



Cell-mediated immune responses in the head-associated lymphoid tissues induced to a live attenuated avian coronavirus vaccine



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ABSTRACT

Humoral immunity is important for controlling viral diseases of poultry, but recent studies have indicated that cytotoxic T cells also play an important role in the immune response to infectious bronchitis virus (IBV). To better understand the cell mediated immune responses to IBV in the mucosal and systemic immune compartments chickens were ocularly vaccinated with IBV. This induced a lymphocyte expansion in head-associated lymphoid tissues (HALT) and to a lesser extent in the spleen, followed by a rapid decline, probably due to homing of lymphocytes out of these organs and contraction of the lymphocyte population. This interpretation was supported by observations that changes in mononuclear cells were mirrored by that in CD3⁺CD44⁺ T cell abundance, which presumably represent T effector cells. Increased interferon gamma (IFN- γ) expression was observed in the mucosal immune compartment, i.e., HALT, after primary vaccination, but shifted to the systemic immune compartment after boosting. In contrast, the expression of cytotoxicity-associated genes, i.e., granzyme A (GZMA) and perforin mRNA, remained associated with the HALT after boosting. Thus, an Ark-type IBV ocular vaccine induces a central memory IFN- γ response in the spleen while the cytotoxic effector memory response, as measured by GZMA and perforin mRNA expression, remains associated with CALT after boosting.

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1. Introduction

Infectious bronchitis (IBV) is a single stranded RNA virus of the Coronaviridae family, which causes a highly contagious respiratory disease in chickens of all ages (Cavanagh and Gelb, 2008; Woo et al., 2010). It causes huge economic losses to poultry industries by reducing the quality and quantity of egg production in layers and breeders and reducing growth rate in broilers (Cavanagh and Gelb, 2008; Dolz et al., 2012). Controlling IBV is difficult due to its high mutation rate, which allows it to quickly evolve into variants or serotypes that can escape the immune response (Cavanagh et al., 1992; Jia et al., 1995; Kottier et al., 1995). IBV infections induce both humoral and cell mediated immune responses (Collisson et al., 2000; Martins et al., 1991; Mockett and Cook, 1986). Although humoral immunity to IBV in the systemic immune compartment is important, it does not correlate with protection against

IBV (Collisson et al., 2000; Ignjatovic and Galli, 1994; Raggi and Lee, 1965). Collisson et al. (2000) showed that an increase in cytotoxic T lymphocyte (CTL) activity in splenocytes correlated with decreased viral loads in lungs and kidneys of IBV infected chickens. Transfer of IBV-specific-CD8⁺ $\alpha\beta$ 1 T cells to naïve chickens provided protection against challenge with a homologous IBV strain. Others investigators have also stressed the importance of cytotoxic T cells in the initial phase of the immune response to IBV (Liu et al., 2012; Seo et al., 2000).

The mucosal immune system is perfectly situated to play a role in antigen-specific immune protection against IBV challenge, since IBV enters the host tissue through the mucosal sites of the ocular and nasal epithelium (Cavanagh and Gelb, 2008). IBV initially replicates in Harderian glands (HG) (Chousalkar et al., 2007) where IBV-specific IgA antibodies are secreted after IBV exposure (Davelaar and Kouwenhoven, 1981; Davelaar et al., 1982; van Ginkel et al., 2008). Like HG, conjunctiva-associated lymphoid tissues (CALT) also play an important role in mucosal immune protection of the eye (Fix and Arp, 1991; van Ginkel et al., 2012). However, little is known about the cell-mediated immune response in these mucosa-associated lymphoid tissues.

In this study the cell mediated immune responses in the mucosal and systemic immune compartments after ocular vaccination of chickens with a live-attenuated Ark-type IBV vaccine were ana-

Abbreviations: IBV, infectious bronchitis virus; CALT, conjunctiva-associated lymphoid tissue; HG, Harderian gland; IFN- γ , interferon gamma; GZMA, granzyme A; CTL, cytotoxic T lymphocyte; EID₅₀, median embryo infectious dose; DPV, days post vaccination; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; HALT, head-associated lymphoid tissues.

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lyzed. Specifically, changes in total mononuclear cells and CD3⁺CD44⁺ T cells and changes in mRNA transcripts of IFN- γ and cytotoxic enzymes, i.e., granzyme A (GZMA) and perforin, were measured during the course of the primary and secondary immune response to IBV in CALT, HG and spleen. Our data demonstrate differences in the IBV-induced immune responses in HG, CALT and spleen, indicating different functions in the IBV specific immune response for these lymphoid organs.

2. Materials and methods

2.1. Chickens and vaccination

Specific pathogen free white leghorn chicken eggs (Sunrise Farms, NY) were hatched and housed in Horsfall-type isolation units maintained at a Biosafety level 2 facility. Live attenuated Ark-DPI IBV vaccine was used at a dose of 3×10^5 median embryo infectious dose (EID₅₀) per bird for ocular immunizations for both the primary and secondary response unless indicated otherwise. Chickens were vaccinated at 3–4 weeks of age. Where appropriate, the chickens were boosted 4 weeks later. CALT and HG from both sides of the head and spleen were collected from these chickens for each experiment. Unvaccinated age-matched chickens were used as negative controls. Experimental procedures and animal care were performed in compliance with federal and institutional animal care guidelines. Auburn University College of Veterinary Medicine has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Mononuclear cell isolation and count

Chickens were vaccinated with 3×10^4 EID₅₀ per bird of Ark-type IBV for this experiment. CALT and HG were obtained from both sides of the head. These lymphoid tissues and spleen were collected in complete RPMI medium and mononuclear cells were isolated from these tissues by density centrifugation as previously described (van Ginkel et al., 2008). The cells were centrifuged over Histopaque Ficoll (1.077) gradient. Live mononuclear cells were counted using trypan blue exclusion on a Bright-Line[®] hemocytometer (Hausser Scientific, Horsham, PA). Cell concentrations were expressed as cells per ml. The total cell counts per organ were determined by multiplying the total volume (ml) of the cell suspension by its concentration (cells/ml).

