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Exploring the role of *ESR1* mutations in metastatic hormone receptor-positive breast cancer T cell immune surveillance disruption

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Abstract

Background Breast Cancer (BC) is the most common type of cancer in women around the world and 70% of cases are hormone-receptor positive (HR+). In 40% of cases, a key mechanism of endocrine resistance to the standard first line is a mutation of the ligand-binding domain (LBD) of *Estrogen Receptor 1* (*ESR1*) encoding estrogen receptor α (ER). Most common *ESR1* mutations that occur at positions 537 and 538 have been associated with poor clinical outcomes. *ESR1* mutations have the potential to provide neoantigens. This study aims to identify if *ESR1* mutations generate specific T cell responses against *ESR1* neoantigens in patients with HR^+ $HER2^-$ BC, and to investigate if *ESR1* mutations might correlate with a gene expression profile related to immune surveillance disruption.

Methods We identified candidate *ESR1*-derived peptides by predictive software (SYFPEITHI and NetMHCpan 3.0). Then the immunogenicity of *ESR1*-derived peptides was assessed in Peripheral-Blood-Mononuclear-Cells from 31 healthy donors (HD) and 25 patients with metastatic HR-positive BC by IFN-γ ELISpot assay. A vaccination assay on a humanized mouse model (HLA-A2/DR1) was used to validate the immunogenicity and the presentation of these peptides. Finally, we used Bulk RNA-Seq sequencing along with MCPcounter, a cellular deconvolution method, to investigate the immune contexture of *ESR1*-mutated BC.

Results Preliminary results showed recognition of *ESR1*-derived peptides by women HD lymphocytes but not in men. Frequencies and intensities of such immune responses were increased in patients with BC. Our results showed that 40% of patients had specific immune responses. In addition, we demonstrated the HLA-A2 *ESR1* peptide immunogenicity in humanized HLA-A2/DR1 mice. In a data set generated from BC patients refractory to conventional therapy we showed that *ESR1* mutations are correlated in advanced diseases with downregulation of molecules

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involved in antigen presentation and with loss HLA Class I gene expression. *ESR1*-mutated BC had a decrease in immune cell infiltration.

Conclusion These results support that common *ESR1* mutations generate neoantigens in hormone-receptor positive metastatic breast cancers. If *ESR1* peptides-restricted lymphocytes were detectable in BC patients, *ESR1* mutations promote immune escape at advanced stages.

Trial registration ClinicalTrials.gov, NCT02838381. Registered on June 2012.

Keywords Hormone-receptor positive breast cancer, *ESR1* mutation, Specific immune responses, Neoantigen, T cell

Background

Breast cancer (BC) is a heterogeneous disease characterized by different molecular subtypes. Hormone receptor-positive (HR^+) tumors represent 70% of breast carcinoma [1], which expresses the estrogen receptor (ER). In a metastatic setting, despite a well-conducted first-line Endocrine therapy (ET), all patients develop resistance to ER blockade. Hormone resistance implies a reduced efficacy of subsequent therapeutic strategies. Today's therapeutic challenge is to extend the hormone-sensitive phase. One established mechanism of resistance is the mutation of the ligand-binding domain (LBD) of Estogen Receptor 1 (*ESR1*), resulting in constitutive transcriptional activity and diminished sensitivity to ET [2].

The *ESR1* mutations occur with a wide-ranging prevalence (10–50%) in metastatic BC (mBC) patients [3–6]. These genomic alterations are mostly missense mutations clustered in codons 537 and 538 of the LBD, resulting in constitutive ligand-independent activation of the receptor. The predominant *ESR1* mutations are Y537S and D538G, while several other mutations have been identified at significantly lower frequencies. These "hot spot" mutations cover 85% of *ESR1* gene mutations. *ESR1* mutations are present in less than 3% of mBC patients at disease onset [6–8] and most of these genomic alterations are mainly detectable in cases of endocrine resistance after exposition to aromatase inhibitors [9, 10]. In these patients resistant to hormone therapy, *ESR1* mutations are common (approximately 1 patient out of 3) and associated with worse outcomes compared with those without *ESR1* mutation detection [11].

Depending on the specific nature of the mutation, *ESR1* mutations have the potential to provide neoantigens [12, 13]. A high tumor mutational burden promotes the generation of neoantigens, and, in breast cancer context, has been associated with endocrine resistance, thus *ESR1* mutations, and poor outcomes [14, 15].

Class I human leukocyte antigen (HLA) plays a pivotal role in the crucial process of presenting neoantigens on the cell surface, making them accessible for T cell recognition. An efficient presentation of neoantigens to HLA proteins is required to elicit effective immune responses. HLA-peptide binding affinity is a predictive factor of neoantigen immunogenicity. Conversely, downregulation of HLA is a cancer-cell intrinsic mechanism of immune evasion and has been associated with poor outcomes [16–18].

Even if *ESR1* mutations could represent an interesting source of neoantigens their capacity to elicit specific T cells responses against *ESR1* derived neoantigens in hormone receptor-positive breast cancer context was barely investigated.

The present study investigated the capacity of *ESR1* mutations to promote immune surveillance by eliciting, among others, specific T cells responses in both healthy donors and patients with $HR^+/HER2^-$ mBC. One immunoprevalent peptide designed from the D538G (P1) *ESR1* mutation was identified. One clone specific for the p1 peptide was polyfunctional but failed to demonstrate the ability of MCF7 $ESR1^{D538G}$ mutant cell to naturally process the p1 peptide. The analysis of immune-related RNA expression profile from advanced mBC patients expressing *ESR1* mutations or not demonstrated that *ESR1* mutations goes hand in hand with the presence of other escape mechanisms in tumor cells, such as the loss of expression of class I and II MHC molecules, an alteration in the processing and presentation of tumor antigens and a decrease in tumor infiltration by CD8 T cells.

Materials & methods

Epitope selection and synthetic peptides

The thirty-four peptides derived from REα were predicted to bind multiples HLA-A/B and HLA-DR molecules using SYFPETHI and/or NetMHCpan 3.0 software (access via www.syfpeithi.de/, www.cbs.dtu.dk/services/NetMHCpan/) according to the mutations in positions 537 and 538 (Y537S, Y537C, Y537N, and D538G). Twenty-two peptides were retained based on their predictive score to bind to HLA-A/B molecules. In addition twelve peptides were selected based on their predictive score to bind to HLA-DR molecules. All synthetic peptides (>80% purity) were purchased from JPT (Berlin, Germany). To assess antiviral T cell immunity, a pool of 23 peptides containing epitopes from influenza virus, Epstein–Barr virus, and cytomegalovirus was used (Cellular Technology Limited, Shaker heights, OH, USA). Lyophilized peptides were dissolved in PBS containing

5% DMSO at a concentration of 4 mg/mL and stored at − 80 °C.

Cell culture

Authenticated Tap-deficient T2 (174x CEM.T2) cell line and MCF7 (HTB-22) was purchased from the ATCC and (upon thawing) never passed for more than 3 weeks before use in experimental determinations. All cell lines were negative for known human's pathogens, including mycoplasma. T2 and MCF7 cells were cultured respectively in RPMI and DMEM (Fisher, France) supplemented with 10% FBS (Fetal Bovine Serum, Eurobio, France), 1% penicillin and 1% streptomycin (Eurobio, Courtaboeuf, France). Cells were pulsed with or without peptides and stained with a FITC-conjugated anti-HLA-A2 mAb (BB7.2, BD Biosciences). Flow cytometry was performed using a DxFlex (Beckman Coulter, Brea, CA, USA) and the data were analyzed using FlowJo software 10.6.2 (Treestar Inc., Ashland, OR, USA).

