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Early immune responses and profiling of cell-mediated immunity-associated gene expression in response to rHVT-IBD vaccination

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ABSTRACT

Infectious bursal disease (IBD) remains a major threat to the poultry industry. Recombinant herpesvirus of turkey (rHVT)-IBD vaccines have been successfully used to induce a protective immune response against IBD. However, the capacity for rHVT-IBD vaccines to induce early protection without detectable antibodies, and the underlying mechanisms mediating specific cell-mediated responses in the early stages following vaccination, have been poorly investigated. Therefore, in this study, specific pathogen-free (SPF) chickens were vaccinated with rHVT-IBD and T-cell subsets were analyzed. Both splenic and circulating $CD8^+$ cell populations increased at 7 days postvaccination (dpv). Next, the expression of adaptive immunity-related genes was analyzed in the spleen and lung of rHVT-IBD-vaccinated chickens. Upregulation of CD8 expression was observed at 7 dpv. Interestingly, a parallel increase in the transcription of granzymes A and K was also detected from 7 dpv. To our knowledge, the latter result is the first to be reported, and it suggests that cytotoxic activity of $CD8^+$ T lymphocytes is activated. In contrast, expression of the innate genes examined remained largely unchanged following vaccination. To further investigate the IBD virus (IBDV)-specific responses triggered by rHVT-IBD vaccination, vaccinated chickens were inoculated with an attenuated IBDV strain with the aim of restimulating induced immune responses *in vivo*. The expression profiles of various genes associated with adaptive immune responses were subsequently analyzed in lung, spleen, and bursa of Fabricius samples. Significant upregulation of CD4, CD8, perforin, and IFNγ expression were observed in the bursa samples 7 days postinoculation (dpi). In the lung, transcript levels of CD8, granzymes and perforin were also significantly higher in the rHVT-IBD-vaccinated chickens at 7 dpi, thereby suggesting that specific cellular immune responses were activated. Overall, these results support the hypothesis that stimulation of specific $CD8^+$ cell-mediated immunity contributes to the response against IBDV in rHVT-IBD-vaccinated chickens.

1. Introduction

Herpesvirus of turkey (HVT) is a *Mardivirus* that belongs to the *Alphaherpesvirinae* subfamily, along with the pathogenic Marek's disease virus (MDV). In field trials, HVT strain FC126 was identified as a good candidate vaccine against MDV based on its efficacy [1]. HVT was also identified as an efficient vector for the delivery of foreign antigens. Consequently, HVT has been used to create recombinant vaccines against several avian pathogens [2–5].

Infectious bursal disease (IBD) is a highly contagious disease and a global threat to the poultry industry. IBD virus (IBDV) targets immature B lymphocytes in lymphoid organs [6] and targets the bursa of Fabricius of young chickens, thereby causing death, disease, or immunosuppression and leading to secondary infections with opportunistic pathogens and vaccination failures [7]. However, recombinant HVT (rHVT)-IBD has been successfully used to protect chickens against IBDV [8–10] by expressing the capsid protein VP2 of IBDV [2]. Moreover, unlike live attenuated IBDV vaccine strains that retain some residual pathogenicity, rHVT-IBD is not associated with a risk of transient immunosuppression [8].

Despite rHVT vaccines being efficacious against a variety of avian pathogens, the immune mechanisms related to the protection elicited by these vaccines have not been entirely elucidated. In chickens vaccinated with rHVT, persistent viremia is established and promotes a long-lasting antibody response that can be measured between 3 weeks postvaccination (wpv) and 30 wpv [11,12]. However, recent studies highlighted good clinical protection against IBDV challenges in the early stages following rHVT-IBD vaccination, despite the detection of low levels of anti-VP2 antibodies, thereby indicating the contribution of other arms of the immune responses [13]. We previously reported the ability of

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rHVT-IBD vaccines to induce cell-mediated immunity at 3 wpv in specific pathogen-free (SPF) chickens based on an analysis of interferon-gamma (IFNγ) production in response to *ex vivo* recall stimulation of splenocytes with a live IBDV strain [14]. Moreover, rHVT-IBD vaccines were demonstrated to induce some cross-protection against IBDV heterologous strains [13]. When rHVT vaccines were used to immunize against avian influenza (AI), this approach was also successful in eliciting protection against a heterologous challenge, despite low hemagglutination inhibition (HI) titers. Furthermore, a cell-mediated cytotoxic immune response was also demonstrated *in vitro* against various subtypes of AI virus with rHVT vaccines [15].

After inoculation of 18 day-old embryos, HVT was shown to initially replicate in the lung before it spreads to peripheral organs, including the spleen, thymus, and bursa [16–18]. In the present study, distinct T cell subsets were first profiled in splenic and peripheral blood lymphocytes populations early after SPF chickens were vaccinated with rHVT-IBD. In addition, the expression of innate and adaptive immunity genes in the lung and the spleen were examined. To further investigate the IBDV-specific responses triggered by rHVT-IBD vaccination, an *in vivo* approach involving vaccination with rHVT-IBD followed by an inoculation with an attenuated IBDV was performed with the goal of specifically re-stimulating immune responses in vaccinated chickens.

