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Comparative analysis of changes in immune cell in the chicken spleen across different ages using flow cytometry

Yeonjae Lee[1†], Rangyeon Lee[1,2†], Jieun Kim[2,4], Yong-Hyun Han[2,3], Christopher Hunter[5] and Jeongho Park[1,2,5*]

Abstract

Background Concurrent emerging and reemerging avian infectious diseases cause multiple risk factors in poultry. A body amount studies attempted to understand pathogen-associated immunity in chickens. Recent research has made progress in identifying immune functions in chicken, there are still gaps in knowledge, especially regarding immune responses during infectious diseases. A deeper understanding in chicken immune system is critical for improving disease control strategies and vaccine development.

Results This study proposes analytical method for chicken splenocytes, enabling the tracking changes in T cells, monocytes, and B cells across three ages. Optimized lymphocyte-activating conditions were suggested using concanavalin A and chicken interleikin-2, which facilitate immune cell activation and proliferation. Next, splenocytes from embryonic day 18, day 5, and day 30 were compared using surface markers and flow cytometry analysis. We observed an increase in T cell subsets, including activated T cells ($CD4^+CD44^+$ and $CD8^+CD44^+$), and B cells, along with a reduced monocyte population after hatching. However, morphological changes and genetic expression of functional immune molecules were limited.

Conclusions The present findings on chicken immune system development offer valuable insights into the avian immune system, including analytical methods and the phenotypic and functional changes in immune cells. Updated immune-boosting strategies during the early stages of life are crucial for developing preventive measures against major infectious diseases in the poultry industry.

Keywords Flow cytometry, Chicken, Immune cell, Development

Background

Concurrent emerging and reemerging infectious diseases, such as avian influenza, Newcastle disease, avian infectious bronchitis, infectious bursal disease, salmonellosis, and avian cholera, cause enormous economic losses to the global poultry industry [1]. The pathogenicity of pathogens differs depending on the age of the infected chickens, as young chickens are more vulnerable to common opportunistic pathogens. For example, fowl adenovirus-4 shows higher mortality rates when young chickens are infected [2, 3]. As birds mature, their susceptibility to disease decreases, likely due to the development and maturation of their immune system [4]. Several

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studies have investigated immune-mediated protection, including immune training, vaccination, and immunomodulating feed additives, to address these problems. Although those studies have utilized various methods for immunological analysis, such as qPCR, immunohistochemistry, and transcriptomic analysis, there is still a need to further explore the avian immune system using updated tools [5–8].

Flow cytometry is widely used in human and mouse immunology research because it is a sensitive technique for both quantitative and qualitative evaluation of immune cells. Flow cytometry using various antibodies, can be employed to not only analyze not just immune cell phenotypes and functions but also identify unknown populations and cells at various developmental stages [9, 10]. This technique has also been applied in avian studies and prompted the development of chicken-specific antibodies, which can enable the investigation of various immune system features [11–14]. For example, previous studies used flow cytometry to analyze the population of leukocytes in peripheral blood and the proliferation of splenic T cells. However, many of them used aged chicken than embryo or newly hatched chicks [11–13, 15]. Chickens have a different immune system than mammals, as they lack peripheral lymph nodes, and the Bursa of Fabricius serves as a major regulator of humoral responses. Therefore, the spleen, as a secondary lymphoid organ in chickens, plays a significant role in cellular immunity for both antibacterial and antiviral activities [7, 8, 16–18].

In newly hatched birds, the activation, phagocytosis, and bactericidal activities of heterophils and macrophages are age-dependent, showing an increase as the birds grow older [19, 20]. Moreover, young chicks have a limited capacity for adaptive immunity, making it crucial to establish immunity to protect against major infectious diseases at an early stage [21–23]. Various strategies are employed to achieve this, including new vaccines and immune-boosting methods. Boosting circulating antibody level by vaccines dose not solely guarantee protective immunity and cellular immunity regulated by T cells and macrophages is essential. Therefore, numbers of studies set the main purpose of vaccination to optimize T cell responses that facilitate prolonged and magnified adaptive immunity [23–25]. Thus, continuous effort to provide safe and efficient protective tools have been made in poultry industry are required. For example, successful vaccine development has been based on a good understandings of immune cell functions and technical procedures, such as drug screening, vaccine production, and cytotoxicity assessment is necessary [11]. Not just in mammals, flow cytometry has also been used to identify avian leukocyte populations in lymphatic tissues of chickens [13, 26, 27] that are vulnerable to multiple infectious diseases at early developmental stages. In our previous studies, we analyzed cellular immune response in chickens using flow cytometry. We observed that enhanced protective immunity, driven by activated T cells and macrophages, was induced by a major gut metabolite, short-chain fatty acids (SCFAs), and an attenuated vaccine candidate against a local fowl adenovirus-4 (FAdV-4) stain [28, 29].

To protect chickens against various pathogen, comprehensive understanding of the immune system at these stages is necessary. Therefore, this study aimed to investigate major chicken immune cell, such as T and non-T cell development from embryonic to early adult stages. First, we proposed optimal conditions for splenocyte culture along with an analytical strategy for flow cytometry. Then, we examined the cellular changes in chicken immune system from aged embryonic day (ED) 18, day 5, and day 30 using the flow cytometry, real-time PCR, and morphology observation. Cells were further cultured for 4–5 days with concanavalin A (Con A) and chicken interleukin-2 (chIL-2), which facilitate lymphocyte activation and proliferation.

Our findings provide valuable insights into the avian immune system, including analytical methods and the phenotypic and functional changes in immune cells during a commercially critical period. We observed drastic changes in the phenotype and population of major immune cells from hatching to a few weeks afterward. This highlights the importance of proper immune-boosting strategies during the early stages of life in the poultry industry. We propose that this study could offer useful information for developing preventive strategies against major contagious diseases.

Materials and methods

Animals

Specific pathogen free (SPF) embryonated chicken eggs were obtained (Sungmin Farm, Korea). The newly hatched White Leghorn layer chicks were housed for 5 days and 30 days. ED 18 chicken embryos, 5-day-old chicks, and 30-day-old chickens were euthanized, and their spleens were collected. The chickens were euthanized with CO2 inhalation and cervical dislocation that we refer to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animal. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (No. KW210401-1).

