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ORIGINAL ARTICLE

T cell subset profile and inflammatory cytokine properties in the gut-associated lymphoid tissues of chickens during infectious bursal disease virus (IBDV) infection

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Abstract

While infectious bursal disease virus (IBDV) mainly targets immature B cells and causes T cell infiltration in the bursa of Fabricius (BF) of chickens, the effect of IBDV infection on the properties of T cells and relevant cytokine production in avian gut-associated lymphoid tissues (GALTs) remains unknown. Here, we show that while the $CD8^+$ T cell subset is not affected, IBDV infection decreases the percentage of $CD4^+$ T cells in the cecal tonsil (CT), but not in esophagus tonsil, pylorus tonsil, and Meckel's diverticulum of GALTs, in contrast to BF and spleen, in which the proportion of $CD4^+$ cells increases upon IBDV infection. Further, IBDV infection upregulates IFN-γ, IL-10, and the T cell checkpoint receptor LAG-3 mRNA expression in BF. In contrast, in CTs, IBDV infection significantly increases the production of IFN-β and CTLA-4 mRNA, while no significant effect is seen in the case of IFN-γ, IL-10 and LAG-3. Together, our data reveal differential modulation of T cell subsets and proinflammatory cytokine production in different lymphoid tissues during the course of IBDV infection.

Introduction

Infectious bursal disease virus (IBDV) is the etiologic agent of infectious bursal disease (IBD), also known as Gumboro disease, a highly contagious and immunosuppressive disease in young chickens [1–3].

IBDV mainly targets the actively dividing B cells in the bursa of Fabricius (BF), a unique primary immune organ of juvenile avian species, leading to depletion of B cells in the bursa via necrosis and apoptotic processes [4, 5]. Bursal edema and atrophy manifest as a major symptom of IBDV infection [2, 6]. As a consequence, IBDV infection can cause morbidity and mortality and impair the immune competence of young birds, which increases the susceptibility of chickens to other infections and compromises vaccination against other diseases [7].

In addition to mainly infecting dividing B cells in the BF, IBDV can also directly target the original immune cells in other immune organs or tissues such as thymus, spleen, bone marrow, and gut-associated lymphoid tissue (GALT), a major component of the highly developed mucosa-associated lymphoid tissue (MALT) of chickens [8–12]. Avian GALT exists as dispersed aggregations of lymphoid cells, or is organized into lymphoid follicles and tonsils [13]. Most of the chicken GALTs, such as cecal tonsils (CTs), esophageal and pyloric tonsils, Meckel's diverticulum, and Peyer's patches [8, 13], play a key role in eliciting protective immune responses against pathogens within the intestinal tract of avian species [14, 15]. Disruption of GALT induces T cell exhaustion and results in the failure of the immune system to mount effective responses against pathogens [16, 17].

Alteration in T cell subsets triggered by IBDV infection contributes to the viral pathogenesis. The infiltration of $CD4^+$ T cells in BF is a hallmark of the progression to IBDV infection [18], which plays a role in the enhancement

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of damage in the BF by induction of inflammatory cytokines such as TNF-α and IL-1β [19]. In addition, upregulation of the T-cell-associated checkpoint receptors LAG-3 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) and changes in immunosuppressive $CD4^+CD25^+$ regulatory T cells in avian immune organs are closely linked to T cell exhaustion and immune suppression during IBDV infection [18, 20]. Interestingly, IBDV replication accompanied by an altered frequency of $CD3^+$ cells has been detected in the germinal centers of CTs in chickens infected with virulent IBDV [9], pointing to the potential influence of IBDV infection-induced alterations in the distribution of immune cells in GALT on the pathogenicity and immunosuppression in infected chickens.

Despite the essential role of GALT in mounting protective immune responses against viral pathogens in the intestinal tract of avian species [13], relatively little information is available regarding the effect of IBDV infection on the balance and properties of T cell subsets and the related inflammatory cytokine response in GALT. In the present study, we sought to understand the dynamics of the T cell response during IBDV infection with respect to T cell subset changes in GALT, including CTs and pyloric tonsils, Meckel's diverticulum, and esophageal tonsils, compared to that in the BF and spleen. Additionally, the effect of IBDV infection on the T-cell-associated cytokines and the expression of T cell checkpoint receptors was examined in these lymphoid tissues. We found that, while $CD8^+$ T cells were not significantly affected, IBDV infection resulted in an increase in the $CD4^+$ T cell subset in the BF and spleen but, conversely, a decrease in the CTs of the GALT. Further, immune cells from these lymphoid organs and tissues displayed a differential expression pattern of the T cell checkpoint receptors LAG-3 and CTLA-4 and genes associated with inflammation. These data contribute to a better understanding of the immunopathogenesis of IBDV.

Materials and methods

Experimental chickens and virus

Specific pathogen-free (SPF) white leghorn chickens were purchased from Zhejiang Lihua Agriculture Co., Ltd. (Hangzhou, Zhejiang province, China). The IBDV strain HZ2 was maintained in our lab as described previously [21]. In each experiment, 24 three-week-old SPF chickens were randomly divided into two groups and inoculated with 0.2 ml of IBDV in Dulbecco's modified Eagle's medium (DMEM) (7×10^7 $TCID_{50}$) (IBDV-infected group) or DMEM (mock-infected group) via the nasal and eyedrop routes, respectively. All animal experiments were carried out with the approval of the Animal Ethics Committee of ZAFU (ZAFU-2017-004).