2. Materials and methods

2.1. Chickens

SPF White Leghorn chickens embryonated eggs were obtained from Lohmann Valo (Cuxhaven, Germany). After hatching, all birds were kept in biosecurity level 3 (BSL-3) isolators, with access to food and water provided *ad libitum* throughout the experimental period. Animal experiments were conducted with authorization and supervision from the Biosafety and Bioethics Committees at the Veterinary and Agrochemical Research Institute (Brussels, Belgium) according to national and European regulations.

2.2. Vaccine and virus

The rHVT-IBD Vectormune® vaccine was provided by Ceva Santé Animale (Lenexa, KS, USA). Classical IBDV strain D78 was purchased from MSD Animal Health (Milton Keynes, UK).

2.3. Experimental design

Protocols for the two animal experiments conducted in this study are summarized in Table 1.

Experiment 1: This experiment was conducted to assess: (i) potential changes in splenic and circulating immune cell subsets by flow cytometry; and (ii) the expression of various genes in spleen and lung of vaccinated chickens. Thus, chickens were immunized at 14 days of age with a subcutaneous injection in the neck of an inoculum of ten commercial doses (3.6 log10 plaque-forming units (pfu)/dose) of vaccine in 100 μl of corresponding vaccine diluent (Ceva-Biomune, KS, USA). Three animals per group were humanely sacrificed at 2, 5, 7 and 9 days postvaccination (dpv). Spleen, lung, and blood samples were collected at each time point.

Experiment 2: This experiment was conducted after Experiment 1 in order to evaluate the expression of several genes related to adaptive immunity in the lung, spleen, and bursa of vaccinated chickens following *in vivo* recall stimulation with an attenuated strain of IBDV. The experimental design was similar

Table 1
Experimental design and summarized protocol of Experiment 1 and Experiment 2.

Animal experiment	Group (chickens/group)	rHVT-IBD vaccination			IBDV inoculation				Sampling timings (chickens/timing)	Samples
		Age at vaccination (days)	Vaccine dose (titer)	Inoculation route (volume)	Age at inoculation (days)	Viral titer	Inoculation route (volume)			
1	Unvaccinated (12)	–	–	–	–	–	–		2, 5, 7, 9 days postvaccination (3)	Spleen, lung, blood
	rHVT-IBD-vaccinated (12)	14	10 commercial doses (3.6 log10 pfu/dose)	Subcutaneous (in 100 μl)	–	–	–			
2	Unvaccinated (9)	–	–	–	–	–	–		2, 5, 7 days postinoculation with IBDV (3)	Spleen, lung, bursa
	rHVT-IBD/- (9)	14	10 commercial doses (3.6 log10 pfu/dose)	Subcutaneous (in 100 μl)	–	–	–			
	rHVT-IBD/IBDV (9)	14	10 commercial doses (3.6 log10 pfu/dose)	Subcutaneous (in 100 μl)	21	10^4 TCID50	Oculo-nasal (in 100 μl)			
	Unvaccinated/IBDV (9)	–	–	–	21	10^4 TCID50	Oculo-nasal (in 100 μl)			

to that of Experiment 1, but also it included an inoculation with live attenuated IBDV strain D78 at 7 dpv with rHVT-IBD. Three animals per group were humanely euthanased at 2, 5 and 7 days postinoculation (dpi) with IBDV. Spleen, lung and bursa samples were collected at each time point. To discriminate responses induced by rHVT-IBD versus the IBDV strain, only chickens vaccinated with rHVT-IBD, or chickens inoculated with an attenuated IBDV, were included as control groups.

2.4. Flow cytometry

2.4.1. Lymphocyte isolation

Single cell suspensions were obtained by mechanical disruption of spleen tissues in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA). Spleen cell suspensions were layered by sedimentation to isolate splenocytes as previously described [19], and heparinized blood samples were centrifuged over Histopaque-1083 density gradients (Sigma-Aldrich, St. Louis, MO, USA) to isolate peripheral blood lymphocytes (PBLs).

2.4.2. Phenotyping

Concentrations of the isolated splenocytes and PBLs were adjusted with ice-cold FACS buffer (0.1% NaN_3, 3% inactivated fetal calf serum, phosphate-buffered saline) and 10^6 cells from each sample were stained with monoclonal antibodies (mAbs) according to the manufacturer's recommendations for direct or indirect immunostaining. The mouse anti-chicken mAbs used for staining were purchased from Southern Biotech (Birmingham, AL, USA): CD4-PE (8210-09), CD8α (CT-8)-UNLB (8220-01), CD45-PE (8270-09), Bu1-UNLB (8395-01), TCRγδ-FITC (8230-02), anti-mouse IgG1-FITC (1072-02), and anti-mouse IgG1-PE (1072-09). Labeled cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences, San José, CA, USA). Lymphocytes were gated on the basis of forward/side scatter characteristics and 10^4 FITC-positive and/or PE-positive staining events were analyzed. For each cell subset tested, the normalized percentages in the rHVT-IBD-vaccinated group relative to that in the unvaccinated group were presented.

Table 2
List of primers used to quantify the relative expression of immune system genes by real-time RT-PCR.

