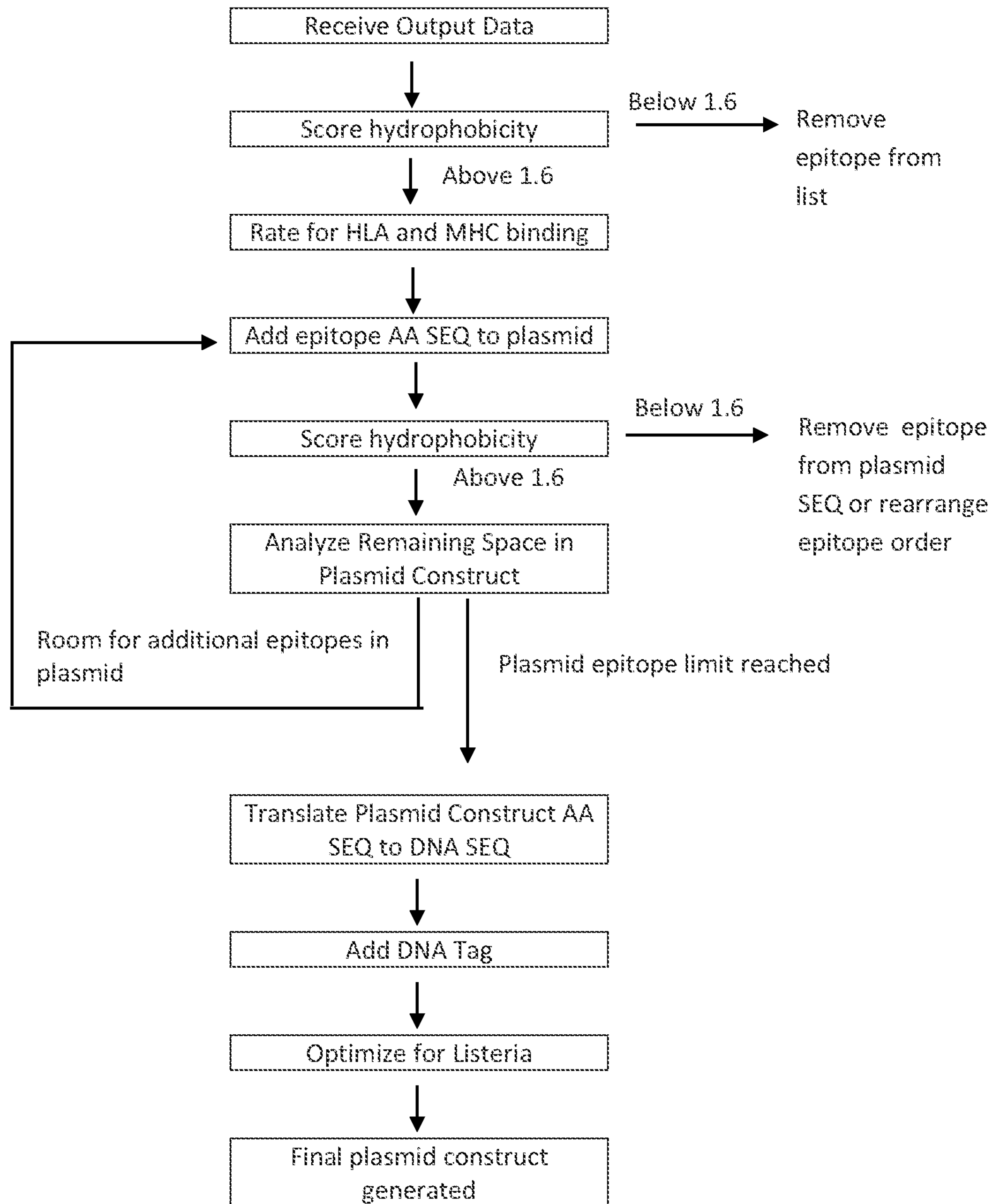


FIGURE 44

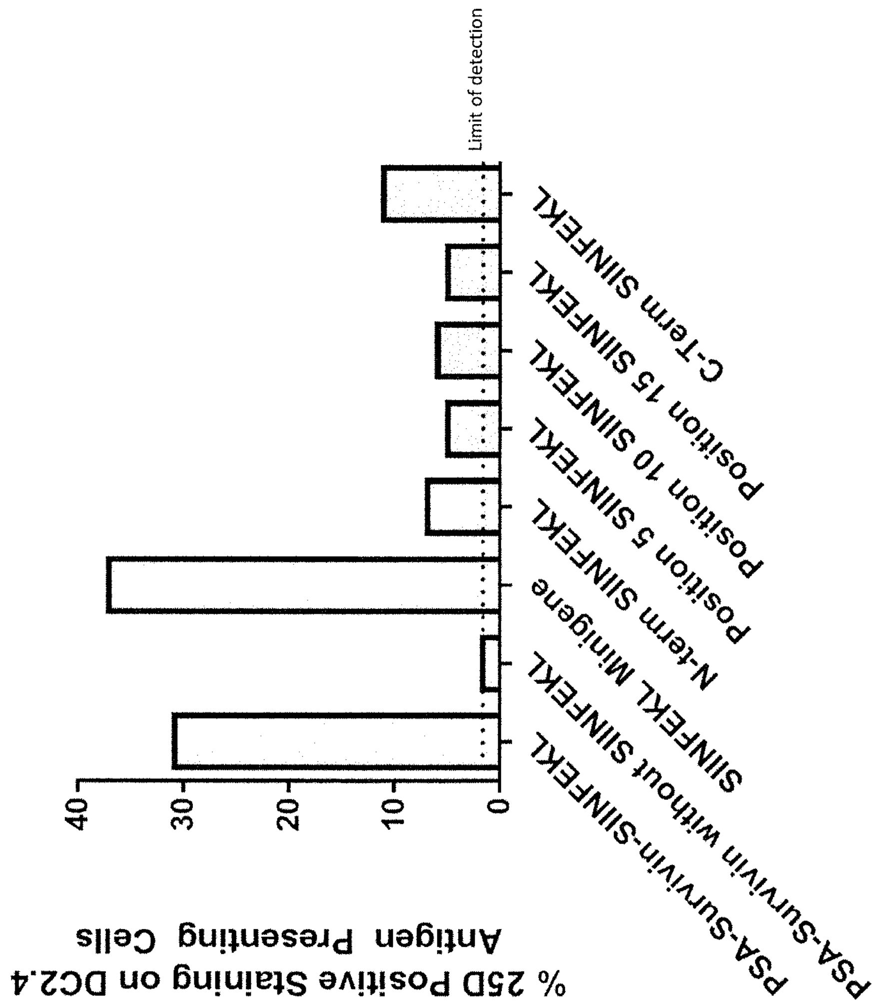


Fig. 45

Fig. 46A

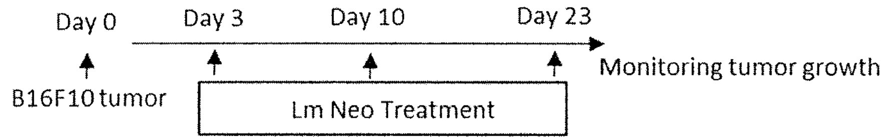


