Cell mediated immunity after ocular Ark-type infectious bronchitis virus vaccination

by

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Abstract

Infectious bronchitis (IB) virus (IBV) is an endemic pathogen of poultry industry causing considerable economic losses by reducing quality and quantity of egg and meat production in chickens. In spite of intensive vaccination programs, outbreaks of IB occur and are difficult to control due to serotypic heterogeneity among IB viruses. Although antibodies are important for controlling IBV, accumulating evidence indicates that cytotoxic T cell responses are very important in the initial phase of an immune response to IBV. To better understand the cell-mediated immune responses induced by IBV vaccination, we evaluated IBV-specific immune responses in mucosal and systemic immune compartments after vaccination with an Arkansas (Ark) type vaccine. Chickens were ocularly immunized with $3x10^5$ or $3x10^4$ 50% embryo infectious doses of the Ark-type IBV vaccine at 3 and 7 weeks of age. Lymphocyte counts in conjunctiva-associated lymphoid tissues (CALT), Harderian glands (HG) and spleen showed that IBV-specific immune response in secondary lymphoid tissues followed the pattern of a lag, expansion, and contraction phase as has been reported for mammals. The proportion of $CD3^+CD44^+$ T cells in the spleen, HG and CALT, as measured by flow cytometry, showed a significant (p value <0.05) increase between 9-11 days after vaccination. In the primary response to IBV vaccination, interferon-gamma (IFN-γ), granzyme A (GZMA) and perforin mRNA expression in CALT and HG displayed significant increases, whereas no significant

response was observed in the spleen. After boosting, the IFN-γ mRNA expression was predominant in the spleen and to some extent in the HG, while a significant increase in GZMA mRNA expression was only observed in the CALT. Hence, the IFN-γ and cytotoxic response to IBV occurs predominantly in the mucosal immune compartment during the primary response, while the secondary IFN-γ response shifts to the systemic immune compartment. Thus, Ark-type IBV vaccination induces a central memory IFN-γ response while the cytotoxic effector memory response as measured by GZMA and perforin mRNA expression, remains associated with CALT. Studies conducted in our laboratory showed that IgA antibody responses to IBV vaccination occurred predominantly in HG. Together, these data indicate that the mucosal-associated lymphoid tissues in chickens may each serve a different function in virus-specific immunity.

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List of abbreviations

Ark – Arkansas

Ark-DPI – Ark serotype from the Delmarva Peninsula Industry

CALT – Conjunctiva associated lymphoid tissue

Conn – Connecticut strain

CTL – Cytotoxic T lymphocytes

DPI – Days post infection

DPV – Days post vaccination

EID_{50} – Median egg infectious dose

E protein – Envelope protein

GZMA – Granzyme A

HALT – Head associated lymphoid tissues

HG – Harderian gland

IB – Infectious bronchitis

IBV – Infectious bronchitis virus

IFN – Interferon

IFN-γ – Interferon gamma

IL – Interleukin

Mass – Massachusetts strain

MBL – Mannose binding lectin

M protein – Membrane protein

NK – Natural killer

N protein – Nucleocapsid protein

PALS – Periarteriolar lymphoid sheath

PAMP – Pathogen associated molecular patterns

PBMCs – Peripheral blood mononuclear cells

qRT-PCR – Quantitative reverse transcription – polymerase chain reaction

S protein – Spike protein

TLR – Toll-like receptor

Chapter 1: Literature review

Introduction

Avian immunology has made important contributions to the science of immunology since the early 1900s and has contributed to the knowledge of the different functions of T and B cells. For example, Murphy observed the graft versus host reaction when rat tissues were transplanted in adult chickens or chicken embryos. He also observed that lymphocytes were the cause of this rejection (1). Furthermore, Bursa of Fabricius helped in differentiating humoral immune response from cell mediated immune response in experiments performed by Bruce Glick (1). More interestingly, the B in B cell is attributed to the Bursa, which coincidentally also stands for bone marrow-derived lymphocytes of mammals. Thus, the avian immune system played an important role early on in the discipline of immunology. The extensive study of avian immunology was in part because of the importance of chickens in food production and their ability to transmit zoonotic diseases such as influenza virus. Food production in the USA in 2010 consisted of 37.2 billion tons of chicken meat derived from 8.6 billion chickens. Georgia, Alabama and Arkansas are top broiler producing states as of 2010 (2). Furthermore, in 2012, 283 million layers produced 6.45 billion eggs in the USA (3). Maintaining these high production numbers will only be possible by the use of effective vaccines against

avian diseases to prevent their spread to both birds and mammals. Hence, knowing the capabilities and limits of the avian immune system is very important.

The avian immune system is considered to be an important model for studying immunology as the core principles of the avian immune system are very similar to those observed in mammals. However, some differences exist. For example, in chickens, B cell development occurs in the bursa of Fabricius as opposed to bone marrow in mammals. The bursa of Fabricius is a sac-like organ connected to the cloaca of chickens and is absent in mammals (4, 5). Also, chickens lack lymph nodes, which are primary sites of antigen presentation in mammals. Instead, chickens have lymphoid tissues or aggregates where antigen presentation and lymphocyte proliferation occurs (6). In chickens, the functional equivalent of neutrophils is heterophils. Both types of cells have similar microbicidal mechanisms, but the heterophil has fewer enzymes, different granules and staining properties (7, 8). Eosinophil-like cells have been identified in chickens and it has been suggested that they play a different role than the mammalian eosinophils (9, 10). Also, some components of T_H2 response, such as interleukin (IL) 5 cytokine and IgE antibody, are not found in chickens (6). Chickens have a lower number of antibody classes – IgM, IgY (IgG) and IgA than mammals (11, 12). Chickens use gene conversion to generate diversity in antibodies and T cell receptors, whereas mammals use gene rearrangement to generate receptor diversity (12). In conclusion, chickens have a different set of cytokines, chemokines, fewer antibodies classes and different types of leukocytes than mammals, but despite these differences the basic principles driving their immune response remain unaltered (6).

Infectious bronchitis virus

Introduction

Infectious bronchitis (IB) virus (IBV) causes a highly contagious disease in chickens called IB. IBV infects the epithelial cells of the respiratory, urogenital and intestinal tract of chickens (*Gallus gallus*) of all ages (13–16). Replication of the virus in the urogenital tract decreases the quality and quantity of egg production in layers and breeders (17). In broilers, the stress of the disease reduces meat production by increasing feed conversion ratio and reducing weight gain. Mortality is mainly observed in young chickens and is largely strain dependent. It increases due to secondary bacterial infection. Control of IBV is difficult due to its rapid transmission rate and prevalence of numerous serotypes (18). Thus, IBV causes huge economic losses to the poultry industry of United States and worldwide. Therefore, a better understanding of characteristics of the virus and the immune response induced to IBV is necessary to establish improved preventive measures.

Classification

Coronaviruses are classified into 4 groups – *Alphacoronaviruses*, *Betacoronaviruses, Gammacornaviruses* and *Deltacoronaviruses*. IBV is a *Gammacoronavirus*. The first two groups represent mammalian coronaviruses and the fourth group represents coronaviruses of wild birds (19). The taxonomic classification of the IBV is as follows:

Order: Nidovirales

Family: Coronaviridae

Genus: Gammacoronaviruses

Species: Avian infectious bronchitis virus

Genome and proteins

IBV is an enveloped, single stranded, non-segmented, positive sense RNA virus. Electron microscopy shows that the virion particles are pleomorphic and range in size from 800 to 1000 Å in diameter (20). The virus consists of structural proteins, a lipid envelope and an RNA genome. Club shaped projections (about 200 Å long) called spike proteins, are attached to the surface of the virus which give it the name Coronavirus (20). In some coronaviruses, including IBV, these spike (S) proteins are post-translationally cleaved into two subunits, S1 and S2. S1 (N terminal) is the outer protein subunit or domain required for attachment to the host cell, whereas the S2 (C- terminal) subunit or domain is inserted in the membrane. The envelope is acquired by the virus from the host cell membrane as it buds out of the cell. IBV makes numerous copies of the membrane (M) protein, compared to the other viral proteins, and the M protein forms a part of the viral envelope (21). The nucleocapsid (N) protein is also an abundant protein and associates with the genome. The genome organization is 5'UTR-1a/ab-Spike-3a-3b-Envelope-Membrane-4b-4c-5a-5b-Nucleocapsid-3'UTR and is 27.6 kbs long (22, 23). Genes 1, 3, 4 and 5 encode non-structural proteins that are needed during replication of the virus. The replicase gene 1 is located at the 5' end as in all positive

sense RNA viruses (24). It is translated using the host machinery to initiate the replication cycle of the virus.

Serotypes

Different strains of IBV have been identified all over the world and are grouped under different serotypes. Serotypes like Massachusetts (Mass), Connecticut (Conn), Arkansas (Ark), Delaware, Georgia, etc. can be found in the United States. M41 is the most common strain found in the world. The RNA dependent-RNA polymerase of IBV causes point mutations in the viral genome during replication which is one of the causes for high mutation rates of IBV (25). Also, recombination events may occur when co-infection with different strains of IBV occurs in the same host cell (25, 26). This leads to the emergence of several strains and serotypes. Differences of even 2-3% in the S1 amino acid sequence of IBV can lead to the generation of different serotypes (27). Thus, IBV has evolved into several different serotypes which may coexist in a region. Virus neutralizing and hemagglutination inhibition antibodies to the S1 protein have been used to classify the IBV strains into different serotypes, i.e., the antibodies generated to the different serotypes of IBV may not necessarily cross react. Hence, it is essential to know which IBV strains are prevalent in a particular region to design an optimal vaccination program.

History

IB was first observed in 1931 by Schalk and Hawn in the United States in 2-3 week old chickens. As a newly observed disease pattern, the nature of the infectious

agent could not be determined at that time (28). Two years later, a similar disease was reported by Bushnell and Brandly and the agent was identified as a filterable virus (29). Soon, similar ailments appeared in chickens all over the United States. For some time there was confusion between laryngotracheitis and IB and the names were frequently used interchangeably, though this confusion was cleared up in a few years. More studies on IB were conducted and van Roeckel made the observation that younger chicks were more susceptible to the disease and died frequently while older chickens between 8-16 weeks were less susceptible to disease. Beaudette and Hudson cultivated the virus in chicken embryos via the chorioallantoic route for the first time. This lead to the observation by Delaplane and Stuart that IBV was attenuated after passage in chicken embryos. These attenuated, embryo passaged strains of IBV are still used for vaccinating chickens today. The economic impact of this virus on the poultry industry was first realized in the late 1930s, since IBV infected layers suffered a permanent drop in egg production which gave an incentive to vaccinate chickens before lay to reduce losses in egg production (30).

Pathogenesis

IBV host tropism is mainly restricted to chickens, but tissue tropism is at least in part dependent on the strain of the virus. IBV spreads by aerosol route, direct contact and through infected equipment. Chicks may contract IBV by vertical transmission through shells of hatching eggs when the virus transfers from ovaries to egg shells (18). Sexual transmission is also possible as was proven experimentally by inseminating hens

with IBV infected semen or semen from IBV infected males (14). Also, experimental inoculations of cloaca with IBV reproduces the disease (31).