Antibodies and reagents

Mouse anti-chicken CD3 PE (8200-09), mouse anti-chicken CD4 APC (8210-11), and mouse anti-chicken CD8 FITC (8220-02) were purchased from Southern Biotech (Southern Biotech, Birmingham, AL USA). Antibodies against IBDV were obtained from Bioss Antibodies (Beijing, China). Alexa Fluor 555–conjugated goat anti-rabbit IgG was obtained from CST (Danvers, MA, USA) and DAPI was obtained from Merck Millipore (Billerica, MA, USA). The H&E staining kit was purchased from Beyotime (Shanghai, China) and used as per the manufacturer's instructions.

Flow cytometry analysis

Single cell suspensions were prepared from BF, spleens, CTs, Meckel's diverticulum, pyloric tonsils, and esophageal tonsils at 1, 3, 5 and 7 dpi. Red blood cells were lysed using ACK lysis buffer (0.15 M NH_4Cl, 1 M K_2CO_3, 0.01 M EDTA, pH 7.2), and the cells were washed three times with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) and stained. Cells (2×10^6) were then incubated with PE-labelled anti-CD3, APC-labelled anti-CD4, and FITC-labelled anti-CD8 mAbs in FACS buffer at 4 °C for 30 min. The gating strategy for flow cytometry analysis of T cell subsets was as follows: Cells were first gated on size and granularity using FSC-A vs. SSC-A to eliminate debris and clumped cells, and, subsequently, single and live cells were gated on expression of CD3 to identify $CD3^+$ cells. T cells were then gated for CD4 or/and CD8 surface expression to identify different T cell subsets. Isotype controls for all antibodies used in the characterization of T cells included mouse IgG1-PE (0102-09), mouse IgG1-APC (0102-11), and mouse IgG1-FITC (0102-02), which were purchased from Southern Biotech. The percentages of T cell subsets in different organs were assessed using a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Data were acquired using CellQuest (Becton Dickinson, CA, USA) and analyzed with FlowJo (version 7.6.1) software.

qPCR analysis

Total RNA was isolated from the cells using TRIzol Reagent and reverse transcribed using a SuperScript First-Strand Synthesis System (Fermentas, Pittsburgh, PA) following the manufacturers' protocols. qPCR analysis was performed using SYBR Premix Ex Taq reagent (TaKaRa, Dalian, China) in an ABI 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA). The fold increase in target gene expression was calculated by the $2^{-\Delta\Delta CT}$ method

by normalizing to the chGAPDH gene and compared to those of mock-infected controls. The primer sequences (5'-3') were as follows: IL-2 sense, CTGGGAGAAGTGGTT ACTCTGA; antisense, ACCCGTAAGACTCTTGAGGTTC; IL-10 sense, GGCGACCTGGGCAACAT; antisense, CCT TGATCTGCTTGATGGCTTT; IFN-γ sense, AAGTCA AAGCCGCACATCAAAC; antisense, CTGGATTCTCAA GTCGTTCATCG; IFN-β sense, CCTCAACCAGATCCA GCATTAC; antisense, CCCAGGTACAAGCACTGTAGTT; TGF-β sense, TGCGGCCAGATGAGCATATAG; antisense, GTGTCGGTGACATCGAAGGA; LAG-3 sense, CCACTT TGCAGGAGGACACT; antisense, GTGACAGCACAG CAATACCG; CTLA-4 sense, AAATGGGACGCAACT CTACG; antisense, CGACAATGGCTGAGATGATG.

Fluorescent immunohistochemistry (IHC) analysis

BFs, spleens, and CTs were harvested and fixed in 4% (w/v) paraformaldehyde (PFA) for 24 hours. For IHC analysis, paraffin-embedded tissue sections (thickness, 4 μm) were prepared by Bioyear Biological Technology Co., Ltd. and were stained with rabbit anti-IBDV overnight at 4°C, followed by incubation with Alexa Fluor 555–conjugated goat anti-rabbit IgG for another 1 h at room temperature. Nuclei were stained with DAPI. Hematoxylin and eosin (H&E) staining was performed on paraffin-embedded tissue sections, following the manufacturer's protocol. Confocal imaging was performed using a Zeiss LSM 880 Airyscan microscope with Zen software (CarlZeiss, Oberkochen, Germany).

Statistical analysis

All data are presented as the mean ± standard deviation (SD) for each group and analyzed using SPSS13.0 (IBM, Armonk, NY, USA). Student's *t*-test was used for comparison between two groups. A *p*-value less than 0.05 was considered statistically significant.

Results

IBDV infection induces infiltration of CD4$^+$ and double positive CD4$^+$CD8$^+$ but not CD8$^+$ T cell subsets in the bursa of Fabricius (BF)

IBDV infection triggers T cell infiltration in the BF [22]. To determine the changes of different T cell subsets during IBDV infection, SPF chickens at 3 weeks of age were challenged with virulent IBDV or mock challenged via the nasal and eyedrop routes. At 1, 3, 5, and 7 days postinfection (dpi), the percentage of total T cells in the BF as well as the percentages of CD4$^+$ and CD8$^+$ T cells were determined