2.3. Flow cytometry

To analyze the T cell composition of CALT, HG and spleen, mononuclear cells were isolated as mentioned above and stained with 0.1 μ g of PE-conjugated mouse anti-chicken CD3⁺ antibody per 10^6 cells for 2 h on ice. Lymphocytes were then stained for CD44⁺ by incubating them with 1 μ g of biotinylated mouse-anti-chicken CD44⁺ antibody per 10^6 cells for 2 h followed by 1 μ g of streptavidin-Alexa660 per 10^6 cells for 1 h on ice. The flow cytometer was set up using single surface marker stained mononuclear cells. Unlabeled cells were used as negative controls. All washes were performed with PBS/1%BSA/0.02%NaN₃. The cells were filtered through a 50 μ m nylon mesh (Small Parts Inc., Miami Lakes, FL) before analysis on a MoFlo high-performance cell sorter (Dako Colorado Inc., Fort Collins, CO). The cells were gated for lymphocytes and analyzed for expression of surface markers with the Summit 4.3 software as previously reported (van Ginkel et al., 2012).

2.4. RNA isolation and qRT-PCR

CALT, HG and spleen tissues were collected to extract total RNA using TRI reagent[®] (Molecular Research Center, Cincinnati, OH) according to the manufacturing company's protocol. The total RNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). A qScript One-Step SYBR[®] Green qRT-PCR kit (Quanta Biosciences) was used to make up the RT-PCR reaction mixture according to the manufacturing company's protocol. IFN- γ , GZMA and perforin primers were based on previous publications (Kaiser et al., 2000; Sarson et al., 2008) and their amplicons were sequenced prior to use. All RT-PCR reactions were confirmed using melting curve analyses. β -actin was used as a housekeeping gene using primers previously described (Abdul-Careem et al., 2006) and the $\Delta\Delta$ (Ct) method was used to calculate the fold change in expression of IFN- γ , GZMA and perforin mRNA compared to one of the controls. This was done for each tissue type. The CFX96[™] system (Bio-rad, Hercules, CA) was used to perform the RT-PCR reactions (Table 1).

2.5. Statistical analysis

The Student's *t*-test or ANOVA were used to compare data to that of control chickens where appropriate. Mann Whitney test was used to perform statistical analysis of the non-parametric RT-PCR data. Depicted are the mean values and the error bars represent one standard error of the mean. Statistically significant differences have a *p* value <0.05.

3. Results

3.1. Lymphocyte counts after primary IBV immunization

Changes in the total number of mononuclear cells in lymphoid tissues over time will give an overall idea of the progression of the immune response as defined by phases, i.e., lag, expansion and contraction phases as have been reported in the mammalian immune response to a pathogen (Kaech et al., 2002). To this end the total number of mononuclear cells in CALT, HG and spleen were counted after ocular immunization with 3×10^4 EID₅₀ per bird of Ark-type IBV. The mononuclear cell counts in CALT and HG increased starting 7–8 days post vaccination (DPV) and peaked on 9 DPV (1.9×10^6 cells in CALT and 5×10^6 cells in HG), representing the expansion phase (Fig. 1A and B). The number of mononuclear cells in the HG and CALT were statistically significantly higher on 9 DPV than in unvaccinated control chickens. The CALT mononuclear cells were also significantly elevated on day 8. An abrupt decline on day 10 post vaccination was observed (going down to 7×10^5 cells in CALT and 5.6×10^5 cells in HG), representing the beginning of the contraction phase combined with homing of mononuclear cells out of these tissues. The mononuclear cell counts in spleen significantly increased from 1.1×10^8 cells on 3 DPV to 1.8×10^8 cells on 9 DPV and decreased to 1.5×10^8 cells on 10 DPV in the contraction phase (Fig. 1C). The changes in mono-

Table 1
RT-PCR reactions used for cDNA synthesis and amplification.

Reaction steps	IFN- γ	Perforin	GZMA
Reverse transcription	55 °C for 10 min	55 °C for 10 min	55 °C for 10 min
Initial denaturation	95 °C for 5 min	95 °C for 1 min	95 °C for 1 min
Denaturation	95 °C for 30 s	95 °C for 30 s	95 °C for 30 s
Annealing	50 °C for 30 s	65 °C for 30 s	55 °C for 30 s
Extension	72 °C for 30 s	72 °C for 30 s	72 °C for 30 s
# of cycles	40	40	40

nuclear cell numbers were not as evident in the spleen as in the CALT and HG relative to the total number of mononuclear cells present. The relative low percentage of change in the number of mononuclear cells of the spleen compared to CALT and HG may be due in part to the ocular immunization route. However, this change still represented the largest decline in cell numbers during the contraction phase of all lymphoid organs analyzed. It decreased by 30×10^6 lymphocytes on average.