Target gene	Sequence	References
CD4	F 5′-GGAGGAAGCTCATGTTTGGA-3′ R 5′-CTGCCACCTCATACCAGTGA-3′	[38]
CD8	F 5′-AGCTCAGAGCCAGGAACAAG-3′ R 5′-GTCTTTTGGCAGAGCACGAT-3′	[38]
GAPDH	F 5′-GACGTGCAGCAGGAACACTA-3′ R 5′-TCTCCATGGTGGTGA AGACA-3′	[39]
Granzyme A	F 5′-ACTCATGTCGAGGGGATTCA-3′ R 5′-TGTAGACACCAGGACCACCA-3′	[38]
Granzyme K	F 5′-CGGGAAGCAACTGTTGAAAT-3′ R 5′-GAGTCTCCCTTGCAAGCATC-3′	[38]
IFNα	F 5′-GACAGCCAACGCCAAAGC-3′ R 5′-GTCGCTGCTGTCCAAGCATT-3′	[40]
IFNβ	F 5′-GCTCACCTCAGCATCAACAA-3′ R 5′-GGGTGTTGAGACGTTTGGAT-3′	[39]
IFNγ	F 5′-GTGAAGAAGGTGAAAGATATCATGGA-3′ R 5′-GCTTTGCGCTGGATTCTCA-3	[41]
IL-13	F 5′-CACCCAGGGCATCCAGAA-3′ R 5′-TCCGCAGGTAGATCTCAT-3′	[42]
IL-2	F 5′-CTGCAGTGTTACCTGGGAGA-3′ R 5′-CTTGCATTCACTTCCGGTGT-3′	[38]
MDA5	F 5′-GGACGACCACGATCTCTGTGT-3′ R 5′-CACCTGTCTGGTCTGCATGTTATC-3′	[43]
Perforin	F 5′-ATGGCGCAGGTGACAGTGA-3′ R 5′-TGGCCTGCACCGGTAATTC-3′	[44]
TLR3	F 5′-GCTATTGAGCAAAGTCGAGA-3′ R 5′-ACAGGGGGCACTTTACTATT-3′	[43]
TLR7	F 5′-TCTGGACTTCTCTAACAACA-3′ R 5′-AATCTCATTCTCATTCATCATCA-3′	[43]

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg of spleen, lung, and bursa tissues as previously described [19] by using the MagMAX-96 Total RNA Isolation kit (AM1830, Ambion, Applied Biosystems, Carlsbad, CA, USA). The purified RNA were then reverse-transcribed to cDNA with oligo$(dT)_{15}$ primers (GoScript™ Reverse Transcription System, A5001, Promega, Madison, WI, USA), according to the manufacturer's instructions. The cDNA products were stored at −20 °C until further use.

2.6. Real-time reverse transcription (RT)-PCR

Expression profiles of immune response-associated genes were obtained as follows: one-step RT-PCR reactions were performed with the Power SYBR® Green RNA-to-CT™ 1-Step Kit (4389986, Applied Biosystems, Carlsbad, CA, USA). Each reaction included: 1 μl experimental cDNA, Power SYBR® Green RT-PCR Mix (2X), 100 μM of forward and reverse primers, and RNAse-free water for a total volume of 25 μl. Sequences of the primers used in this study are listed in Table 2. RT-PCR amplification and detection were performed with a LightCycler®480 Real-Time PCR system (Roche, Mannheim, Germany): 94 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. At the end of the amplification program, a melting curve analysis was performed from 65 °C to 97 °C at a rate of 0.11 °C/s with continuous acquisition, and this was followed by a final cooling step to 40 °C.

Data were normalized to levels of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and relative quantification of each gene's expression level was evaluated according to the $2^{-\Delta\Delta CT}$ method [20]. Fold change in immune-related gene expression in rHVT-IBD-vaccinated (Experiment 1), rHVT-IBD/-, rHVT-IBD/IBDV and unvaccinated/IBDV groups (Experiment 2) was presented as fold-increase of the expression level of the unvaccinated group.

To quantitate relative IBDV load in the organs of interest: total RNA was extracted as described above and was used to determine IBDV D78 VP3 RNA load in tissue samples as previously described [21]. Reverse transcription of total RNA into cDNA was performed with Random primers (GoScript™ Reverse Transcription System, Promega, Madison, WI, USA), according to the manufacturer's instructions. IBDV load in the tissue samples was determined by relative quantification according to a standard curve prepared from 2-fold serial dilutions of IBDV D78. A value of 0.0 TCID50/30 mg tissue was assigned to the samples with an undetectable relative virus load.

2.7. Statistical analysis

Statistical analyses were performed using R statistical software. Analysis of the flow cytometry data was performed with one-way analysis of variance (ANOVA) or Student's paired *t*-test. Fold changes were analyzed with one-way ANOVA followed by Duncan's test. If normality and homogeneity of variance were not demonstrated, the non-parametric Kruskal-Wallis test was used. Differences with a p-value $\leq .05$ were considered statistically significant.