Peptide binding assay

T2 cells are a hybrid human cell line which lack most of the MHC class II region including the known TAP (transporter proteins for antigenic peptide) and proteasome genes. They contain the gene HLA-A*0201, but express very low levels of cell-surface HLA-A2.1 and are unable to present endogenous antigens [19]. T2 binding assays were performed to test peptide binding affinity to HLA-A*02:01 molecules. 500 000 T2 cells were incubated with 50 µg/mL of mutant, wild-type, or control peptides (V8L for negative control and L10L for positive control) for 12 h at RT in RPMI1640 media supplemented with 10% FBS. Then, T2 cells were incubated with 10 µg/mL of peptide for 30 min at RT. Surface expression of HLA-A*02:01 in T2 cells was determined by staining with an anti-HLA-A2 mAb (BB7.2, BD Biosciences). The median fluorescence intensity (MFI) was measured using a FACSCanto II (BD Biosciences).

Mice model

HLA-DRB1*0101/HLA-A*02:01 transgenic mice C57BL/6J (A2/DR1 mice) described in [20] were used to demonstrate in vivo the immunogenicity of ERα-derived peptides. Female mice aged of 8 to 10 weeks were used in the experiments. Hockpad vaccination [21] was performed on D0 with 100 µg of ERα-derived peptides and 2 µM of CpG (Invivogen, USA). Three other peptide injections were performed on D7, D14 and D121 with 50 µg of peptides and 1 µM of CpG. The mice were sacrificed on D23 by euthanasia, in order to remove the splenocyte and popliteal lymph nodes. The presence of T lymphocytes specific for mutated REα in these nodes was assessed ex vivo by 24 h IFN-γ ELISpot assay.

All experiments were carried out according to the good laboratory practices defined by the animal experimentation ethic rules in France.

Patients and healthy donors

Breast cancer patients ($n = 25$) were recruited from the department of Oncology at the Franche-Comté Hospital in the CRC01 cohort D. Patients' characteristics are detailed in additional Table **S3**. This trial was registered with ClinicalTrials.gov (https://clinicaltrials.gov/) numb ers (NCT02838381). All patients were enrolled after the signature of informed consent, in accordance with the French regulation and after approval by the local and national ethics committee. Blood was collected after cancer specific treatment.

Blood cells from anonymous healthy donors (HD) were collected at the *Etablissement Français du Sang* (EFS, Besançon, France) from apheresis kit preparations after the signature of informed consent and following EFS guidelines. The major exclusion criteria for blood collection in HD were minors (< 18 years), age over 65 years, dehydration, fatigue, low levels of hemoglobin (> 120 g/L for women and 130 g/L for men), flu symptoms, HIV, HTLV or hepatitis B/C positive status, autoimmune diseases, surgical procedures in the last 4 months and vaccine less than 4 weeks old. Blood collection allows the storage and use of this sample in anonymous fashion precluding clinical characterization of these donors.

PBMC (peripheral blood mononuclear cells) from HD and cancer patients were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Eurobio, France).

Isolated PBMC were cryopreserved at a cell density of $8–15 \times 10^6$ cells per vial in CryoStor (CS10 and CS5) cell preservation media (Sigma-Aldrich, St. Louis, MO, USA) and were conserved at − 196 °C for IFN-γ ELISpot analysis.

IFN-γ ELISpot assay

T cell responses were assessed by IFN-γ ELISPOT after a short in vitro stimulation of fresh or thawed PBMC (viability > 80%) with *ESR1*-derived peptides during 7 days. For in vitro stimulation, at day 0, PBMC were plated at 1.10^6 cells/well for 7 days in 48-well plates with the pool of peptides derived from *ESR1* (5 µg/ml). Recombinant interleukin IL-7 (5 ng/mL; PeproTech, Neuilly-sur-seine, France) was added at day 1. Recombinant IL-2 (20 UI/mL; PROLEUKIN aldesleukin, Novartis Pharma, CH, EU) was added at day 3. At day 7, the specificity of T cells was investigated by IFN-γ ELISpot, according to the manufacturer's instructions (Diaclone, Besançon, France). Briefly, T cells (10^5 per well) were cultured in anti-human IFN-γ monoclonal antibody pre-coated IFN-γ ELISpot plates with *ESR1*-derived peptides (5 mg/mL) in X-vivo 15 medium (Lonza, Bâle, Switzerland) for 18 h at 37 °C.

Cells cultured with medium alone or phorbol myristate acetate (PMA; 1 ng/ml)/ionomycin (500 ng/ml) Sigma -Aldrich, St. Louis, MO, USA) were used as negative and positive controls, respectively. The IFN-γ spots were revealed following the manufacturer's instructions (Diaclone, Besançon, France). The number of specific T cells expressed as spot forming cells/ 10^5 cells was calculated after subtracting negative control values (background). Spot-forming cells were counted using the C.T.L. Immunospot system (Cellular Technology Limited, Shaker heights, OH, USA). Responses were considered positive when IFN-γ spots numbers were more than 10 and more than two times the background. All the experiments were conducted in duplicates.

Production of lentiviral vector

For production of *ESR1* D538G lentiviral vectors, 2.6×10^6 293FT cells were plated into a 100-mm dish (131050 C Culture TC ClearLine 95.6 × 22 mm, sterile). Next day cells were transfected with 30 µg of lentiviral "*ESR1* D538G" (VectorBulder, Neu-Isenburg, Germany), 2.7 µg of PMD2G (intern production), 8.6 µg of pSPAX2(intern production), using calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO, USA) in the presence of 25 µ*M* chloroquine (Sigma-Aldrich St. Louis, MO, USA). Eleven hours after transfection, the medium was changed with Optimem medium (Fisher, Courtaboeuf, France), thereafter virus containing supernatant was collected 2 days after transfection (Amicon Ultra-15 Centrifugal Filters, Sigma-Aldrich, St. Louis, MO, USA), and stored at − 80 °C until further use. A small aliquot from each production was used to determine viral titers by transduction of 293 cells with serially diluted amounts of virus supernatant. The virus supernatant was used for MOI (Multiplicity of Infection) at 15.

Lentiviral transduction

For each lentiviral transduction, 5×10^5 tumor cells per well were seeded in a 24-well plate and mixed with an appropriate amount of virus supernatant in the presence of 8 µg/ml of hexadimethrine bromide (Polybrene®) (2 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in a final volume of no more than 500ul. Plates were centrifuged at 3000×RPM for 15 min at room temperature. After centrifugation, without removing viral supernatants, the plates were incubated at 37 °C, 5% CO_2 for 12 h. At the end of the incubation, the supernatants were removed from the wells and 1 ml of fresh tumor cell growth medium (RPMI supplemented with 10% fetal serum and 1% PS, Gibco, Illkirch, France) per well was added. The cells were maintained in this medium for at least 2 days before acquisition of enhanced green fluorescent protein (eGFP) expression was carried out. If the number of translated cells is suitable, the cells are sorted (SONY SH800S, Puteaux, France) then amplified and cloned at 1 cell/well in a 96-well plate.

Generate a T cell clone

T cell clones were generated either from HLA-A*02:01 HD PBMC. PBMC (2×10^7) were cultured in 24-well plates (4×10^6 cells per well), in RPMI medium containing 10% of human serum, 1% PS enriched with R-848 (resquimod, 3 µg/ml; Invivogen), high–molecular weight poly-IC (polyinosine-polycytidylic acid) (50 µg/ml; Sigma-Aldrich, St. Louis, MO, USA), interleukin-2 (20 IU/ml; IL-2; PROLEUKIN aldesleukin, Novartis Pharma, CH, EU), at day 0. At day 1, the peptide of interest (10 µg/mL) was added. After 2, 6, and 12 days, 100 µl of medium were replaced by enriched fresh medium (IL-7 (50 ng/mL; PreproTech 200-07) only at day 2 and IL-7 (5 ng/mL) and IL-2 (50 UI/mL) at day 6, 12) and splited if necessary. On day 14, cells were collected and functional analyses of T-cell were done by using intracytoplasmic IFN-g staining. *ESR1*-reactive T cells were isolated by IFN-γ T cell sorting (Miltenyi Biotec, 130-054-201), according to manufacturer's instructions. *ESR1*-specific HDB.5 T cell clone was generated by limiting dilution in terasaki plates (T/C Microtitre 60 well plate, Sarstedt, Germany) and amplified after stimulation by PhytoHémaglutinnine (PHA) (Sigma-Aldrich, St. Louis, MO, USA, 1 µg/mL) and 150 UI/mL of IL-2 in presence of irradiated allogenic PBMC (25 Gy), B-EBV cell line (50 Gy).