Fig. 46B

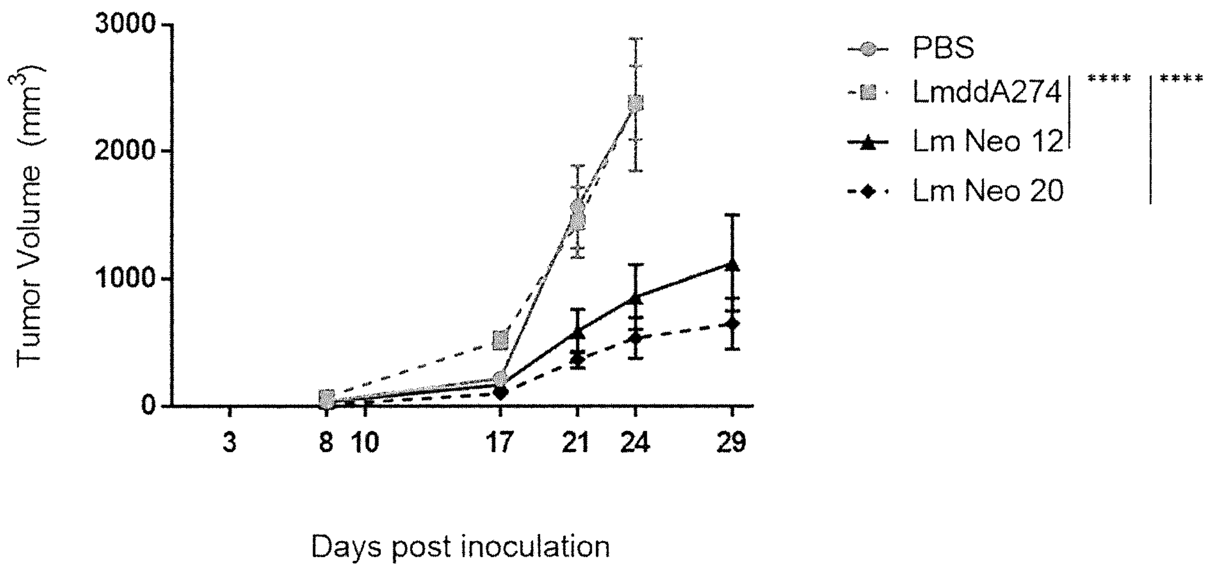
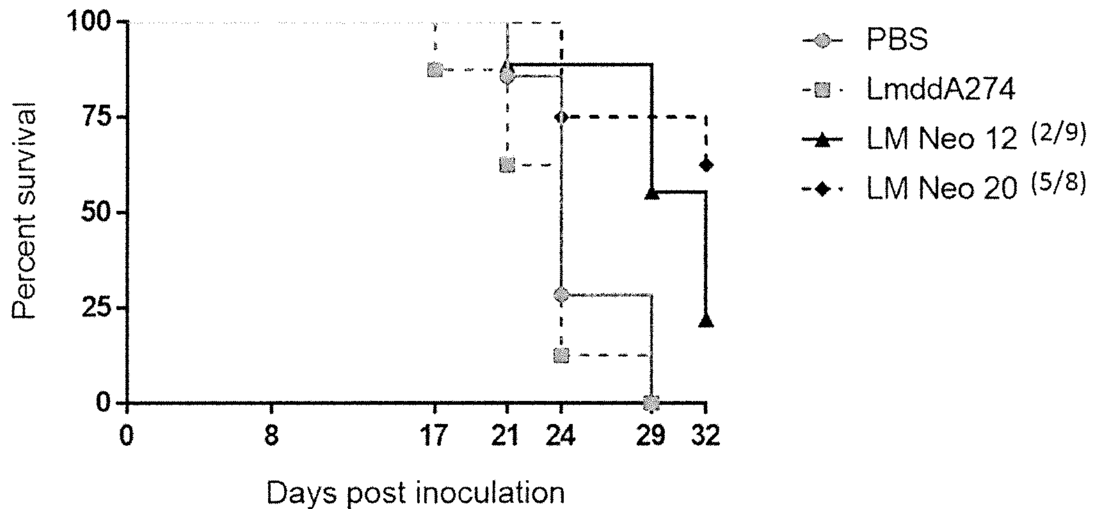


Fig. 46C



25D Assay

Fig. 47A

PSA-Survivin-SIINFEKL