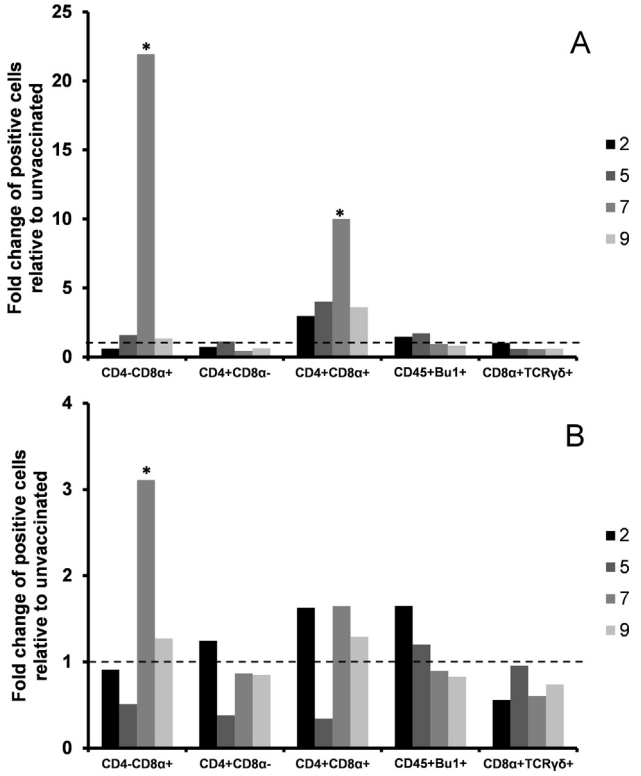

Fig. 1. Early changes in the proportions of T and B cell populations in PBLs (A) and splenocytes (B) following vaccination with rHVT-IBD (Experiment 1). PBLs and splenocytes were isolated at 2, 5, 7 and 9 dpv with rHVT-IBD. Lymphocytes were gated on the basis of forward/side scatter characteristics and then 10^4 events for $CD4^-/CD8\alpha^+$, $CD4^+/CD8\alpha^-$, $CD4^+/CD8\alpha^+$, $CD45^+/Bu1^+$, and $CD8^+/TCR\gamma\delta$ cells were analyzed. The data presented are the average of three rHVT-IBD-vaccinated chickens relative to the percentages obtained for the unvaccinated control birds that were analyzed in parallel. The percentage of cells in the unvaccinated chickens samples were normalized to 1 (indicated by the dotted line). The asterisk indicates that a significant increase in percentage was observed between the vaccinated and unvaccinated groups ($p < .05$).

3. Results

3.1. Flow cytometric immunophenotyping of PBLs and splenocytes following rHVT-IBD vaccination

The response to rHVT-IBD vaccination by distinct lymphocyte subsets was evaluated at 2, 5, 7, and 9 dpv. Cytotoxic T cell ($CD4^-/CD8\alpha^+$), helper T cell ($CD4^+/CD8\alpha^-$), double positive T cell ($CD4^+/CD8\alpha^+$), γδ T cell ($CD8\alpha^+/TCR\gamma\delta^+$), and total B cell ($CD45^+/Bu1^+$) populations were examined in both PBLs and splenocytes (Fig. 1). The vaccinated group had a significantly higher percentage of $CD4^-/CD8\alpha^+$ and double positive $CD4^+/CD8\alpha^+$ cells among the PBLs compared with unvaccinated group at 7 dpv. In the splenocytes, only the $CD4^-/CD8\alpha^+$ subset appeared to be affected. In contrast, there were no marked changes observed in the percentage of helper T cells, B cells, and γδ T cells in the PBL and splenocyte cell populations between the vaccinated and unvaccinated chickens at all of the time points examined.

3.2. Early immune responses to vaccination with rHVT-IBD

To characterize the early immune response following vaccination with rHVT-IBD, changes in transcription of early immune response-associated genes were evaluated in the lung and spleen. CD4 expression was unchanged, while expression of CD8 was significantly upregulated in both spleen and lung, at 7 dpv (Fig. 2). Expression of IFNγ and IL-2 were used as indicators of a Th1 response, and IL-13 was used as an indicator of a Th2 response. None of these markers exhibited significant changes throughout the duration of Experiment 1. However, in the lung and spleen samples of the vaccinated birds, granzyme A was upregulated at 7 dpv and 9 dpv, while mRNA levels of granzyme K gradually increased until they reached a significantly higher level at 9 dpv. In contrast, perforin expression was unchanged in both organs in the samples from vaccinated group compared to the samples from unvaccinated chickens at all of the time points examined.

The innate immune response was evaluated by measuring the expression of antiviral genes (type I IFNs, IFNα and IFNβ) and innate sensors (TLR3, TLR7, and MDA5) in the spleen and lung. The transcription levels of these genes did not significantly differ between the unvaccinated and vaccinated chickens at any of the time points tested (data not shown).

3.3. In vivo IBDV inoculation affects IBDV-specific adaptive immunity in rHVT-IBD-vaccinated chickens

Based on the results of the first experiment, adaptive immunity-related genes were examined to characterize the IBDV-specific responses that are triggered in rHVT-IBD-vaccinated birds. After applying the same immunization scheme used in the first experiment, immune responses were subsequently re-stimulated with inoculation of a live attenuated IBDV at 7 dpv. Levels of IBDV RNA remained undetectable in the spleen, lung, and bursa samples collected from the rHVT-IBD/IBD-vaccinated chickens. In contrast, IBDV RNA was detected in all three organs in the unvaccinated/IBDV group at 5 dpi and 7 dpi (Fig. 3).