Cell culture

Spleens from embryos, chicks, and chickens were pooled and homogenized using a 40 μm cell strainer to obtain single-cell suspensions. Splenocytes were collected in

Table 1 Antibodies used for flow cytometry analysis

Panel 1	Target	Clone	Fluorescence
	CD3	CT-3	PacBlue
	CD4	CD-4	PC7
	CD8a	CT-8	AF647
	CD44	AV6	PE
	TCRγδ	TCR-1	AF488
	Live/Dead	-	KO525
Panel 2	CD45	LT40	APC
	MHCII	2G11	FITC
	Monocyte/macrophage	KUL01	PE
	Bu1	AV20	PacBlue
	Live/Dead	-	KO525

Table 2 Primers used for RT-qPCR

β-actin	**F: TTGTCCACCGCAAATGCTTC** ***R*: AAGCCATGCCAATCTCGTCT**
IFN- γ	F: GATGACTTGCCAGACTTACAAC R: TAGGTCCACCGTCAGCTACA
TNF-α	F: AGATGGGAAGGGAATGAACC R: ACTGGGCGGTCATAGAACAG
IL-10	F: CTGTCACCGCTTCTTCACCT R: ATCAGCAGGTACTCCTCGAT
IL-1β	F: ACAGAGATGGCGTTCGTTCCCGA R: TCAGCTCGACGCTGTCGATGT
IL-6	F: CCAGAAATCCCTCCTCGCCAATC R: GCCCTCACGGTCTTCTCCATAAAC
IL-12	F: CCTGTGGCTCGCACTGATAA R: TCTTCGGCAAATGGACAGTA
Inos2	F: GGACCGAGCTGTTGTAGAGA R: AGCAGCTGAGTGATGATCCA

PBS (Gibco, USA) using centrifugation for 4 min at 300 x g. The erythrocytes were lysed using 1mL of RBC lysis buffer for 2 min, and then washed with 10mL of PBS using centrifugation for 4 min at 300 x g. We used a lysis buffer commonly used for erythrocyte removal [27, 30]. When evaluating trypan blue-treated cells using a hemocytometer, we did not observe significant cell damage. The lysed splenocytes were resuspended in RPMI-1640 (Corning, USA) and maintained at 4 °C.

The collected splenocytes were seeded on 96-well U-bottom plates (0.5×10^6 cells/well). The cells were cultured in 37 °C incubator with 5% CO^2 for 5 days with chIL-2 (10 ng/ml) (Sigma Aldrich, USA) and Con A (5 µg/ml) (Kingfisher Biotech, USA) in complete RPMI-1640, containing 10% FBS and 1% antibiotic-antimycotic solution (Gibco, USA). The experiments were repeated for five times.

Morphological analysis of splenocytes

Cultured splenocytes were resuspended in PBS and the monolayers of splenocytes were obtained using a Cytospin 4 centrifuge (Thermo Fisher, USA). The cells were stained using Diff-Quik solution (Sysmex Corporation, Japan), and the cellular morphology was assessed using a bright-field microscope.

Flow cytometry

The splenocytes were harvested and stained using a LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fisher) and two panels of chicken antibodies (Southern Biotech, USA) as shown in Table 1. The cells were stained with antibodies using MACS buffer (0.5% BSA, 2mM EDTA in PBS) for 30 min. The stained cells were washed with MACS buffer for 4 min at 300 x g and then fixed with 1% PFA. The data was acquired using Cytoflex (Beckman Coulter, USA) and analyzed wtih CytExpert Software (Beckman Coulter).

RNA isolation and cytokine gene expression analysis

Total RNA was isolated from splenocytes using Trizol reagent (Invitrogen, USA). The quantity and purity of isolated RNA were measured using a Nanodrop (Thermo Fisher). The purity of the RNA was assessed using the A260/A280 ratio and 1 µg of the RNA and Bioneer RT premix (Bioneer, Korea) were used to produce the complementary DNA (cDNA) to a final volume of 20 µl. The cDNA was diluted in 100 µl of DEPC-treated water and cDNA products were stored at − 20 °C until further use. The 2 µl of the diluted cDNA was used for real-time qPCR with Maxima SYBR Green qPCR Master Mix (Thermo Fisher) to a final volume of 20 µl. All samples were performed in duplicate using a QuantStudio 3 Real-Time PCR system (Thermo Fisher). The gene expression levels were determined using the formula $2^{-\Delta\Delta CT}$. The results were normalized to chicken β-actin expression and the primer sequences used are listed in Table 2.

Various reference genes, such as β-actin, 18s rRNA and GAPDH can be altered under experimental conditions and there is considerable debate about which is most suitable for qPCR. β-actin is used as a suitable internal control for studying gene expression [31] and it was also used to examine genetic expression of immune-molecules in chickens [32]. We followed the MIQE guidelines and performed qPCR using β-actin as a reference gene in previous studies [28, 29] and the same method was employed in this study.

Statistical analysis

Flow cytometry data were analyzed using the Kruskal-Wallis test (the nonparametric equivalent of one-way