3.2. Changes in the CD3⁺CD44⁺ T cell population after primary IBV vaccination

Dual staining for CD3 and CD44 was used to compare changes in the activated T cell population in mucosal and systemic lymphoid tissues after ocular vaccination with Ark-type IBV. Lymphocytes from CALT, HG and spleen were stained for flow cytometric analyses on 3, 7, 9, 11 and 14 DPV. In CALT the level of CD3⁺CD44⁺ T cells significantly decreased from 25% of the total lymphocytes in

controls to 9% on day 3. This was followed by a gradual increase back to pre-vaccination levels on day 11, followed by another significant decline on day 14 (Fig. 2A). Thus, this T cell population increased in CALT from day 3 until day 11, the peak of the immune response. In the HG CD44⁺CD3⁺ T cells increased from 5% in controls to significantly elevated peak levels on day 9 representing 22% of the HG lymphocyte population (Fig. 2B). This significantly elevated level was maintained on day 11 and then dropped back to control levels by day 14. The spleen displayed a similar but delayed profile as was seen in CALT, but the splenic CD44⁺CD3⁺ T cells did not display any significant changes relative to controls. Approximately 50% of control spleen-derived lymphocytes were CD3⁺CD44⁺ T cells and they reached the highest level at 11 DPV at 57% (Fig. 2C).

3.3. IFN- γ mRNA expression after primary IBV vaccination

The mRNA expression of IFN- γ increased significantly over age-matched controls in CALT starting on 4 DPV and remained significantly elevated during the period CALT was monitored (Fig. 3A). Tissues from a control chicken were collected every other sample

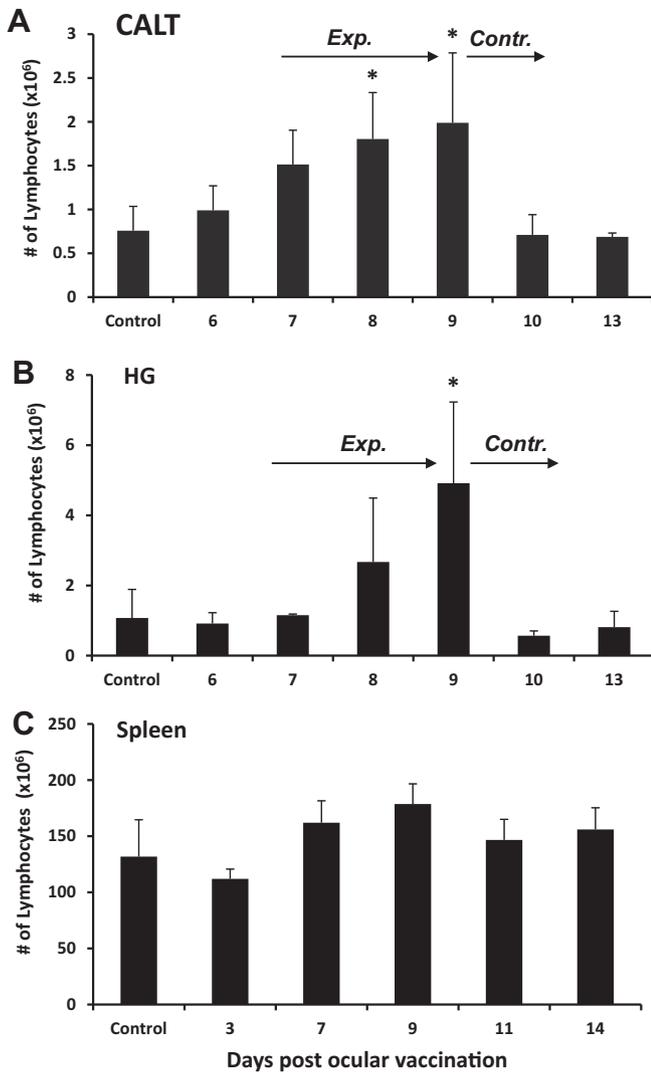


Fig. 1. Mononuclear cell count after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and whole tissues were collected on both sides of the head to isolate the total number of mononuclear cells in the CALT (A), HG (B) and spleen (C) using trypan blue exclusion. The phases of the immune response are indicated as expansion phase (Exp.) and beginning of the contraction phase (Contr.) which coincides with homing of lymphocytes to mucosal surfaces. Arrows indicate the progression of the phases. Six control and three IBV vaccinated chickens were used per time point. Depicted are the means and one standard error. A significant increase from control chickens is indicated by * ($p < 0.05$).