IBV interacts with the host cell through mannose containing carbohydrate residues in the IBV S proteins (32). Immunohistochemistry shows that S1 protein of IBV-M41 specifically binds to the base of the cilia and goblet cells in the trachea and the peribronchial epithelial cells in the lungs of chickens (33, 34). (Neu5Acα2,3Galβ1,4)-GlcNAc is one of the α-2,3-linked sialic acids found in host cells that binds the S1 protein of IBV as determined by a glycan array analysis performed by Wickramasinghe *et al.* (34). This binding specificity explains the restricted host tropism of IBV as the S1 protein bound to only 1 of the 450 glycans used in the study (34). Another study shows that dendritic cell- specific Intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and liver/lymph node specific ICAM 3-grabbing non-integrin are two of the C-type lectins that are a part of the IBV receptor complex involved in uptake of the virus into the host cells. IBV uptake is reduced in the cell lines in which DC-SIGN is blocked (32).

Upon entering the host via ocular route, IBV first multiplies in Harderian glands (HG) (16). Regardless of the tissue tropism of the infecting IBV strain, it initially infects the upper respiratory tract and then spreads through blood and lymph to other tissues (16, 18). Viral genomes have been detected in the epithelial cells of the nasal turbinates, trachea, lungs, kidneys, cecal tonsils, cloaca and bursa of Fabricius of day old chicks inoculated with IBV. Extensive replication was seen in nasal turbinates at 1 day post vaccination (DPV) in day old chicks inoculated with IBV (Spain/00/337) (18, 35). IBV

infections may be followed by secondary bacterial infection which increases the severity of the disease.

The main lesions in 10-day-old chicks infected with M41 include loss of cilia, degenerative and hyperplastic changes in epithelial cells and depletion of goblet cells and alveolar mucous glands. There is infiltration of heterophils, plasma cells and lymphocytes into the mucosa of the trachea. These lesions were more severe in the susceptible lines than the resistant lines of chickens and duration of infection was longer in the former. The progression of the lesions can be divided into three stages: degenerative, hyperplastic and recovery (36). Some variants of IBV may be nephropathogenic causing mainly pathological abnormalities in kidneys and a little inflammation in trachea and alimentary tract, although high titers of IBV have been observed in gut with nephropathogenic strains of IBV (13). The main kidney lesions include necrosis of proximal convoluted tubules, distension of distal convoluted tubules, necrotic foci, infiltration of lymphocytes in the interstitial space, edema of Bowman's capsule, urates, and granulocytic casts in collecting ducts (16). IBV RNA and infectious IBV particles have been detected in lachrymal fluids after vaccinations. Also IBV RNA and infectious IBV particles are found more in immunocompromised birds than immunocompetent birds (37). Immunocompromised birds succumb to secondary bacterial infections and show airsacculitis, pericarditis and perihepatitis (38).

Clinical disease

Susceptibility to IBV is determined by a combination of the following factors: the strain of IBV, age of the chicken, health status, the major histocompatibility complex haplotype of the chicken and housing conditions (39–41). IBV causes a highly contagious respiratory disease. It infects epithelial tissues of the trachea, ovaries, oviduct, testis, kidney, and intestine (13–16). It has an incubation period of 18-36 hours (18). IBV infected chickens show clinical signs including depression, ruffled feathers, gasping, coughing, sneezing, nasal and ocular discharge, tracheal rales, conjunctivitis etc. (35, 42). Damage to the oviduct causes a decrease in egg production in layers, which may decrease by 90%. The eggs from an IBV infected hen may be misshapen, have poor shell quality, poor internal egg quality or can even be shell-less. Hens recovered from IBV may bring up their egg production to about 75% of the original values (35). The stress of the disease and damage to internal organs decreases weight gain in broilers. Secondary bacterial infections may follow and increase the severity of the disease, which may also lead to death. Most adult birds survive IBV infections, but chicks less than 6 weeks of age generally succumb to the disease (18).

Vaccination

Vaccination and good management practices help in controlling the spread of viral infections. Both live attenuated vaccines and inactivated vaccines are used in controlling IBV. World organization for animal health (OIE) recommends that a live vaccine for IBV be given first either through drinking water, aerosol or eye drop route,

followed by an inactivated vaccine given by intramuscular or subcutaneous route (43). Usually, in layers and breeders, live vaccines are given at 1 day of age and at 2-3 weeks of age with or without booster doses of live vaccines, followed by a dose of an inactivated vaccine before onset of lay. Broilers routinely receive the first two vaccines of the live attenuated IBV vaccine.

Vaccination with different serotypes either sequentially or simultaneously provides better protection against IBV than vaccination with a single serotype. Epidemiological studies for prevailing IBV strains can best determine the combination of IBV serotypes to be used for vaccination. However, not all vaccinated chickens develop protective immunity against IBV. Vaccine failure is partly attributed to genetic variations in the field virus in addition to the genetic makeup of the chicken (18). Point mutations of the virus within the host may also contribute to immune escape.

The Mass serotype is the most commonly used IBV serotype in vaccines around the world. In the USA, Mass, Ark and Conn serotypes are frequently found. Ark strain was the most frequently isolated type of IBV as observed in an 11 year-long study conducted from 1994 to 2004. Analysis of the samples collected from South-Eastern USA indicated that the Ark strain was the most prevalent strain in this region (44). An Ark strain reported in the Delmarva Peninsula, was named as the Ark-DPI (Ark strain from the Delmarva Peninsula Industry) strain. It was isolated and passaged 50 times in eggs and then provided to companies, who passaged it further to manufacture and supply the live attenuated Ark-DPI vaccine (45). Immunization with Ark-DPI provided complete protection against Conn, 63% against JMK and no protection against M41

serotype (46). There are several problems associated with the protection induced by Ark-type IBV vaccines. The study by Jackwood *et al.* (44) reported that new Ark-like viruses continue to emerged every year. Several studies also report the persistence of Ark serotypes (41, 47). Furthermore, Jackwood reported that the Ark serotype remains persistent in the flock longer than the Delaware serotype (DE072 strain in this case). Persistence of the virus might be due to the induction of incomplete immunological protection. Also, persistence of the virus may reduce the protective immune response generated against reinfections with IBV (48). A study using 4 different commercial Ark-DPI vaccines found that a subpopulation with an S1 sequence differing in 5 to 11 codons from the original predominant vaccine strain, was positively selected within 3 days after vaccination (45). Thus, Ark-type IBV undergoes changes in its genome (here S1 sequence) in the host. The type of progeny viruses selected are mostly likely determined by the selective pressure of microenvironment including the tissues targeted and the immune status of the bird (49, 50). In conclusion, subtypes of the Ark strain continue to emerge and cause economic losses despite vaccination with Ark type vaccine strains.

Immune response to IBV

Maternal immunity

Maternal antibodies are very important in protecting progeny chickens in early life when their immune system is not fully developed and is limited in its ability to combat pathogens. Maternal antibodies are transferred from mother to offspring as a

form of passive immunity. In birds, chicks receive passive protection from hens through maternal antibodies in the egg (51, 52). The egg yolk contains IgY whereas, IgM and IgA antibodies can be found in the egg white (11, 53, 54). However, the embryo doesn't absorb all of the maternal antibodies present in the egg. Only 10% of the total amount of IgY present in the yolk is absorbed by the embryo and the rest is most probably digested (11). On an average, maternal antibodies are metabolized in the chick within 2 to 3 weeks post hatch (52). Chicks with maternal antibodies to IBV are protected from IBV infections up to 4 weeks of age (55). IBV-specific maternal antibodies have been found in serum, tears and intestines. The concentrations of maternal antibodies in tears is higher than in serum (56). Maternal antibodies against IBV were noted from day 1 to 30 days post hatch in serum. Their levels increased from 1 day of age and peaked at 6 days of age after which they declined and became undetectable by 30 days of age. After day 22, their levels were so low that protection seemed unlikely (57).

Vaccination in the presence of maternal antibodies resulted in low levels of pathogen-specific antibody production by the chick. This was the case because the pathogen was neutralized by the maternal antibodies and the immune system of the chick was not exposed to the virus to induce an adequate adaptive immune response. IBV vaccination of chicks with low levels of IBV-specific maternal antibodies resulted in a prompt and high production of IBV-specific antibodies (58). Therefore, IBV neutralizing antibodies increased progressively only in birds that were vaccinated at 20 days of age when the concentration of maternal antibodies was low (57, 59), whereas vaccination

with IBV at 7, 14 and 17 days of age in the presence of maternal antibodies, resulted in limited protection (59).

Despite the fact that maternal antibodies inhibit humoral antibody production, IBV vaccination at 1 day of age resulted in high humoral antibody levels at 2 weeks post vaccination (57). It also yielded 95% protection 4 DPV with almost no detectable virus in trachea and serum (59). As mentioned earlier, at 1 day of age, the maternal antibodies in the chick are not at their highest concentration and peak only by day 6 post hatch which allows the virus to generate an adaptive immune response when chickens are vaccinated at day 1 post hatch (57). A drawback to vaccinating at this early age is that it hastens the decline of maternal antibodies and results in incomplete protection (57, 60). Vaccination of chickens at 1 day of age resulted in virus shedding in the trachea and cloaca from 21 to 83 DPV, which also indicated incomplete protection (61).

Innate immunity

Innate immunity is the first line of defense against any invading pathogens. However, unlike adaptive immunity, it lacks specificity and immunological memory. Physical barriers like epithelium and epithelial junctions, body secretions, cilia, etc. are the first components of innate immunity that the pathogen has to overcome (62). Infection of the mucosal epithelium, such as the lining of the trachea, by IBV leads to the secretion of mucous which prevents the virus from attaching and infecting other epithelial cells. Hyperplastic changes in the goblet cells and alveolar mucous glands leading to nasal discharge and catarrhal exudates in trachea are the first signs of innate

immunity against IBV infections (36, 63). However, these physical barriers are very limited in their capabilities of eliminating IBV. A few days after IBV infection, there is loss of cilia, degenerative changes in the epithelium and depletion of goblet cells and alveolar mucus glands indicating that other immunological components have to be activated (36).

Changes in the cell populations belonging to the innate immune system have been observed in IB. An increase in the number of macrophages was found in spleens of chickens inoculated with IBV-M41 from 1 to 7 DPV (63). In addition, heterophils infiltrate the site of inflammation along with other lymphocytes in HG, trachea, spleen, etc. of IBV-infected chickens (36, 64). However, they have no effect on viral replication and only contribute to the inflammatory response and further damaging the trachea (65). Inflammatory cytokines like IL-1β, IL-6 and the chemokine CXCL8 secreted by macrophages increase within 6 hours after IBV infection (66–70). Highly susceptible chicken lines generate a greater inflammatory response to IBV. These lines showed severe damage to tissues and higher viral loads than resistant lines of chickens (70, 71). The inflammatory cytokines and chemokines stimulate the production of acute phase proteins like mannose binding lectin (MBL), C-reactive protein, α I acid-glycoprotein, fibrinogen, etc. (72). MBL binds to foreign particles and damaged cells to initiate their elimination by phagocytic cells. In chickens, MBL increases by 1.2 to 2 fold after inflammation (72). Lower levels of MBL leads to higher replication of IBV in chickens (73). Higher levels of α I acid-glycoprotein have also been found in IBV infections (70, 72). Furthermore, an increase of natural killer (NK) cells is observed after IBV infections.