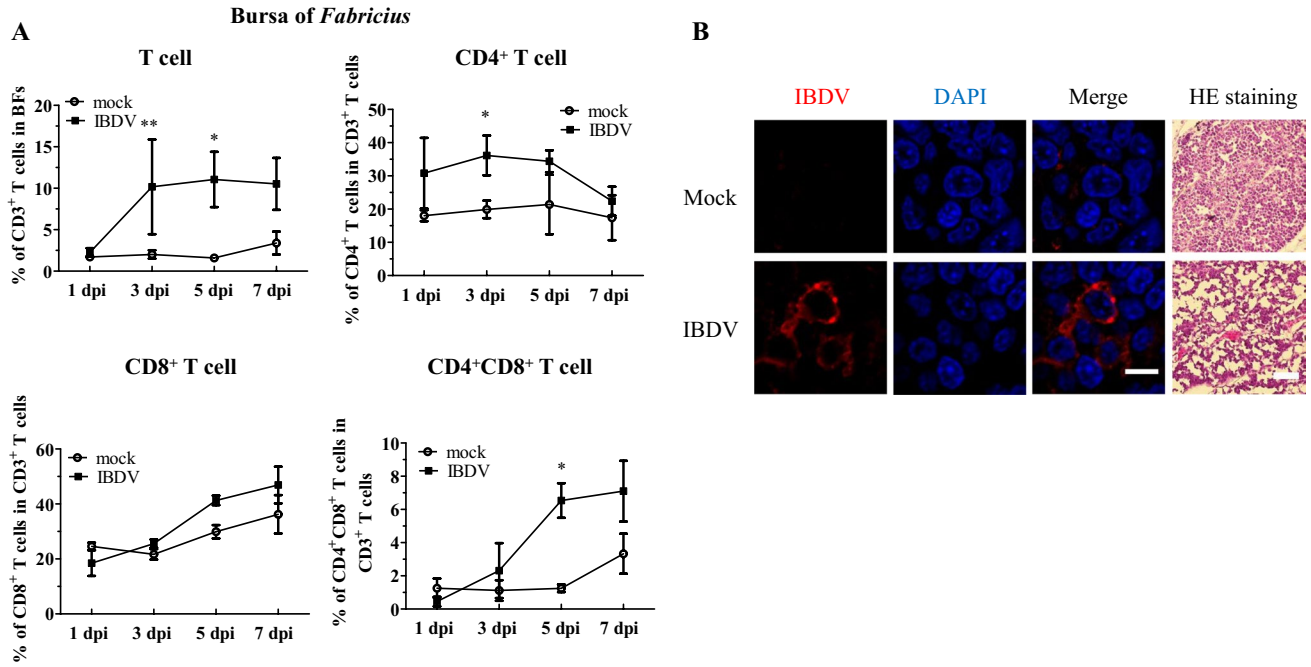

Fig. 1 IBDV infection induces infiltration of CD4$^+$ and CD4$^+$CD8$^+$ double-positive but not CD8$^+$ T cell subsets in the bursa of Fabricius (BF). SPF chickens 3 weeks of age were challenged with virulent IBDV or mock control via the nasal and eyedrop routes. (A) Percentages of total T cells, CD4$^+$ T cells, CD8$^+$ T cells, and CD4$^+$ CD8$^+$ double-positive T cells in the BF at 1, 3, 5, and 7 dpi (n = 3). (B) H&E staining and fluorescent IHC staining of BF from mock- and IBDV-infected chickens at 1dpi with IBDV antibody (red) and DAPI (blue). Scale bars in H&E staining images and IHC images are 25 μm and 5 μm, respectively. *, $p < 0.05$; **, $p < 0.01$

using flow cytometric analysis. As shown in Fig. 1A, in IBDV-infected chickens, the percentage of total T cells in the BF increased rapidly starting at 3 dpi and reached a peak at 5 dpi. These values were 5.05-fold and 6.95-fold higher, respectively, than that in mock control chickens ($p < 0.01$, $p < 0.05$). Interestingly, while the percentage of $CD8^+$ T cells showed no significant changes after infection, the increase in the total T cells in the BF was mainly due to an increase in the $CD4^+$ T cell subset and in $CD4^+$ $CD8^+$ double-positive T cell subset. Of note, in comparison, the increase of $CD4^+$ T cells occurred more rapidly than the increase in $CD4^+$ $CD8^+$ T cells. Interestingly, the percentage of $CD4^+$ $CD8^+$ double-positive T cells in the IBDV-infected group was about 5.24-fold higher than that in the mock-infected control group at 5 dpi ($p < 0.05$). Further, while the percentage of $CD4^+$ T cells recovered to the level of the mock-infected control at 7 dpi, the increase in $CD4^+$ $CD8^+$ T cells was sustained until 7 dpi, which was similar to the trend with total T cells. Confocal microscopy of bursal sections stained with anti-IBDV antibody confirmed that IBDV antigens were detected in the cytoplasm of bursal cells from IBDV-infected chickens, but not in bursal cells from mock-infected control chickens at 1 dpi (Fig. 1B, left panels). In addition, bursal sections of chickens that were either mock infected or infected with IBDV were prepared for histopathological examination by hematoxylin and eosin (H&E) staining. As expected, IBDV caused characteristic lesions such as necrosis of bursal follicles and hemorrhage at 1 dpi (Fig. 1B, right panels). Together, the data revealed that the percentage of $CD4^+CD8^-$ and double-positive $CD4^+$ $CD8^+$ T cell subsets in IBDV group significantly increased in the BF at 3 dpi, while there were no marked changes observed in the percentage of cytotoxic $CD8^+$ T cells when compared to the mock-infected control group.