Flow cytometry

To discriminate living from dead cells, PBMC were first washed in 1× phosphate-buffered saline (PBS) (Gibco, Grand Island, NY, USA) and stained with eFluor 780 viability dye according to the manufacturer's instructions (eBioscience, Villebon-sur-Yvette, France). For T cell clones analysis, samples were surface-stained in the dark for 30 min at 4 °C with the following antibodies: BV605-CD3 (BD Biosciences clone HIT3a), BV421-CD4 (SBD Biosciences clone RPA-T4), BV786-CD8 (BD Biosciences clone RPA-T8). For intracytoplasmic cytokine staining, the cells were fixed and permeabilized using cytofix/CytoPerm Plus KIT (BD Bioscience) before staining with APC-IFN-γ (BD Pharmingen, clone B27), FITC-TNFα (BD Pharmingen, clone Mab11) and PE-IL2 (Sony clone MQ1-17H12) and washed. The samples were directly acquired on DxFlex (Beckman Coulter, Brea, CA, USA) and data analyzed with FlowJo software 10.6.2 (Treestar Inc., Ashland, OR, USA).

For *ESR1*-tetramer staining: *ESR1*^{D538G}-HLA-A2 tetramer was provided by the peptide and Tetramer Core Facility, UNIL-CHUV, Epalinges, Switzerland. One million cells were incubated with 2 µL phycoerythrin (PE)-labeled tetramers loaded with HLA-A2-derived peptide in 100 µL FACS buffer for 20 min at RT (Room

Temperature). Thereafter, cells were stained with anti-CD3-BV605, anti-CD8-BV786 for 20 min at 4 °C. Cells were analyzed on a DxFlex flow cytometer and FlowJo software.

Peptide-MHC tetramer

ESR1^{D538G}-HLA-A2 tetramer was designed using the p1 peptide (*ESR1*^{D538G}).

Bulk RNA-sequencing analysis

Bulk RNA-Seq reads were aligned using STAR [22] v2.7.9a to GENCODE Human Release 39 (GRCh38.p13) and read counts matrix produced with FeatureCounts [22] v2.0.1, both with default parameters. The Bioconductor RNA-seq workflow was followed to detect differential expression genes with DESEQ2 [23] v1.34.0. A total of 19,258 coding genes filtered with biomaRt 2.5.3 were analyzed for transcript abundance and poorly expressed genes were eliminated based on the criteria of a maximum read count > 10 for all samples. Data visualization was achieved in R v4.1.1 with ComplexHeatmap package v2.11.1 and EnhancedVolcanoPlot [24] v1.12 using DESeq 2's regularized-logarithm transformation (rlog) of the count data. HLA Typing has been performed by ArcasHLA [25] v0.5.0 and been use with default parameters. TCR Analysis has been executed by the tool MIXCR [26] v3.0.13 with the default parameter "analyze shotgun". Gene set enrichment analysis was performed with FGSEA R package v1.20.0 on a gene set subset from msigdb including "ANTIGEN_PROCESSING" in pathways from c2 collection CP: KEGG subcollection. Figure showing differential expression of pathway genes using pathview R package v1.34.0. Computations have been performed on the supercomputer facilities of the *Mésocentre de calcul de Franche-Comté*.

Availability of RNA-seq data and materials

The dataset used in this study is the property of the Gustave Roussy Institute and is available upon reasonable request.

METAPRISM (PMID: 36862804) EGAD00001009684

Statistics

Statistical analyses were carried out with Prism 8 software (San Diego, CA, USA). Continuous parameters were summarized with median and interquartile range (IQR) and compared between subgroups of interest using the Wilcoxon-Mann–Whitney test. Proportions were compared using the Chi2 test (or Fisher exact test, if appropriate). The level of significance was set at $p < 0.05$ for all tests ($*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, and $****p \le 0.0001$).

Results

Identification of HLA-A/B and HLA-DR restricted peptides derived from *ESR1* -mutated oncogene

Since *ESR1* oncogenic variants fulfil the criteria of shared neoantigens, we aim to characterize if *ESR1* mutations derived peptides display immunogenic properties. We first established a list of potential peptides generated by the most prevalent *ESR1* mutations occurring in breast cancer patients treated by hormonotherapy. *ESR1* mutations are most commonly clustered in codons 537 and 538 (Y537C, Y537N, Y537S and D538G) of the LBD, and these mutations represent 85% of *ESR1* gene mutations. The amino acid sequence of *ESR1* was examined to identify the existence of specific T cell epitopes able to bind HLA-A/B or HLA-DR molecules using the results obtained from 2 algorithms (SYFPEITHI and NetMHCpan3). Common alleles encoded by HLA-A/B genes were included for this analysis (HLA-A*01:01, HLA-A*02:01, HLA-A*24:02, HLA-B*07:02) [27].

First, we selected in silico twenty-one peptides' sequences derived from the predominant *ESR1* mutations that had at least a low score for HLA-A or B allele commonly found in humans. Six peptides derived from *ESR1*^{D538G} (p1, p2, p3, p4, p5, p6), six peptides derived from *ESR1*^{Y537S} (p7, p8, p9, p10, p11, p12), four peptides derived from *ESR1*^{Y537N} (p13, p14, p15, p16) and five peptides derived from *ESR1*^{Y537C} (p17, p18, p19, p20, p21) were selected. Peptide p0 corresponded to the wild type sequence of *ESR1* (Table 1). Then, we selected in silico eleven peptides' sequences derived from the predominant *ESR1* mutations that had at least a low score for HLA-DR alleles commonly found in humans. Three peptides derived from *ESR1*^{D538G} (pA, pB, pC), three peptides derived from *ESR1*^{Y537S} (pD, pE, pF), two peptides derived from *ESR1*^{Y537N} (pG, pH) and three peptides derived from *ESR1*^{Y537C} (pI, pJ, pK) (Additional Table S1). Peptide pZ corresponded to the wild type sequence of *ESR1*. Of note, BLASTs with human genome were performed for each designed peptides and sequences producing significant alignments were the one of estrogen receptor only.

ESR1 -derived peptides are recognized by human CD8 T cells from breast cancer patients

To determine the immunogenicity of *ESR1*-derived peptides, the presence of a specific T cell was investigated in women and men HD and mBC patients. *ESR1*-derived peptides were used to stimulate CD8 T cells in vitro as previously described [28]. *ESR1* specific CD8 T cells were revealed using IFN-γ ELISpot assay (Fig. 1.A). As shown in Table 2, specific T cell responses in HD was observed almost only in women expect for one response observed in a man for the p3 peptide. Thus, further result's description was conducted only among women HD.