Five week old chickens receiving IBV-M41 via intratracheal and intranasal routes showed an increase of $CD107^{+}CD3^{-}$ NK cells in lungs only on 1 day post infection (DPI), whereas the change of NK cells in peripheral blood mononuclear cells (PBMCs) levels was biphasic with an increase on 1 and 4 DPI but not 2 DPI. These increases correlated with increases of granzyme A and Fas mRNA transcripts in the lungs indicating cytotoxic activity (74).

Toll-like receptors (TLRs) belong to a family of genes that recognize different types of pathogen associated molecular patterns (PAMPs). Recognition of PAMPs by TLRs leads to the activation of an intracellular signaling pathway resulting in the production of inflammatory cytokines and chemokines. In chickens, the currently known TLRs are TLR-1 LA, TLR-1 LB, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-15 and TLR-21. TLR-3 and TLR-7 recognize dsRNA and ssRNA molecules respectively in the host cells (72, 75). Analysis of IBV-infected tissues found an increase in the mRNA transcripts of different TLRs and protein expression in the TLR signaling pathway. Specifically, intranasal inoculation with IBV-M41 resulted in increased mRNA expression of TLR-2 and TLR-3 three DPI (68). Intranasal inoculation of 3 week old chicken with attenuated Mass strain increases TLR-2, TLR-3, TLR-6 and TLR-7 transcripts levels in the tracheal tissues (76). The chicken TLR-21 is a functional homologue of mammalian TLR-9, which induces NFκ-B production after stimulation with deoxyoligonucleotides containing CpG motifs (77). Treatment of 18 day old embryos with deoxyoligonucleotides containing CpG motifs prior to inoculation with IBV, decreased viral load (69). An increase in the RIG-I transcript, which is an important PAMP recognizing molecule involved in the pathway of

type I IFN induction, was observed after intranasal inoculation of 3 week old chicken with attenuated an Mass strain (76). However, a more rigorous study failed to detect any RIG-I or RIG-I related gene in chickens (78). Beside PAMP receptors, an increase in expression of interferon regulatory factor gene – 1 was also reported (67). Interferon gene expression, normally activated through TLR stimulation, plays an important role in IBV infections. Type I IFNs inhibit IBV replication in chicken kidney cells infected with the Beaudette strain of IBV (79). Chicken IFN-α reduced IBV-associated respiratory illness (79). Type II IFN, i.e., IFN-γ, expression increased in the lungs 2 to 4 DPV with M41 IBV and in the PBMCs within 1 DPV (74). The interferon induced Mx gene expression was also increased (68).

Studies indicate that IBV can alter certain components of the innate immune system in such a way that it facilitates secondary bacterial infections. Twenty seven day old broiler chickens were inoculated with an IBV strain (either H120 or M41). Two DPV the nitric oxide production decreased in splenocytes and PBMCs of these chickens compared to the phosphate buffer saline (PBS) treated group. Decreased nitric oxide production resulted in reduced killing of *Escherichia coli* in the IBV inoculated groups. In addition, an increase of inflammatory cytokines such as IL-8, IL-1β was observed starting 1 DPV (66). Inflammation and associated damage to cilia and epithelium induced by IBV provides opportunities for bacterial attachment and invasion.

Humoral immunity

The humoral immune response to IBV has been extensively studied over the past years. In early publications the antibody response to IBV was described in terms of levels of neutralizing antibodies and hemagglutination inhibition antibodies (80–84). This was in part driven by the development of IBV neutralization tests (85). This was later followed by measuring the different classes of IBV-specific antibodies over time and later their presence or absence in particular tissues after an IBV infection. The development of IBV-specific ELISAs and immunohistochemistry techniques enabled a more detailed analysis of IBV-specific antibodies and their distribution in different chicken tissues (86).

IgM is the first antibody detected in serum after IBV infection. Its concentration is maintained in blood for a shorter period than other antibody classes after primary and secondary vaccination (87, 88). Thus, IBV-specific IgM antibodies in serum can serve as an indication for a recent IBV infection (87). Following vaccination with IBV-M41, IgM antibodies can be detected at 1 week post vaccination in the serum (88, 89). However, IgM antibody has been detected as early as 3 DPV with IBV-M41 (88). IgM antibodies reach their highest concentration by 2 weeks and then gradually decline until they became undetectable by 21 DPV (88, 89). A second inoculation induces a similar IgM response with no significant changes in the antibody concentrations as observed in the primary response throughout the observation period. In similar experiments, IgM was noted in serum by 10 DPV with a combination vaccine containing H120 and D274. The IgM response in serum correlated with an 89-100% protection to challenge with IBV-

M41 in chickens. This conclusion was made by comparing the IgM concentration in plasma with the amount of ciliostasis in the trachea of IBV vaccinated chickens after challenge (90).

The kinetics of the IgG response to IBV is very different than for the IgM response. IgG was detected on 6 DPV and peaked on 9 to 14 DPV in chickens vaccinated with IBV-M41. There was a gradual decline in IgG antibody concentration after day 14, but significant amounts of IgG were still detected in serum until 42 DPV. Thus, the primary IgG response lasted much longer than the IgM response (which was undetectable by 21 DPV). After boosting, IgG levels in sera were much higher than those observed in the primary response but followed a similar pattern as observed after priming (88). Analyzing IBV-specific IgG secreting plasma cells, low numbers were detected 3 DPI in spleen and in PBMCs on 7 days post intraocular-nasal inoculation of IBV-Gray strain. The IgG plasma cells peaked on 7 DPI in the spleen and sharply declined on 14 days post inoculation and maintained this level until 10 weeks post inoculation. In PBMCs IBV-specific IgG-secreting plasma cells reached a peak on 10 days post inoculation and then gradually declined. Memory B cells were also detected by 3 weeks post inoculation in the spleen (91).

IgA antibodies are important for mucosal immunity to IBV (37, 92, 93). IgA antibodies can be found in HG and tears after IBV infections (93–95). IBV-specific IgA antibodies are first detected in tears and later in serum (96). IBV-specific IgA is also present in saliva and tracheal washes after an IBV infection (92). More importantly, lachrymal IgA correlates with resistance to reinfection with IBV (93). Ocular vaccination

with a live-attenuated H120 vaccine induced IgA-positive plasma cells in HG 2 weeks post vaccination (96, 97). IBV-specific IgA and IgM secreting cells increased on days 9 and 14 post vaccination in HG with a low passaged, mildly pathogenic Ark-type IBV isolate. This antibody response correlated with an overall increase in B cells in HG. In addition, IBV-specific IgA was also found in cecal tonsils 14 days post inoculation, representing a delay in the IgA response in the cecal tonsils compared to that in HG after ocular vaccination (37).

Different IBV proteins have been used to induce IBV-specific antibody responses. For example, the S1 and N proteins induced a higher antibody response than the M protein using a peptide vaccine (98). *In ovo* vaccinations with the S1 subunit have also proven successfully. Eighteen day old chicken embryos were vaccinated *in ovo* with an adenoviral vector expressing the S1 subunit of the spike protein derived from IBV-XDC2 and were revaccinated with the same vector intramuscularly at 15 days post hatch. This resulted in high titers of IgG S1-specific antibodies in serum within a week, which remained significantly elevated until 28 DPV. Boosting of the immune response brought down the mortality to zero after challenging the chickens with IBV-XDC2. This showed that mortality to IBV can be reduced using DNA vaccines encoding the S1 subunit (99). The N protein, particularly the amino terminal part of the N protein of IBV contains B cell epitopes (100). A DNA vaccine encoding this segment of the N protein induced the highest protection in terms of morbidity and mortality rate after challenge with IBV-M41, when compared to other IBV protein components (98). Since this segment of the

N protein is conserved among coronaviruses, it has been suggested to be used in the production of subunit and DNA vaccines (100).

Antibody responses are very prevalent in the immune response to IBV. However, Raggi *et al.* (101) were the first to determine that there was no correlation between the level of antibodies in serum and protection against IBV infections. Furthermore, vaccination with an S1 subunit peptide induced antibody levels that did not correlate with protection against IBV (102). In addition, increased antibody concentrations in serum did not correlate with a decrease in viral loads in the respiratory tract and cecal tonsils. However, they correlated with decreased viral load in remote organs like the kidney (103). Collisson *et al.* (104) showed that the cytotoxic T cell response peaked before the IgG and IgM response and concluded that the cytotoxic T cell response is more important than the antibody response in the initial control of an IBV infection.

<u>Cell mediated immune response</u>

Intratracheal inoculation of an attenuated IBV-V1648 strain leads to the accumulation of mucus and edema in the trachea. Five days post vaccination there is infiltration of leukocytes in the trachea, the majority of which are $CD3^+$ T cells. No B cells are detected at this stage. Seven days after vaccination, the number of leukocytes in the trachea increases and after 11 days B cells are detected among these leukocytes. A similar pattern of leukocyte accumulation is noticed in kidneys, one of the target organs of IBV (105). Adoptive transfers of IBV stimulated T lymphocytes to naïve chickens that were challenged the next day with a homologous IBV strain, showed that the mortality,

viral loads and clinical signs decreased in the lungs and kidneys compared to the control chickens receiving naïve lymphocytes (104, 106). Protection to IBV challenge increased in proportion to the total number of lymphocytes transferred (106). In a chromium release assay chicken kidney cells infected with IBV were predominantly lysed by $CD8^+$ T cells and also by a minor population of either $CD4^+CD8^+$ or $CD4^+CD8^-$ T cells (104). Adoptive transfer of $CD8^+$ T cells and $\alpha\beta$ T cells helped in diminishing viral loads 5 days post challenge. In contrast, adoptive transfers of $CD4^+$ T cells and $\gamma\delta$ T cells decreased viral load by less than 11%, and no change in clinical illness was apparent after challenge. Thus, it was concluded that T cells with a $CD8^+TCRV\beta1^+$ phenotype were most protective in an IBV infection (104). Adoptive transfer of immune T cells indicates that $CD8^+$ memory T cells can be detected even after 6 weeks post IBV infection. Transfer of these memory T cells provided the protection against subsequent challenge (107).

Boots *et al.* (108) described the first IBV-specific T cell epitope in the IBV nucleoprotein (IBV $N_{71\text{-}78}$) from an IBV H52 strain. Only the S1 and N proteins of IBV generated cytotoxic T cell responses, but not the M protein of IBV. The whole N protein and its carboxy-terminal region induced a CTL response, but not its amino terminal region (104, 109). Chickens vaccinated with a DNA vaccine containing the sequence of N protein increased the number of $CD4^+$ and $CD8^+$ T cells in the PBMCs (98). Immunization of chickens with a DNA vector expressing the granulocyte macrophage-colony stimulating factor and S1 subunit of the spike protein, resulted in high antibody levels in serum, lymphocyte proliferation including $CD4^+$ and $CD8^+$ T cells in PBMCs, and reduced the severity in clinical signs and mortality rate after challenge (110).

IFN-γ is an inflammatory cytokine secreted by various cells of the immune systems but predominantly by T cells and NK cells (111). Eye drop vaccination of chickens with a recombinant S1 and N protein of IBV or the whole virus induced secretion of IFN-γ as measured by an ELISpot assay (112, 113). Post hatch boosting after an *in ovo* vaccination with recombinant vaccines expressing S1 and N proteins, also leads to secretion of IFN-γ (99). The immune responses to IBV infections contribute to IFN-γ and IL-2 production, reminiscent of a cell mediated immune response (114).