Chickens in the rHVT-IBD/IBDV group also exhibited significant upregulation of CD4 in the bursa samples at 7 dpi, although the levels were significantly lower than those of the unvaccinated/IBDV group. In contrast, the IBDV inoculation did not significantly affect CD4 expression in the spleen and lung in the rHVT-IBD/IBDV group compared to the unvaccinated control group (Fig. 4). Regarding CD8 expression, significant upregulation was detected in the lung, spleen, and bursa in the rHVT-IBD/IBDV chickens at 2 dpi, and again in the lung at 5 dpi, and in the lung and bursa at 7 dpi, compared to the unvaccinated and unvaccinated/IBDV groups. In contrast, no significant difference in CD8 expression was detected between the rHVT/IBDV and rHVT-IBD/- groups in all of the organs tested.

Expression of the Th1 cytokine, IFNγ, was upregulated in the bursa samples collected from the rHVT-IBD/IBDV group at 7 dpi. At the same time point, the levels of IFNγ were similarly upregulated in the lungs of the rHVT-IBD/IBDV chickens, although the upregulation was not significant compared to the unvaccinated group. Furthermore, the inoculation of IBDV did not affect the expression of the lymphocyte proliferative cytokine, IL-2, yet its expression significantly increased in the spleens of the unvaccinated/IBDV chickens at 5 dpi and 7 dpi. In the bursa samples, IL-2 expression was upregulated at 7 dpi in the rHVT-IBD/- and unvaccinated/IBDV groups.

IL-13 expression in the spleen and lung remained unchanged in the rHVT-IBD/IBDV group following inoculation with IBDV. Meanwhile, IL-13 expression was only upregulated at 5 dpi in the spleens of the unvaccinated/IBDV group. A similar tendency was observed in the lungs from the unvaccinated/IBDV group at 5 dpi, despite the levels not being significantly different from those of the unvaccinated control group.

Chickens in the rHVT-IBD/IBDV group exhibited a significant increase in granzyme A expression compared with the unvaccinated and unvaccinated/IBDV groups that was measurable at 2 dpi in the spleen and bursa, and at 7 dpi in the lung. Granzyme K exhibited a different expression pattern in the rHVT-IBD/IBDV

