Heliyon 10 (2024) e25366

Contents lists available at ScienceDirect

Heliyon

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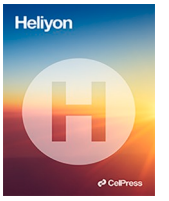

Research article

Protective immune response induced by Leghorn male hepatoma cell-adapted fowl adenovirus-4

Rangyeon Lee [a], Haan Woo Sung [a,c], Hee-Tae Cheong [a,c], Jeongho Park [a,b,*]

[a] College of Veterinary Medicine, Kangwon National University, Chuncheon, Republic of Korea
[b] Multidimensional Genomics Research Center, Kangwon National University, Chuncheon, Republic of Korea
[c] Institute of Veterinary Science, Kangwon National University, Chuncheon, Republic of Korea

A B S T R A C T

Fowl adenovirus-4 (FAdV-4) is a highly contagious virus that causes acute and lethal hepatitis. It leads to substantial economic losses in the poultry industry. Among the structural proteins of FAdV-4, hexon and fiber2 are associated with immunopathogenesis. A frameshift mutation was generated in the fiber2 protein by seral passages in the Leghorn male hepatoma (LMH) cell line. Immunization using the attenuated virus (80 times passaged) before the virulent FAdV-4 challenge protected hosts from the infection and cleared the invading virus. In immunized animals, activated $CD4^+$ and $CD8^+$ T cell populations were larger during the FAdV-4 challenge. The change in the B cell population was similar. Myeloid cells were highly increased during FAdV-4 infection after the immunization, but the immunization inhibited the expansion in both liver and spleen. The functional gene expression for immune modulation was strongly associated with immune cell changes in the liver, however, this association was not strong in the spleen. The present findings imply that genetic modification by cellular adaptation regulates immune cell phenotype and function in the target organ. In addition, we suggest the attenuated virus as a protective strategy against the novel FAdV-4 strains.

1. Introduction

Fowl adenovirus (FAdV) is a non-enveloped dsDNA virus that belongs to the Aviadenovirus genus within the Adenoviridae family. FAdVs are grouped into 5 species (FAdV-A-E) and 12 serotypes (FAdV-1–7, 8a, 8b, and 9–11). FAdV-4 infection affects broilers aged 4–8 weeks and involves severe pathology, including hepatomegaly, hepatic hemorrhage, inclusion body hepatitis (IBH), hydropericardium, and nephritis [1,2]. The duration of infection is relatively short, it has a high mortality rate, and outbreaks are still reported worldwide [3–5].

The host recognizes pathogens through pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) [6]. Various cytokines and chemokines induced by the TLR response led to the activation or differentiation of specific immune cells and promote their migration to the inflammatory site. Macrophages reside in most tissues and are essential for the initial defense response during pathogens invasion [7]. They can be classified into M1-type macrophages, which produce inflammatory cytokines, and M2-type macrophages, which are involved in tissue repair and the resolution of inflammatory responses. The initial TLR response to the pathogen induces macrophages polarize into the M1-like phenotype. Differentiated macrophages migrate to infected or damaged sites, and they produce cytokines such as IL-1β, IL-6, and TNF-α. Their proper response helps pathogen clearance while minimizing tissue damage [8–10].

However, excessive cytokine production induces unwanted pathogenesis in uncontrolled immune responses [11,12]. For example, aberrant production of IL-6 and type 1 IFN causes tissue damage and severe inflammatory reactions in the infected area [13]. During

* Corresponding author. College of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Republic of Korea.
E-mail address: jhp@kangwon.ac.kr (J. Park).

https://doi.org/10.1016/j.heliyon.2024.e25366
Received 29 October 2023; Received in revised form 4 January 2024; Accepted 25 January 2024
Available online 1 February 2024

avian influenza virus infection, excessive effector cytokines (IFN-α, IFN-γ, IL-1, IL-6, IL-12 and TNF-α) levels can induce severe tissue damage and high mortality in chickens [14]. Hepatitis is the leading cause of high mortality during FAdV-4 infection, which is also accompanied by superfluous production of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-6 [15,16]. Therefore, regulating the immune response during viral infection is essential for host protection.

Vaccines reduce the immunopathology generated by pathogenic antigens by adjusting abnormal immune responses [17]. Recent strategies including inactivated viruses, live-attenuated viruses, subunit antigens, and combined vaccines have been used to deal with FAdV-4 outbreaks and severe hydropericardium hepatitis syndrome (HHS) [18]. For example, inactivated vaccines derived from the liver suspension of the disease flocks and attenuated virus vaccines derived from the embryonated eggs or cell culture-based virus propagation have been used. A cell-culture-propagated vaccine was evaluated to be capable of cross-defense against various serotypes of FAdV-4 [3]. The attenuated live vaccine induced a higher antibody reaction and successful protection than the inactivated vaccine [6]. Moreover, deletion of fiber2 protein in FAdV-4 provided protection against a lethal challenge [19]. Recent studies indicated that local immune responses by vaccination have a pivotal role instead of neutralizing antibodies [20,21]. However, vaccine-induced cellular immune response along with various immune genes has not been thoroughly investigated.

In a previous study, we developed an attenuated FAdV-4 virus [22]. The genetic modification that attenuated the virulence was generated through serial passages (using a virulent FAdV-4 strain isolated in Korea) in the Leghorn male hepatoma (LMH) cell line. The immunization protected the hosts against FAdV-4 infection. In protected animals, T and B cell population were expanded, while myeloid cell populations were not in both peripheral and lymphoid tissues. Genetic levels of functional cytokines were maintained at basal levels during the infection in immunized animals. The present study will contribute to understanding the immune response induced by live-attenuated virus and the immune function of attenuated vaccine during avian viral infection.

2. Materials and methods

2.1. Virus preparation

The FAdV-4 strain was isolated from the FAdV-4-infected layers (45-week-old) with hydropericardium syndrome (HHS) in the commercial chicken flock (Yeoju-city, Gyeonggi-do, Korea). The FAdV-4 virus was propagated in the LMH cell line (ATCC, USA). The virus was passaged 10 times (LMH10) and 80 times (LMH80), and the LMH cells were maintained in Waymouth's media (Gibco, USA) supplemented with 1 % antibiotic-antimycotic solution (Gibco, USA).

2.2. Animal experiments

Thirty SPF chickens were divided randomly into two groups: LMH80-immunized group and control group. For the immunization, 15 chickens (1 day old) were injected intramuscularly with 0.1 ml of 80-times passaged FAdV-4 (LMH80) and 15 chickens in control group were injected with the same volume of non-passaged culture media (Control). At 7 days after injection, 10 chickens (8 days old) in the LMH80 immunized group and control group were challenged with 0.1 ml of virulent FAdV-4 (LMH10, 10^5 $TCID_{50}$). The remaining 5 chickens in each group were injected with 0.1 ml of non-passaged culture media. The chickens were housed in separated isolators with adequate temperature and had free access to food and water. The chickens were euthanized with CO_2 inhalation and cervical dislocation 3 days post-challenge, and the tissue samples were collected. The experiments were repeated three times. The animal experiment was approved by the Institutional Animal Care and Use Committee of Kangwon National University (No. KW-210401-1).