As mentioned earlier, the cell-mediated immune response is very important in the initial control of the virus. IBV-Gray strain specific CTL activity was noted as early as 3 DPV, peaking at 10 DPV and gradually decreasing over time after day 10. The IBV viral loads in this study increased until day 8 and declined dramatically in lungs and kidneys after day 8 in the response (104). In addition, the $CD8^{+}$ T cell response in the blood and spleen occurred prior to the serum IgG antibody response to IBV (42). Thus, the CTL response correlated with decreased viral load and an improved clinical response.

IBV-specific immunity in the head associated lymphoid tissues

Harderian glands

The Harderian glands (HG) contain lymphoid tissues that are a part of head-associated lymphoid tissues (HALT) (95). They were first observed by J. J. Harder in a deer in 1694 ((115) cited in (116)). HG are tubuloacinar glands located in the orbit behind the eye and are roughly divided into a head and body. Their main function is to

lubricate the nictitating membrane (117). However, their immunological role is also acknowledged by several papers (96, 118). Lymphoid tissues of HG consist of a follicle associated epithelium, T cell areas with a few macrophages and germinal centers. Germinal centers are located in the head of HG and plasma cells can be found in the body. Plasma cells are closely associated with the excretory and secretory duct of HG allowing secretion of antibodies into tears. Leukocyte infiltration into the gland increases with age and with antigen stimulation (117). Majority of the T cells in HG are $CD4^+$ and $\alpha\beta_1TCR^+$ and a few $CD8^+$ $\gamma\delta$ T cells. HG are a major source of IgA in tears, but plasma cells in HG can be IgG, IgM or IgA positive. (95)

The HG play a major role in the mucosal immune response to IBV (37, 64, 93, 97). Ocular vaccination with IBV generated extensive accumulation of plasma cells in HG by 2 weeks post inoculation (96, 97). IBV-specific IgA first appeared in tears and then in serum (96). HG contain IgA-positive plasma cells, which are the major source of IgA in tears (94, 95). IBV-specific IgA and IgM secreting cells in HG increased on 9 and 14 DPV with Ark-type IBV, which correlated with an overall increase in B cells in HG (37). Lachrymal IgA correlated with resistance to reinfection with IBV (93). The IBV-specific IgA spot forming cells appeared in the cecal tonsils 14 days after vaccination with IBV, representing a delay in the IgA response in the cecal tonsils compared to that observed in the HG after ocular vaccination (37, 92).

Conjunctiva-associated lymphoid tissues

Conjunctiva-associated lymphoid tissues (CALT) are located in the conjuctival folds of the lower eyelid and the upper eyelid of chickens and thus are, like HG, a part of the HALT (119, 120). The reddish inflamed appearance after stimulation with an antigen/virus makes it easier to locate CALT. CALT contains a population of lymphocytes and macrophages as was first described by Fix and Arp in 1991 (121). They also contain germinal centers which develop 2 weeks post hatch and plasma cells are detected 4 weeks post hatch (121). Germinal centers, surrounded by $CD4^+$ and $CD8^+$ T cells, can be found at 4 weeks of age in chickens (120). In addition, high endothelial venules, which serve as a port of entry of circulating lymphocytes into lymphoid tissue, were seen at the base of lymphoid nodules (119). The number of lymphocytes increases in CALT after ocular immunization or contact with antigen. IgA positive B cells can be seen in germinal centers along with a few IgG and IgM positive B cells. T cells in CALT are mostly TCR$\alpha\beta_1$ and can be either $CD4^+$ or $CD8^+$. Also, a few $\gamma\delta$ T cells and $\alpha\beta_2$T cells can be found (119). However, IBV-specific immune responses in CALT have not been studied up until now.

Chapter 2: Cell-mediated immunity to IBV in HALT and spleen

Introduction

IBV is a single stranded RNA virus of the Coronaviridae family (19, 20). It causes a highly contagious respiratory disease in chickens of all ages (18). IBV 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 (18, 35). Controlling this virus is difficult due to its high mutation rate resulting in serotypic heterogeneity, which allows IBV to quickly evolve into variants or serotypes that can escape the immune response (25–27).

IBV infections lead to the induction of both humoral and cell mediated immune responses (87, 88, 97, 104). Although humoral immunity to IBV in the systemic immune compartment is important, it does not correlate with protection against IBV (101, 102, 104). Collisson *et al.* (104) have shown that increase in cytotoxic T lymphocyte (CTL) activity in splenocytes correlated with decreased viral loads in lungs and kidneys of IBV infected chickens. They also showed that transfer of IBV-specific-$CD8^{+}\alpha\beta_1$ T cells to naïve chickens provided protection against challenge with a homologous strain (104). Others have also stressed the importance of cytotoxic T cells in the initial phase of the immune response as opposed to antibody responses (42, 106). These observations

illustrate the importance of a better understanding of the cell mediated immune response induced after IBV vaccination in chickens.

Expression of anti-viral cytokines like interferon gamma (IFN-γ), which are predominantly secreted by natural killer (NK) cells and T cells, gives us an overall perspective of the immune response to viral pathogens (122, 123). A detailed analysis of the expression of cytotoxic enzymes GZMA and perforin, which are secreted by cytotoxic cells such as CTLs, γδ T cells, NK cells etc., help measuring the cytotoxic activity induced following exposure to a pathogen (68, 74, 124, 125). Perforin creates pores in the cell membrane through which granzymes and other proteases are delivered into the target cell cytoplasm (126). GZMA induces caspase-independent cell death characterized by single stranded nicks in the DNA (127). Although granzyme B is more commonly analyzed in mammals, GZMA expression is studied in chickens since there are no published records of the existence of granzyme B in chickens (68, 74).

Mucosal immunity is an important component of protection against IBV. IBV enters the host tissue through mucosal sites of the ocular and nasal epithelium (18). Studies show that IBV initially replicates in HG (16) where IBV-specific plasma cells and IgA antibodies are detected after infection with IBV (37, 96, 97). Like HG, CALT also plays an important role in mucosal immunity of the eye (119, 121). Thus, it is important to study the parameters of the cell-mediated immune response in these two tissues and compare it with the spleen as a measure of the systemic adaptive immunity.

In our study we analyzed the cell mediated immune responses in the mucosal and systemic immune compartments induced after ocular vaccination of chickens with a

live-attenuated Ark type IBV vaccine. Specifically, we measured changes in lymphocyte numbers and $CD3^{+}CD44^{+}$ T cells in CALT, HG and spleen after IBV vaccination. Furthermore, we measured changes in mRNA transcripts of IFN-γ and cytotoxic enzymes, i.e., GZMA and perforin during the course of the primary and secondary immune response following IBV vaccination in HG, CALT and spleen. A shift of the primary IFN-γ response in mucosal HALT tissues to a spleen-associated secondary IFN-γ response was observed. However, the cytotoxic response, based on GZMA and perforin mRNA expression, during the primary response was almost exclusively associated with the mucosal lymphoid tissues, i.e., the HALT, and this association was maintained after boosting.

Materials and methods

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 $3x10^5$ EID_{50} per bird for ocular immunizations unless indicated otherwise. Chickens were vaccinated at 3-4 weeks of age. Where appropriate, the chickens were boosted 4 weeks later. 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.

Lymphocyte isolation

Tissues were collected in complete RPMI medium as previously described (128). CALT, HG and spleen were collected after ocular vaccination and lymphocytes were isolated as previously described (37). In brief, tissues were mechanically disrupted to obtain a single cell suspension in complete RPMI medium. Lymphocytes were isolated by density centrifugation at 2000 rpm (930x*g*) at 24˚C for 20 min over a Ficoll-Histopaque® gradient (1.077g/mL) (Sigma-Aldrich, St. Louis, MO). Lymphocytes collected from the interface after centrifugation were washed with complete RPMI and used for further analysis.

Lymphocyte counts

For this experiment, chickens were vaccinated with $3x10^4$ EID_{50} per bird of Ark-type IBV. Lymphocytes from CALT, HG and spleen were collected on various days after ocular immunization. Live lymphocytes were counted on a Bright-Line® haemocytometer (Hausser Scientific, Horsham, PA) using trypan blue exclusion. 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).

Flow cytometry

To analyze the T cell composition of CALT, HG and spleen, lymphocytes were collected on various days after ocular immunization with Ark-type IBV. The lymphocytes were incubated with 0.1 μg of biotinylated mouse anti-chicken $CD3^+$ per 10^6 cells for 2 hours followed by 1 μg of streptavidin-Alexa660 per 10^6 cells for 1 hour on ice. Lymphocytes were then stained for $CD44^+$ by incubating them with 1 μg of biotinylated mouse-anti-chicken $CD44^+$ per 10^6 cells for 2 hours followed by 1 μg of streptavidin-PE per 10^6 cells for 1 hour on ice. Unlabeled cells were used as controls. All washes were performed with PBS/1%BSA/0.02%NaN_3. The cells were filtered through a 50 μm nylon mesh (Small Parts Inc., Miami Lakes, FL) before analysis on a MoFlo high-performance cell sorter (Dako Colorado Inc., Fort Collins, CO). Summit 4.3 software was used for data analyses.

RNA isolation and quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR)

Tissues were collected daily in microcentrifuge tubes containing zirconium oxide beads (Next Advance, Averill park, NY) and TRI reagent®(Molecular Research Center, Cincinnati, OH) between 2 to 14 days post primary IBV vaccinations and between 4 to 11 days post boosting. Tissues were homogenized by placing the microcentrifuge tubes in a tissue homogenizer (Bullet blender, Next Advance, Averill Park, NY). Each tube containing about 1 mL of homogenized tissue with TRI reagent® and 200 μL of chloroform was subjected to centrifugation at 12,000x*g* for 15 min at 4 ˚C. The aqueous layer containing RNA was collected into a new tube and precipitated using 500 μL of isopropanol at 12,000x*g* for 8 min at 4 ˚C. The RNA pellet was washed using 1 mL of 75% cold ethanol by centrifugation for 5 min at 4 ˚C at 7500x*g*. The RNA pellets were then air dried by tilting the tubes on a stand and resuspended in 10 μL of nuclease free water. The total RNA concentration was measured using a Nanodrop 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 (Table 1) used were based on previous publications (129, 130). β-actin was used as a house keeping gene; β-actin cDNA was amplified using primers previously described (Table 1) (131) and the ΔΔ(Ct) method was used to calculate the fold change in expression of IFN-γ, GZMA and perforin mRNA. The

details of qRT-PCR amplification protocol are outlined in Table 2. The CFX96^{TM} system (Bio-rad, Hercules, CA) was used to perform the RT-PCR reactions.

Statistical analysis

The student t-test or ANOVA were used to compare data to that of control chickens where appropriate. When the data were not normally distributed, the Mann Whitney test was used to perform statistical analysis for non-parametric data. Data shown are mean values and the error bars represent positive standard error of the mean. Statistically significant differences have a p value <0.05.