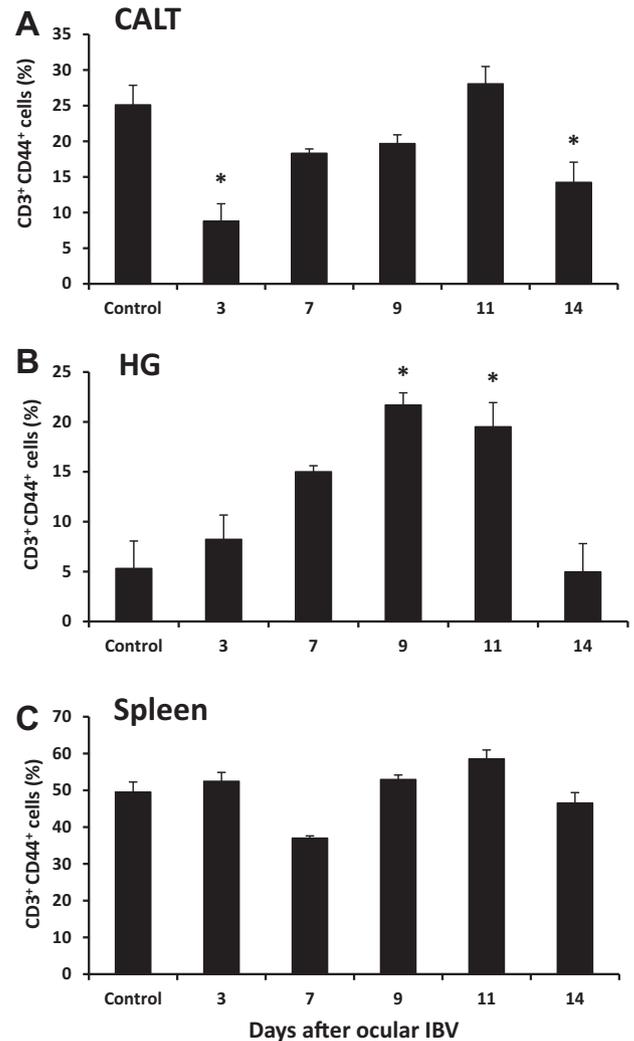


Fig. 2. CD3⁺CD44⁺ T cells after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and tissues were collected to measure the percent CD3⁺CD44⁺ T cells of the total lymphocyte population in the CALT (A), HG (B) and spleen (C) using flow cytometry. Values significantly different from controls are indicated by * and have a p value < 0.05 . Five control and three IBV-vaccinated chickens were used per time point. Depicted are the mean values and one standard error.

day. Two peaks in IFN- γ mRNA expression were observed, i.e., on 6 and 10 DPV. The CALT demonstrated by far the highest fold increase in IFN- γ mRNA expression when compared to HG and spleen in the primary IBV response. In HG IFN- γ mRNA expression significantly increased over control values on 2, 5 and 6 DPV (Fig. 3B). In the primary IBV response, unlike CALT and HG, the spleen did not show any significant changes in IFN- γ mRNA expression compared to control spleens over the period it was monitored (Fig. 3C).

3.4. GZMA and perforin mRNA expression during the primary response to IBV

The expression of GZMA and perforin mRNA was measured since it emulates the induction of cytotoxic lymphocytes in the IBV immune response. Total RNA was isolated on 2–11 and 14 DPV from CALT, HG and spleen after primary ocular immuniza-

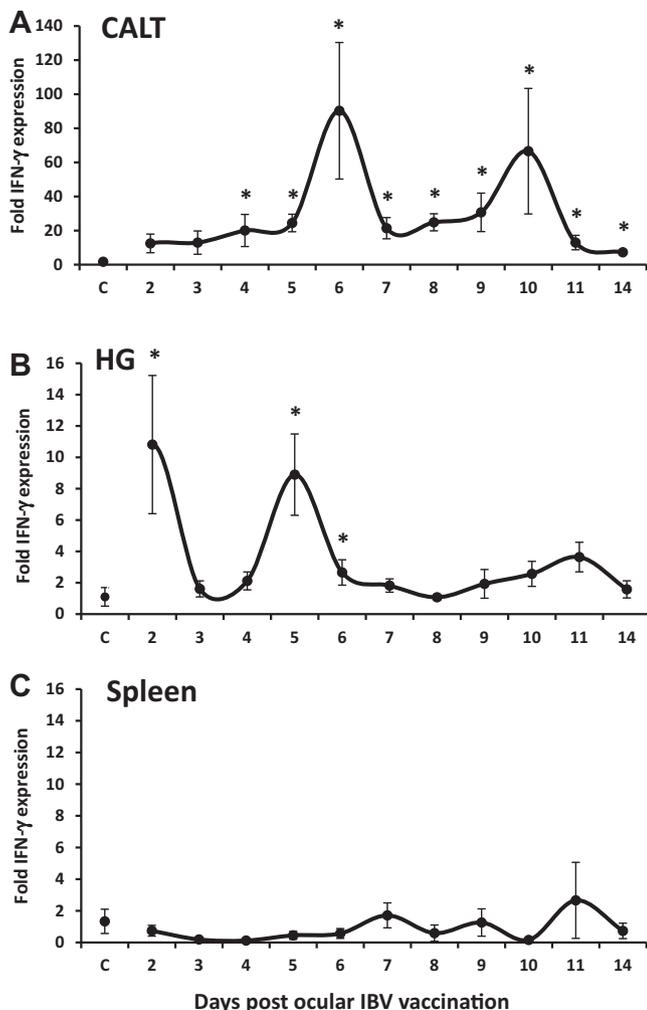


Fig. 3. IFN- γ mRNA expression after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and CALT (3A), HG (3B) and spleen (3C) were collected on various days after vaccination for RNA extraction to measure IFN- γ mRNA expression by qRT-PCR. RNA levels were normalized using β -Actin mRNA levels. Values are indicated as fold mRNA expression compared to age-matched, unvaccinated control values represented as 'C' on the x axis. Controls were collected every other sample day and were used as the control group. The number of chickens analyzed per time point varied between 5 and 10 for the IBV vaccinated chickens and 6 for the non-vaccinated control chickens. Depicted are the means and one standard error. Values significantly different from control are indicated by * and have a $p < 0.05$.