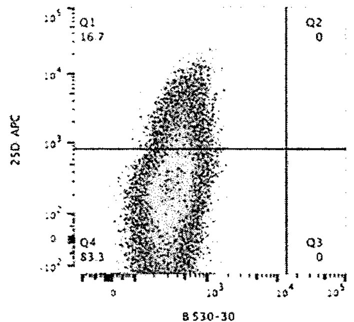


Fig. 47B

PSA-Survivin Without SIINFEKL

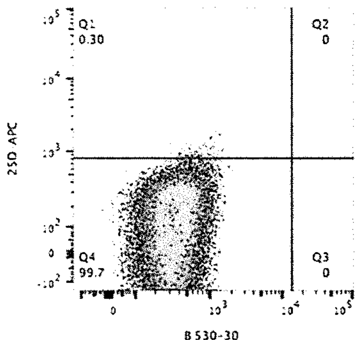
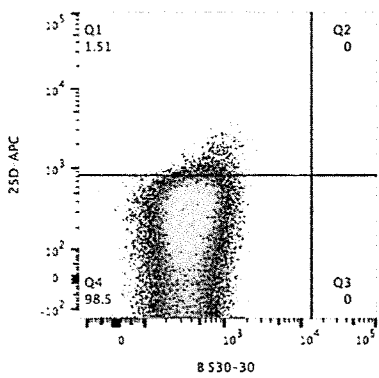