Results

Lymphocyte counts after primary IBV immunization

The total number of lymphocytes in CALT, HG and spleen were counted after ocular immunization with $3x10^4$ EID_{50} per bird of Ark-type IBV. Changes in the total number of lymphocytes in lymphoid tissues over time give an overall idea of the progression of the immune response as defined by phases, i.e., lag, expansion and contraction phases as has been reported in the mammal immune response to a pathogen (132). The lymphocyte counts in CALT and HG increased starting 7-8 DPV and peaked on 9 DPV ($1.9x10^6$ cells in CALT and $5x10^6$ cells in HG), representing the expansion phase (Figure 1A & 1B). However, only in HG was the number of lymphocytes statistically significantly higher at 9 DPV than in unvaccinated control chickens. An abrupt decline on day 10 post vaccination was observed (going down to $7x10^5$ cells in CALT and $5.6x10^5$ cells in HG), representing the contraction phase probably combined with homing of lymphocytes out of these tissues. The lymphocyte counts in spleen significantly increased from $1.1x10^8$ cells at 3 DPV to $1.8x10^8$ cells at 9 DPV and decreased to $1.5x10^8$ cells at 10 DPV in the contraction phase (Figure 1C). These changes in lymphocyte numbers were not as marked in the spleen as in the CALT and HG relative to the total number of lymphocytes present. The low magnitude of change of the lymphocyte counts in the spleen compared to CALT and HG may be in part due to the ocular immunization route used in this study. However, they still represented the largest

change in cell numbers during the contraction phase for the lymphoid organs analyzed. They decreased by 30x10^6 lymphocytes on average.

Changes in the $CD3^+CD44^+$ T cell population after primary IBV vaccination

CD44 is a cell adhesion molecule, which displays increased expression in activated effector and memory T lymphocytes after antigenic stimulation (133–135) and is the main surface receptor for hyaluronate (136, 137). CD44 expression in chickens during development has been studied (136) and has also been analyzed as a marker for activated lymphocytes in challenged or vaccinated chickens (138). In our study 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. Birds were vaccinated with a dose of 3x10^5 EID_{50} IBV per bird to induce the primary T cells response. This higher IBV dose, to induce a more robust T cell response, when compared to our first experiment for the lymphocyte counts extended the expansion phase by 1-2 days (data not shown). Lymphocytes from CALT, HG and spleen were isolated and stained for flow cytometric analyses on 3, 7, 9, 11 and 14 days post vaccination. In CALT the level of $CD3^+CD44^+$ T cells significantly decreased from 25% of the lymphocytes in controls to 9% on day 3 suggesting an initial efflux of this lymphocyte population out of CALT. This was followed by a gradual increase back to pre-vaccination levels on day 11, followed by another significant decline that coincides with the contraction phase (Figure 2A). Thus, this lymphocyte population increased in CALT during the peak of the T cell response. In contrast, the HG displayed a small, statistically

insignificant increase in $CD44^+CD3^+$ T cells on day 3 (Figure 2B). In HG, the $CD3^+CD44^+$ T cells increased from 5% in controls to peak levels on day 9 representing 22% of the lymphocyte population. A similar level was maintained on day 11 and then dropped back to control levels on day 14. The spleen displayed a similar profile as seen in CALT, but the initial decline in $CD3^+CD44^+$ T cells was delayed by a few days to 7 DPV, which may be due to the ocular route of vaccination. Approximately 50% of control spleen-derived lymphocytes were $CD3^+CD44^+$ T cells and the $CD44^+CD3^+$ T cells peaked at 11 DPV at the end of the expansion phase (Figure 2C).

IFN-γ mRNA expression after primary IBV vaccination

IFN-γ mRNA expression was measured by qRT-PCR in CALT, HG and spleen on days 2-11 and 14 post ocular IBV vaccination. The mRNA expression of IFN-γ increased significantly over age-matched controls in CALT starting at 4 DPV and remained significantly elevated during the period the CALT was monitored (Figure 3A). Two peaks in IFN-γ mRNA expression were observed, i.e., at day 6 and day 10. 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 at 2, 5 and 6 DPV (Figure 3B). The increase in IFN-γ mRNA expression at 2 DPV was in part due to γδ T cells as their count increased on day 2 post vaccination and they expressed higher IFN-γ RNA levels than controls (data not shown). In the primary IBV response, unlike CALT and HG, the spleen did not show

any significant changes in IFN-γ mRNA expression over the period it was monitored (Figure 3C).

GZMA and perforin mRNA expression during the primary response to IBV

GZMA and perforin are enzymes secreted by cytotoxic cells. Their expression has been measured previously to understand the cytotoxic mechanisms of T cells in Marek's disease and infectious bursal disease (129, 139). Similarly, we measured the expression of the mRNA encoding these enzymes to estimate the induction of cytotoxic lymphocytes in the immune response to a live-attenuated Ark-type IBV vaccine. RNA was isolated at 2-11 and 14 DPV from CALT, HG and spleen after primary ocular immunization with IBV. An initial increase in the average mRNA expression of GZMA was observed at 4-5 DPV in CALT along with a concomitant increase in perforin mRNA expression (Figure 4A & 4B). A significant increase in GZMA mRNA expression was observed during the peak response at 8-9 DPV (90 fold higher than controls) after which it declined. Although, the pattern of GZMA mRNA expression was mimicked by perforin mRNA expression in CALT, the changes in perforin mRNA expression were of lower magnitude and were not significantly increased over control values. The significant decline of perforin mRNA expression on days 2-3 post vaccination (Figure 4B) in CALT coincided with a decrease of $CD44^{+}CD3^{+}$ T cells (Figure 2A). The HG displayed a significant, concomitant increase in mRNA expression of both GZMA (18 fold increase over controls) and perforin (7 fold increase over controls) 2-3 DPV (Figure 4C & 4D). The HG also displayed a peak GZMA mRNA expression on days 8-9, which was not significant

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. It is not clear why the variation in response on days 8-9 was greater than on the days preceding or following this time point. Perforin mRNA expression in HG also had a second significantly higher response 14 DPV (8 times higher). Unlike 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 (Figure 4E & 4F). It is not clear whether the decline of mRNA expression of these enzymes was related to homing of cytotoxic lymphocytes out of the spleen to other sites or whether other mechanisms were at play.

IFN-γ mRNA expression during secondary response to IBV

IFN-γ mRNA expression was measured in CALT, HG and spleen by qRT-PCR after a booster dose of Ark-type IBV 4 weeks after the primary immunization. CALT, HG and spleen were isolated on days 4-11 after boosting. As opposed to the primary response, no significant increase in mRNA expression of IFN-γ was observed in CALT (Figure 5A). A significant increase in IFN-γ mRNA expression was seen on day 4 after boosting in HG and spleen, i.e., ~2 fold higher than the control in HG and ~24 fold higher in the spleen (Figure 5B & 5C). 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.

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 was measured after ocular boosting with IBV vaccine. RNA was isolated from CALT, HG and spleen on days 4-11 after boosting. The fold changes in the level of GZMA and perforin mRNA expression were measured using qRT-PCR and were compared to age-matched, unvaccinated control birds. No significant changes in GZMA and perforin mRNA expression were observed in HG and spleen (Figure 6C-F). Although perforin mRNA expression increased to 2.5 fold in the spleen on 4-5 days post boosting, this increase was not significant. The only significant changes observed were in the CALT on days 4-7 post boosting in mRNA expression of GZMA (Figure 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 increased on days 10-11 in CALT, but due to higher variation in the response the increases were not significant.

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 exhibited a response with a noticeable increase in the total number of lymphocytes, $CD3^{+}CD44^{+}$ T cells, and IFN-γ, GZMA and perforin mRNA expression while little change was noticed in the spleen measuring these variables. The route of vaccination, i.e., ocular vaccination, may have contributed to this lack of responsiveness of the spleen when compared to the HALT. However, upon ocular boosting of the IBV-specific immune response four weeks after priming, the IFN-γ response shifts from the mucosal to the systemic immune compartment. However, the cytotoxic T cell response as measured by the mRNA expression of GZMA and perforin was still associated with CALT in the secondary response. One of the models for memory T cells development in mammals states that an effector T cells receiving an antigen for a shorter duration differentiate into $CCR7^{+}CD62L^{hi}$ central memory cells and circulate within the lymphatic system. In contrast, if the effector T cells receive antigen for a longer duration, they differentiate into $CCR7^{-}CD62L^{lo}$ effector memory T cells that travels to effector sites. These effector memory T cells reside at the "frontline" of pathogen invasion and initiate a faster and better response upon reinfection with the pathogen (132, 140). Thus, the presence of GZMA-expressing cells in CALT following secondary immunization suggests the presence of effector memory cells and the IFN-γ expression in the spleen is consistent with the presence of central memory cells.

The mRNA expression of IFN-γ, GZMA and perforin in CALT, HG and spleen were analyzed to get a better understanding of the magnitude of the cell mediated immune response to Ark-type IBV vaccine in mucosal versus systemic lymphoid tissues after ocular vaccination. IFN-γ is an antiviral cytokine essential for both innate and adaptive anti-viral immune responses. NK cells and NK T cells are important IFN-γ producers in the innate response, while T cells, such as $CD4^+$ T_H1 cells and $CD8^+$ cytotoxic effector T cells, are important IFN-γ secreting lymphocytes in the adaptive immune response (141). GZMA and perforin mRNA expression have been used previously to indicate the induction of NK cells and CTLs in response to Marek's disease virus, infectious bursal disease virus and IBV in chickens (68, 129, 139). Collisson *et al*. (104) observed that $CD8^+$ memory T cells are important in the control of IBV. This was based on transfer of splenic $CD8^+$ T lymphocytes from IBV immunized chickens into syngeneic chickens, which were protected upon subsequent IBV challenge (104, 107). This urged us to take a closer look at systemic versus mucosal mRNA expression of GZMA and perforin as a measure for the induction of cell mediated immunity after IBV vaccination. No data is available for CALT and very little data have been reported for HG on cell mediated immune response to IBV, despite the fact that HG are known targets for initial replication of IBV in chickens (11). We found that after ocular IBV vaccination, CALT shows an extended increase of IFN-γ mRNA expression, i.e., from 4 to 14 DPV during the primary response to Ark-type IBV. A concomitant increase in GZMA and perforin mRNA expression from 4 DPV to 9 DPV in CALT was observed. These observations combined with the IFN-γ mRNA

expression pattern, are consistent with a T_H1-driven cytotoxic T cell response in CALT following primary IBV vaccination.

During the primary response to IBV, two periods of increased IFN-γ mRNA expression are observed in HG. The peak response on day 2 represents, at least in part, IFN-γ production by $\gamma\delta$ T cells. This statement is based on sorting HG lymphocytes using a flow cytometer and analyzing their IFN-γ mRNA expression by qRT-PCR (data not shown). Avian $\gamma\delta$ T cells are known to secrete IFN-γ upon viral infections (106, 142) and are capable of displaying cytotoxic activity (143). This is consistent with our observation that HG display an increased mRNA expression of GZMA and perforin on day 2 post vaccination. This early innate immune response in HG may also involve NK cells in addition to $\gamma\delta$ T cells (74). Future studies may be able to confirm this. A second increase of IFN-γ mRNA expression in the HG occurs on day 5 to 6 post vaccination and may coincide with induction of a T_H1 response. In addition to IFN-γ secreted by T helper cells providing support to the cytotoxic response, T helper cells may also support antibody production as has been reported for mammals. Our observation that IBV-specific IgA secreting cells are observed starting from day 7 post ocular IBV vaccination in HG (manuscript in preparation) would be consistent with this notion. Circumstantial 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.