Table 1 Characteristics of HLA-A/B *ESR1*-specific-restricted peptides predicted by immune informatics database. *ESR1* amino acid sequences were submitted to two prediction algorithms (NetMHCpan3 and SYPEITHI) and peptides binding prediction to HLA-A/B were classified as strong (+++), moderate (++), weak (+) or null (-)

Peptide	Sequence	Mutation	Binding predictions							
			A1		A2		A24		B7	
			NetMHCpan3	SYFPEITHI	NetMHCpan3	SYFPEITHI	NetMHCpan3	SYFPEITHI	NetMHCpan3	SYFPEITHI
P0	PLYDLLLEM	WT	-	-	++	++	-	-	-	-
P1	VPLY**G**LLLEM	D538G	-	-	-	-	-	-	+++	+
P2	NVVPLY**G**LL	D538G	-	-	-	+	-	+	-	-
P3	VVPLY**G**LLL	D538G	-	-	+	+	-	+	-	+
P4	PLY**G**LLLEM	D538G	-	-	+	++	-	-	-	-
P5	LY**G**LLLEML	D538G	-	-	-	+	+++	++	-	-
P6	KNVVPLY**G**L	D538G	-	-	-	+	-	-	-	+
P7	NVVPL**S**DLL	Y537S	-	-	+	+	-	+	-	-
P8	KNVVPL**S**DL	Y537S	-	-	-	+	-	-	+	+
P9	VPL**S**DLLLE	Y537S	-	-	-	-	-	-	-	+
P10	PL**S**DLLLEM	Y537S	-	-	++	++	-	-	-	-
P11	L**S**DLLLEML	Y537S	+++	+	-	+	-	-	-	-
P12	VVPL**S**DLLL	Y537S	-	-	-	+	+	+	-	+
P13	NVVPL**N**DLL	Y537N	-	-	++	+	-	+	-	-
P14	PL**N**DLLLEM	Y537N	-	-	++	++	-	-	-	-
P15	VPL**N**DLLLE	Y537N	-	-	-	-	-	-	-	+
P16	KNVVPL**N**DL	Y537N	-	-	-	+	-	-	-	+
P17	KNVVPL**C**DL	Y537C	-	-	-	+	-	-	-	+
P18	VVPL**C**DLLL	Y537C	-	-	+	+	++	+	-	+
P19	L**C**DLLLEML	Y537C	++	+	-	+	-	-	-	-
P20	PL**C**DLLLEM	Y537C	-	-	++	++	-	-	-	-
P21	NVVPL**C**DLL	Y537C	++	-	-	+	-	+	-	-

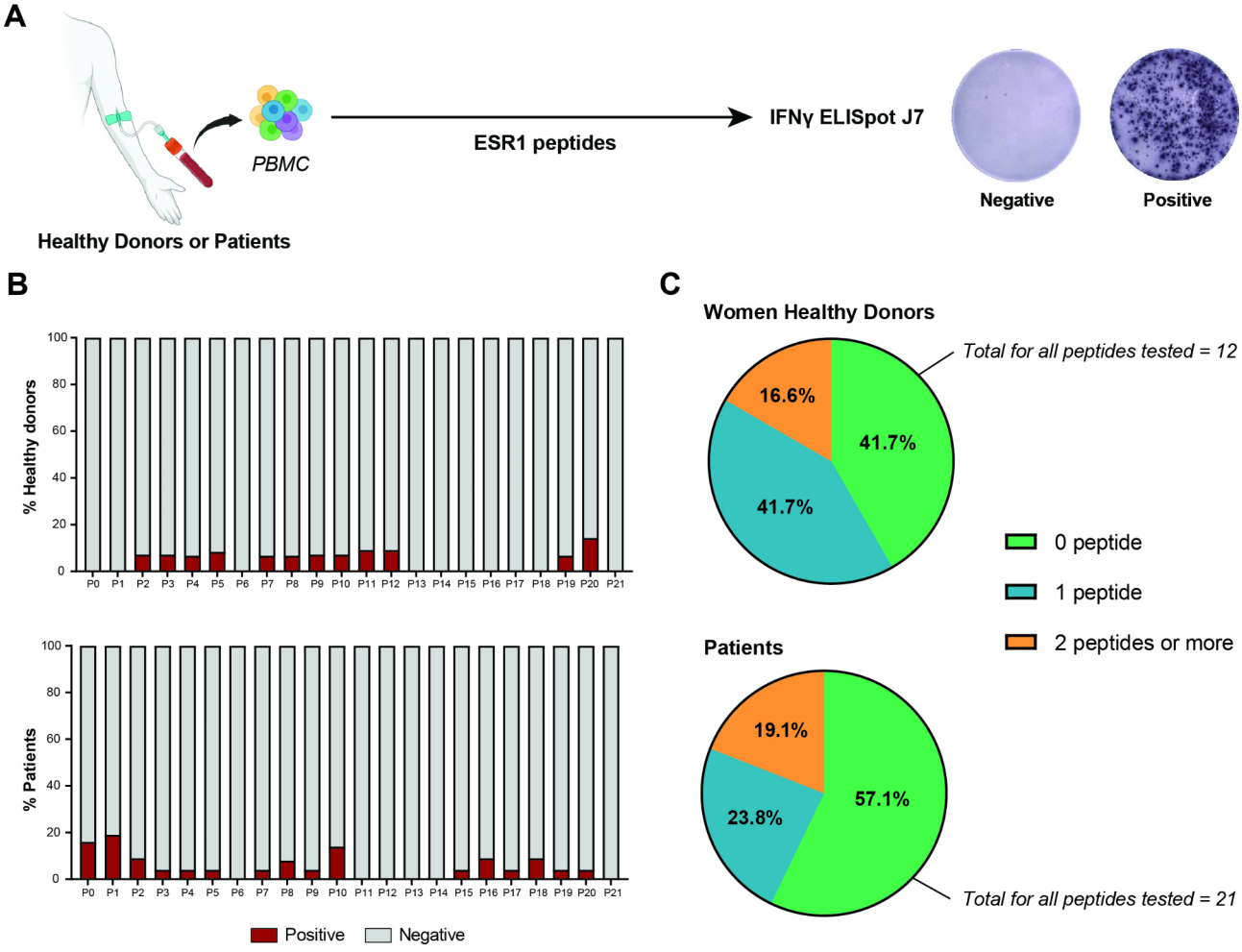

Fig. 1 Specific T cell responses induced by*ESR1* -derived peptides in healthy donors and metastatic breast patients. (**A**) Peripheral blood mononuclear cells (PBMC) from healthy donors and breast cancer patients were stimulated using *ESR1* derived peptides, and an IFN-γ ELISpot assay was performed. (**B**) Frequency of positive responses induced by *ESR1*-derived peptides in healthy donors and metastatic breast cancer patients. (**C**) Distribution of women healthy donors ($n=12$) vs. patients ($n=21$) according to their T cell responses against 0, 1, and 2 or more *ESR1* derived peptides

An *ESR1* specific CD8 T cell response was observed in 7 out of 19 (36.8%) women HD and against at least one peptide from each mutation studied (Fig. 1.B). Among women HD tested for all peptides, respectively 5 out of 12 (41.7%) and 2 out of 12 (16.6%) mounted specific CD8 T cell responses for respectively 1 and 2 or more peptides (Fig. 1.C). Median intensities of positive responses were low, inferior to 50.0SFC/10^5 cell, with the exception of the p15 peptide (80.5 SFC/10^5 cells) and the p11 peptide (136.5SFC/10^5 cells) (Table 2). Altogether, these results imply that it exists precursor T cells specific for mutated *ESR1* epitopes within the human T cell repertoire.

Then, the existence of *ESR1* specific CD8 T cell responses among mBC patients was investigated. Our results showed that an *ESR1*-specific T cell was present in 9 out of 25 (36.0%) mBC patients. Interestingly, 5 out of 21 mBC patients (23.8%) tested for all peptides presented a specific CD8 T cell response for 1 peptide, and 4 out of 21 (19.1%) for two or more peptides (Fig. 1C). Interestingly, the P1 peptide was more immunogenic among mBC patients when compared to HD. The analysis of biological data regarding patient's clinical characteristics demonstrated that *ESR1* specific CD8 T cell responses were overwhelmingly presented in patients with progressive disease after the first line of hormone therapy in 6 out of 9 cases (66.7%) (Additional table S2). In addition, *ESR1* peptides tend to induced stronger cellular responses (superior to 50 spots/10^5 cells) in patients than in women HD. The assessment of MHC class II restricted peptides immunogenicity did not reveal any difference between HD and mBC patients (Additional Table **S2**). This lack of specificity prompted us to focus further analyses on MHC class I restricted *ESR1* peptides.