Fig. 47C

Neo 20-SIINFEKL



SIINFEKL-specific CD8⁺ T cell Response To a "low secretion" Lm Construct

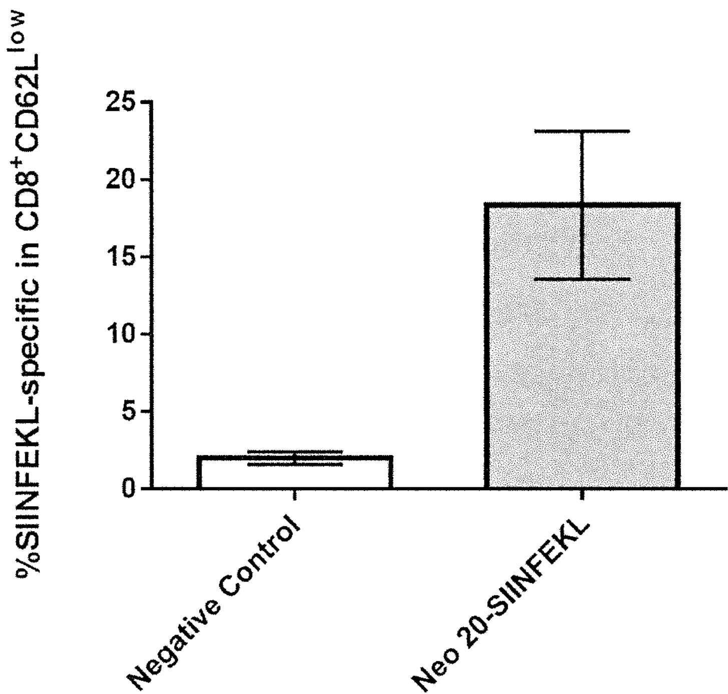


Fig. 48

Fig. 49A

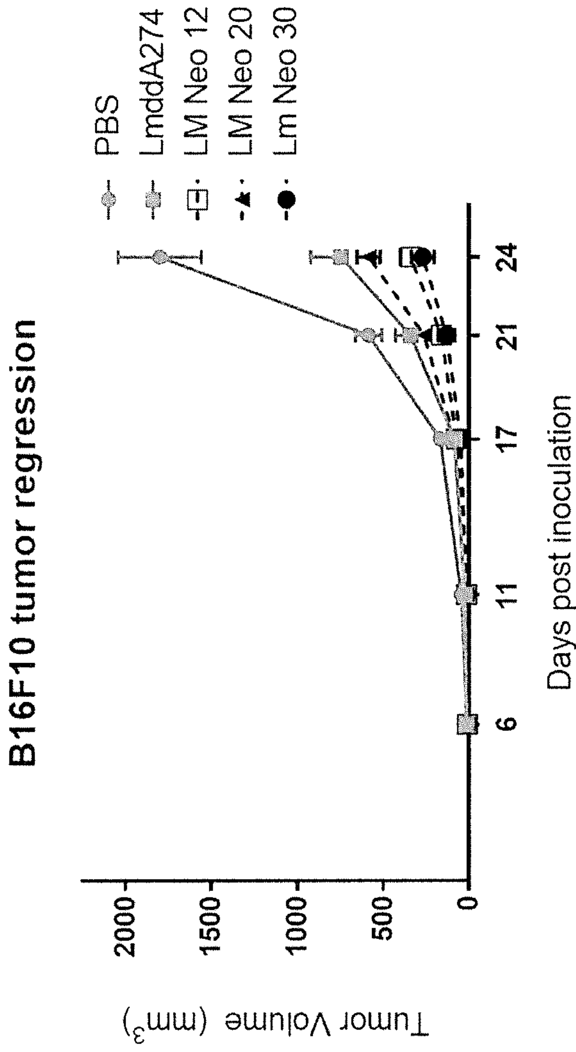
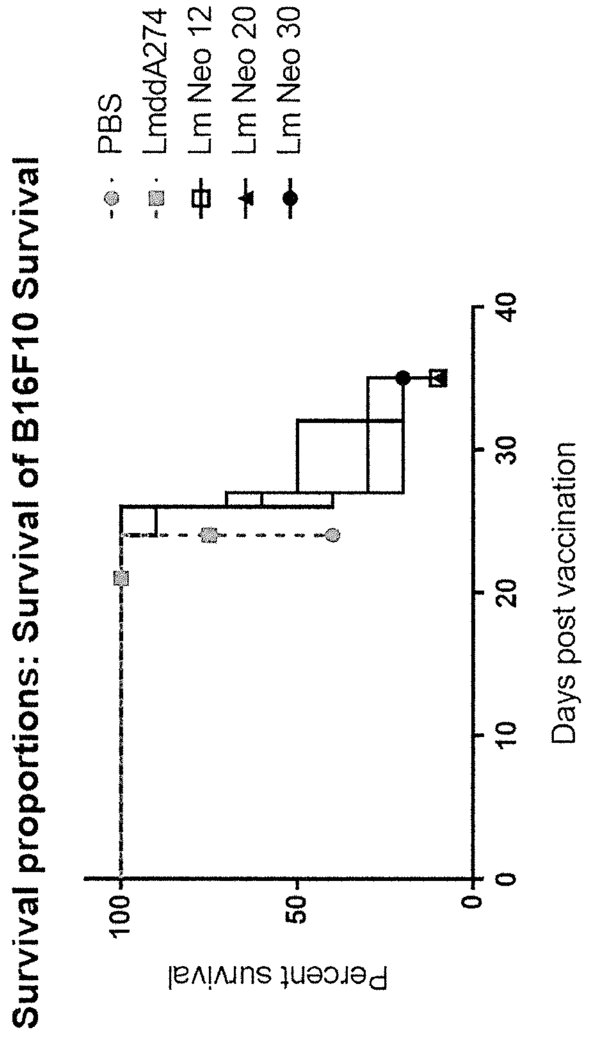