2.3. Viral detection

The feces (2.5 mg) were collected from euthanized chickens at 3 dpi and homogenized with 300 μl of PBS. The homogenized samples were centrifuged at 3000 rpm for 10 min and the 200 μl of supernatant was used for viral gene detection. The viral nucleic acid was extracted using the Maxwell RSC Viral Total Nucleic Acid Purification kit (Promega). FAdV-4 was detected by conventional PCR using Hexon A and Hexon B primers, as described in the previous report [23]. The PCR was performed as follows: pre-denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 90 s, 45 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 3 min.

2.4. Cell isolation

The livers and spleens were collected from three chickens per group. The chopped liver tissues were digested in RPMI media containing collagenase type IV (Worthington Biochemical Corporation, USA) for 30 min in at 37 °C. The dissociated tissues were homogenized with 40 μm cell strainer (SPL Life Sciences, Korea). The mononuclear cells were obtained by centrifugation with 30 % Percoll solution (Sigma-Aldrich, USA). Red blood cells were lysed with chicken RBC lysis buffer for 2 min and then washed with PBS. Isolated cells from each tissue were centrifuged and suspended in RPMI media containing 10 % FBS (Corning, USA) and 1 % antibiotic-antimycotic solution (Gibco, USA). The liver cells and splenocytes were prepared using the Cytospin and the cell morphology was examined using Diff-Quik staining.

2.5. RNA extraction and quantitative PCR

Total RNA was extracted from cut liver tissues and splenocytes using Trizol reagent (Invitrogen, USA). Then, cDNA was synthesized with 1.5 μg of total RNA using a RT premix (Bioneer, Korea) according to the manufacturer's protocol. Real-time qPCR was performed in total 20 μl reaction mixture using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher, USA). All samples were analyzed in duplicate. The threshold cycle (CT) values were examined using (QuantStudio™ 3 Real-Time PCR System, Thermo Fisher, USA) and the expression level was calculated using the formula $2^{-\Delta\Delta CT}$. Relative gene expression levels were normalized using the chicken β-actin gene levels. The primer pairs were synthesized based on the genome sequences in the NCBI. The sequence of primers was taken from previously published papers [24,25] or designed using Primer 3 and BLAST (NCBI). The primer sequences used are listed in Table 1.

2.6. Cell staining and flow cytometry

Mononuclear cells isolated from the liver and spleen were stained for 30 min using fluorescent antibodies. The following antibodies were used: LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fisher) and anti-chicken CD4 (CT-4), CD8α (CT-8), CD44 (AV6), TCRgd (TCR1), Monocyte/Macrophage (KUL01), MHCII (2G11), Bu-1 (AV20), CD45 (LT40), all from Southern Biotech. Stained cells were acquired using flow cytometry (Cytoflex, Beckman Coulter) and the data were analyzed using CytExpert Software (Beckman Coulter).

3. Statistics

Student's unpaired *t*-test was used to determine the statistical significance between groups in the flow cytometry analysis. The quantitative PCR results were analyzed using the one-way ANOVA. P values less than 0.05 were considered significant.

4. Results

4.1. Protective efficacy of the attenuated virus against FAdV-4 infection

We previously observed the protective action of attenuated FAdV-4 after serial passages in LMH cells when the virus was injected intramuscularly [22]. Here, we set up a harsher condition: we immunized 1- or 2-day-old chicks using the attenuated virus (LMH80) and challenged them with a virulent strain (LMH10) 7 days later. As expected, most immunized animals survived and stayed active,

Table 1
Primer information for real-time PCR.

Genes	Sequence
B-actin	F: TTGTCCACCGCAAATGCTTC
	R: AAGCCATGCCAATCTCGTCT
TNF-a	F: AGATGGGAAGGGAATGAACC
	R: ACTGGGCGGTCATAGAACAG
IFN-a	F: CATCCTGCTGCTCACGCTCCTTCTG
	R: ATCCTGGACACCAGCAACACCCA
IFN-b	F: CCTCCAGCTCCTTCAGAATACG
	R: ACAGCCTCCTCAACCAGATCCAGC
IFN-g	F: GATGACTTGCCAGACTTACAAC
	R: TAGGTCCACCGTCAGCTACA
IL-1b	F: ACCCGCTTCATCTTCTACCG
	R: TCAGCGCCCACTTAGCTTG
IL-6	F: CCAGAAATCCCTCCTCGCCAATC
	R: GCCCTCACGGTCTTCTCCATAAAC
IL-8	F: GCTCTGTCGCAAGGTAGGA
	R: TGGCGTCAGCTTCACATCT
IL-10	F: CTGTCACCGCTTCTTCACCT
	R: ATCAGCAGGTACTCCTCGAT
IL-12b	F:CCTGTGGCTCGCACTGATAA
	R: TCTTCGGCAAATGGACAGTA
IL-22	F: CAATGCCCATCAAGCCTGCA
	R: ATGCTGAGGATGTGGCACAG
IL-6R	F: CGCCTGCTGGTGGAAGA
	R: TTCACCCGGCAGACGAATTT
CX3CR1	F: TCCAGAACGATCAAGCACAG
	R: CGGTGTTCAGTTCCACATTG
TGFB3	F: GAGTCCGAGTACTACGCCAAAGA
	R: CACGTTAAAGCGGAACACATTG
iNOS2	F: GGACCGAGCTGTTGTAGAGA
	R: AGCAGCTGAGTGATGATCCA