Altogether, our first results evidenced that CD8 T cells from mBC patients recognized *ESR1* derived peptides. Levels and frequencies of *ESR1*-derived peptides

Table 2 Mean, median and frequencies of T cells responses according to HLA-A/B *ESR1*-specific-restricted peptides in healthy donors and metastatic breast cancer patients. *ESR1* amino acid sequences were submitted to two prediction algorithms (NetMHCpan3 and SYPEITHI) and peptides binding prediction to HLA-A/B were classified as strong (+++), moderate (++), weak (+) or null (-)

Mutation	Peptides	Healthy Donors				Patients		
		Intensity, IFN-y spots/10^5 cells (median [min-max])	Frequency (%)	Women	Men	Intensity, IFN-y spots/10^5 cells (median [min-max])	Frequency (%)	Women
Wild Type	P0	0.00	0.00 (0/31)	0/19	0/12	28.50 [10.00–84.50]	16.00	4/25
D538G	P1	0.00	0.00 (0/26)	0/14	0/12	26.00 [11.50–70.00]	19.00	4/21
	P2	13.50	3.84 (1/26)	1/14	0/12	60.75 [15.50–106.00]	9.50	2/21
	P3	29.50 [19.50–39.50]	8.33 (2/24)	1/14	1/10	695.00	4.16	1/24
	P4	17.00	3.84 (1/26)	1/15	0/11	97.50	4.00	1/25
	P5	13.00	4.34 (1/23)	1/12	0/11	12.00	4.16	1/24
	P6	0.00	0.00 (0/23)	0/12	0/11	0.00	0.00	0/23
Y537S	P7	10.00	3.70 (0/27)	1/15	0/12	116.00	4.00	1/25
	P8	26.50	3.84 (1/26)	1/15	0/11	14.75 [13.50–16.00]	8.30	2/24
	P9	14.00	4.16 (1/24)	1/14	0/10	106.00	4.16	1/24
	P10	15.50	3.84 (1/26)	1/14	0/12	15.00 [11.00–115.50]	14.30	3/21
	P11	136.50	4.34 (1/23)	1/11	0/12	0.00	0.00	0/23
	P12	21.50	4.34 (1/23)	1/11	0/12	0.00	0.00	0/23
Y537N	P13	0.00	0.00 (0/22)	0/11	0/11	0.00	0.00	0/24
	P14	0.00	0.00 (0/23)	0/12	0/11	0.00	0.00	0/23
	P15	80.50	3.22 (1/31)	1/19	0/12	81.50	4.00	1/25
	P16	0.00	0.00 (1/31)	0/18	0/13	65.75 [12.50–119.00]	9.52	2/21
Y537C	P17	0.00	0.00 (0/27)	0/15	0/12	131.50	4.00	1/25
	P18	0.00	0.00 (0/26)	0/14	0/12	68.25 [24.50–112.00]	9.50	2/21
	P19	18.50	3.70 (1/27)	1/15	0/12	88.50	4.00	1/25
	P20	30.50 [20.00–41.00]	3.84 (0/26)	2/14	0/12	695.00	4.16	1/24
	P21	0.00	0.00 (0/23)	0/12	0/11	0.00	0.00	0/23

recognition were enhanced in mBC patients compared to women HD.

Validation of HLA-A2-mediated presentation of *ESR1* derived peptides

To determine whether the *ESR1* peptides we designed in silico had the potential to bind MHC class I we selected the most promising candidate using T2 binding assays followed by in vivo experiments using A2/DR1 transgenic mice. CD8 T cell specific responses were observed following stimulation of PBMC with peptides restricted by MHC class I, we controlled the HLA-A2 restriction of the selected peptides. To assess the HLA-A*02:01 binding affinity of the *ESR1* candidate peptides, T2 binding assays were performed. In comparison with negative HLA-A*0201 pulsed T2 cell peptide, six (neo)peptides (p0, p1, p4, p5, p10 and p20) showed an average fold change in MFI > 1, indicating affinity peptide binding to surface HLA-A*02:01 on T2 cells (Fig. 2.A). These (neo) peptides were further selected to be tested in an HLA-A2 transgenic mouse model to validate in vivo their immunogenicity. Because p1, p4 and p5 were very similar and designed from the same D538G mutation, only the p1 and p4 (initially selected based on its ability to bind the HLA-A2 in silico) peptides were selected to be tested in vivo. Mice were vaccinated with selected peptides and the presence of *ESR1*-specific lymphocytes was assessed directly ex vivo using IFN-γ ELISpot assay (Fig. 2.B).

Mice stimulated with p0, p1, p4, p10 and p20 were able to mount an important *ESR1*-specific T cell immune response. As shown in Fig. 2.C, selected (neo)peptides induced *ESR1* lymphocytes detectable in splenocytes (respectively 37.5 SFC/10^5 cells [17.0-244.5], 48.5 SFC/10^5 cells [11.0-379.5], 45.0 SFC/10^5 cells [11.0–73.0], 36.5 SFC/10^5 cells [11.5–239.0] and 507 SFC/10^5 cells [15.5-1015.5]), the popliteal lymph nodes (respectively 26.5 SFC/10^5 cells [12.0-125.0]), 20.0 SFC/10^5 cells [12.0–60.0], 27.8 SFC/10^5 cells [10.0-136.0], 16.5 SFC/10^5 cells [10.0–75.0], and 171.5 SFC/10^5 cells [18.5–1169]) (Fig. 2.D).

These results point out that *ESR1* selected peptides were able to bind to MHC-I and to elicit specific $CD8^+$ T cell responses in HLA-A2 transgenic mice.

ESR1^{D538G} specific T cells are polyfunctional producing cells

The next set of experiments was designed to evaluate the functional activity of *ESR1*-specific T cells. To this end, HLA-A2 positive PBMC from HD were stimulated using a pool of the previously selected peptides for 14 days. Specific T cells were isolated from one HD using an IFN-γ capture method. Following an amplification step, T cells were cloned by limiting dilution using terasaki plates. A $CD8^+$ T cell clone specific for the p1 peptide, entitled HDB.5 clone, was isolated. To confirm its HLA allele restriction, a moderate to high-resolution HLA typing was performed (data not shown). Additionally, HDB.5 T cell clone showed over 80% of p1-tetramer-positivity confirming both its restriction for the HLA-A2 alleles and its restriction for p1 (Fig. 3.A). HDB.5 avidity was next determined by incubating HDB.5 clone overnight using a range of p1 peptide concentration. As shown in Fig. 3.B, half-maximal IFN-γ secretion was observed at 10 µg/mL (Fig. 3.B). To further substantiate the ability of HDB.5 clone to be activated by the p1 peptide in an HLA-A2 dependent manner, its polyfunctionality was tested by flow cytometry. Thus, the clone was stimulated overnight with p1 peptide, and the cytokine secretion was evaluated. As demonstrated in Fig. 3 the HDB.5 clone was able to produce IFN-γ (80.5%) (Fig. 3.C), TNF-α (78.4%) (Fig. 3.D) and IL-2 (29.9%) (Fig. 3.E). Then, the p1 peptide natural presentation by tumor cells was investigated. To this end, the HDB.5 clone was co-cultured with MCF7 *ESR1*^{D538G} mutant pulsed with the p1 peptide (Fig. 3F). An IFN-γ secretion equal to 1051.5SFC/105 cells was detected suggesting a specific peptide recognition by the clone. However, at respectively 10:1 and 20:1 effector: target ratios, the MCF7 *ESR1*^{D538G} mutant were not able to naturally process the p1 peptide (Fig. 3.F). Altogether, these results demonstrated that p1 is an immunogenic peptide eliciting *ESR1* polyfunctional secreting T cells. Nevertheless, we failed to demonstrate its natural presentation using a tumor cell line model transduced with an *ESR1*^{D538G} mutant.