The $CD4^+$ T cell subset, but not the $CD8^+$ T cell subset, is significantly reduced in cecal tonsils (CTs) at the early stage of IBDV infection

The cecal tonsil (CT), one of the immunologically essential components of avian GALT, elicits protective immune responses against pathogens within the intestinal tract of avian species [8]. To investigate changes in the percentages of different T cell subsets in the CT during IBDV infection, samples of CTs were collected from both IBDV-infected and mock-infected chickens at 1, 3, 5 and 7 dpi. As shown in Fig. 2A, despite no overall change in the percentage of total T cells, the percentage of $CD4^+$ T cells in the CT was decreased by 2.86-fold ($p < 0.05$) during the early phase of IBDV infection, at 1 dpi, recovered by 3 dpi and was maintained until 7 dpi. In contrast, the $CD8^+$ subset and the double-positive $CD4^+$ $CD8^+$ T cells remained steady throughout the course of the 7-day infection.

Further, immunohistochemistry was performed to confirm the presence of IBDV infection on CT sections from IBDV-infected chickens but not from the mock-infected controls (Fig. 2B, left panels). H&E staining of histological sections of CT showed that punctate hemorrhage, submucosal edema, hyperemia, and a significant decrease in the total thickness of the mucosa were observed in the cecum of IBDV-infected chickens compared to that of the mock-infected controls (Fig. 2B, right panels).

In contrast, in other lymphoid tissues of GALT, including esophagus tonsil, pylorus tonsil, and Mekel's diverticulum, percentages of both the $CD4^+$ and $CD8^+$ T cell subsets were not significantly affected upon IBDV infection over a 7-day time course (Fig. 3). The only exceptions were the double-positive $CD4^+$ $CD8^+$ T cells in pyloric tonsils, which were 3.10-fold higher in the IBDV-infected group than the mock-infected group at 7 dpi (Fig. 3C, $p < 0.05$).

Together, these data demonstrate that IBDV infection specifically causes a dramatic decrease in the frequency of the $CD4^+$ cell subset, but not $CD8^+$ T cells, in CTs of GALT at the early stage of infection.

IBDV infection potentiates $CD4^+$ T cell subset infiltration in the spleen

The spleen is one of the major peripheral lymphoid organs in chickens [23]. To investigate the effect of IBDV infection on the distribution of T cell subsets in the spleen, we purified splenocytes from IBDV-infected or mock-infected chickens, stained them with anti-CD4 and anti-CD8 mAbs, and analyzed them by flow cytometry. As shown in Fig. 4A, IBDV infection did not cause an obvious change on the percentage of total T cells (upper left panel) or the $CD8^+$ T cell subset (lower left panel) monitored over a time course of 7 dpi. In contrast, like the situation in the BF, there was a rapid increase in the $CD4^+$ T cell subset at 1 dpi (upper right panel), which returned to the normal level by 3 dpi. Interestingly, however, differing from that in BF, where IBDV infection induced an increase in double-positive $CD4^+$ $CD8^+$ T cells, IBDV infection caused no changes in the number of $CD4^+$ $CD8^+$ T cells in the spleen over time (upper right panel). Thus, IBDV infection induced helper T cell ($CD4^+$ $CD8^-$) infiltration in the spleen at the beginning of the infection (Fig. 4A, $p < 0.05$), but without affecting the percentages of $CD8^+$ T cells and double-positive T cells ($CD4^+$ $CD8^+$). Confocal microscopy of spleen sections stained with anti-IBDV antibody showed that IBDV antigen was detected in spleens from virus-infected chickens, but not in those from the control chickens at 1dpi. However, H&E staining of spleen sections showed no significant change in spleens from IBDV-infected chickens compared to those of mock-infected controls (Fig. 4B).