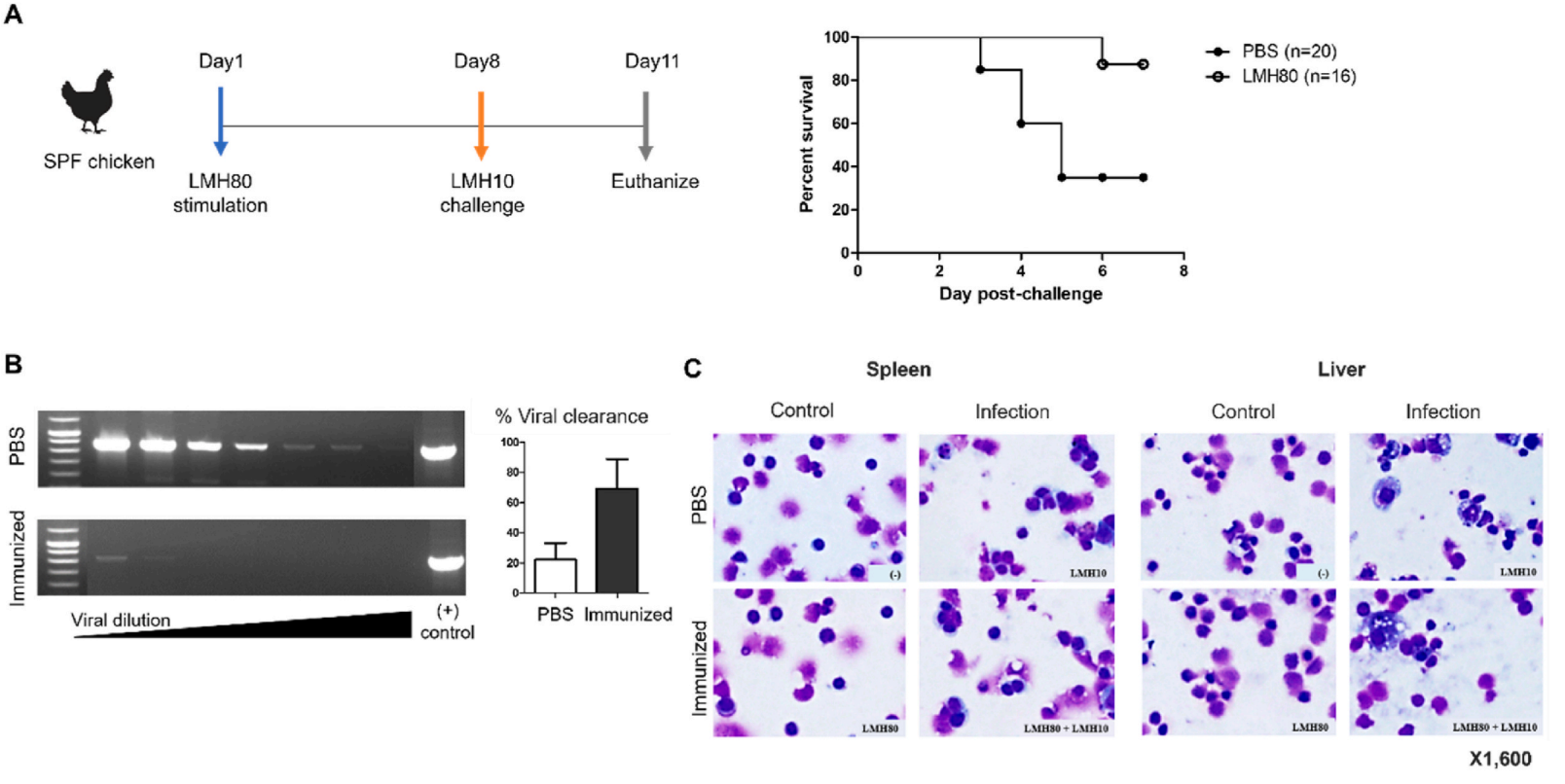

Fig. 1. Effect of the attenuated virus on survival rate, viral clearance, and cytology during FAdV-4 infection. (A, left panel) A schematic of the study design. Chickens were immunized in the presence or absence of the attenuated FAdV-4 (LMH80) and were challenged by LMH10 after a week. (A, right panel) The survival was examined (n = 16–20 per group). (B) Viral clearance was assessed using conventional PCR. (n = 4 per group). Data are representative of three independent experiments. Non-adjusted image is shown in the supplementary material. (C) Representative cellular morphology of the spleen and liver at 3 days after FAdV-4 infection. The experiments were performed in triplicate.

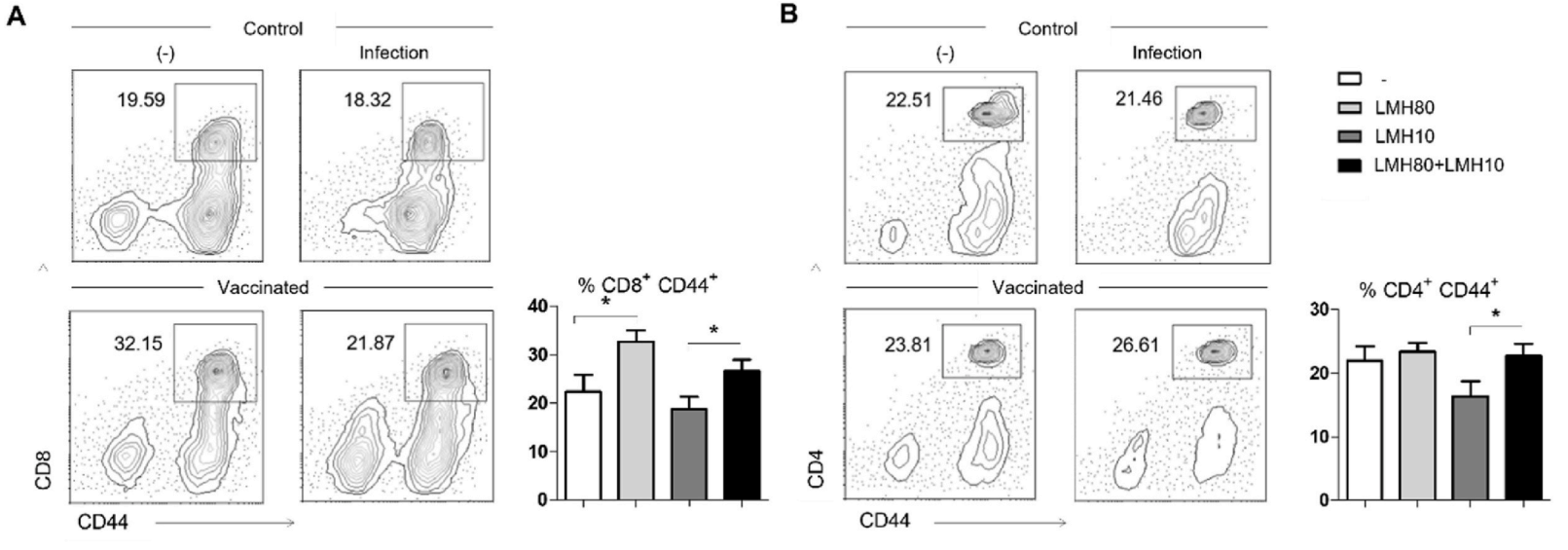

Fig. 2. Effect of the attenuated virus on T cell phenotype in the spleen during FadV-4 infection. Frequencies of activated $CD8^+$ (A) and $CD4^+$ (B) T cells in the spleen at 3 days after FAdV-4 infection. Splenocytes from 3 to 4 chickens per group were analyzed using flow cytometry. Independent experiments were repeated three times, and representative dot plots and pooled data are shown. Statistical significance in the unimmunized group was analyzed using an unpaired *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