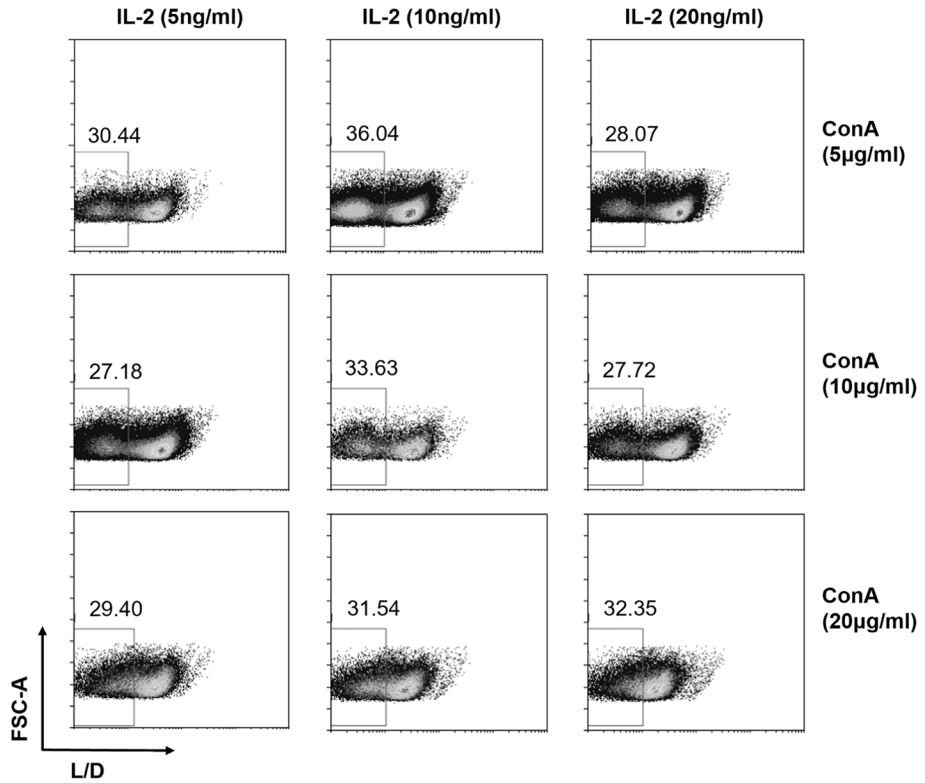


Fig. 1 The cell viability of splenocytes after stimulation using ConA and chIL-2. The splenocytes from day 30 chickens were cultured with ConA (5, 10, and 20 μg/ml) chIL-2 (5, 10, and 20 ng/ml) for 5 days. The stimulated cells were stained with live / dead cell staining kit and the cell viability was examined using flow cytometry

ANOVA) and compared using Dunn's multiple comparisons test in GraphPad Prism software. *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$. Combined data from 5 to 6 experiments are shown, and the error bars represent the SD.

Results

Optimization of lymphocyte activating condition

Con A and IL-2 are widely used for lymphocyte activation, differentiation, and proliferation in both mammals and chickens [33]. We collected 30-day-old chickens and stimulated them using Con A (5, 10, and 20 μg/ml) and chIL-2 (5, 10, and 20 ng/ml) to identify the optimal culture condition. The highest cell viability (36%) was observed when 5 μg/ml of Con A and 10 ng/ml of chIL-2 were used (Fig. 1).

Morphological changes in splenocytes with aging

Next, we examined the cytology of splenocytes from three different ages (ED18, day 5, and day 30; embryo, chick, and adult hereafter) in the presence or absence of Con A and chIL-2. Most embryonic cells showed a myeloid cell-like shape with lobular nuclei and extensive cytoplasm with granules. The lymphocyte-like cells were frequently observed at higher ages, but the stimulation (Con A and chIL-2) did not lead to any cytological changes (Fig. 2A).

Gating strategy of chicken immune cells by flow cytometry

We studied the transition of the cellular phenotype using flow cytometry because the cytological analysis provided limited information of immune cells. Adult splenocytes were examined in the presence and absence of stimulators (Con A and chIL-2) for immune cell phenotyping. Antibodies against CD3, CD4, CD8a, CD44, and TCRγδ were used with a live/dead cell staining kit (Table 1) for T cell identification. Lymphocyte population was selected using forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot gating. Then, the live cell population was separated using the discrimination dye (L/D KO525-A) versus SSC-A density plot gating. For T cell selection, the anti-CD3 antibody was used, and CD4- and CD8-positive cells were identified among γδ-negative T cells. Most $CD4^+$ and $CD8^+$ T cells expressed CD44 on their surfaces among the stimulated cells (Fig. 2B). The non-T cell population was characterized using antibodies against CD45, B cell (Bu-1), and macrophages/monocytes (KUL01) along with a live/dead cell staining kit (Table 1). Initial gating

was performed using FSC-A and SSC-A gating followed by live cell selection. B cell and monocyte populations were selected among $CD45^+$ cells (Fig. 2C).

T cell populations at different ages

Applying the gating strategy from the previous section, we studied changes in T cell populations before and after the stimulation at different ages. Few $CD3^+$ T cells were detected in the embryonic stage under both conditions. However, 20–40% of live splenocytes were identified as T cells in chicks. The proportion of T cells were increased up to 60% on stimulation in adult splenocytes (Fig. 3A). γδ-T cells were hardly detected in the embryonic stage, but a sizable population (approximately 20%) of these cells was present in chicks. This population further expanded on stimulation in adult animals (Fig. 3B). Among αβ-T cells, $CD4^+$ T cells were present in chicks, but the stimulation did not lead to the expansion of this population. Unlike other T cell subsets, $CD8^+$ T cells were detected even in the embryonic stage, and the population was bigger in aged animals. Moreover, the stimulation did not affect the population size (Fig. 3C). These findings indicate that T cell populations, including αβ- and γδ-T cells, expanded with aging, and this expansion can be marginally facilitated using stimulation factors.

Activated T cells, which express CD44 on their surface, are essential for antigen-specific immune response [34]. We examined the populations of activated $CD4^+$ and $CD8^+$ T cells at three ages. CD44-expressing cells were detected at the embryonic stage; however, they were not $CD4^+$ cells. Moreover, 10–20% of chick and adult $CD4^+$ cells expressed CD44 on cell surfaces. CD44 expression was slightly lower in activated cells, and the difference in CD44 expression between chicks and adults was minimal (Fig. 4A). The embryonic $CD8^+$ T cells did not show CD44 expression in unstimulated splenocytes. However, CD8 expression was observed after stimulation in the embryo stage and the population of CD8-expressing cells subsequently expanded with aging (Fig. 4B). These findings indicated that CD44 expression increased as the chickens aged and the shift in the expression was more prominent in $CD8^+$ T cells.