ESR1 mutations in advanced mBC patients are correlated with a gene expression profile related to immune surveillance disruption

The lack of natural presentation observed in previous experiments raised the hypothesis that *ESR1* mutations might impair the immune recognition of mBC. Genomic alterations interfering with antigen presentation have been previously described in many cancer types, including breast cancer [29]. Given the relevance to immune evasion and high incidence of LOH in HLA genes, we first asked whether HLA LOH was observed in *ESR1* mutated breast carcinoma. To test this hypothesis, we interrogated the META-PRISM dataset which includes tumor RNA samples collected from mBC patients who receives at least one line of treatment and had no other available therapeutic option. A total of 91 Bulk RNA-Seq samples from mBC patients were selected among which 54 exhibited HR+/HER2- profile. Overall, we selected 33 mBC for whom we had both whole exome sequencing and Bulk RNA-Seq samples. Among these 33 patients 12 displayed *ESR1* mutations (Fig. 4.A). We observed a statistically significant decreased expression of both MHC class I and MHC class II alleles in the presence of *ESR1* mutations in advanced BC tumor samples (Fig. 4.B).

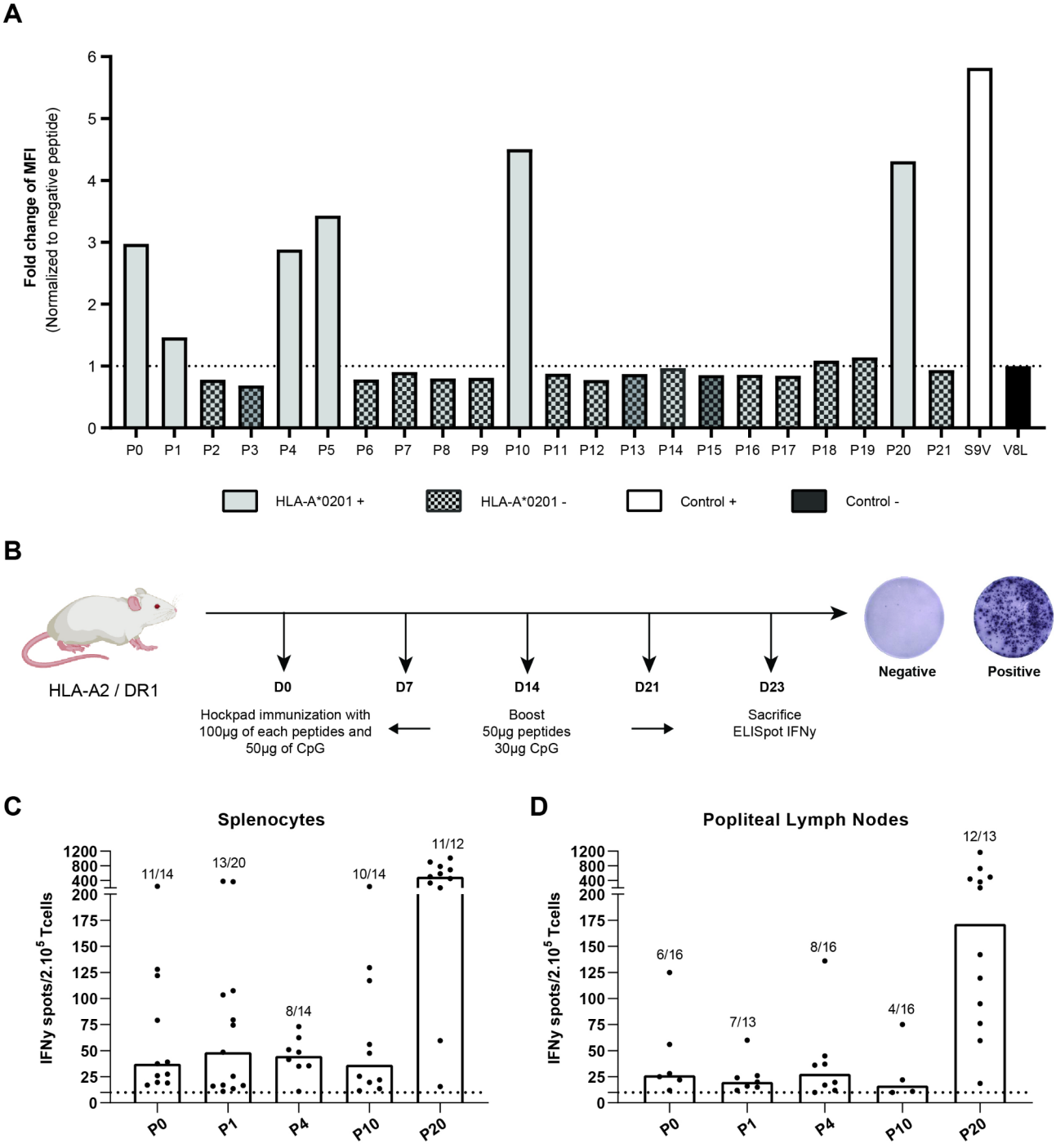

Fig. 2 Validation of HLA-A2-mediated presentation of *ESR1*. **A.** Peptides bind to HLA-A*02:01. T2 cells were pulsed with the indicated peptide for 12 h and surface HLA-A*02:01 was stained. MFI of pulsed T2 cells were normalized to experimentally matched non-pulsed T2 cells, and data were expressed as fold change in MFI. The L10L peptide was used as a positive control and V8L murine peptide was used as a negative control. The dashed horizontal line indicates negative peptide (V8L) pulsed T2 HLA-A*02:01. **B.** Vaccination assay on humanized mice at days 0, 7, 14 and 21. **C**-**D** Magnitude and frequency of spontaneous T cell responses against *ESR1* derived peptides in (**C**) splenocytes from humanized HLA-A2/DR1 mice and (**D**) popliteal lymph nodes

Then, we further investigated the immune infiltration in the microenvironment using MCPcounter algorithm (Fig. 4.C). In line with the lack of MHC class I expression, CD8 T cell infiltration levels were decreased in all *ESR1*-mutated tumor samples compared to non-*ESR1*-mutated mBC. The analysis of the number of TCR clonotypes established that advanced *ESR1*-mutated mBC had a lower number of TCR clonotypes detected compared

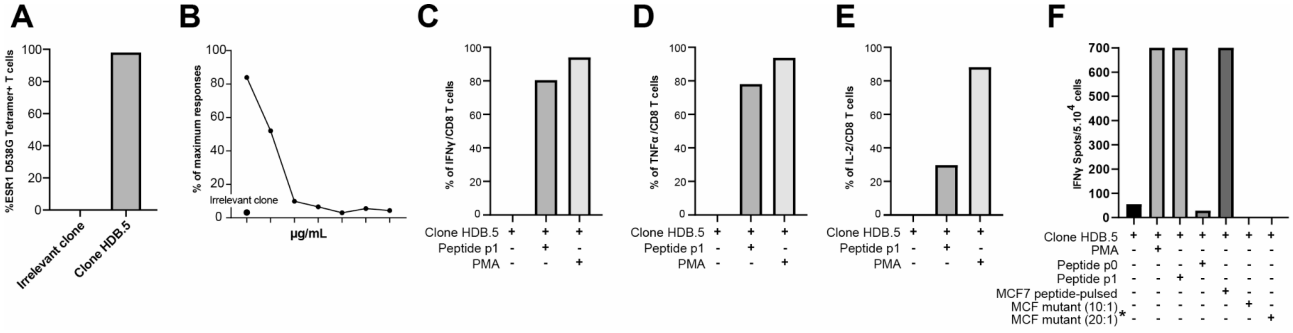

Fig. 3 *ESR1*^{D538G} specific T cells are polyfunctional producing cells. (**A**) Representative FACS histogram of *ESR1*^{D538G} (p1) tetramer staining of respectively irrelevant T cells and specific p1 clone (HDB.5). (**B**) Functional avidity of HDB.5 T cell clone was evaluated after stimulation with a range of the indicated increasing p1 peptide concentrations (p0 was used as an irrelevant peptide). **C-E.** Representative histograms of *ESR1* specific CD8 T clone cytokine production upon p1 peptide unstimulated (medium), stimulated, and PMA/Iono stimulated; IFN-γ (**C**), TNF-α (**D**) and IL-2 (**E**). **F.** Representative histograms of HDB.5 T cell clone IFN-γ production unstimulated (medium), stimulated with PMA/Iono (positive control) stimulated with p1 or p0 peptides, stimulated with MCF7 mutant pulsed with p1, stimulated with MCF7 mutant E: T 10:1 or MCF7 mutant E: T 20:1. All data are representative of at least 3 independent experiments

to *ESR1* wild type mBC (Fig. 4.D). Finally, a differential gene expression analysis showed that expression of molecules involved in antigen processing and presentation was decreased in *ESR1* mutated mBC patients (Fig. 4.E).