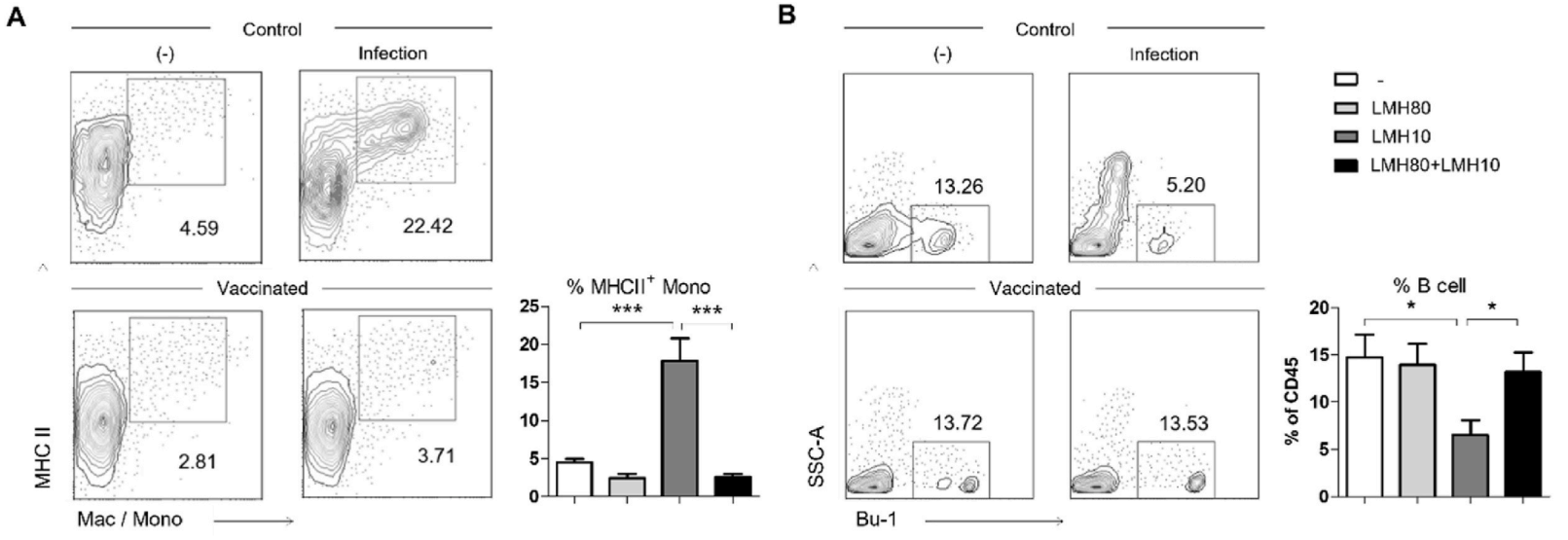

Fig. 3. Effect of the attenuated virus on myeloid and B cell phenotypes in the spleen during FAdV-4 infection. Phenotypic changes of MHCII-expressing macrophages/monocytes (A) and B cells (% of live cells) (B). Splenocytes from 3 to 4 chickens per group were analyzed using flow cytometry 3 days after FAdV-4 infection. Representative dot plots and pooled data are shown from three individual experiments. Statistical significance in the unimmunized group was analyzed using unpaired *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

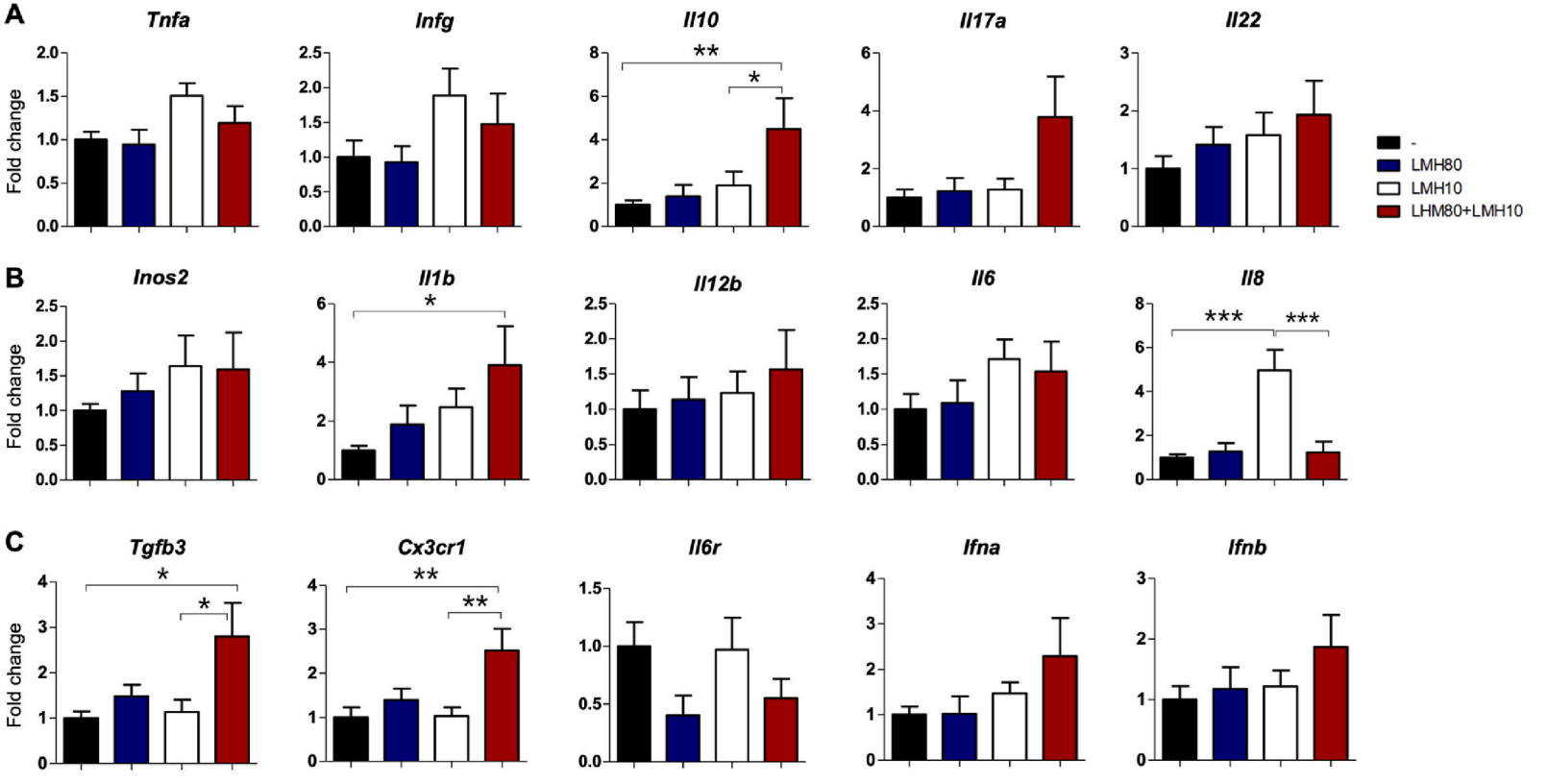

Fig. 4. Effect of the attenuated virus on immune functions in the spleen during FAdV-4 infection. (A) mRNA expression of T cell-associated cytokines in the spleen during FAdV-4 infection. (B and C) Expression of effector genes of innate immunity and myeloid cells. Spleens were obtained from 3 to 4 chickens each group at 3 days after FAdV-4 infection. cDNA was synthesized from each sample, and real-time qPCR was conducted in duplicate. Three independent experiments were performed, and the pooled data are shown. Relative gene expression was normalized using the control group expression. Statistical significance was analyzed using one-way ANOVA (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