Non-T cell populations at different ages

Most embryonic splenocytes were observed to be myeloid-like cells in the cytology analysis. However, a drastic transition to lymphocytes was observed after the birth stage (Fig. 2A). After screening the transition of T cell populations (Figs. 3 and 4), we studied the transition of non-T cell using anti-macrophage/monocyte and B cell antibodies. In line with the cytological analysis, 20–30% of embryonic splenocytes were positive for anti-macrophages/monocytes staining. The population of such cells gradually decreased after hatching and this decrease was accelerated by Con A and chIL-2 stimulation (Fig. 5A). Chicken B cells are a major lymphocytes in the spleen [35]. We studied changes in the B cell population using the Bu-1 antibody. Notably, embryonic spleens lacked B cells. However, a distinct B cell population was detected in the adult spleen both in the presence and absence of stimulators (Fig. 5B). This finding indicates that myeloid-like cells are decreasing but B cells are expanding after hatching.

mRNA expression changes for major cytokines with aging

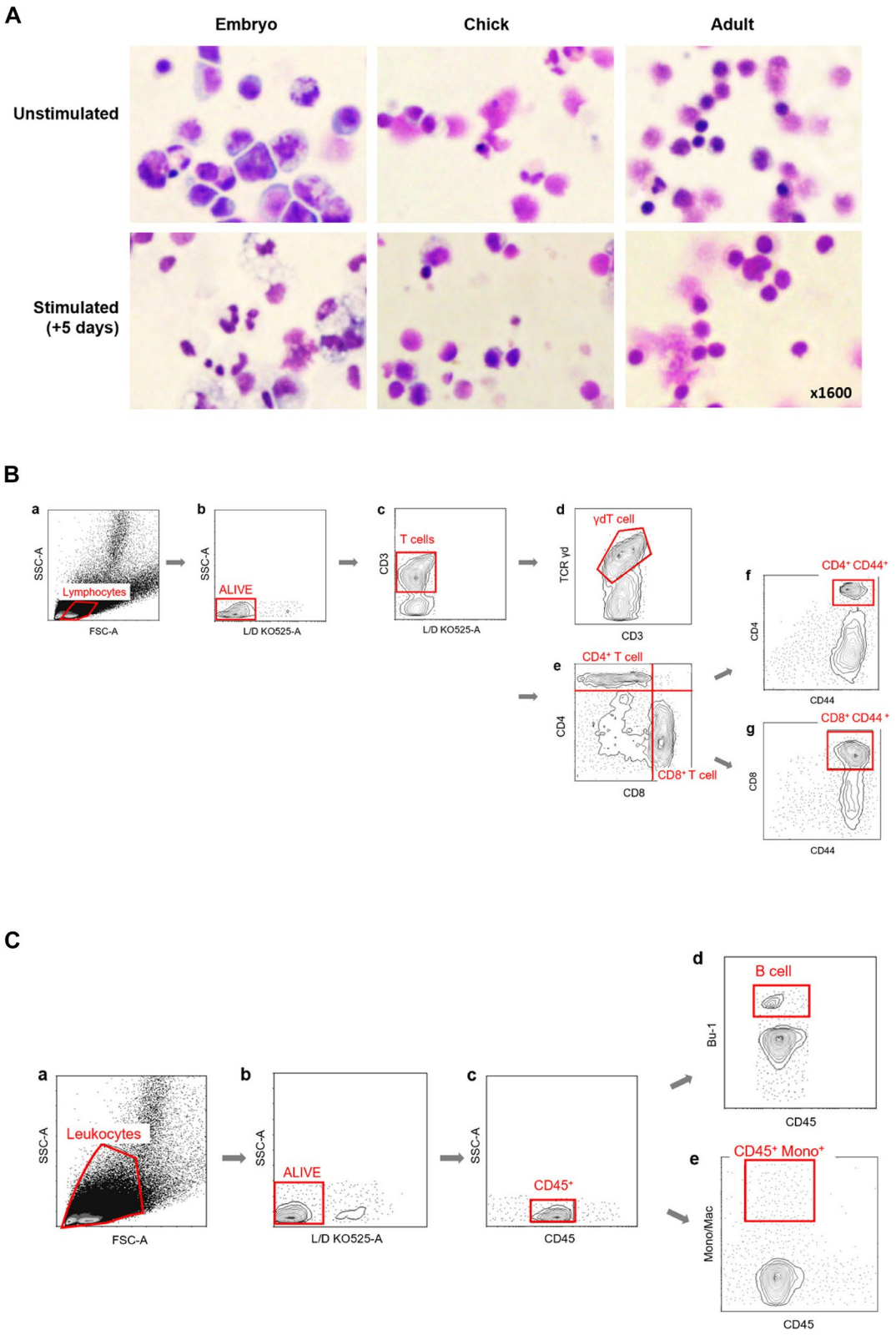
Next, we sought to investigate whether aging affects cytokine expression of immune cells. The expression of major effector and regulatory cytokines was examined at the mRNA level. Although significant changes were not detected, unstimulated splenocytes expressed more genes for T cell-mediated cytokines and the expression levels were slightly suppressed with aging in the stimulated cells (Fig. 6A). The expression trend of non-T cell-associated molecules, such as IL-1β, IL-6, IL-12a, and iNOS2 was consistent with that of T cell-associated molecules. Cytokine expression changes were not detected during the developmental stages without antigenic stimulation (Fig. 6B). In general, no significant changes at the genetic level were observed.

Discussion

The constitution of a robust immune system in the early developmental stages is critical for protection against various infectious diseases in poultry [28, 36, 37]. Here, we propose to observe the population changes of major

(See figure on next page.)

Fig. 2 Cytology and gating strategy. (A) The morphology of splenocytes from different ages Splenocytes from the three different ages were cultured in the presence or absence of ConA (5 μg/ml) and chIL-2 (10 ng/ml). Cytology of splenocytes by ages is shown after 5-day culture. (B) Gating strategy for T cell analysis using flow cytometry; (a) Forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot for the lymphocyte population. (b) Live and dead discrimination dye (L/D KO525-A) versus SSC-A density plot gating. (c) CD3 versus L/D density plot gating for the $CD3^+$ T cells. (d) γδT cells were gated by TCRγδ versus CD3. (e) The $CD3^+$ cells are separated into $CD4^+$ and $CD8^+$ T cells. f and g) gating for activated $CD4^+$ and $CD8^+$ T cells. (C) Gating strategy for non-T cell analsysis (a) Forward scatter area (FSC-A) versus side scatter area (SSC-A) density plot for the leukocyte population. (b) Live and dead discrimination dye (L/D KO525-A) versus SSC-A density plot gating. (c) CD45 versus SSC-A density plot for the pan leukocytes. (d) B cells were gated by Bu-1 versus CD45. (e) Myeloid cells were gated using Monocytes/macrophages versus CD45