Fig. 49B



Strain	Secretion/Presentation
2712 1-20	No
2712 21-40	No
2712 41-60	Yes
2712 61-80	Yes
2712 81-100	Yes
2712 101-120	Yes
2712 121-140	Yes
2712 141-160	No
2712 161-180	Yes
2712 181-200	No
2712 random1	Yes
2712 random2	Yes
2712 random4	No
2712 random5	Yes

Strain	Secretion/Presentation
2712 1-20	No
1-5	Yes
6-10	Yes
11-15	Yes
16-20	Yes
1-20 random1	Yes
1-20 random1 1-10	Yes
1-20 random1 11-20	Yes

Strain	Secretion/Presentation
2712 21-40	No
21-40 1-5	No
21-40 6-10	No
21-40 16-20	Yes
21-40 random1	No
21-40 random1 1-10	No
21-40 random1 11-20	Yes
21-40 random1 1-5	Yes
21-40 random1 6-10	No
21-40 random1 11-15	Yes
21-40 random1 16-20	Yes
21-40 random2 11-20	No
21-40 random2 6-10	Yes

Fig. 50

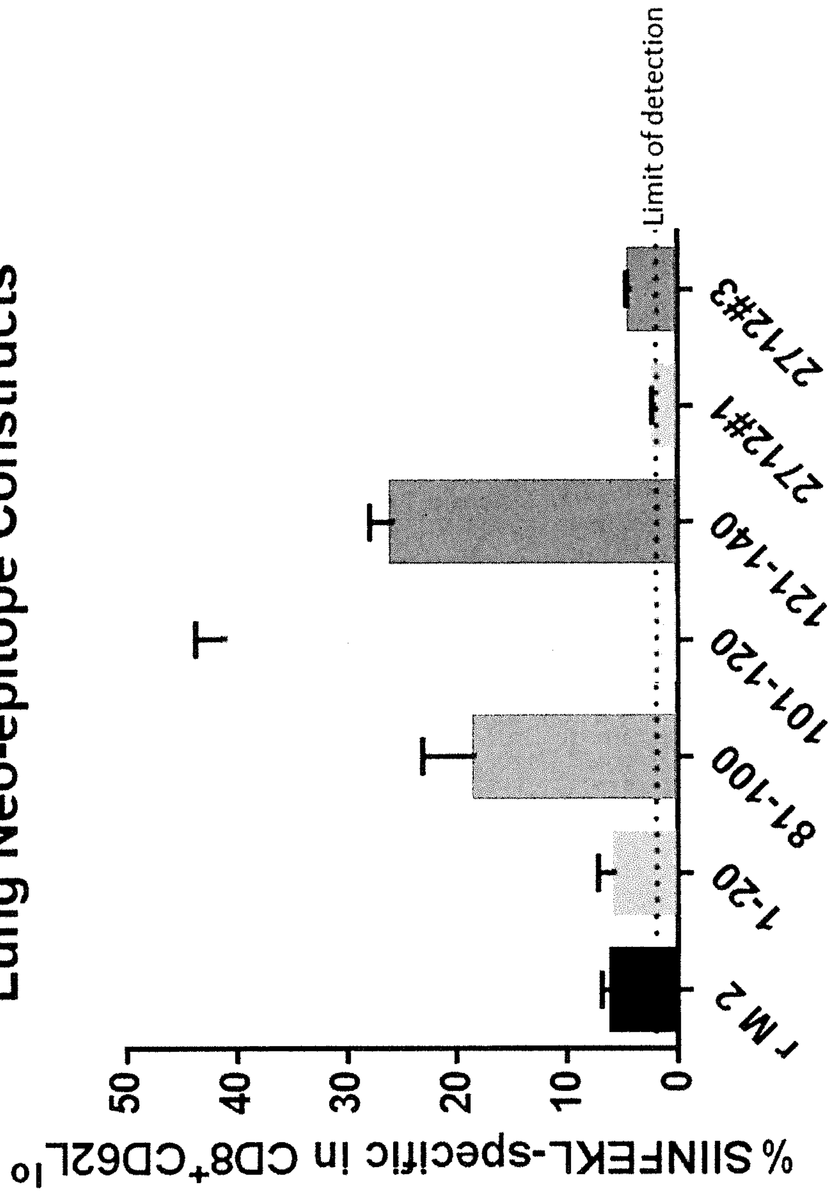


Fig. 51

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/034301

A. CLASSIFICATION OF SUBJECT MATTER

A61K 48/00(2006.01)i, A61K 38/16(2006.01)i, C07K 14/195(2006.01)i, C12N 15/74(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K 48/00; A61K 39/02; A61P 35/00; A61K 39/00; C12Q 1/68; A61K 38/16; C07K 14/195; C12N 15/74

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: listeria, immunogenic, neo-epitope, LLO, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SINGH, R. et al., 'Fusion to Listeriolysin O and delivery by Listeria monocytogenes enhances the immunogenicity of HER-2/neu and reveals subdominant epitopes in the FVB/N mouse' The Journal of Immunology, 2005, Vol. 175, pp. 3663-3673 See abstract; pages 3663, 3668 and 3669.	125-127,132(1)-138
Y	WO 2014-052707 A2 (THE UNIVERSITY OF CONNECTICUT) 03 April 2014 See paragraphs [0049]-[0051]; claims 1, 8.	125-127,132(1)-138
A	HACOHEN, N. et al., 'Getting personal with neoantigen-based therapeutic cancer vaccines' Cancer Immunology Research, 2013, Vol. 1, No. 1, pp. 11-15 See the whole document.	125-127,132(1)-138