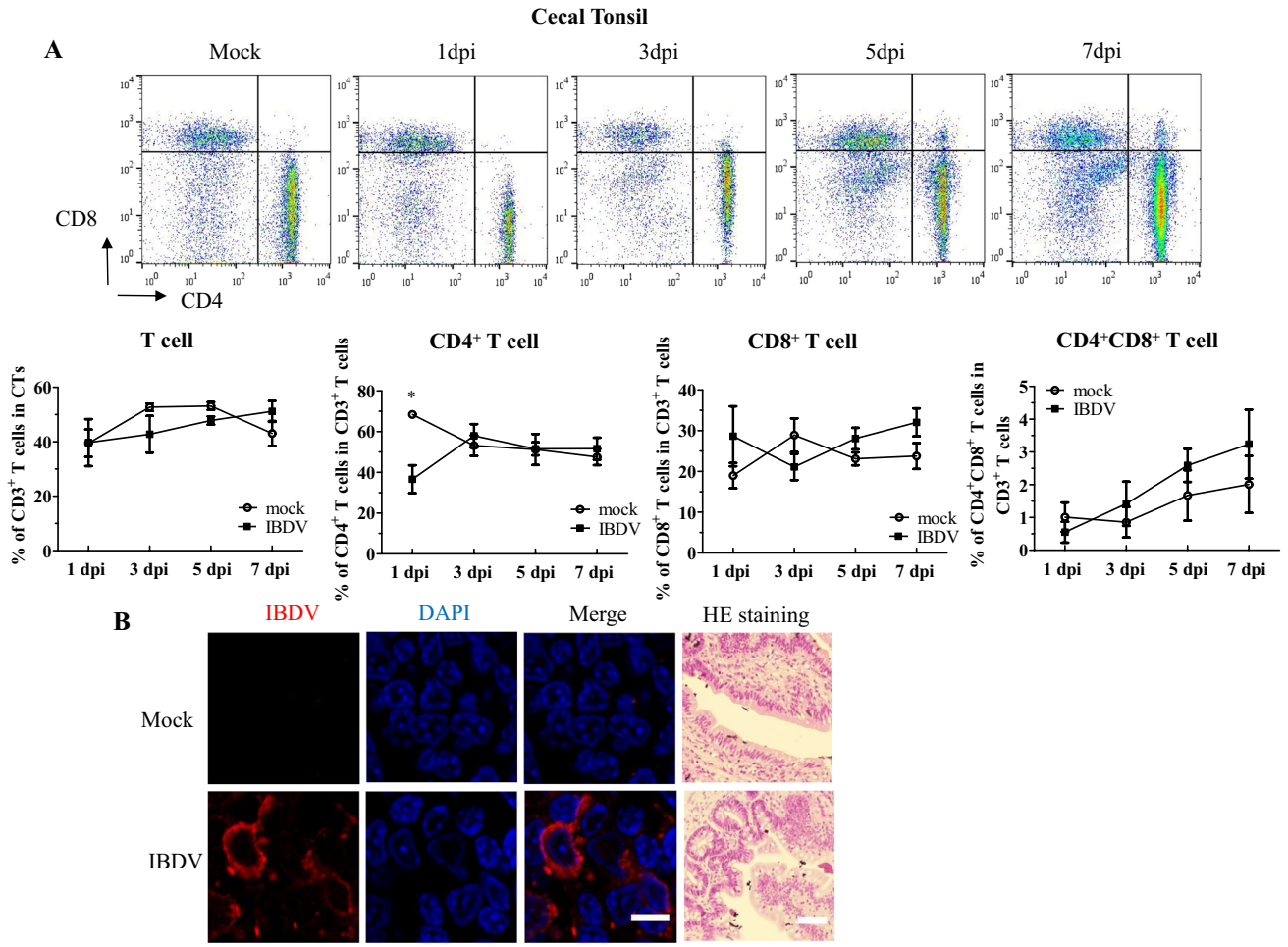

Fig. 2 The $CD4^+$ T cell subset, but not the $CD8^+$ T cell subset, is significantly reduced in CTs at the early stage of IBDV infection. (A) Percentages of total T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+$ $CD8^+$ double-positive T cells in CTs at 1, 3, 5, and 7 dpi (n = 3). Representative FACS profiles of $CD4^+$ and $CD8^+$ T cells in CTs are shown at the top panel. (B) H&E staining (right) and fluorescent immunohistochemistry staining (left) of CTs from mock- and IBDV-infected birds at 1 dpi with IBDV antibody (red) and DAPI (blue). Scale bars in H&E staining images and IHC images are 25 μm and 5 μm, respectively. *, $p < 0.05$

Distinct expression pattern of the genes of T cell checkpoint receptors and genes associated with inflammation in different lymphoid tissues from IBDV-infected chickens

To further assess whether IBDV infection affects cytokine production by T cells, the expression of the pro-inflammatory cytokines IL-2 [24], IFN-γ and IFN-β [25] the anti-inflammatory cytokines IL-10 and TGF-β, and the T cell checkpoint receptors CTLA-4 and LAG-3 were analyzed on days 1 and 5 after IBDV infection by qPCR. As shown in Fig. 5, while not affected at 1 dpi, the relative mRNA levels of IFN-γ was markedly increased in both the BF and spleen, but not in CTs, at 5 dpi. In the BF, in addition to the upregulation of mRNA expression of the pro-inflammatory cytokine IFN-γ, the mRNA levels of the anti-inflammatory cytokine IL-10 and LAG-3, a checkpoint receptor on T cells, were also markedly increased (Fig. 5A, $p < 0.05$). In CTs, the mRNA levels of IL-2, IL-10, TGF-β, IFN-γ, and the LAG-3 receptor from the IBDV-infected birds did not differ (Fig. 5C, $p > 0.05$) when compared to those of mock-infected controls. However, the IFN-β mRNA levels increased at 1 dpi (Fig. 5C, $p < 0.05$). Interestingly, at 1 dpi, when the percentage of $CD4^+$ T cells was substantially reduced in the CTs (Fig. 2A), there was a significant upregulation of mRNA production from the CTLA-4 gene, a marker of regulatory T cells, which returned to its normal level by 5 dpi. Splenocytes from IBDV-infected chickens also exhibited an increase in IFN-γ mRNA expression (Fig. 5B). However, the effect of IBDV infection on IL-2, IL-10, IFN-β, IFN-γ and TGF-β gene expression in cells from esophageal tonsils, Meckel's diverticulum, and pyloric tonsils was marginal (Supplementary Fig. 1).