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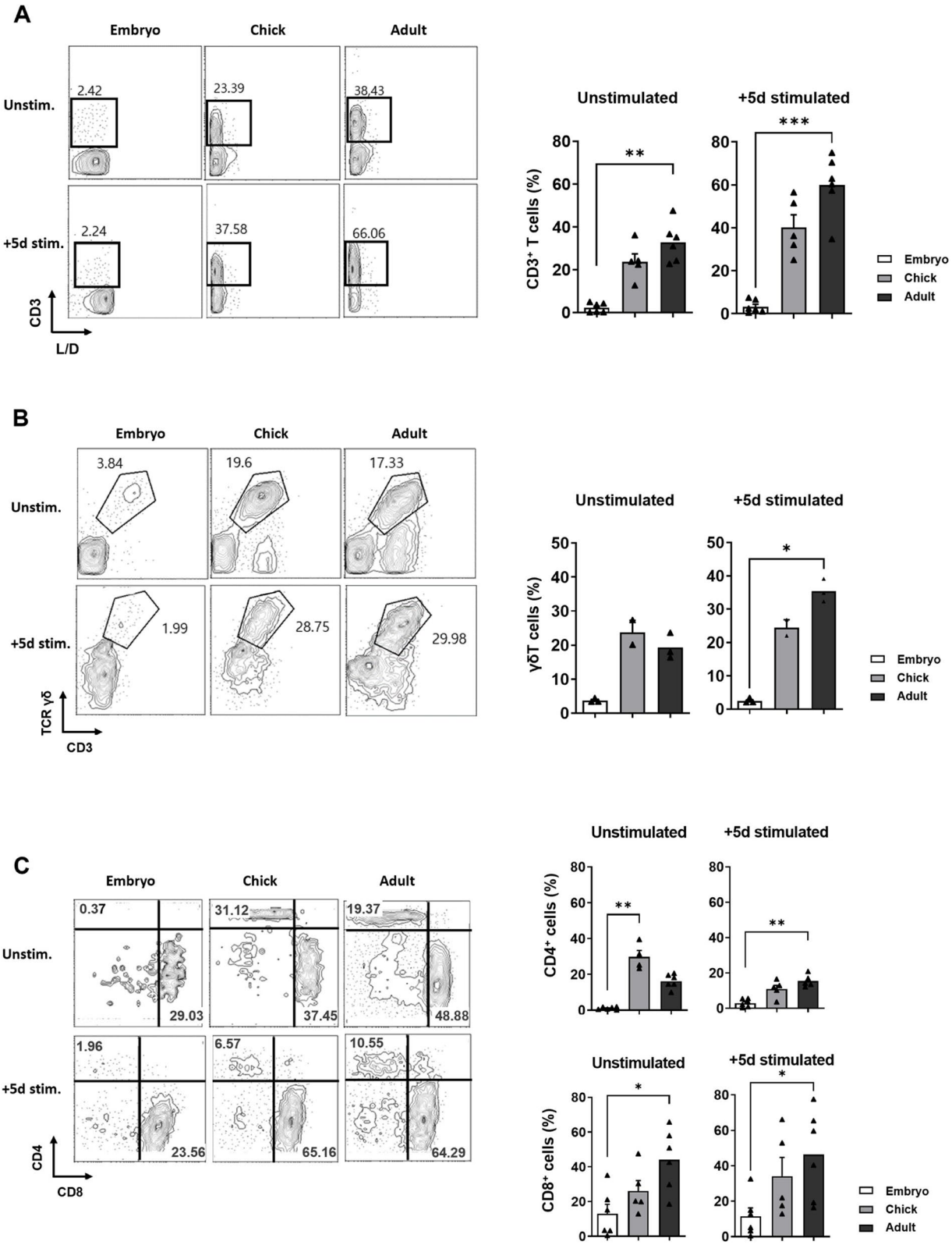

Fig. 3 Phenotypic changes of splenic T cells at three developmental ages. Splenocytes were collected at each age (Embryo, Chick, and Adult) and cultured with ConA and chIL-2. After 5 days, T cell populations were examined in the presence and absence of stimulators. Proportion of $CD3^+$ cells (**A**), TCRγδ and (**B**) and $CD4^+$ or $CD8^+$ T cells (**C**) are shown. The data from 3–6 experiments were combined and representative plots are shown. Statistical significance between ages is indicated as *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$