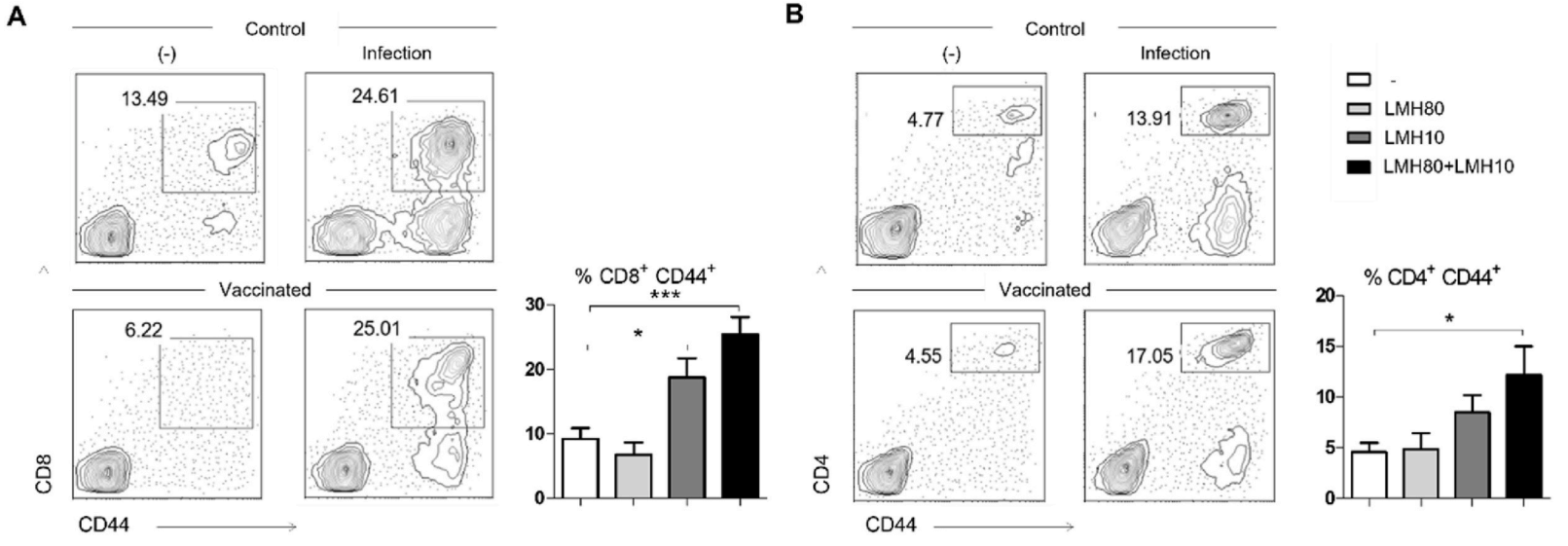

Fig. 5. Effect of the attenuated virus on T cell phenotype in the liver during FadV-4 infection. Frequencies of activated $CD8^+$ (A) and $CD4^+$ (B) T cells in the liver 3 days after FAdV-4 infection. Splenocytes from 3 to 4 chickens per group were analyzed using flow cytometry. Independent experiments were repeated three times, and representative dot plots and pooled data are shown. Statistical significance in the unimmunized group was analyzed using an unpaired *t*-test (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

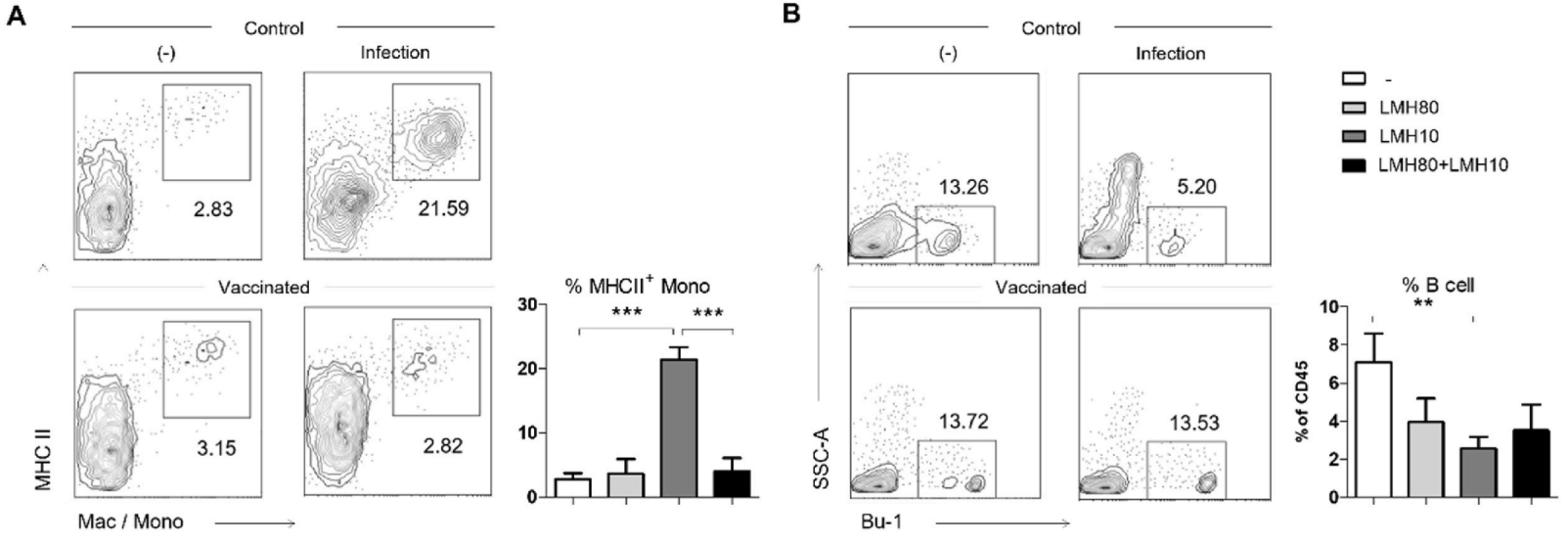

Fig. 6. Effect of the attenuated virus on myeloid and B cell phenotypes in the liver during FadV-4 infection. Phenotypic changes of MHCII-expressing macrophages/monocytes (A) and B cells (% of live cells) (B). Mononuclear cells were isolated from livers. Three or four chickens per group were analyzed using flow cytometry at 3 days after FAdV-4 infection. Representative dot plots and pooled data are shown from three individual experiments. Statistical significance in the unimmunized group was analyzed using an unpaired *t*-test (*p $\leq$ 0.05, **p $\leq$ 0.01, and ***p $\leq$ 0.001).