tion with IBV. A gradual increase in the average mRNA expression of GZMA was observed starting on 4–5 DPV in CALT (Fig. 4A) resulting in a significant increase in GZMA mRNA expression during the peak of the response on 8–9 DPV (90 fold higher than controls), after which it declined. This pattern was mimicked by perforin mRNA expression in CALT, but the changes were of lower magnitude and were not significantly increased over control values (Fig. 4B). The significant decline of perforin mRNA expression on days 2–3 post vaccination (Fig. 4B) in CALT coincided with a decrease of CD44⁺CD3⁺ T cells seen on 3 DPV (Fig. 2A). The HG displayed significant increases in mRNA expression of both GZMA (18 fold increase over controls) and perforin (7 fold increase over controls) 2–3 DPV (Fig. 4C and D). The HG also displayed a peak GZMA mRNA expression on days 8–9, which was not significantly elevated over controls due to the high degree of variation in mRNA expression. However, the time points prior to this peak, days 6–7, and following this peak, days 10–11, displayed a significantly elevated GZMA mRNA expression over controls. Perforin mRNA expression in HG also had a significantly higher response 14 DPV (8 times higher). Unlike in CALT and HG, no significant increase in GZMA or perforin mRNA expression was observed in the spleen. In contrast, a significant decline in mRNA expression of GZMA was observed on days 6–7 and 10–11 and for perforin on days 2–7 (Fig. 4E and F).

3.5. IFN- γ mRNA expression during secondary response to IBV

CALT, HG and spleen were isolated on days 4–11 after boosting to measure the IFN- γ mRNA expression. As opposed to the primary response, no significant increase in mRNA expression of IFN- γ was observed in CALT (Fig. 5A). A significant increase in IFN- γ mRNA expression was seen on day 4 after boosting in both HG and spleen, i.e., ~2 fold higher than the control in HG and ~24 fold higher in the spleen (Fig. 5B and C). In addition, a significant 11 fold increase in IFN- γ mRNA expression in the spleen was also observed on day 10 post boosting. Unlike the IFN- γ mRNA expression in the primary response, in which the spleen displayed no significant increase of IFN- γ , the secondary IFN- γ response to IBV was more prevalent in the spleen than HALT after ocular boosting.

3.6. GZMA and perforin mRNA expression after secondary IBV vaccination

To analyze cytotoxic activity in the IBV memory response, the expression of mRNA encoding the cytotoxic enzymes GZMA and perforin were measured after ocular boosting with the IBV vaccine. Total RNA was isolated from CALT, HG and spleen on days 4–11 after boosting. No significant changes in GZMA and perforin mRNA expression were observed in HG and spleen (Fig. 6C–F). The only significant changes observed were in the mRNA expression of GZMA in CALT on days 4–7 post boosting (Fig. 6A). These 4–5 fold increases were quite modest compared to the 90 fold increase in GZMA mRNA expression in CALT observed after primary immunization (Fig. 4A). Both GZMA and perforin mRNA expression reached the highest means on days 10–11 in CALT, but due to high variation in the mRNA levels the increases were not significant.

4. Discussion

In the primary IBV-specific immune response, induced by ocular vaccination with an Ark-type IBV live attenuated virus, only the HG and CALT exhibit a response with a noticeable increase in the total number of mononuclear cells, CD3⁺CD44⁺ T cells, and IFN- γ , GZMA and perforin mRNA expression while little change is noticed in the spleen measuring these variables. The route of vac-