A	US 2010-0189739 A1 (FRANKEL, F. R. et al.) 29 July 2010 See the whole document.	125-127,132(1)-138
A	WO 2011-020604 A1 (PEVION VIOTECH AG) 24 February 2011 See the whole document. Note : The second claim 132 is renumbered as claim 132(1) by this authority because claim 132 is found twice.	125-127,132(1)-138

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
21 October 2016 (21.10.2016)

Date of mailing of the international search report
21 October 2016 (21.10.2016)

Name and mailing address of the ISA/KR

International Application Division
Korean Intellectual Property Office
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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-58,60-66,68-87,89-123,131,132
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-58, 60-66, 68-87, 89-123, 131 and 132 pertain to a method for treatment of the human body therapy or include a method for treatment of the human body by surgery, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

2. ☒ Claims Nos.: See below.
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 6,9,13,15,16,29,30,32-39,48,53,56,58,61-64,76,78,82,83,85,93,95,100-106,108,111,113,114,116,118,120-122,129,130 and 141 are unclear, since they refer to one of claims which are not drafted in accordance with PCT Rule 6.4(a) (PCT Article 6).

3. ☒ Claims Nos.: See the extra page.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

3. Claims Nos.:
5,7,8,10-12,14,17-28,31,40-47,49-52,54,55,57,60,65-67,74,75,77,79-81,84,86,87,89-92,94,96-99,107,109,110,112,115,117,119,123,124,128,131,132,139,140,142-147

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2016/034301

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