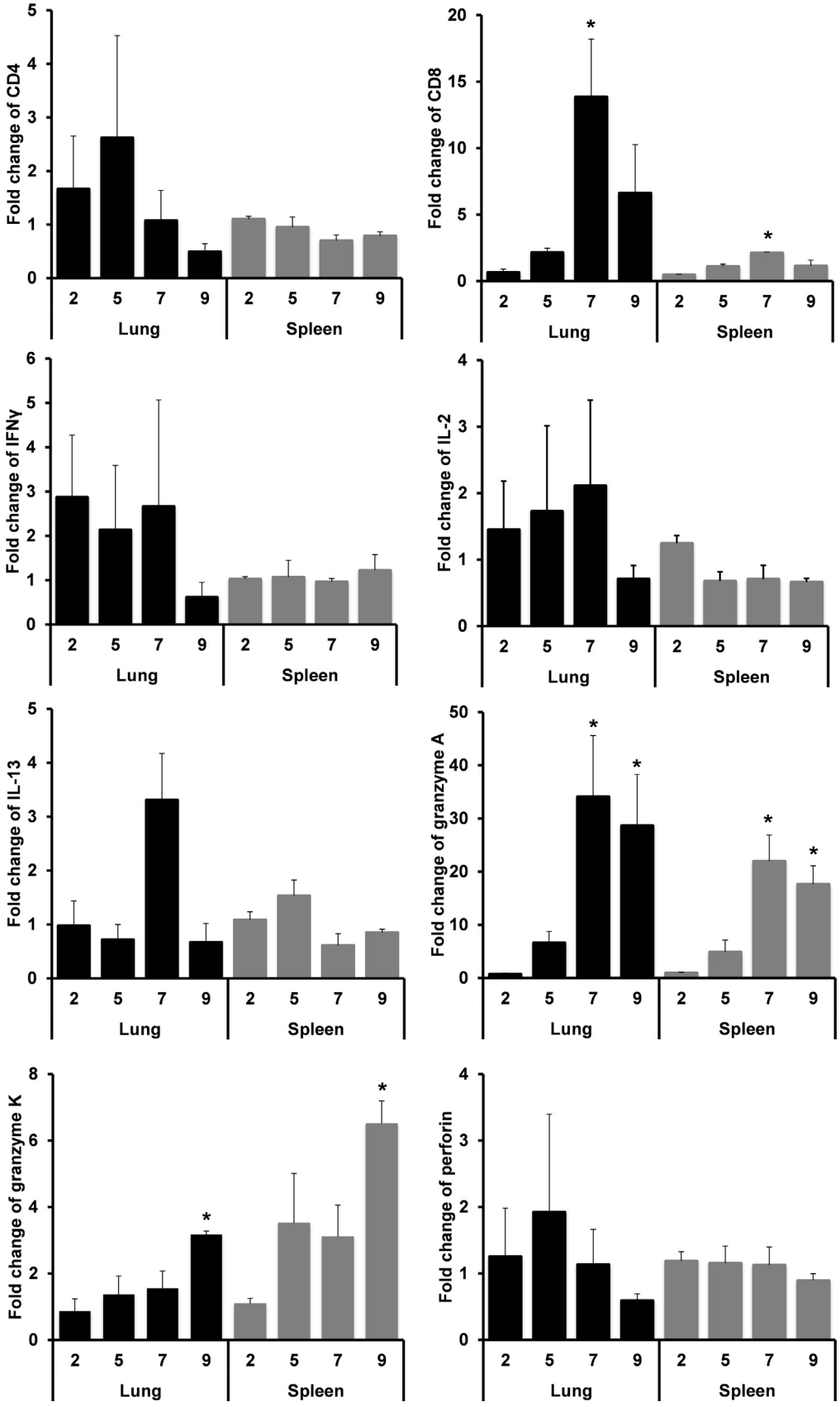

Fig. 2. Gene expression of IFNγ, IL-2, CD4, IL-13, CD8, perforin, granzyme A, and granzyme K detected in rHVT-IBD-vaccinated chickens in Experiment 1. Spleen and lung tissues were collected at 2, 5, 7, and 9 dpv. Fold change in immune-related gene expression relative to the levels in the unvaccinated chickens (controls) are presented. The data were normalized to *GAPDH* expression, calculated according to the $2^{-\Delta\Delta CT}$ method [20], and presented ±standard error of the mean. An asterisk indicates that a significant increase in gene expression was observed between the vaccinated and unvaccinated samples ($p < .05$).

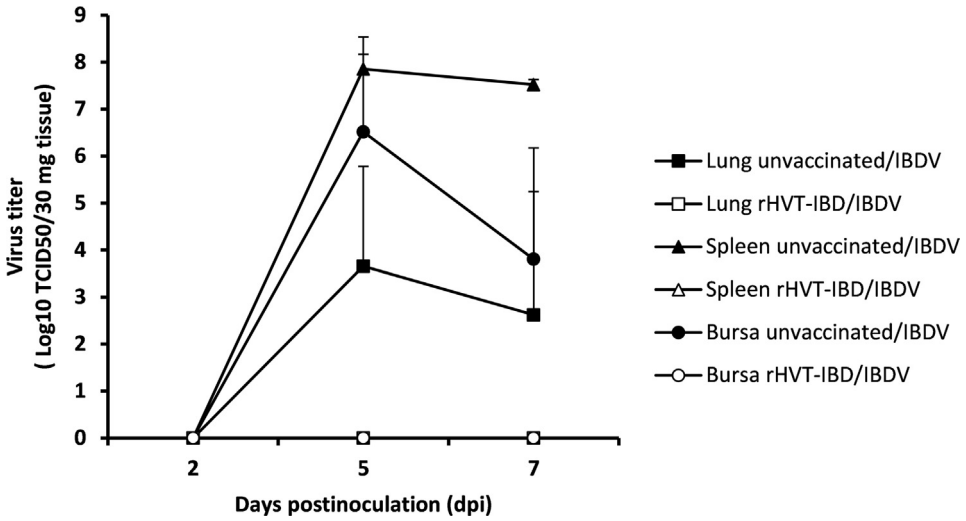

Fig. 3. Detection of IBDV RNA in spleen, lung, and bursa tissues of unvaccinated/IBDV and rHVT-IBD/IBDV chickens in Experiment 2. Organs were collected at 2, 5, and 7 dpi with live IBDV. Titer data are presented standard error of the mean.

chickens with its transcript levels being significantly higher in the lung and the spleen at 7 dpi. Nonetheless, no significant difference in granzyme A and K expression was detected between the rHVT-IBD/IBDV and rHVT-IBD/- groups, thereby making it difficult to confirm an impact of the IBDV inoculation on granzymes expression. Meanwhile, perforin expression was higher in the lung and bursa of the rHVT-IBD/IBDV chickens at 7 dpi, yet diminished expression was detected in the bursal samples, compared to the unvaccinated/IBDV birds.