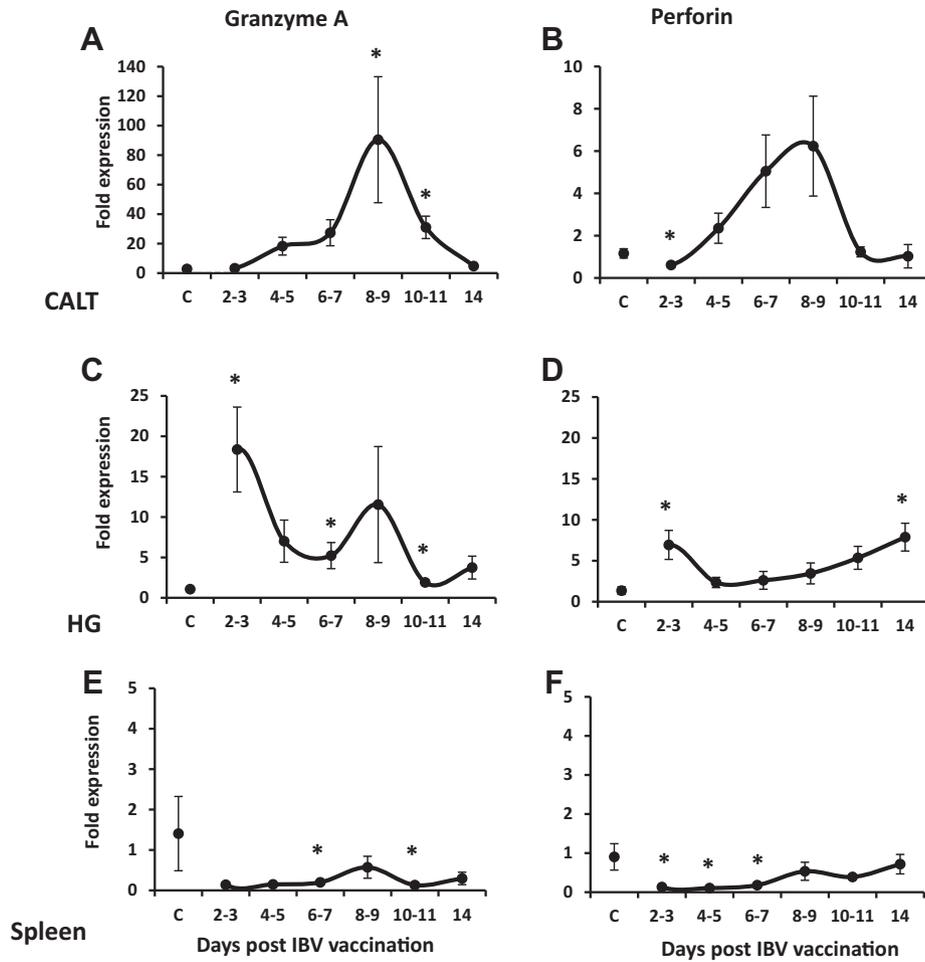


Fig. 4. GZMA and perforin mRNA expression after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and CALT, HG and spleen were collected on various days after vaccination for RNA extraction and measuring the GZMA and perforin mRNA expression by qRT-PCR. The graphs represent GZMA mRNA expression in CALT (A), HG (C) and spleen (E), and perforin mRNA expression in the CALT (B), HG (D) and spleen (F). RNA levels were normalized using β -Actin mRNA levels. Values are indicated as fold mRNA expression compared to a control. The control group is represented as 'C' on the x axis. Six non-vaccinated control chickens were collected on the same days as experimental chickens, i.e., between 5 and 10 DPV. One control chicken was collected on each sampling time point when vaccinated birds were collected. Indicated are the mean and one standard error. Values significantly different from the control group are indicated by * and have a $p < 0.05$.

ination, i.e., ocular vaccination, most likely contributed to this lack of responsiveness in the spleen compared to the HALT. Upon ocular boosting of the IBV-specific immune response 4 weeks after priming, the IFN- γ response shifts from the mucosal to the systemic immune compartment. Although the cytotoxic T cell response, as measured by the mRNA expression of GZMA and perforin, remains associated with CALT in the secondary response.

Effector memory T cells (T_{EM}) are known to home to effector sites and contain subsets of T_H1 and CTLs in response to a virus challenge. In contrast, central memory T cells (T_{CM}) home to the lymph nodes and spleen, proliferate and secrete IFN- γ in response to stimulation with antigen (Sallusto et al., 2004). The expression of GZMA in CALT after boosting suggests the induction or presence of cytolytic T_{EM} , while IFN- γ expression without GZMA expression in spleen is consistent with proliferating T_{CM} . T_{EM} reside at the "frontline" of pathogen invasion and initiate a quick response upon reinfection with the pathogen (Kaech et al., 2002; Masopust and Picker, 2012). Modification of IBV vaccines to generate greater numbers of these T_{EM} may reduce the severity of an IBV infection.

Relatively little research exists regarding CD44 expression in chickens and even less on its expression on avian lymphocytes. CD44 expression has been analyzed as a marker for detection of activated T cells in challenged or vaccinated chickens without finding a clear correlation (Shimizu et al., 1989; Dalgaard et al., 2010).

In our study, the increase of $CD3^+CD44^+$ T cells follows the increase in the number of the lymphocytes in HG. Both total lymphocytes and $CD3^+CD44^+$ T cells numbers drop during the contraction phase. Based on this observation, CD44 is expressed on activated or effector T lymphocytes. CALT follows a similar pattern except that it initially undergoes a drop in $CD3^+CD44^+$ T cells followed by an increase during the expansion phase and a second drop during the contraction phase. This indicates that effector T cells initially leave the CALT upon IBV exposure, followed by a repopulation during the expansion phase and a subsequent decline during the contraction phase, which coincides with homing out of CALT to infection sites. The $CD3^+CD44^+$ T cells in the spleen seem to follow a similar pattern as CALT, only with lower amplitude relative to the total number of lymphocytes.

The mRNA expression of IFN- γ , GZMA and perforin in CALT, HG and spleen are analyzed in order to get a better understanding of the magnitude of the cell mediated immune response to an Ark-type IBV vaccine in mucosal versus systemic lymphoid tissues after ocular vaccination. The CALT shows an extended significant increase of IFN- γ mRNA expression, i.e., from 4 to 14 DPV, during the primary response to ocular Ark-type IBV vaccination. A concomitant increase in GZMA and perforin mRNA expression from 4 to 9 DPV in CALT is also observed. These observations, combined with the IFN- γ mRNA expression pattern, are consistent with a