Altogether, our results demonstrate that the occurrence of *ESR1* mutations might be a source of tumor specific neoantigen. The detection of *ESR1* neoantigen-restricted T cells in women HD and mBC patients supports the role of such lymphocytes in early immune surveillance. However, in advanced disease, *ESR1* mutations were associated with an immune evasion profile, the loss of MHC-I and II expression and the decrease of intratumoral CD8 T cells infiltration.

Discussion

Hotspot somatic mutations that cluster in the LBD region of the ER gene are a common molecular mechanism that leads to antiestrogen resistance. Mutations in the *ESR1* gene possess some characteristics of an ideal source of tumor neoantigens. In this study, we demonstrated the immunogenicity of *ESR1*-derived CD8 peptides in both HD and mBC patients. Our results led to the identification of one HLA-A2 immunoprevalent *ESR1*-derived peptide, *ESR1*^{D538G} (p1) in mBC patients. HLA-A2 restriction of the immunogenic peptides was confirmed in HLA-A2 transgenic mice. Using a gene set enrichment analysis, we showed that antigen processing and presentation alteration of HLA class I goes hand in hand with *ESR1* mutation. Indeed, *ESR1* mutations were associated with reduced expression of MHC molecules which could explain the low immunogenicity of neoantigens derived from the protein.

The immunogenicity of *ESR1* peptides has been described recently by Goldberg et al. [12]. They identified five immunogenic *ESR1*-derived peptides including two peptides like the ones present in our study (p4 and p10). Moreover, Shafer et al. demonstrated the immunogenicity of different *ESR1*$^{D538G \text{ and } Y537S}$ peptides [13]. Like in our study, they isolated specific T cell clone able to kill target cells pulsed with *ESR1* peptides. However, they failed to demonstrate the natural presentation of these peptides. Their results support our data suggesting that *ESR1* mutation derived peptides are immunogenic but naturally poorly processed. The lack of processing and presentation might contribute to a polyclonal evolution of mBC under the pressure of endocrine therapy.

ESR1 mutations are more prevalent in metastatic ER + breast cancer tissues than in primary tumors (< 1%) [5]. Recently, the BOLERO-2 trial ($n = 541$) showed an OS of 32.1 months for patients without mutation vs. 25.9 months and 19.9 months for patients with respectively *ESR1* D538G or Y537S mutation [11]. Similar results have been reported by Clatot et al. highlighting the association between "circulating" *ESR1* mutations and significantly worse outcomes with a difference equal to 8.3 months for OS and to 1.1 months for PFS compared to patients without mutation (D538G and Y537S/N/C) [30]. In the SoFEA trial assessing a maximum double endocrine targeting approach combining fulvestrant with continued oestrogen deprivation ($n = 383$) the PFS was equal to 2.4 months in the exemestane arm and 3.9 months in the fulvestrant arm for patients with *ESR1* mutations (including E380Q, L536R, Y537C, D538G, S463P, Y537N and Y537S) vs. 4.8 months and 4.1 months without *ESR1* mutations [31]. Finally, in the PALOMA-3 trial ($n = 395$) the PFS was equal to 9.2 months for patients without mutation vs. 7.3 months in cases of Y537S mutation suggesting a potential Palbociclib resistance uniquely gained in tumors bearing the Y537S mutation [32]. Altogether, both Y537S and D538G *ESR1* hotspot mutations were associated with a significantly worse outcome in contrast with mBC patients without *ESR1* mutation. Nevertheless,