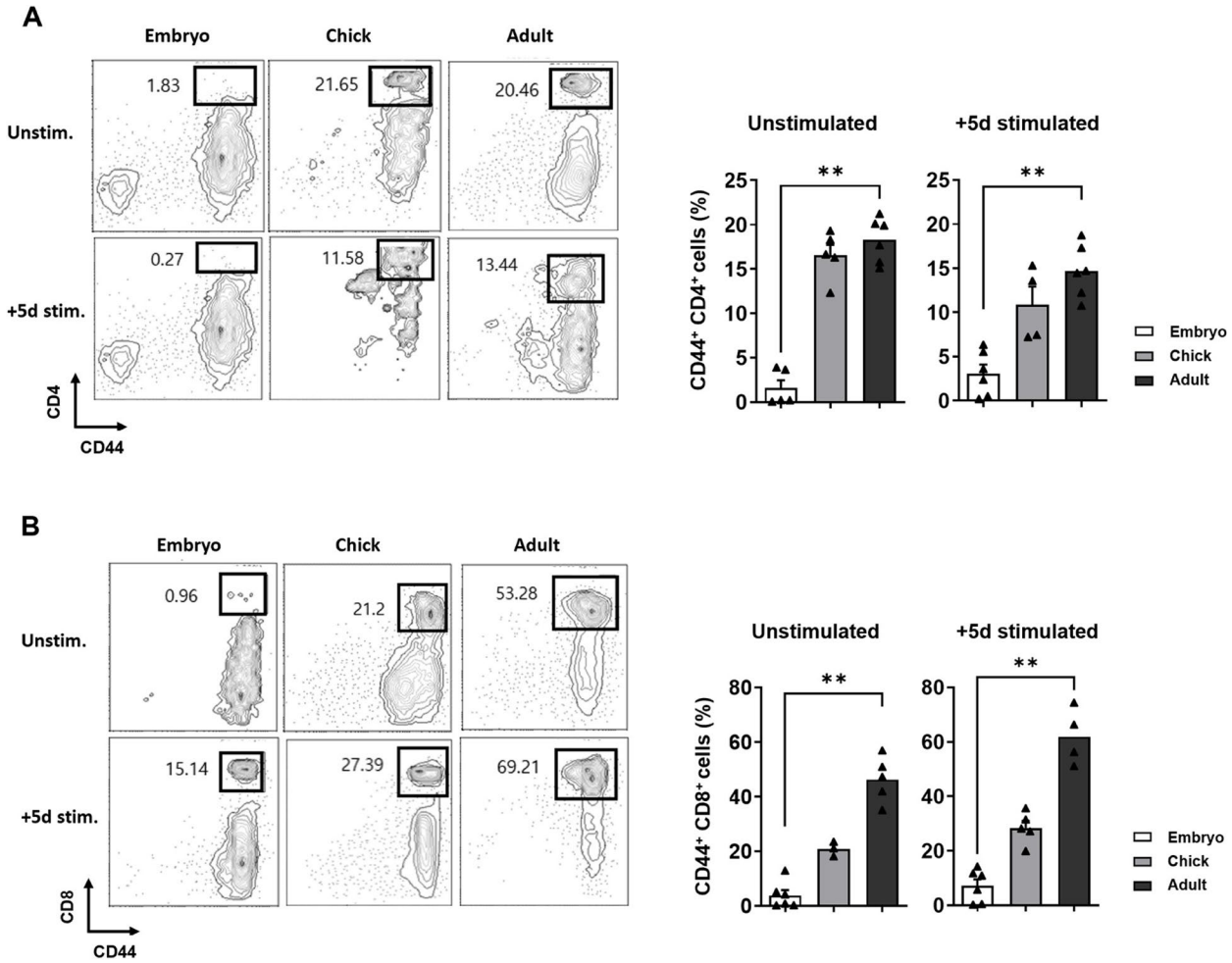

Fig. 4 Population of activated T cells at three developmental ages. CD44-expressing T cells were examined before and after 5 day-stimulation. **A** $CD44^+$ $CD4^+$ and **B** $CD44^+$ $CD8^+$ T cells at each age (Embryo, Chick. And Adult) were examined. Pooled data ($n=6$) and representative plot are shown. Statistical significance between ages is indicated as *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$

immune cells in the spleen across three different ages. Avian immune cell changes were examined from embryo to 1-month-old chickens using flow cytometry, real-time PCR, and morphological observation. Populations of $CD3^+$ T cells, including $CD4^+$, $CD8^+$, and γδ-T cells, were markedly increased after hatching. The increase was apparent among CD44-expressing cells. Moreover, a similar trend was observed in splenic B cells. By contrast, myeloid-like cells were frequently observed in the embryonic stage, but the population drastically decreased after hatching. Unlike lymphocytes, stimulation with Con A and chIL-2 did not show significant differences in these populations. Additionally, changes at the genetic level of immune molecules and morphological changes were minimal. For more refined assays, cell-specific stimuli need to be used in future studies.

T cell precursors are produced in the bone marrow and migrate to the thymus for their development. T cells express T cell receptors (TCRs) during maturation, and the mature T cells migrate to secondary lymphoid organs, such as spleen. This process is initiated during the embryonic stage [38]. CD3 is a major T cell marker that forms the TCR complex, which is associated with antigen recognition and signal transduction [39]. In chickens, TCRs consist of three subsets: γδ (TCR1), αβ1 (TCR2), and αβ2 (TCR3), which are commonly found. Between 2 and 7 weeks of age, $CD4^+CD8^+$ cells significantly decrease in the spleen while being maintained in the thymus [40]. We observed that the proportion of CD3-expressing splenocytes is low at the embryonic stage but increases after hatching. Moreover, stimulation using Con A and chIL-2 further increased this population. This finding infers that either T cell maturation is restricted in the embryonic stage and accelerated following hatching or migration of mature T cells to secondary lymph nodes is not complete in the embryonic stage.