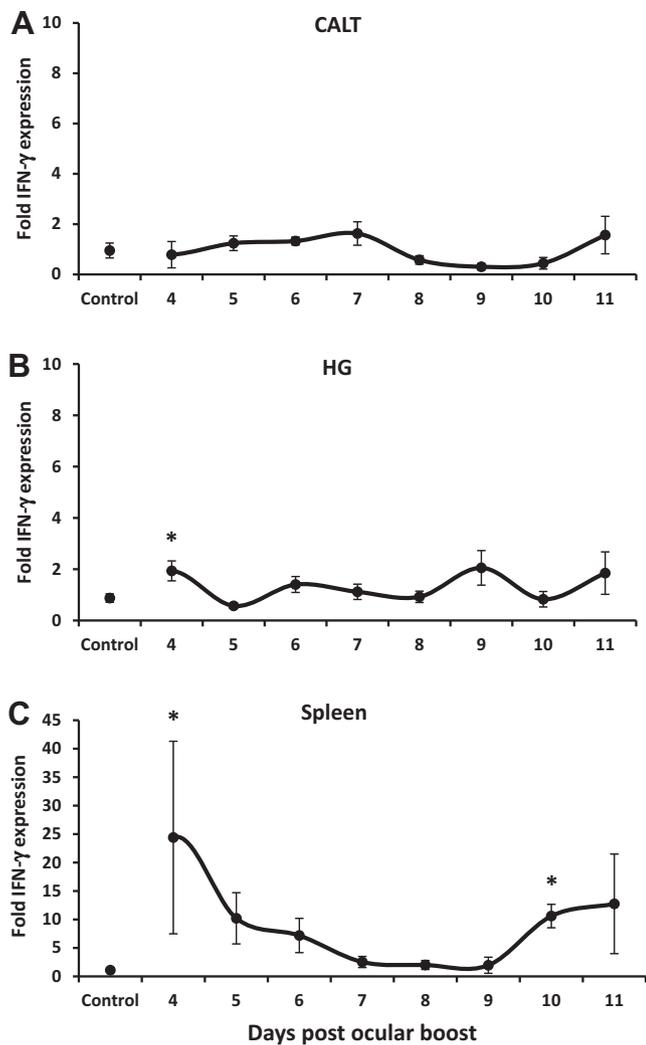


Fig. 5. IFN- γ mRNA expression after secondary IBV vaccination. Chickens were vaccinated at 3 and 7 weeks of age and CALT (A), HG (B) and spleen (C) were collected on various days after boosting to measure IFN- γ mRNA expression by qRT-PCR. RNA levels were normalized using β -Actin mRNA levels. Values are indicated as fold mRNA expression compared to a control. The age-matched, unvaccinated control group is represented as 'C' on the x axis. The number of chickens used per time point varies between 5–10 IBV vaccinated and a total of 6 non-vaccinated control chickens. Controls were collected at the same time vaccinated bird samples were collected during days 5 through 10 DPV. One control chicken was collected per collection day. Values indicate mean and one standard error. Significant changes in expression, when compared to the control group, are indicated with * and have a $p < 0.05$.

T_H1 -driven cytotoxic T cell response in CALT following primary IBV vaccination.

During the primary response to IBV in the HG two periods of increased IFN- γ mRNA expression are observed. The peak response on day 2 may represent an early innate immune response in HG involving NK cells and/or $\gamma\delta$ T cells capable of displaying cytotoxic activity (Pieper et al., 2008; Seo et al., 2000; Choi and Lillehoj, 2000; Vervelde et al., 2013). Future studies may be able to confirm this. A second increase of IFN- γ mRNA expression in HG occurs on day 5–6 post vaccination at the beginning of the expansion phase and may coincide with induction of a T_H1 response. T_H1 cells, in addition to providing support to cytotoxic T cells, also support antibody production by B cells as is documented for mammals (Jiang and Dong, 2013). Our observation that IBV-specific IgA secreting cells are observed starting from 7 days post ocular IBV vaccination in HG would be consistent with this notion (unpub-

lished observations). It is interesting that the viral load of IBV in the HG starts to decrease after day 7 (Ndegwa et al., 2012), which coincides with increased IgA production (unpublished observations) and Granzyme A and perforin expression (Fig. 4). In addition, the largest decrease in virus load occurs when the $CD3^+CD44^+$ T cells are significantly elevated in the HG (Fig. 2), indicating these cells may also function as T effector cells in the chicken. These observations indicate that both cell mediated and humoral immune responses to IBV may contribute to decreased virus load in the HG after IBV vaccination. Indirect evidence that IFN- γ may also support the cytotoxic T cell response comes from a steady increase of perforin mRNA expression from 6 to 14 DPV and of GZMA mRNA expression from 6 to 9 DPV in the primary IBV response.

The observation that HG and CALT rather than the spleen display the strongest IFN- γ response following primary immunization is consistent with the observation that HG are the initial site of replication for IBV (Chousalkar et al., 2007). The increase in IFN- γ mRNA expression in the HG at 5–6 days post primary vaccination could be indicative of a T_H1 -dominated immune response. Since the viral replication is restricted to mucosal tissues in the first few days after vaccination, the immune response to IBV prevails in these sites as opposed to the systemic compartment, which the virus has not yet reached (Davelaar and Kouwenhoven, 1981). Although the spleen does not display a significant increase in IFN- γ mRNA expression in the primary IBV response, an increase in IFN- γ mRNA expression by sorted $CD8^+$ T cells in the spleen is observed on 10 days post primary vaccination (data not shown). This is consistent with the finding that $CD8^+$ T cells increase 10 days post infection following challenge with the IBV-Gray strain (Collisson et al., 2000). In addition, the mRNA expression of GZMA and perforin was significantly decreased in the spleen on day 6–11 and 2–7 post vaccination, respectively. This indicates that lymphocytes in the spleen are producing lower levels of mRNA for expression of cytotoxic enzymes or alternatively are homing to mucosal tissues after primary IBV vaccination, reducing expression of these enzymes in the spleen. Since there is no precedent for splenic lymphocytes producing lower levels of cytolytic enzymes after exposure to a virus, the latter option seems the most likely scenario.