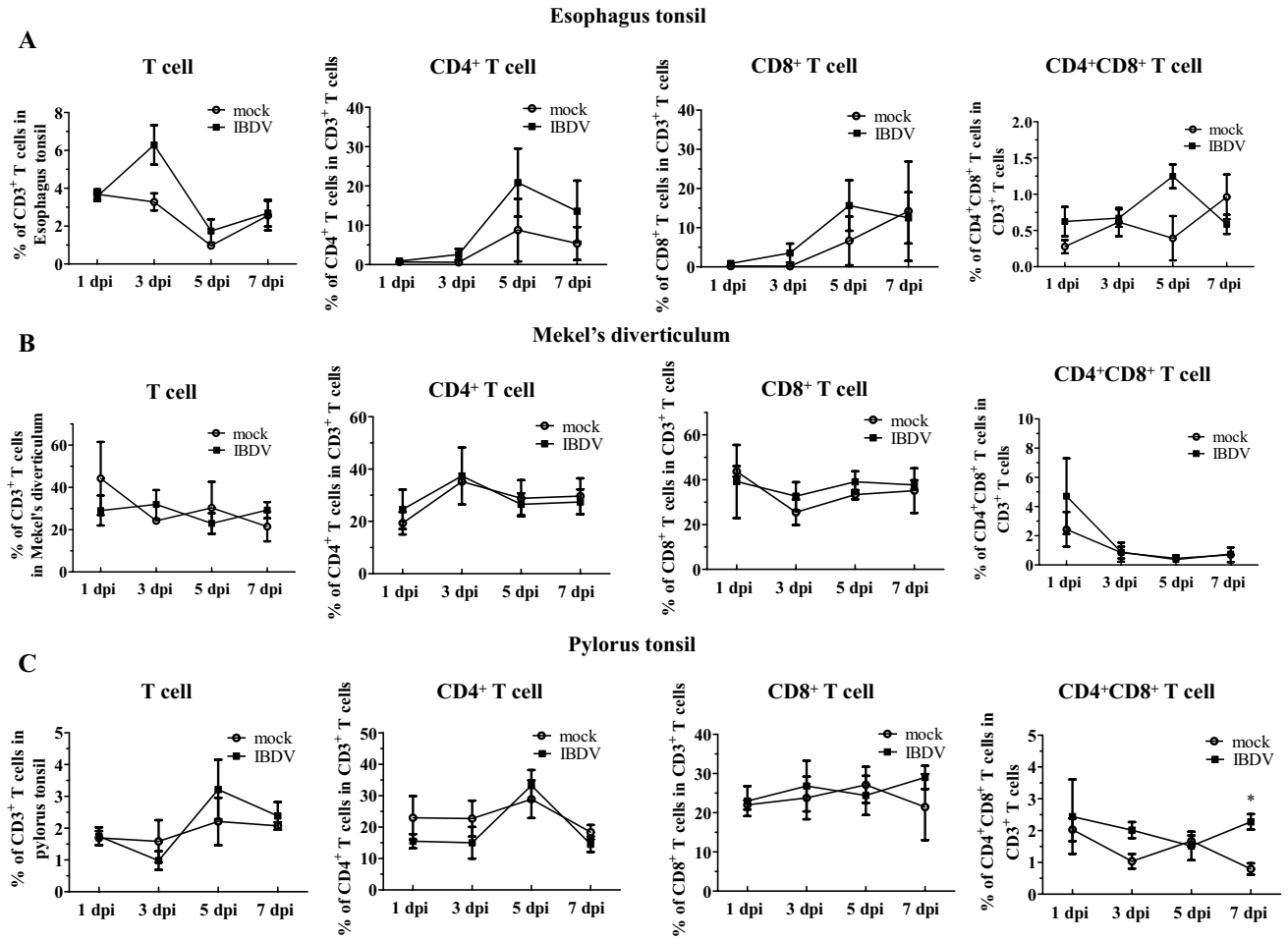

Fig. 3 Frequencies of $CD4^+$ and $CD8^+$ T cell subsets were not affected during IBDV infections in esophagus tonsil, Mekel's diverticulum, and pylorus tonsil. Percentages of total T cells, $CD8^+$ T cells, $CD4^+$ T cells, and $CD4^+$ $CD8^+$ double-positive T cells in the esophagus tonsil (A), Mekel's diverticulum (B), and pylorus tonsil (C) at 1, 3, 5, and 7 dpi (n = 4)

Together, these data suggested that IBDV infection causes a differential mRNA expression pattern of the genes associated with inflammation and genes of T cell associated checkpoint receptors in different lymphoid organs and tissues of GALTs.

Discussion

We investigated and compared changes in T cell subsets and in the gene expression of T-cell-associated checkpoint receptors and inflammatory cytokines in the lymphoid organs BF and spleen and in GALTs including CT, esophagus tonsil, Meckel's diverticulum, and pylorus tonsil, over a 7-day time course of IBDV infection. Here, we make three major points about these findings: 1) IBDV infection causes changes in the percentage of $CD4^+$ T cells, but not $CD8^+$ T cells. The number of $CD4^+$ T cells increased in the BF and spleen but, conversely, decreased in the CT during IBDV infection. 2) Immune cells from these lymphoid organs or tissues display a differential expression pattern of the genes associated with inflammation and genes of T-cell-associated checkpoint receptors. The reduction in the number of $CD4^+$ T cells in the CT is simultaneously accompanied by an upregulation of expression of the T cell checkpoint receptor CTLA-4, whereas in the BF, the increased frequency of $CD4^+$ T cells is accompanied by a significant upregulation of mRNA expression for the T cell checkpoint receptor LAG-3. 3) IBDV induces a distinct interferon gene expression response pattern in different lymphoid tissues. Whereas the expression of IFN-γ is elevated in both the BF and spleen, by contrast, it is IFN-β but not IFN-γ whose expression is specifically induced in the CT during IBDV infection. These findings provide new clues about the pathogenic and immune evasion mechanisms of IBDV infection.