Although the spleen did not display a significant increase in IFN-γ mRNA expression in the primary IBV response, an increase in IFN-γ mRNA expression by $CD8^+$ T cells in the spleen on day 10 post vaccination was observed after sorting these cells using a flow cytometer and isolating their RNA to measure IFN-γ mRNA expression (data not shown). This is consistent with the finding that $CD8^+$ T cells increase 10 days post infection with IBV-Gray strain (104). 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.

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 (16). Since the viral replication is restricted to mucosal tissues in the first 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 (97).

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 mRNA expression, the mRNA expression of GZMA in CALT is significantly elevated between 4-7 days and also on day 11 post boost, while no significant increases 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 effector memory T cell response in CALT. Effector memory T cells are known to home to effector sites such as CALT and this would represent a scenario consistent with observations made for mammals (132). Interestingly, no increase of GZMA mRNA expression is observed in HG, indicating that effector memory cytotoxic cells may preferentially home to CALT rather than HG.

CD44 is a cell adhesion receptor for hyaluronate and is found in activated effector and memory T cells in mammals (133, 134). Relatively little research exists regarding CD44 expression in chickens and even less on its expression on avian lymphocytes. CD44 expression has been analyzed in chicken embryos (136). Furthermore, it was analyzed as a marker for detection of activated T cells in challenged or vaccinated chickens without finding a clear correlation (138). In HG the increase of $CD3^{+}CD44^{+}$ T cells follows the increase in the number of the lymphocytes. Both total lymphocyte and $CD3^{+}CD44^{+}$ T cells numbers drop during the contraction phase. Based on this observation, CD44 may be 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 pattern would be more consistent with effector T cells leaving the lymphoid tissue upon virus exposure followed by a repopulation during the expansion phase and a decline during the contraction phase. The $CD3^{+}CD44^{+}$ T cells in the spleen seem to follow a similar pattern as CALT, only delayed and with lower amplitude.

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 in CALT. Since, CALT is the main site for cytotoxic immune responses to IBV in both the primary and secondary immune response based on GZMA mRNA expression, it would argue for a functional divergence of HG and CALT in the IBV specific immune response. This is further supported by the observation that upon ocular IBV vaccination HG generates the highest number of IBV-specific IgA and IgG secreting cells, while CALT response is very limited (manuscript in preparation). Our data indicate a specialization of HALT in which HG preferential generates humoral immune responses and CALT cell mediated immune responses to IBV. However, both display an increase in IFN-γ mRNA expression at 5-6 days post vaccination, consistent with the induction of a T_H1-dominated immune response. One could speculate that CALT T helper cells may

focus their helper activity more towards cytotoxic responses, unlike HG T helper cells, which are geared to provide help for antibody responses.

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Appendix I: Tables

Table 1: Primers used for RT-PCR amplification

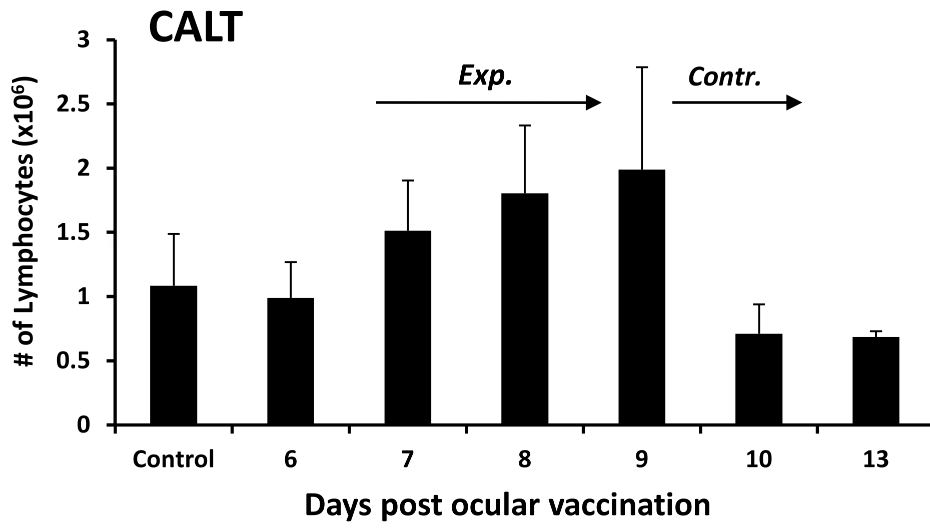
Target	Primer sequence	Amplicon size (bp)	Reference
β-Actin	F – CAACACAGTGCTGTCTGGTGG R – ATCGTACTCCTGCTTGCTGAT	205	(131)
IFN-γ	F – GTGAAGAAGGTGAAAGATATCATGGA R – GCTTTGCGCTGGATTCTCA	714	(130)
Granzyme A	F – TGGGTGTTAACAGCTGCTCATTGC R – CACCTGAATCCCCTCGACATGAGT	454	(129)
Perforin	F – ATGGCGCAGGTGACAGTGA R – TGGCCTGCACCGGTAATTC	390	(129)

Table 2: RT-PCR reactions used for cDNA synthesis and amplification

Reaction steps	IFN-γ	Perforin	Granzyme A
Reverse transcription	55^0C for 10 min	55^0C for 10 min	55^0C for 10 min
Initial Denaturation	95^0C for 5 min	95^0C for 1 min	95^0C for 1 min
Denaturation	95^0C for 30 sec	95^0C for 30 sec	95^0C for 30 sec
Annealing	50^0C for 30 sec	65^0C for 30 sec	55^0C for 30 sec
Extension	72^0C for 30 sec	72^0C for 30 sec	72^0C for 30 sec
# of cycles	40	40	40

Appendix II: Figures

A



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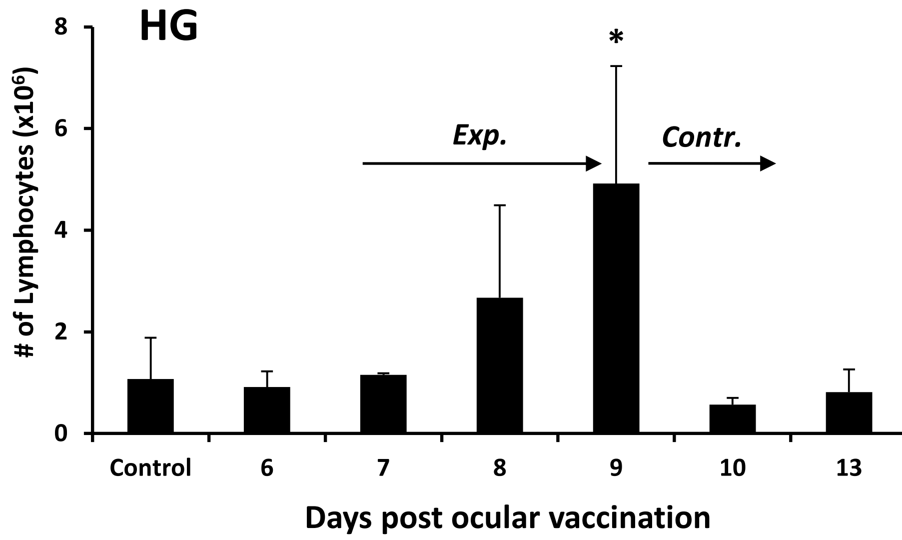

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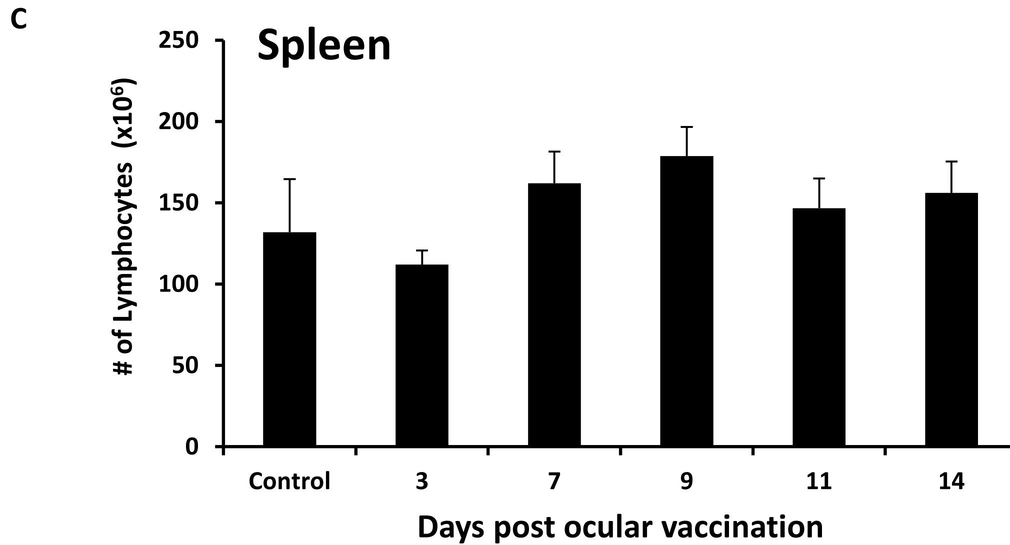
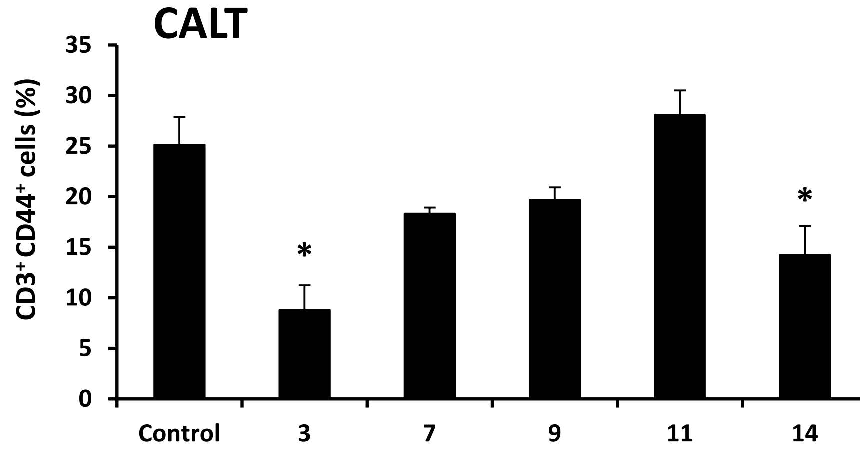


Figure 1: Lymphocyte counts after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and tissues were collected on various days after vaccination to measure the total number of lymphocytes in the CALT (A), HG (B) and spleen (C) using trypan blue exclusion. The phases of the immune response are indicated as expansion phase (*Exp.*) and contraction phase (*Contr.*). Arrows indicate time and direction of the phase. n = 6 controls, 3 IBV vaccinated chickens per time point. Depicted are the means and one standard error. A significant increase from controls is indicated by ***** ($p < 0.05$)