4. Discussion

The first aim of this study was to measure early cellular immune responses following a subcutaneous rHVT-IBD vaccination. A flow cytometric analysis of lymphocyte subsets present in splenocytes and PBLs suggested that the vaccination stimulated the proliferation of $CD8^+$ cells, thereby triggering an increase in the $CD8^+$ cell population in the spleen and their subsequent circulation in peripheral blood. A similar increase was previously observed for blood $CD8^+$ cells in susceptible B13/B13 chickens following their inoculation with HVT [22]. A recent study reported contradictory findings and demonstrated a decreased splenic $CD8^+$ T cells number at day 8 of age following *in vivo* vaccination with rHVT-IBD [23]. Further studies examining the influence of age and route of immunization on the number and biological activities of $CD8^+$ T cells should be carried out. Among the $CD8^+$ subpopulations of the cells examined in the present study, an increase in circulating double-positive $CD4^+/CD8^+$ cells was notably observed. Mature $CD4^+/CD8^+$ T cells have been detected in the spleen, blood, and intestinal epithelium of chickens, and it is suggested that they provide a similar functional role as $CD4^+$ T lymphocytes [24]. However, their biological function in the immune response to a rHVT-IBD vaccination remains to be elucidated. In parallel, the expression levels of immune-related genes were profiled in spleen and lung samples, and CD8 was also found to be upregulated at 7 dpv. This result correlates with the increase in $CD8^+$ T cells detected by flow cytometry as described above. Additionally, the upregulation of granzymes A and K that was observed in the spleen and lung following vaccination in the present study is in agreement with the results of Parvizi et al. which demonstrated that an *ex vivo* re-stimulation of spleen cells isolated from HVT-vaccinated birds with an inactivated HVT tended to enhance granzyme A expression [25]. Granzyme K specificities are still poorly documented in chickens, although in mammals, this serine protease is thought to be functionally redundant to granzyme A [26,27]. In contrast with the granzyme profiles, perforin expression remained at basal levels in the rHVT-IBD-vaccinated chickens. These findings suggest that vaccination with rHVT-IBD did not elicit noticeable *de novo* transcription of perforin during the time points assayed. Differential expression of granzyme A and perforin has previously been observed in chicken bursal mononuclear cells following their infection with a classical IBDV strain. The authors suggested that these results were associated with the stage of activation of cytotoxic T lymphocytes (CTLs) [28]. In humans, distinct profiles for granzyme and perforin expression have been observed in memory CD8 T cells in response to different viruses, including influenza virus and human immunodeficiency virus type 1 [29]. Surprisingly, despite the CD8 response observed, expression of the Th1 cytokines, IFNγ and IL-2, remained unchanged after rHVT-IBD vaccination. Because of the short half-life of cytokine mRNAs [30], detection of an increase in levels of IFNγ and IL-2 may have been missed due to the time points that were selected for examination. Consequently, future studies may want to consider daily time points for analysis. In addition, rHVT-IBD vaccination was reported to induce low IBDV-specific antibody titers during the first three weeks [13]. In agreement with these findings, the analysis of IL-13 expression in the present study indicates that rHVT-IBD does not appear to promote strong Th2-oriented immune responses in the early stages following vaccination.

Innate immunity is indispensable for mounting a quick and efficient response to infection. It also contributes to the initiation of adaptive responses. Previously, the infection of chicken embryo fibroblasts with HVT was shown to activate the IFN regulatory factor pathway, which may help induce expression of type I IFN to activate antiviral innate responses [31,32]. However, the *in vivo* model established in the present study did not provide experimental evidence to indicate an influence of rHVT-IBD vaccination on the expression of the innate immune genes tested.

In the second part of the present study, specific cellular immune responses were analyzed in lung, spleen, and bursa tissues of rHVT-IBD vaccinated-chickens following inoculation with an attenuated IBDV. The greatest response was observed in the bursa at 7 dpi with reduced levels of CD4, IL-2, and perforin mRNAs detected in the rHVT-IBD-vaccinated birds compared with the unvaccinated/IBDV group. In combination with an absence of IBDV VP3 RNA, these results suggest that virus replication was blocked