Atrophy accompanied by T cell infiltration in the BF is a hallmark of the progression to IBD [22]. However, despite the fact that the total T cell count progressively increases in

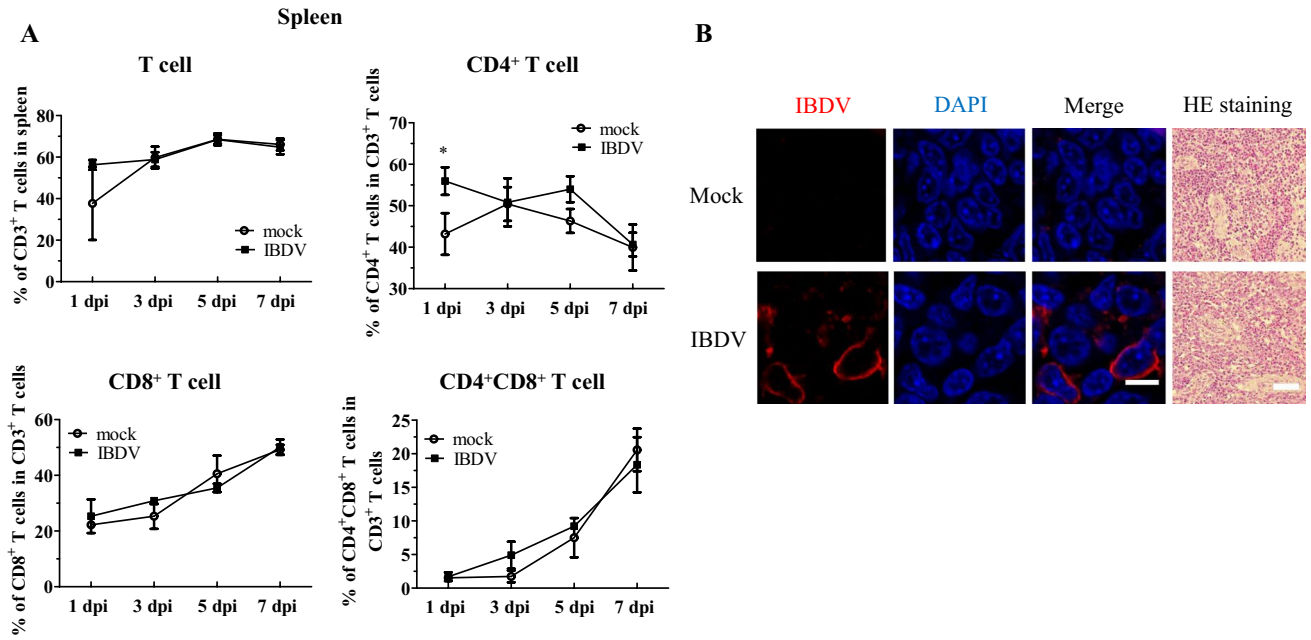

Fig. 4 IBDV infection potentiates $CD4^+$ T cell subset infiltration in the spleen. (A) Percentages of total T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+$ $CD8^+$ double-positive T cells in the spleen at 1, 3, 5, and 7 dpi (n = 3). (B) H&E staining and fluorescent immunohistochemistry staining of BF from mock- and IBDV-infected birds at 1 dpi with IBDV antibody (red) and DAPI (blue). Scale bars in H&E staining images and IHC images are 25 μm and 5 μm, respectively. *, $p < 0.05$

the BF as infection progresses, the relative ratio of $CD4^+$ to $CD8^+$ T cells in the BF remains unchanged upon infection with virulent IBDV [26]. Further, Williams and Davison reported that the number of $CD4^+$ and $CD8^+$ cells increases significantly at 3 and 5 dpi, respectively [27]. In contrast, our data clearly demonstrate that IBDV infection results in a marked increase in the percentage of $CD4^+$ single-positive T cells, whereas the $CD8^+$ single-positive (SP) T cell subset remains unchanged in the BF. Interestingly, the number of $CD4^+$/ $CD8^+$ double-positive (DP) T cells increased by approximately 5.24-fold at 5 dpi in the BF after IBDV infection. Thus, the IBDV-induced infiltration of T cells in BF is attributed to increased both $CD4^+$ SP T cells and $CD4^+$/ $CD8^+$ DP T cells. Peripheral extra-thymic $CD4^+$/ $CD8^+$ DP T cells are mainly functional effector/memory T cells specific for antigens of viral pathogens [28], and an increase in the frequency of $CD4^+$/ $CD8^+$ DP T cells during infections by other viruses such as human immunodeficiency virus (HIV) and Epstein-Barr virus has been reported [29, 30].

While the number of $CD4^+$ T cells increases progressively during IBDV infection in the BF and spleen, surprisingly, the number of $CD4^+$ T cells decreases significantly in the CT of IBDV-infected chickens in the early stage of infection (1 dpi). This is in agreement with a previous report that, during the acute phase of IBDV infection, a significant reduction in lymphocyte number in CT was observed [31]. Given that the CT is the nearest immune tissue to the BF [8], it is likely that the $CD4^+$ T cell subset might migrate from CTs to the BF during IBDV infection, resulting in an increased $CD4^+$ T cell population in the BF and a decrease in $CD4^+$ T cells in the CTs. In contrast, the frequencies of $CD4^+$ and $CD8^+$ T cell subsets were both unchanged in the other lymphoid tissues of GALT that were tested, including esophagus tonsil and Mekel's diverticulum, in response to IBDV infection, suggesting that the IBDV-mediated reduction in the number of $CD4^+$ T cells in CTs is specific.

The expression of IFN-γ in both the BF and spleen was significantly upregulated after IBDV infection. As IFN-γ is an essential mediator of inflammation and recruitment of $CD4^+$ T cells and macrophages during infections with many pathogens [32], the $CD4^+$ T-cell infiltration in the BF induced by IBDV infection could be a consequence of the enhanced expression of IFN-γ. Further, IBDV infection stimulates IL-10 expression in the BF, but not in spleen or the lymphoid tissues of GALT. Given the anti-inflammatory and immunoregulatory properties of IL-10 and TGF-β [33], the increase in IL-10 might alleviate acute inflammation in the BF in the late stage of IBDV infection. Interestingly, at 1 dpi, when the percentage of $CD4^+$ T cells was markedly reduced in the CT, there was a significant increase in mRNA levels of the CTLA-4 gene, a major immune checkpoint receptor that is highly expressed on the surface of regulatory T cells (Tregs) in chickens [34]. This may link CTLA-4 to the reduction of $CD4^+$ T cells in IBDV-infected CTs, given that Tregs disrupt effector T cell metabolism and the polarization of $CD4^+$ helper T cells [35, 36].