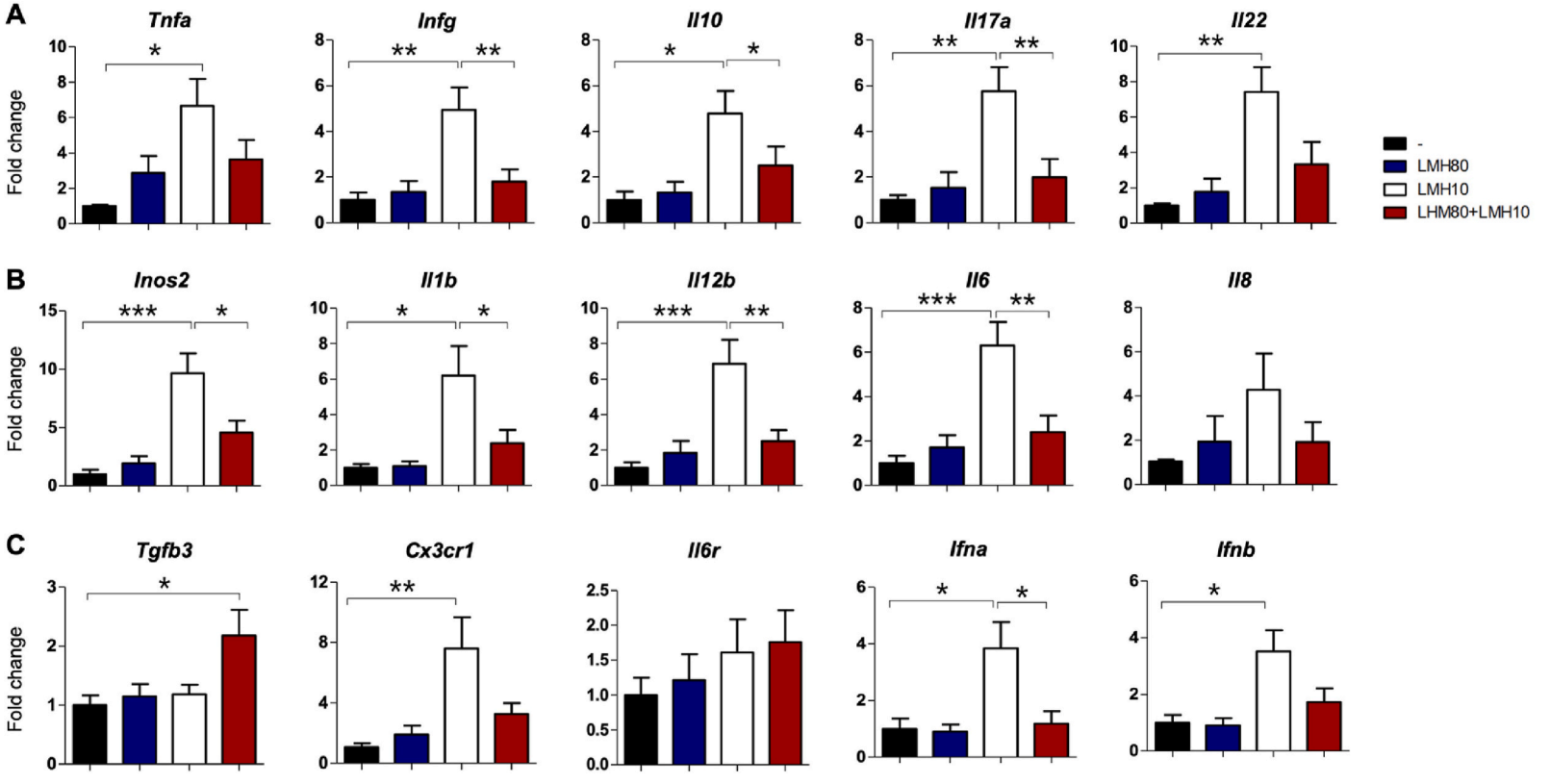

Fig. 7. Effect of the attenuated virus on immune functions in the primary tissue during FAdV-4 infection. (A) mRNA expression of T cell-associated cytokines in the liver during FAdV-4 infection. (B and C) Expression of effector genes of innate immunity and myeloid cells. Liver tissues were obtained from 3 to 4 chickens per group 3 days after FAdV-4 infection. cDNA was synthesized from each sample, and real-time qPCR was conducted in duplicate. Three independent experiments were performed, and the pooled data are shown. Relative gene expression was normalized using the control group expression. Statistical significance was analyzed using one-way ANOVA (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$).

whereas LMH10 was fatal to chicks in the absence of LMH80 pre-treatment (Fig. 1A). The effect of LMH80 immunization on viral clearance was evaluated. The virus was less frequently detected among immunized animals, in line with the survival rate. Furthermore, the amount of remaining virus in the immunized group was lower than in the control group (Fig. 1B and S. Fig. 2). Because immune cells can regulate viral clearance, we examined immune cells from the spleen and liver in each group. Cellularity and cell size appeared to be increased by infection, but the morphologic changes were not evident (Fig. 1C).

4.2. Phenotypic analysis of splenocytes following attenuated virus immunization and FAdV-4 challenge

Previously, we observed the protective action of the attenuated virus (LMH80) during acute FAdV-4 infection, but microscopic cytology carries limited information regarding immune responses. Thus, we identified the immune cell phenotype using flow cytometric analysis. We examined the population of activated T cells in the spleen, which is a carinal lymphoid tissue in chicken. $CD8^+$ T cells have cytotoxic activity and $CD4^+$ T cells are populated when immunity to FAdV is established [26,27]. In line with those, CD44-expressing $CD8^+$ T cells were increased by LMH80 immunization, regardless of the virulent FAdV-4 challenge (Fig. 2A). The activated form of helper T cell population was significantly increased by LMH80 immunization only when followed by the virulent FAdV-4 challenge (Fig. 2B). However, such changes were not evident for total T ($CD3^+$) and γδ-T cells (S. Fig. 1A).