A



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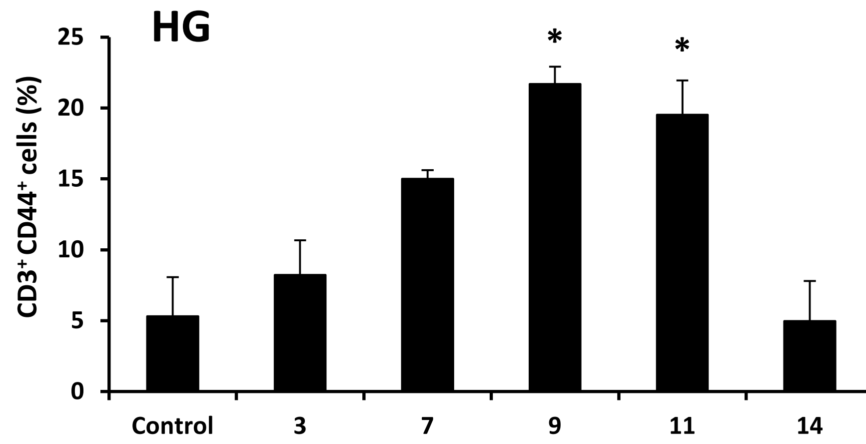

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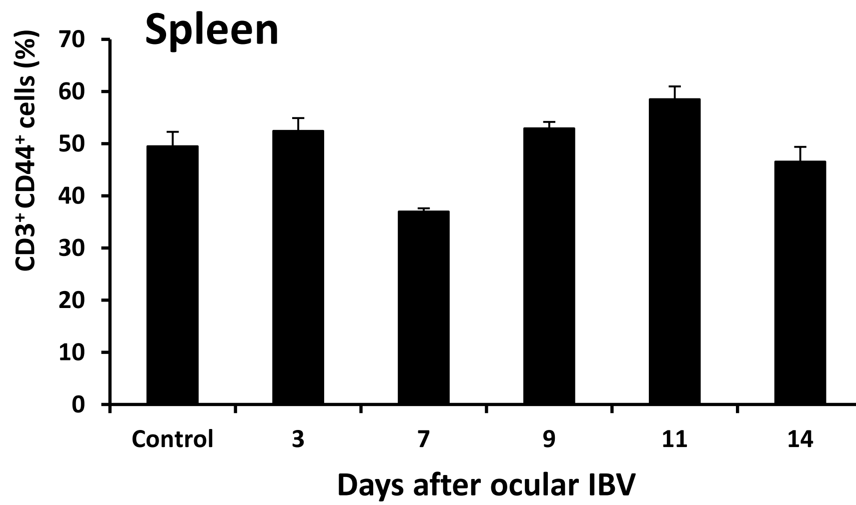
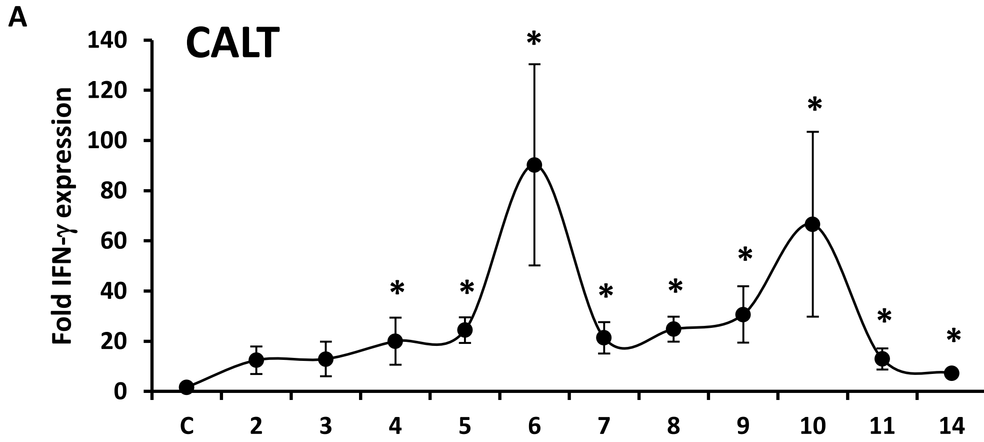
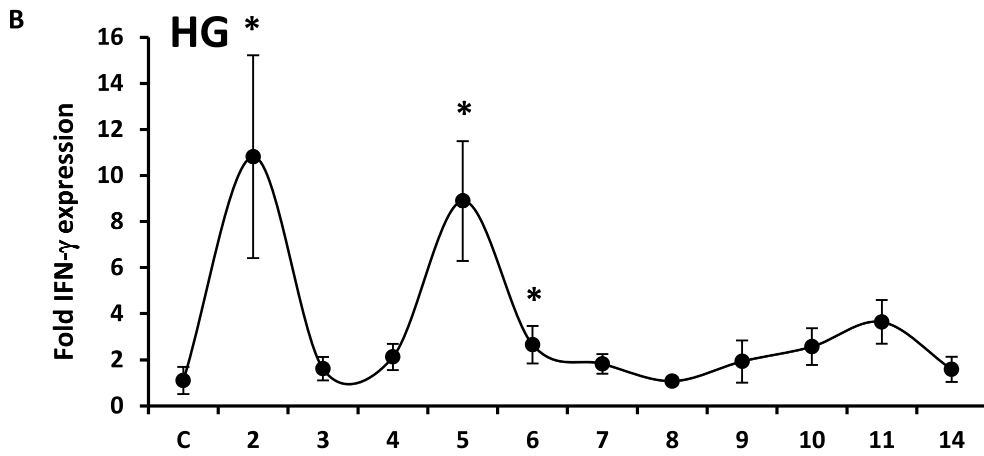


Figure 2: Percent $CD3^+CD44^+$ T cells after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and tissues were collected on various days after vaccination to measure the $CD3^+CD44^+$ T cells out of the total lymphocyte population in the CALT (A), HG (B) and spleen (C) using flow cytometry. Values significantly different from control are indicated by ***** and have a p value <0.05. n = 5 controls, 3 IBV-vaccinated chickens per time point. Depicted are the mean values and one standard error.


A
CALT
Fold IFN-γ expression
140
120
100
80
60
40
20
0
C
2
3
4
5
6
7
8
9
10
11
14
*


B
HG
Fold IFN-γ expression
16
14
12
10
8
6
4
2
0
C
2
3
4
5
6
7
8
9
10
11
14
*

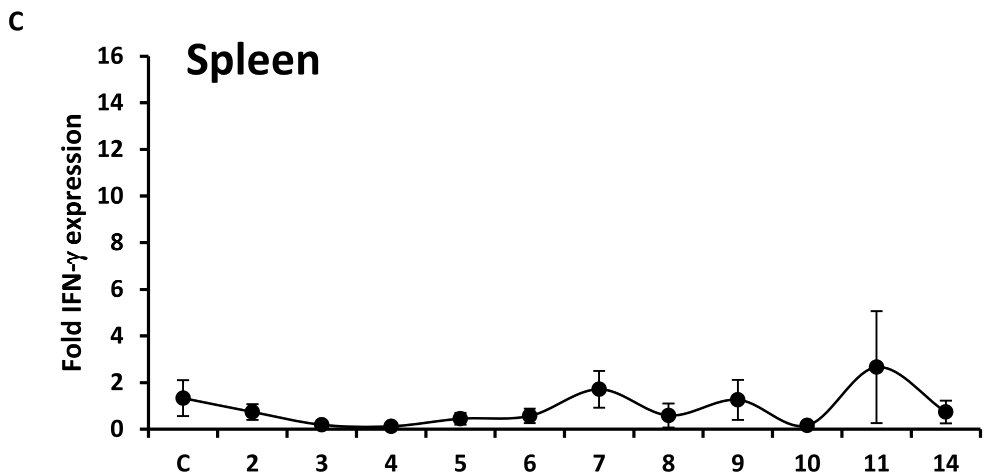
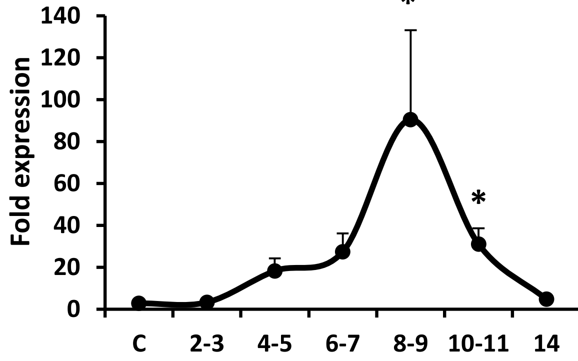

C
Spleen
Fold IFN-γ expression
16
14
12
10
8
6
4
2
0
C
2
3
4
5
6
7
8
9
10
11
14
Days post ocular IBV vaccination

Figure 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. n is between 5 and 10 for the IBV vaccinated chickens and 6 non-vaccinated control chickens. Depicted are the means and one standard error. Values significantly different from control are indicated by ***** and have a p value <0.05.

Granzyme A **Perforin**

CALT

A



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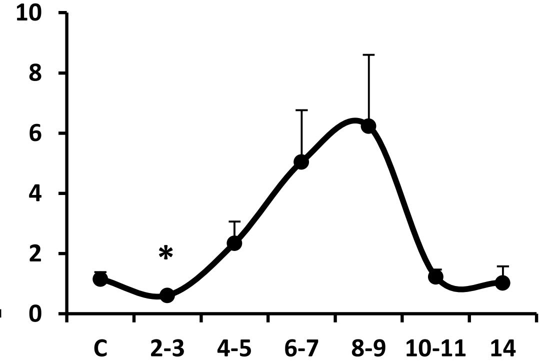
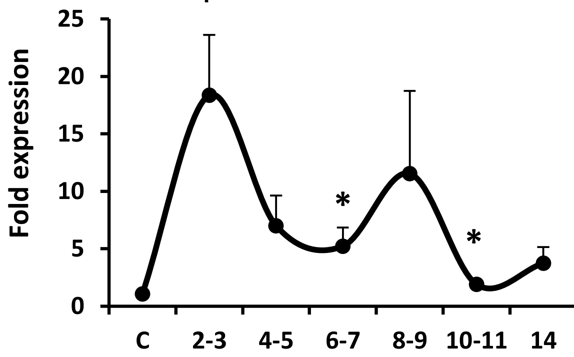
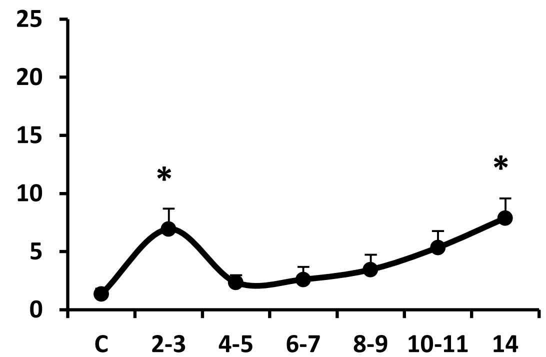