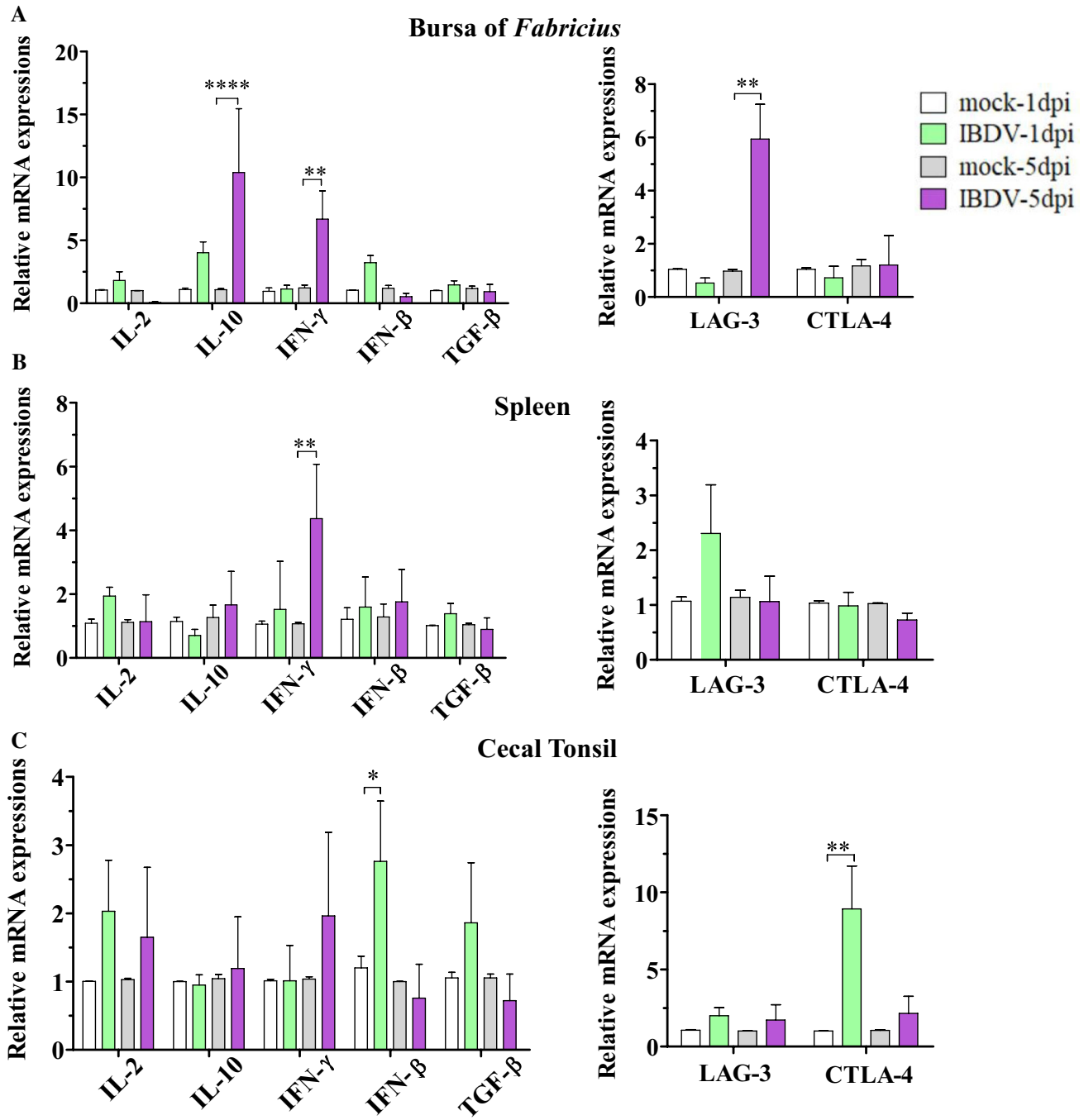

Fig. 5 Distinct mRNA expression pattern of inflammation-related genes and T cell checkpoint receptors in BF, spleen, and CT from IBDV-infected chickens. SPF chickens (n =12) were either infected with 0.2 ml of IBDV-containing DMEM (7×10^7 $TCID_{50}$) or mock infected with sterile DMEM. At 1 and 5 dpi, the mRNA expression levels of the inflammation-related genes IL-2, IL-10, IFN-γ, IFN-β and TGF-β (left), as well as the T-cell-associated checkpoint receptors LAG-3 and CTLA-4 (right) in BF (A), spleen (B) and CT (C) were determined by qPCR. Data represent the mean ± SEM from 5-6 individuals. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$

In summary, we show that IBDV infection is associated with rapid changes in the $CD4^+$ T cell subset, but not in the $CD8^+$ T cell subset, in the BF and the lymphoid tissues of GALT. There is an association between CTLA-4 levels in the early stage of infection and depletion of the $CD4^+$ T cell subset in CTs. Our findings provide a new insight into immunomodulatory processes that occur during IBDV infection via modulation of the infiltration patterns of T cell subsets, the production of inflammation-associated

cytokines, and T cell checkpoint receptors in lymphoid organs and GALTs of IBDV-infected chickens.

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