Stimulation by pathogenic antigens leads to the differentiation of monocytes into macrophages. Phagocytosis, which has a critical role in innate immunity during viral infection is subsequently initiated. The activated monocytes express MHCII molecules on their surfaces and secrete effector cytokines [28]. A few MHCII-expressing myeloid cells were detected before the infection and immunization in the spleen. Although pre-immunized chickens did not show an increase in monocyte number, monocytes were greatly expanded when the animals were challenged with the virulent FAdV-4 (Fig. 3A). Antibody production is also closely associated with protective immunity [29]. Thus, we examined the B cell population in the spleen. On FAdV-4 infection the splenic B cells were decreased threefold, but LMH80 pre-treatment inhibited this decrease (Fig. 3B). These findings implied that LMH80 is a valid regulator of primary immune cells, such as T cells, macrophages, and B cells during FAdV-4 infection; lymphocytes activity was supported but uncontrolled myeloid cell expansion was suppressed in the spleen.

4.3. Effect of LMH80 treatment on immune-regulating genes in the spleen

LMH80 immunization increased the memory T cell subset and the numbers of $CD44^+$ $CD4^+$ and $CD8^+$ cells but maintained the numbers of $MHCII^+$ macrophages to the basal level during FAdV-4 infection. We probed whether functional changes were induced in splenocytes by examining the mRNA levels of key effector molecules. Because CD44-expressing T cells were expanded by immunization and infection, we expected the stimulation of effector cytokine genes. However, the immunization minimally affected type I cytokine genes, including *tnfa* and *ifng*. Instead, genes for regulatory cytokines, such as *il10* and *tfgb3* were upregulated by immunization with challenge. Other T cell-associated effector molecules, such as *il17a* and *il22*, were also slightly upregulated (Fig. 4A). Both antigen presentation and cytokine production by monocytes are required for T cell differentiation [10,30]. The LMH80 immunization followed by virulent FAdV-4 challenge enhanced gene expressions of *il1b*, *il10*, and type I IFNs, but not other effector molecules, such as *inos2*, *il12b*, and *il6*. Notably, mRNA levels of *il8* were downregulated with immunization and challenge but *cx3cr1* level was upregulated (Fig. 4B and C). We suggest that LMH80 immunization might have a regulatory role in splenocytes, but the impact was insufficient.

4.4. Phenotypic analysis of hepatocytes following LMH80 immunization and FAdV-4 challenge

FAdV-4 generates primary pathology in the liver [1]. We investigated whether LMH80 pre-treatment regulates the population of immune cells during an acute FAdV-4 infection. Like the findings in the spleen, the FAdV-4 challenge enhanced the infiltration of activated $CD4^+$ and $CD8^+$ T cells in the liver. Administration of attenuated virus before the virulent infection, further expanded these cell populations (Fig. 5A and B). In addition, fewer γδ-T cells were observed on LMH80 immunization, but the changes were masked by CD3 gating (S.Fig. 1B). This data indicates that LMH80 immunization enhanced T cell infiltration in the primary peripheral organ during the FAdV-4 infection.

In the steady state, limited numbers of $MHCII^+$ macrophages were observed, which were greatly increased by the virulent FAdV-4 infection. However, LMH80 pre-treatment inhibited the surge of $MHCII^+$ macrophages in the liver (Fig. 6A). Only a few B cells were observed in the intact liver, and no significant cell population changes were induced by immunization and/or infection (Fig. 6B). This finding indicates that the viral antigen induced excessive infiltration of the macrophages, which was regulated by the preexisting immune priming.

4.5. Regulation of immune genes in the liver by LMH80 immunization

We analyzed the genetic regulation of functional molecules for principal immune cells. We applied the same staining panel of flow cytometry as that used for the spleen, but more dynamic gene regulation was examined in the liver. Although activated $CD4^+$ and $CD8^+$ T cells were less frequently observed in the LMH80-immunized group during the infection, the genetic expression of effector cytokines was suppressed. For example, genes for T cell-associated cytokines, such as *tnfa*, *ifng*, *il10*, *il17a*, and *il22* were downregulated, whereas *tgfb3* expression was the highest in this group (Fig. 7A). The activation of TLRs on chicken monocytes enhances both effector cytokine levels and phagocytosis [30]. The FAdV-4 infection stimulated pro-inflammatory molecules, including *inos2*,

il1b, *il12b*, *il6*, *il8*, and type I IFNs. However, they were maintained at the basal level in the LMH80-immunized animals. Unlike that in the spleen, the *cx3cr1* mRNA level of was similarly regulated as the macrophage population (Fig. 7B and C). Taken together, the expression of immune-regulating molecules was in line with the infiltration of macrophages in the liver during immunization and viral infection.

5. Discussion

In the previous study, we adapted a virulent FAdV-4 strain in the LMH cells using serial cell passages. The adaptation generated a frameshift mutation in the fiber2 protein, and the resulting virus functioned as a live-attenuated vaccine. Although immunization using the attenuated virus protected animals from virulent FAdV-4 infection [22], a thorough understanding of the regulation of the protective immune system was lacking. In the present study, we investigated the immune regulation caused by the attenuated virus during a pathogenic FAdV-4 challenge. Activated $CD8^+$ and $CD4^+$ T cell populations were expanded following the attenuated virus administration and the virulent virus challenge. However, the number of myeloid cells, did not increase on immunization. The expression of the immune-regulating genes was partially associated with phenotypic change in splenocytes, and this association was stronger in hepatocytes.

FAdV-4 infection is acute and highly pathogenic because the viral factors facilitate fast genetic replication in host cells. Immune system has critical roles in control of the hypervirulent virus-induced pathogenesis [31]. The innate immune system detects viral antigens via TLRs and initiates pro-inflammatory responses. Excessive cytokine production such as IL-1β, IL-6, IL-8, and TNF-α along with reduced metabolism and apoptosis can be observed. These are correlated with severe tissue damage such as HHS and liver degeneration [15,32–35]. The primary function of innate immunity is mediated by macrophages, which express the MHCII molecule on their surface. They migrate to the site of pathogen invasion and then produce inflammatory cytokines and initiate phagocytosis [10]. The present study showed that the lethal FAdV-4 infection generated MHCII-expressing myeloid cells in lymphoid and peripheral tissues. The pre-treatment of the attenuated virus protected hosts from the virulent challenge, wherein $MHCII^+$ myeloid cells were maintained at the basal level. The gene expression pattern of immune molecules implies that the function of cells in the liver is distinct from that in the spleen. The gene expression levels for pro-inflammatory cytokines were associated with the population of MHCII-expressing cells in the liver; however, such complementary gene expressions were not observed in the spleen. For example, gene expression for effector molecules, such as iNOS2, IL-1, Il-6, and IL-12 was highly upregulated during the infection and was assumed to occur in inflammatory monocytes. The hyperactivation of these monocytes is fatal because it can induce both abnormal innate immunity and autoimmune diseases by increasing aberrant effector T cell differentiation [11,30]. The virulent FAdV-4 challenge led to expansion of $MHCII^+$ cells, which might have induced inflammatory cytokine production. Thus, the host could be experiencing the macrophage activation syndrome (MAS), which is a life-threatening condition. We suggest that LMH80 pre-treatment regulates the differentiation into $MHCII^+$ myeloid cells, wherein the excessive expansion of inflammatory monocytes is prohibited.