HG

C

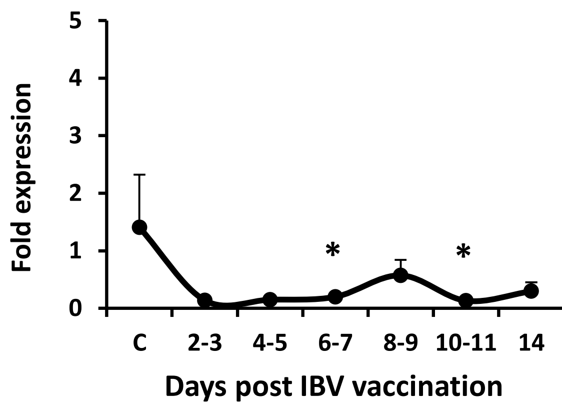


D



Spleen

E



F

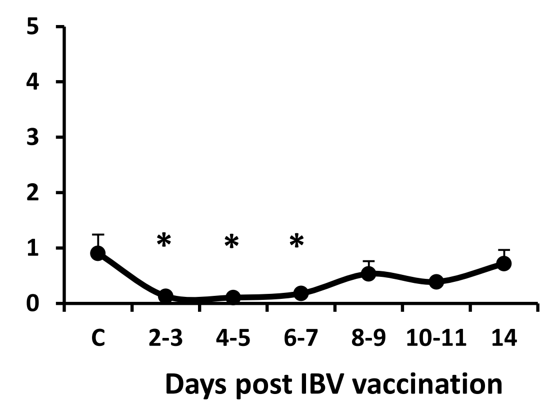
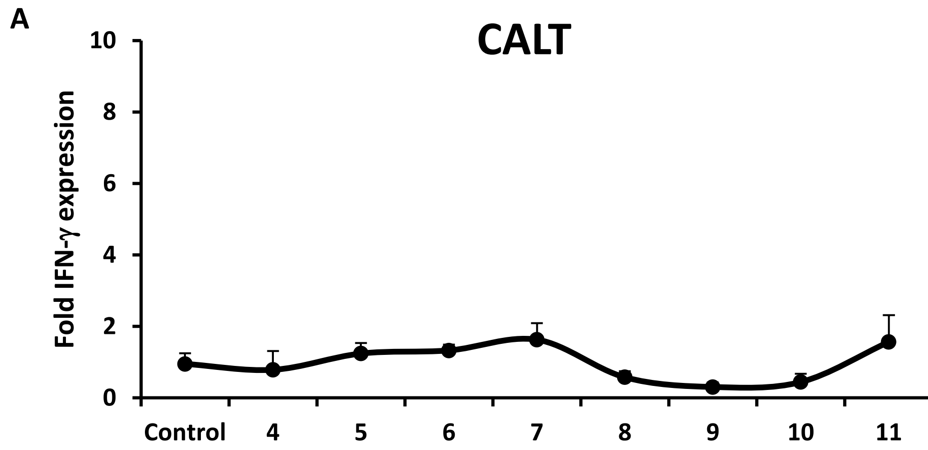


Figure 4: GZMA and perforin mRNA expression after primary IBV vaccination. Chickens were vaccinated at 3 weeks of age and CALT, HG and spleen were collected on various days after vaccination for RNA extraction and measuring the GZMA and perforin mRNA expression by qRT-PCR. The graphs represent GZMA mRNA 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 the control represented as 'C' on the x axis. n is between 5 and 10 IBV vaccinated chickens per time point and 6 non-vaccinated control chickens. Indicated are the mean and one standard error. Values significantly different from control are indicated by ***** and have a p value <0.05.

A



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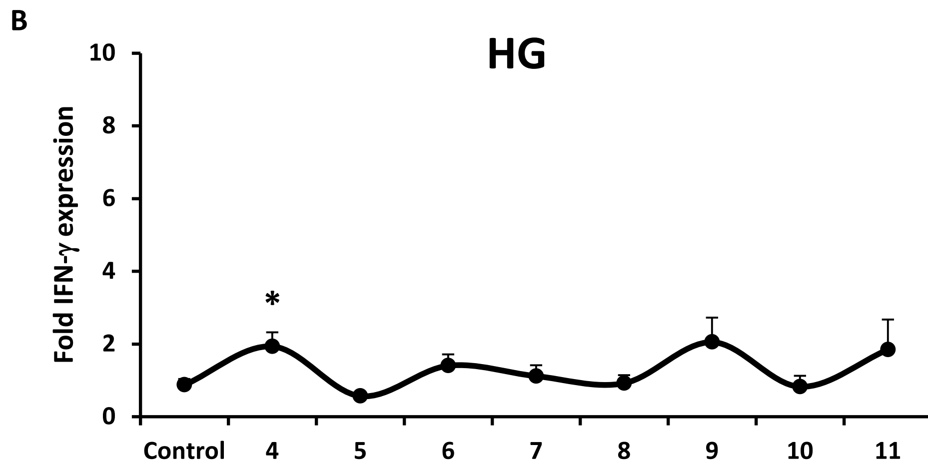

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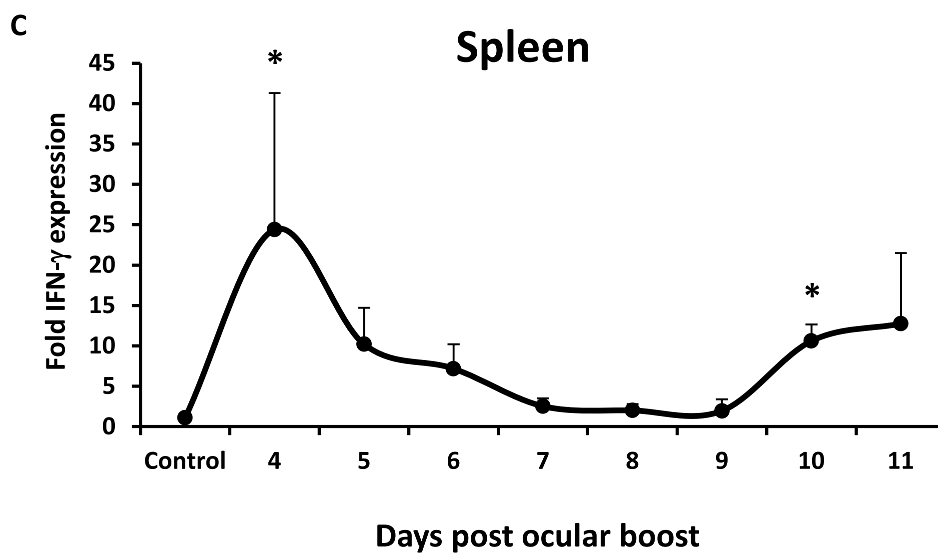


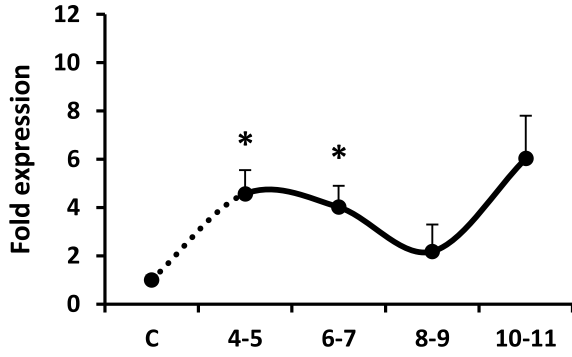
Days post ocular boost

Figure 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 the age-matched, unvaccinated controls which are represented as 'C' on the x axis. n = 5-10. IBV vaccinated and 6 non-vaccinated control chickens. Values indicate mean and one standard error. Significant changes in expression, when compared to controls, are indicated with ***** and have a p value <0.05.

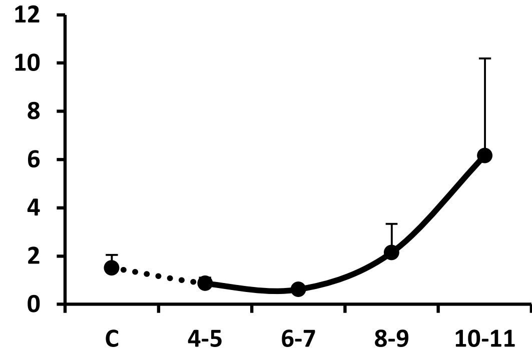
Granzyme A **Perforin**

CALT

A

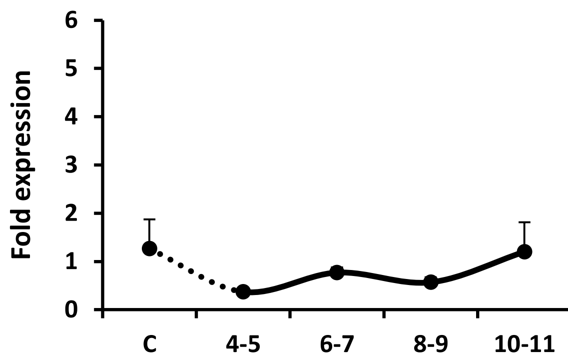


B

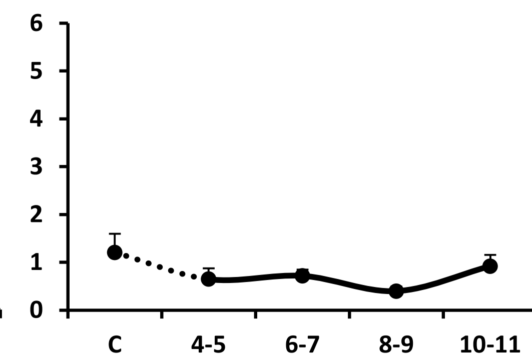


HG

C

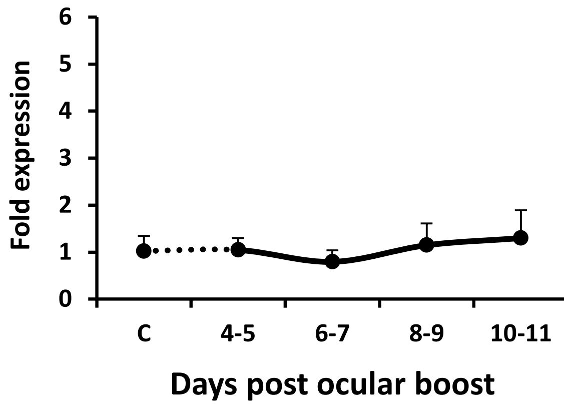


D



Spleen

E



F

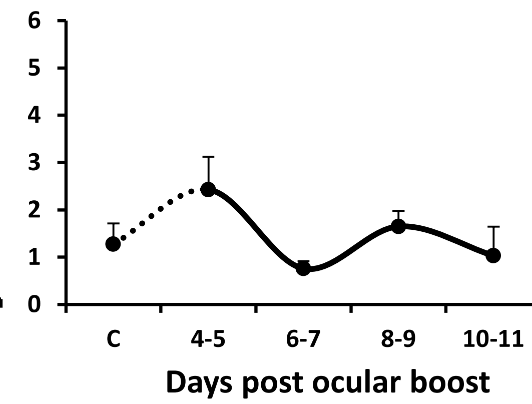


Figure 6: GZMA and perforin mRNA expression after secondary IBV vaccination. Chickens were vaccinated at 3 weeks of age and boosted 4 weeks later. RNA was extracted from CALT, HG and spleen on various days after vaccination. GZMA and perforin mRNA expression was measured by qRT-PCR. The graphs represent 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 the control values, which are indicated as 'C' on the x-axis. Data points on two consecutive days are pooled to reduce the variability and make the graphs more clear and concise. n = 5-10 IBV vaccinated chickens per time point and 6 non-vaccinated control chickens. Indicated are the mean and one standard error. Significant changes in expression, when compared to controls, are indicated with ***** and have a p value <0.05.