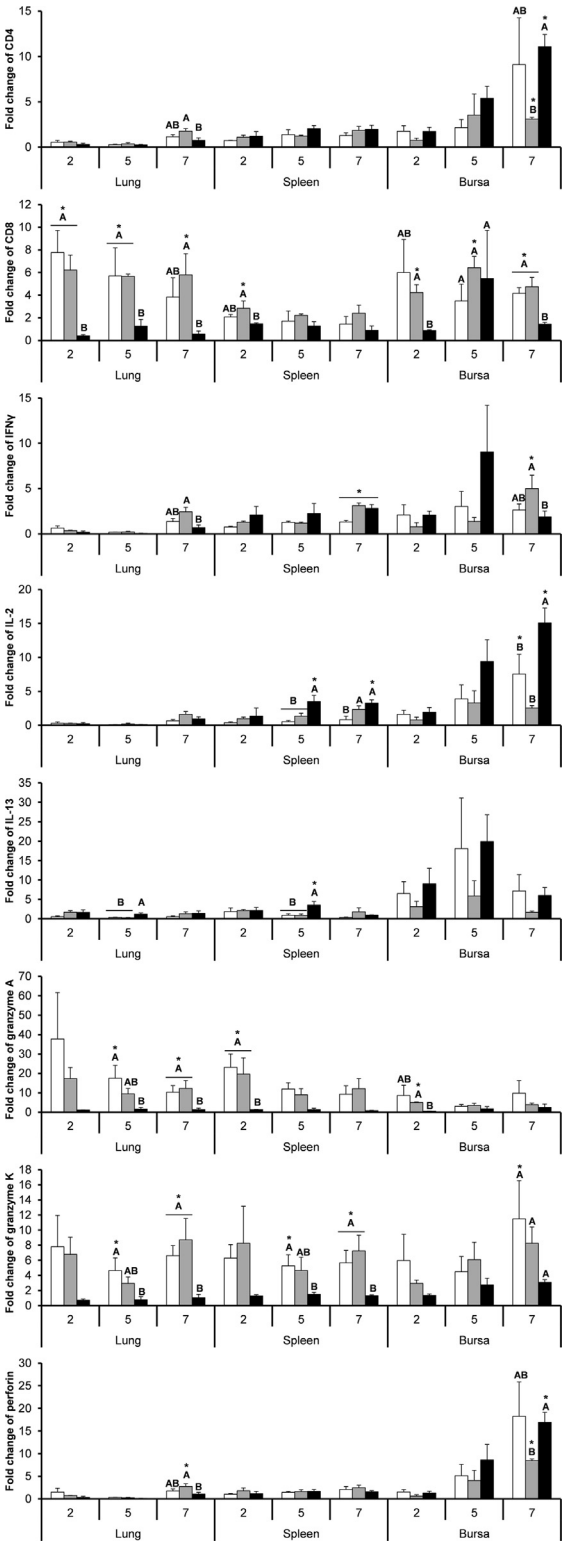

Fig. 4. Gene expression of IFNγ, IL-2, CD4, IL-13, CD8, perforin, granzyme A, and granzyme K in rHVT-IBD/- (white), rHVT-IBD/IBDV (grey), and unvaccinated/IBDV (black) chickens in Experiment 2. Organs were collected at 2, 5, and 7 dpi with live IBDV. Fold change in immune-related gene expression relative to the levels in the unvaccinated chickens are presented. The data were normalized to *GAPDH* expression, calculated according to the $2^{-\Delta\Delta CT}$ method [20], and presented ±standard error of the mean. An asterisk indicates that a significant increase in gene expression was observed between the vaccinated and unvaccinated samples ($p < .05$). Different letters indicate statistically significant differences between the groups at a specific time point.

in the bursa as a result of a rapid host immune response that was triggered by rHVT-IBD vaccination. Indeed, inoculation of an attenuated IBDV strain into unvaccinated chickens triggered an immune response that was predominantly localized in the bursa, and this is consistent with the selective tropism of IBDV for this organ. Moreover, upregulated expression of IFNγ in the bursa of the rHVT-IBD/IBDV chickens at 7 dpi, coupled with upregulation of both CD4 and CD8, indicates that a T cell-specific reactivation occurred in response to the IBDV inoculation. However, further studies are needed to identify these IFNγ-producing cells.

Interestingly, CD8 expression was also upregulated in the lungs of the rHVT-IBD/IBDV group at 7 dpi, and this result is consistent with the upregulated expression observed for perforin. Moreover, these results, in combination with the detection of IBDV RNA in the lungs of the unvaccinated/IBDV chickens, suggest that an IBDV-specific cytotoxic response may have been induced in this organ. These findings are in accordance with those of a previous study where augmented host immune responses against MDV were detected in the lung, and they were characterized by an infiltration of $CD4^+$ and $CD8^+$ T cells when the chickens were vaccinated with HVT, suggesting that HVT-induced memory cells may play a role in an immune response against infection [33]. The key role that CTLs have been found to have in protective immunity and in clearance of IBDV [28,34] also highlights the potential for vaccines to initiate a cellular response against this pathogen. Monitoring CTL activity in response to infectious pathogens requires the use of inbred chickens and MHC-matched target cells. These cell lysis-based assays are the most accurate methods currently available, yet they are expensive and technically challenging [35]. Further studies are needed to elucidate the potential mechanisms that mediate the specific activation of $CD8^+$ T cells following IBDV inoculation in rHVT-IBD-vaccinated chickens.

We previously demonstrated that rHVT-IBD can be detected in the spleen in the early stages after vaccination [14]. With an absence of well-developed lymph nodes, the spleen is a major secondary lymphoid organ in birds and it provides a microenvironment that is favorable for interactions between T lymphocytes and antigen-presenting cells [36]. In the present study, an inoculation of IBDV elicited measurable changes in CD8 and granzyme A expression in the spleen at 2 dpi, contrasting with the response observed in the bursa. These results suggest that cytotoxic T cell responses may be induced in the spleen of rHVT-IBD-vaccinated chickens following inoculation with attenuated IBDV. Moreover, lesions in the spleen have been observed in the early stages following immunization with an intermediate IBDV vaccine [37], thereby indicating that the spleen is a site for viral replication, similar to the bursa. In agreement with these results, IBDV RNA was detected in the spleen and the bursa following the IBDV inoculation of unvaccinated chickens.

The expression profiles obtained for the organs analyzed in the rHVT-IBD/IBDV group demonstrated that the inoculation of IBDV had an effect compared to the unvaccinated/IBDV group. However, these profiles did not significantly differ from those of the rHVT-IBD/- group. Therefore, it would be useful for future studies to include a greater number of individuals at each time point and to perform an inoculation of IBDV at a later time point following vaccination in order to dissociate VP2-specific responses from those associated with the vaccine vector, and also to evaluate the ability of IBDV to activate IBDV-specific memory cells in rHVT-IBD-vaccinated birds. Additionally, stimulation of immune responses in rHVT-IBD-vaccinated chickens in response to a pathogenic IBDV strain should be evaluated.

To our knowledge, the results of the present study demonstrate for the first time that rHVT-IBD vaccination leads to an increase in the numbers of circulating and splenic $CD8^+$ T cells and to an upregulation of granzyme A and K expression in the lung and

spleen from 7 dpv. Previously, protection against IBDV has consistently been correlated with the presence of neutralizing antibodies. However, upregulation of CD4, CD8, perforin, and IFNγ expression in the bursa of rHVT-IBD-vaccinated chickens at 7 dpi with IBDV provides evidence that specific cellular responses can also play important roles in host protection. Moreover, these specific cellular responses could be measured as correlates of protection in rHVT-IBD-vaccinated chickens following IBDV *in vivo* recall stimulation.

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Conflict of interest

None.

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