CX3CR1 is expressed both on myeloid and T cells. CX3CR1-expressing monocytes migrate into the antigen-invading region and initiate phagocytosis. On the other hand, $CX3CR1^+$ T cells are terminally differentiated effector cells that secrete effector molecules during infection [36,37]. We observed that the hepatic *cx3cr1* gene expression was compatible with the frequency of MHCII-expressing cells and inflammatory gene levels., while the splenic *cx3cr1* regulation was in accordance with the population of T cells. Therefore, we conclude that the attenuated virus immunization inhibited excessive infiltration of inflammatory myeloid cells in the liver but supported effector T cell generation in the lymphoid tissue. For a precise analysis, avian-reactive CX3CR1 antibody development is necessary to identify the functional subsets of avian immune cells.

Antigen presentation via MHCII-expressing myeloid cells directs $CD4^+$ T cell differentiation. In humans, adenovirus-specific $CD4^+$ T cells produce effector cytokines and facilitate B cells to secrete antigen-specific antibodies [38]. Active regulation of T and B cells during FAdV-4 infection is essential for protective immunity. A previous study reported protection of the virulent FAdV-4 infection with an attenuated virus. Similar to the ours, the immunization supported the conservation of T and B cells [17]. The LMH80 immunization positively regulated $CD8^+$ and $CD4^+$ T cells in the spleen and liver during infection. The phenotypic changes were more evident in the liver than in the spleen. However, the observed pattern of effector molecule expression was unexpected. In the spleen, regulatory and effector cytokine genes, such as *il17a*, *il22*, *il10*, and *tgfb3*, were enhanced by LMH80 immunization followed by FAdV-4 challenge, but no significant changes were observed in type I cytokine expression. Instead, the immunization limited the variation in the expression of the T cell-associated genes, except *tgfb3*, during FAdV-4 infection. The regulatory action of T cells, which was unexpected, has been observed in a murine model of viral infection. The study analyzed the function of intrahepatic $CD8^+$ T cells during hepatitis B and C virus infections. Although T cells did not express Foxp3, they showed suppressive function by expressing inhibitory molecules such as programmed cell death protein/programed cell death ligand 1 (PD-1/PD-L1) and Tim 3. In turn, the Tim-3 molecules bound to the high-motility group box-1 (HMGB-1), which limited effector T cell activation and proliferation [39]. Hepatic PD-L1 can also be stimulated by adenovirus infection, type I and II IFNs, and activated T cells. Enhanced inhibitory receptors restrict protective immunity against pathogen invasion [40]. Although the mechanism of LMH80-mediated immunity is unclear, a balanced immune response between regulatory and effector T cell responses appeared to be established by LMH80 immunization. A recent report showed the activation of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathway in the liver during FAdV-4 infection. This activation was associated with IL-6, IL-1β, and Type-I IFN mRNA levels. Moreover, arginine and JAK2 inhibitor treatments downregulated the intracellular pathways activated by FAdV-4 infection in LMH cells [41].

Novel FAdV strains have been isolated in East Asian countries, which actively proliferate in the liver and generate uncontrolled inflammatory immune molecules. Although the dominant isolates belong to serotype 4, the inclusion body hepatitis (IBH)-inducing strains, such as serotypes 8b and 11, have also been reported [42,43]. Therefore, several vaccine strategies against FAdV infections,

including inactivated with adjuvants, live attenuated, and protein subunit vaccines have been used [18]. A formaldehyde-inactivated vaccine protected the host from liver swelling with necrotic foci and hydropericardium. The antigen-specific antibody response was unregulated, but the immune-related gene expressions were controlled in the case of this vaccine [43]. Inactivated vaccines can provide efficient protection, but they are costly, and lower humoral and cellular immune responses are expected for such vaccines than those for live-attenuated vaccines. Thus, attempts for protection against FAdV-4 infection were made using attenuated viruses. A virulent FAdV-4 strain was adapted to a fibroblast cell line (QT35), and animals were immunized in advance to the virus challenge. The immunization protected the host from the infection, resulting in a sizable amount of neutralizing antibodies. Moreover, the attenuated virus prohibited the proliferation of the virulent virus [29]. Genetic changes were assumed to be generated during the adaptation because previous studies showed that genetic modification of the attenuated virus supported the protective immunity. For example, 188R of the hexon protein was found to be a critical amino acid for the virulence of the novel FAdV-4, and thus, the hexon protein mutant virus (rR188I) was created. The rR188I mutation protected the host from the virus challenge by lowering the viral load, inflammatory cell infiltration in the liver, and giant cell hyperplasia in the spleen and bursa [44]. Fiber proteins in FAdV-4 are major regulators for pathogenicity during infection, and recombinant vaccines have been developed using fiber1 and fiber2 proteins. A recombinant virus with an edited N-terminus region of the fiber1 protein protected chickens from a virulent FAdV-4 infection [45]. The fiber2 construct from a novel FAdV-4 strain was used as a vaccine and showed high protection efficacy. $CD4^+$ T cells and T cell-associated cytokines were generated by the vaccination, which showed slightly higher protection than the inactivated vaccine [26]. We generated an attenuated FAdV-4 virus strain through serial passage in the LMH cell line, inducing a genetic modification in the fiber2 protein. The immunization actively regulated key immune cells, including T, myeloid, and B cells, which protected chickens from a virulent FAdV-4 challenge. Our findings are consistent with those of previous studies that affirm the critical role of the fiber2 protein and the validity of the attenuated vaccine.

Viral propagation in cell lines has led to significant advances in vaccine development. Attenuated vaccine exhibits dynamic immunity to viral infections by generating cellular and humoral immunity. Here, we evaluated immune cell regulation caused by an attenuated FAdV-4 immunization that protected hosts against the pathogenic strain. The immunization generated balanced T cell immunity and regulated inflammatory monocyte expansion. Although the intracellular mechanisms mediated by the immunization still need to be established, this study suggests a potential FAdV-4 vaccine candidate against emerging and preexisting variants.

CRediT authorship contribution statement

Rangyeon Lee: Validation, Methodology, Investigation, Formal analysis, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Haan Woo Sung:** Validation, Methodology. **Hee-Tae Cheong:** Methodology, Data curation. **Jeongho Park:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by National Research Foundation of Korea (NRF-2022R1C1C1002793 and RS-2023-00301850 to JP).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25366.

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