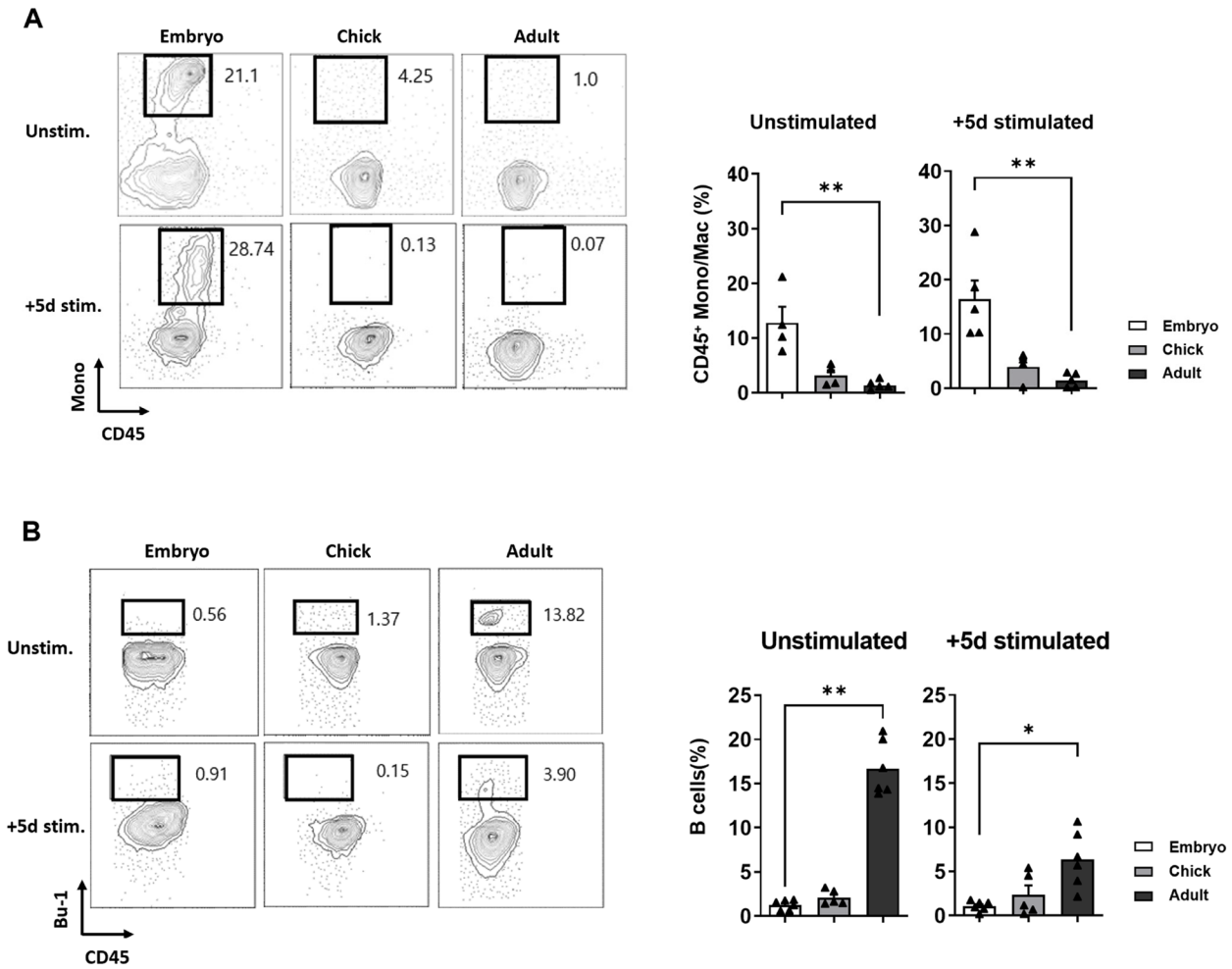

Fig. 5 Population of myeloid and B cells at three developmental ages. Proportion of non-T cells are examined before and after 5 day-stimulation. The population of Macrophage/monocytes (**A**) and B cells (**B**) were observed. The data combined from 6 experiments and representative plots are shown. Statistical significance between ages is indicated as *; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$

Native T cells in the secondary lymph nodes, await antigens that are presented by antigen-presentation cells (APCs). Antigenic peptides, which are presented via the major histocompatibility complex (MHC) of APCs to the T cells directly signal through the TCRs. This interaction is facilitated by numerous costimulatory molecules and inhibitory receptors [1, 41]. Chickens are exposed to enormous amounts of antigens after hatching, which results in increased T cell subset populations. αβTCRs, such as TCR2 and 3, recognize MHC-mediated antigen and undergo maturation process [42]. Notably, we examined that aging led to an increase in both αβ- and γδ-T cells, whereas CD8-expressing cells were observed even in the embryonic stage. Antibodies against chicken CD8 molecule can bind either thymocytes or mature T cells. Moreover, the $CD4^{-}CD8^{+}$ T cell ratio was increased in the spleen at an early age [43, 44]. We speculate that CD8 molecules from thymocytes were detected in the embryonic stage.

The γδ-T cells are the first-observed T cells during the T cell development and maturation in the thymus. These cells constitute approximately 50% of the total T lymphocytes in chickens, whereas they constitute only 2–10% of peripheral lymphocytes in human and mouse [45, 46]. Although it is unclear whether chicken γδ-T cells behave similarly to human and mouse cells, a recent study reported the capability of chicken γδ-T cells to mediate cytotoxic effects against target cells and the enhancement of these effects by chicken IL-2 and IL-12. During Marek's disease virus infection, majority of γδ-T cells expressed CD8 molecules, suggesting an effector function for γδ-T cells. These cells begin appearing in the embryonic spleen and intestinal epithelium from ED15. γδ-T cells are transported to these tissues immediately after and 6–8 days post-hatching [47, 48]. We observed