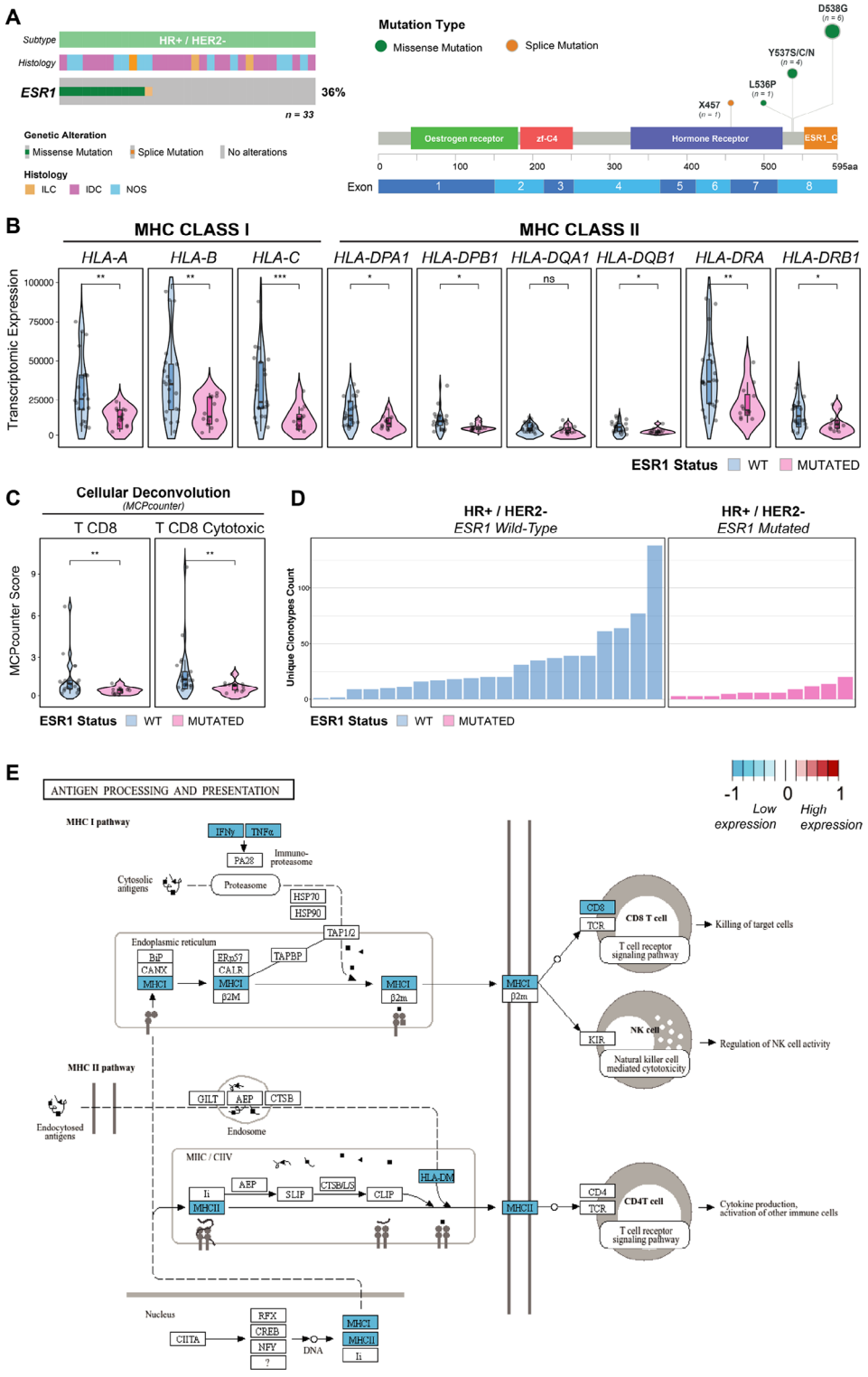

Fig. 4 MHC Class I alterations in breast cancer *ESR1* mutated patients. (**A**) Oncoprint of *ESR1* Mutated tumors (33 h+/HER2- patients, 12 of which are *ESR1* mutated). All mutations are missense. Overall, 20.4% of HR+/HER2- metastatic tumors harbor *ESR1* mutation, uniquely missense (**B**) ViolinPlot of transcriptomic expression of MHC Class I (left) and MHC Class II (right). *ESR1* mutated tumors have a significantly lower expression of every gene in those antigen-presentation mechanisms. **C.** Expression of *ESR1* status in CD8 T cells and CD8 cytotoxic T cells. Blue: *ESR1* Wild Type; Pink: *ESR1* mutated. **D.** Number of unique TCR clones established that advanced *ESR1*-mutated BC (pink) had a lower number of TCR clonotypes detected compared to *ESR1* wild type BC (blue). **E.** Antigen processing and presentation (KEGG) pathway regulation. Genes with lower expression in *ESR1* mutated vs. *ESR1* wild type IFNγ and TNFα; TAP1/2; the MHCI molecule and the class II molecule (HLA-DM) are depicted in blue

the existence of a specific T cell targeting these hotspot mutations in patients according to the presence or not of the mutation(s) has never been investigated. Our results suggest that *ESR1* restricted CD8 immune responses might be initiated early during the immune surveillance phase since detectable immune responses occurred in women HD. The negative prognosis of *ESR1* mutation during the course of the metastatic disease might rely on an escape immune mechanism as suggested by the META-PRISM gene expression analysis (Fig. 4).

Expression of MHC-I by the tumor cell is essential for the effective killing of the tumor [33]. As part of our initial data, we compared the antigen processing and presentation with patients $HR^+/HER2^-$ with or without *ESR1* mutations. Interestingly, the *ESR1* mutated tumors downregulate molecules involved in the antigen processing (TAP1/2) and presentation (MHC I). Previous studies have revealed that loss of HLA presentation is a frequent escape mechanism for neoantigen-specific immunity [17, 34–36]. McGranahan and colleagues found that HLA LOH occurs in 40% of non-small-cell lung cancers (NSCLCs) despite a high subclonal neoantigen burden [36]. In BC context, Sinn et al. analyzed HLA-I expression in 732 patients $HR^+/HER2^-$ and showed that patients with low (34%) or total loss (9%) of HLA-I molecule was associated with worse prognosis when compared to patients with high HLA expression was associated with better response to chemotherapy and elevated TIL density [37]. Therefore, the correlation between *ESR1* mutations and hampered antigen presentation capacities in our gene expression analyses sustains that *ESR1* mutations generate potent neoantigens targeted by these immune escape mechanisms. Further in vivo experiments in mBC patients in the absence and presence of *ESR1* mutations are warranted to decipher these immune evasion mechanisms.

Immune evasion mechanisms may allow the emergence of new *ESR1* mutated tumor cell clones triggered by hormone therapy. In this context, ELISPOT IFN-γ assay has the potential to discriminate between primary and acquired *ESR1* mutations. However, distinguishing these two types of mutations would require monitoring of *ESR1* mutation-specific T cells before and after the initiation of hormone therapy to differentiate between pre-existing and newly generated specific T cell memory populations. Additionally, it is important to keep in mind the limitations of the ELISPOT IFN-γ assay, which depend on the immunogenicity of *ESR1* mutations (as a mutation may not be immunogenic) and the functional state of the patient's immune system. A recent study demonstrated the potential of using ctDNA to prospectively monitor the emergence of *ESR1* mutations in the blood of mBC patients [38]. By using dynamic biomonitoring throughout the first line metastatic treatment of hormone-sensitive BC patients, our research approach could be valuable when combined with ctDNA analysis to prospectively assess emerging *ESR1* mutations in patients' blood, thereby demonstrating their immunogenicity. From a clinical perspective, the emergence of both *ESR1* mutation-specific T cell and the rising levels of ctDNA associated with *ESR1* mutations suggests the presence of an active immunosurveillance in patients. This immunosurveillance could potentially be enhanced through immunotherapies, such as immune checkpoint inhibitors or cancer vaccines, in combination with hormone therapy. Nevertheless, based on the results obtained from the META-PRISM dataset, assessing HLA-I expression by tumor cells would be crucial to achieving effective immunotherapy outcomes. Conversely, the absence of *ESR1* mutations specific T cells might challenge the potential benefit of such therapies and suggest alternative therapeutic strategies.

Altogether, our work demonstrates that mutations occurring in *ESR1* gene generate immunogenic peptides. The presence of specific immune responses in women HD supports the immunogenicity of these mutations and their possible contribution to immunosurveillance. However, the hampered MHC-I expression prevent neoantigens natural presentation in mBC patients and maybe the use of *ESR1* mutations as target for personalized immunotherapy.

Abbreviations

BC	Breast cancer
ER	Estrogen receptor
ESR1	Estrogen receptor 1
ET	Endocrine therapy
HD	Healthy donors
HLA	Human leukocyte antigen
HR	Hormone-receptor
LBD	Ligand-binding domain
mBC	Metastatic breast cancer
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
PBMC	Peripheral-blood-mononuclear-cells
PFS	Progression free survival
RT	Room Temperature

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13058-025-01962-6.

Supplementary Material 1: **Lopez_BCR_Additional_1**: Characteristics of HLA-DR ***ESR1***-specific-restricted peptides predicted by immune informatics database. ***ESR1*** amino acid sequences were submitted to two prediction algorithms (NetMHCIIpan 3) and peptides binding prediction to HLA-DR were classified as strong (+++), moderate (++), weak (+) or null (-).

Supplementary Material 2: **Lopez_BCR_Additional_2**: Mean, median and frequencies of T cells responses in healthy donors and breast cancer patients according to ***ESR1*** HLA-DR peptide.

Supplementary Material 3: **Lopez_BCR_Additional_3**: Clinical characteristics of patients with and without T cell responses to ***ESR1*** hot spot mutations.

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Author contributions
Conception and design: C.B and L.M. Development of methodology: C.B, L.M, M.K and M.L. Acquisition of data: M.L, L.S, A.M, E.S. Analysis and interpretation of data: M.L, M.K, L.S, J.V. Writing, review and/or revision of the manuscript: M.L, M.K, L.S, L.M, C.B. Administrative, technical, or material support: C.B, E.C, G.M, E.D, S.L, R.B, V.D, FC.B. Study supervision: C.B, L.M and M.K.

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Data availability
The datasets used in this manuscript are available upon reasonable request (METAPRISM (PMID: 36862804) EGAD00001009684).

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the ethical standards set forth by the CRC01 protocol, cohort D, and was approved under NCT02838381 number. All participants provided written informed consent prior to their inclusion in the study. The consent process included a detailed explanation of the study. Participants were assured that their participation was voluntary and that they could withdraw from the study at any time without any consequence to their medicam care or legal rights. The confidentiality of the participants' information was strictly maintained throughout the study. The datasets used in this manuscript are available upon reasonable request (METAPRISM (PMID: 36862804) EGAD00001009684). For more detailed information, see the methods section, Bioinformatics procedure/Analysis of database part.

Consent for publication
All authors have given consent for publication.

Competing interests
The authors declare no competing interests.

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