In the secondary immune response to Ark-type IBV vaccination, the IFN- γ response was most prominent in the spleen, when compared to HG and CALT, and peaked on days 4 and 10 after boosting. This coincides with an increase in perforin mRNA expression in the spleen (although not significant). A significant increase in IFN- γ mRNA expression is observed in HG four days after the booster vaccine was given, while no significant increase in IFN- γ mRNA expression is observed in CALT. In contrast to IFN- γ , the GZMA mRNA expression in CALT is significantly elevated between 4–7 days and is also increased on day 11 post boosting. However, no significant increases in GZMA mRNA expression are observed in HG or spleen. Thus, after boosting, the observed data is consistent with the induction of a central memory-dominated T cell response in the spleen, and induction of a cytotoxic T_{EM} response in CALT. Interestingly, no increase of GZMA mRNA expression is observed in HG, indicating that the cytotoxic T_{EM} cells may preferentially home to CALT rather than HG.

Thus, ocular vaccination with a live attenuated Ark-type IBV vaccine generates a primary immune response, which is predominantly observed in HALT. The IBV-induced IFN- γ memory response is mainly located in the spleen, indicating a central memory IFN- γ response while the cytotoxic memory response, as seen in both the primary and secondary response, is predominantly located in CALT indicating an effector memory GZMA response. Thus CALT is the main site for cytotoxic immune responses to IBV in both the primary and secondary immune response based on GZMA mRNA expression. Upon ocular IBV vaccination HG also expresses some GZMA and perforin in the primary response but not the secondary

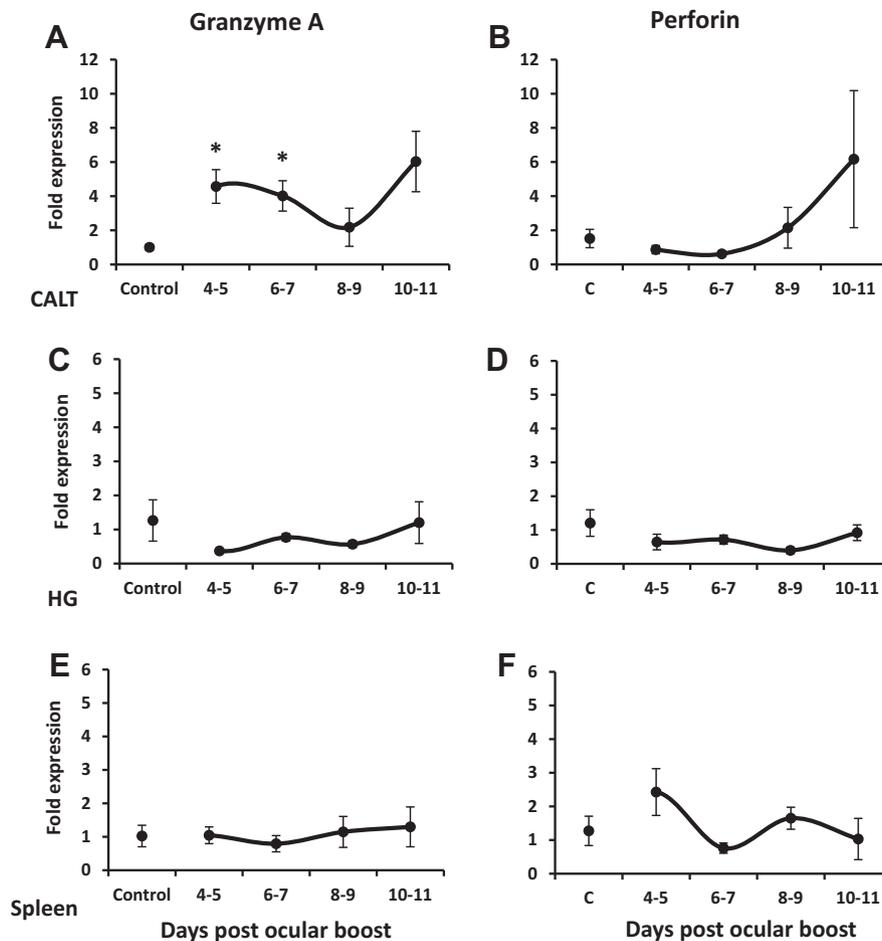


Fig. 6. GZMA and perforin mRNA expression after secondary IBV vaccination. Chickens were vaccinated at 3 weeks of age and boosted 4 weeks later. RNA was extracted from CALT, HG and spleen on various days after vaccination. GZMA and perforin mRNA expression was measured by qRT-PCR. The graphs represent GZMA mRNA expression in CALT (A), HG (C) and spleen (E), and perforin mRNA expression in the CALT (B), HG (D) and spleen (F). RNA levels were normalized using β -Actin mRNA levels. Values are indicated as fold change in mRNA expression when compared to a control. The control group is indicated as 'C' on the x-axis. The number of chickens used per time point varies between 5–10 IBV vaccinated chickens and 6 non-vaccinated control chickens. Tissues from one control bird were collected on the first and the last day of sampling and two control birds on each of the two sampling days in between. Indicated are the mean and one standard error. Significant changes in expression, when compared to the control group, are indicated with * and have a $p < 0.05$.

response. When we analyze IgA and IgG spot forming cells (SFC) to IBV after ocular immunization in the HG and CALT, the HG contain considerably higher numbers of IgA and IgG SFC than CALT (unpublished observation). The same was observed for the IgA SFC response to an adenovirus vector after ocular application (van Ginkel et al. 2012). This indicates that there may be some functional differences between CALT and HG in generating an immune response after ocular vaccination.

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