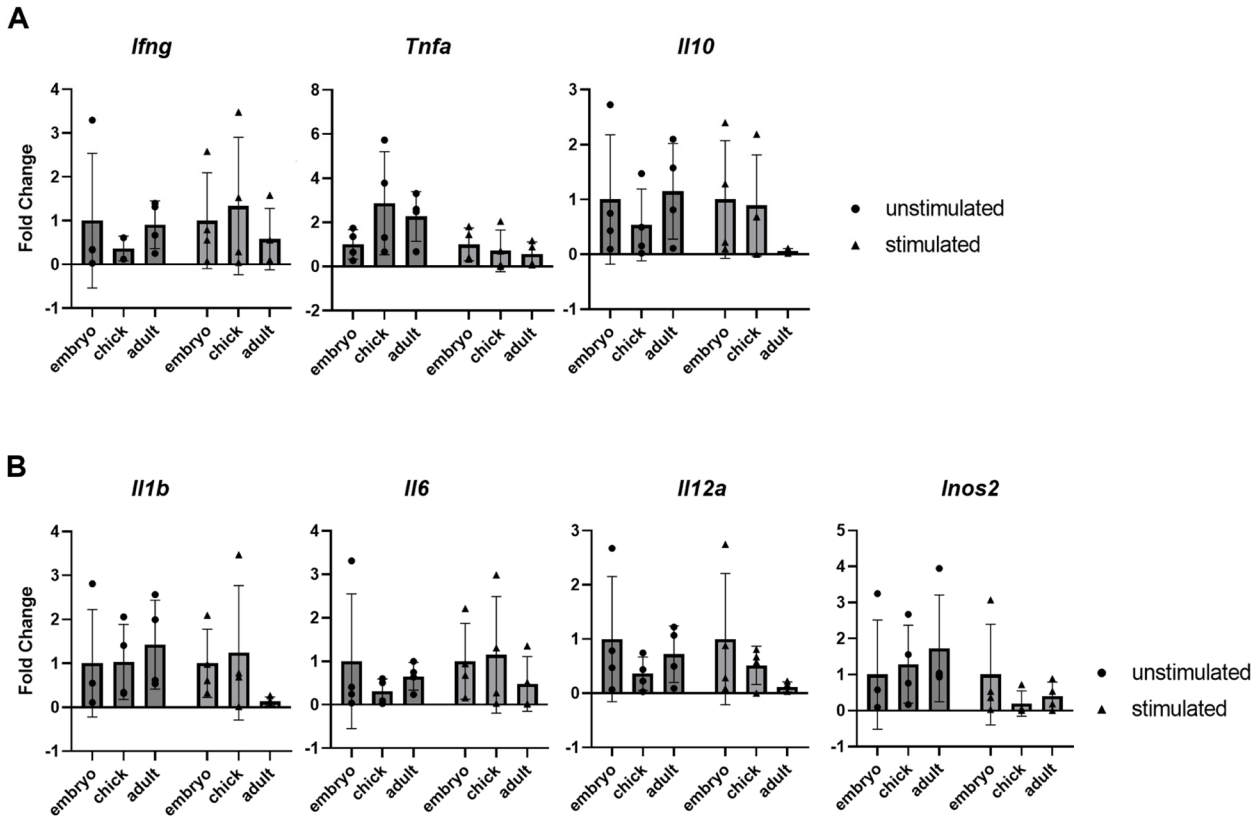

Fig. 6 Immune molecule expressions at mRNA level. The splenocytes from the three developmental ages are shown. Cells were stimulated with ConA and IL-2 for 5 days and genetic levels of cytokines were examined using qPCR. **A** Genes for T cell cytokine (IFN-γ. TNF-α, and IL-10) and **B** Myeloid cells-associated cytokines (IL-1β, IL-6, IL-12a, and iNOS2) were exmained. The data from each group ($n = 3$) were combined and qPCR was conducted in duplicates. The gene expression levels are presented as fold changes compared with the β-actin gene

that additional stimulation using Con A and chIL-2 expanded γδ-T cell population. This finding is consistent with previous reports showing that Salmonella challenge expanded γδ-T cells in the spleens on days 9 and 12 [49]. Therefore, exposure to antigens during early developmental stages is critical for establishing γδ-T cells.

CD44 is a prominent activation marker that distinguishes memory and effector T cells from their naïve counterparts. It also plays a role in early T cell signaling events because it is bound to the lymphocyte-specific protein kinase and thereby enhances TCR signaling [50]. The CD44 molecule, a cell-surface receptor for hyaluronate, is expressed on the surface of various cell types, including hematopoietic and embryonic cells. During infectious bursal disease virus (IBDV) infection, CD44 is suggested to facilitate viral binding on B cells [42]. However, this study observed CD44 as an activation marker for T cells. Using a CD44 antibody, we observed that the populations of activated CD4 and CD8 T cells expanded with aging, but the lymphocyte stimulators did not further increase. CD44-expressing T cells are critical for pathogen immunity [28, 51], and this cell population appears to be established robustly and immediately after hatching even in the absence of additional antigen stimulation. In mouse studies, various surface antibodies have enabled to the identification of T cell subsets, such as central and effector memory types among activated T cells [52, 53]. Moreover, a recent publication analyzed chicken T cell populations using multiple antibodies, including CD25, CD28, CD5, and MHC-II. The kinetic changes in activated T cells were examined during in vitro stimulation by flow cytometry [15]. For a deeper understanding of avian immunology, applying new combinations of markers and developing a broader range of antibodies is necessary.

Avian leukocytes express the CD45 antigen, which can be analyzed by flow cytometry using monoclonal antibodies in combination with various subset markers [15, 54]. Thus, we first gated splenocytes using a CD45 antibody and then used antibodies for B cells (Bu-1) and macrophages/monocytes (KUL01) for additional classification. In the embryonic stage, many KUL01$^+$ cells were detected among leukocytes. Consistent with a previous finding [13], these myeloid cells were reduced in the

spleen of adult animals. KUL01 is co-expressed with the TIM4 molecule at the interface between the periellipsoidal white pulp and red pulp. These markers are detected from the early embryonic stage and potentially differentiate into phagocytic cells [55]. We assume that either myeloid cells rapidly migrate to peripheral organs or undifferentiated primitive immune cells are present in the embryo. Additional antibodies, such as those targeting hematopoietic stem cells and activation markers, can be used to further dissect the subsets of these myeloid cells.

B cell development in chickens has three distinct stages, namely prebursal, bursal and post-bursal stages. Prebursal B cells are dominant in the bursa in the embryonic stage. However, they migrate to lymphoid organs after hatching. The involvement of gut antigens is considered as key factors in the development of B cells [17, 56, 57]. The origin of T and B cells is different, although the process of their expansion in secondary lymphoid tissues appears similar. We speculate that the limitation in vaccine efficacy is due to the biased focus on B cell-mediated humoral responses than the cellular immunity. Therefore, we examined the genetic expression of effector molecules in splenocytes at different ages using real-time PCR. No significant changes were detected at the mRNA level for the immune molecules regardless of age and stimulation. In a human study, the level of effector cytokines such as, IFN-γ and TNF-α were low in the cord blood but highly increased after birth [58]. The results imply that the either developmental age or experimental condition for our study might not be optimal to determine the immune molecule regulation at the mRNA level. Unlike flow cytometry analysis, this analysis was performed using combined immune cells, which could have masked changes.

For more specific analysis, a recent study tried intracellular staining of IFNγ- and TGFβ-expressing T cells in chickens [45]. Direct fluorescence antibodies for different cytokines are required for a more reliable analysis.

Conclusion

Developing effective preventive strategies against infectious diseases, including vaccination and immune boosters, is a major goal in the poultry industry. Chickens are more susceptible to major infections at an earlier age. Therefore, a deeper understanding of the chicken immune system is necessary. Specifically, detailed information on the development of immune cell types across different ages would be particularly helpful. The spleen is a major secondary peripheral lymphoid organ that plays a crucial role in regulating cellular immunity during infectious diseases [7]. We collected and examined phenotypic and functional changes in immune cells from the spleen. Using flow cytometry, we investigated population transition of T cell subsets and non-T cells with aging in the presence or absence of lymphocyte stimulators. We report that drastic changes in immune cell populations are observed during the early stages, particularly after hatching. This could be valuable for evaluating disease prevention strategies and enhancing our basic understanding of the early developmental stages of chickens.

Author contributions

JP designed study and drafted the manuscript. YL and RL performed all experiments (Figs. 1, 2, 3, 4, 5 and 6), contributed for experimental design, and data analysis. JK, YH, and CH analyzed data and participated in manuscript revision. YL and RL contributed equally to this work.

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Availability of data and materials

The data used in this study are available on request to the corresponding author.

Declarations

Ethics approval and consent to participate

This work was approved by the Institutional Animal Care and Use Committee (IACUC) of Kangwon National University (No. KW210401-1).

Consent for publication

All authors reviewed the final version of manuscript and agreed to publish the manuscript.

Competing interests

The authors declare no competing interests.

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