IDENTIFYING THE UNDERLYING MECHANISMS OF MAREK'S DISEASE VACCINE SYNERGY

By

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ABSTRACT

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Marek's disease virus (MDV; *Gallid herpesvirus 2*, aka, serotype 1) is a ubiquitous and highly oncogenic α-herpesvirus that causes Marek's disease (MD), a lymphoproliferative disorder affecting chickens with estimated annual costs to the poultry industry of ~$2 billion worldwide. Since 1970, MD has been largely controlled through widespread vaccination. While MD vaccines are very successful in preventing tumors, they do not prevent viral replication and spread. As a consequence, new and more virulent MDV strains have repeatedly emerged in vaccinated flocks. Thus, there is a need to understand how MD vaccines work in order to design future vaccines that are more protective, especially against more virulent MDVs. One promising insight for vaccine development is based upon protective synergism, a phenomenon where two vaccines when combined provide greater protection compared to either original vaccine when administered alone as a monovalent vaccine. The mechanism that underlines the synergistic effect between SB-1 (a *Gallid herpesvirus 3*, aka, serotype 2 strain) and HVT (herpesvirus of turkey, aka, *Meleagrid herpesvirus 1* or serotype 3), two of the most widely used MD vaccines, has never been investigated, and thus, provides a highly relevant and useful model to explore. To investigate the mechanisms of protective synergy of SB-1 and HVT, we used three approaches. First, we investigated how monovalent SB-1 or HVT replicates when they were alone in the host or together as a bivalent vaccine. We observed that the replication patterns of SB-1 and HVT were

different with respect to time after administration into the bird and the organs that they were found to replicate in regardless if the other vaccine were present. Based on the observation that HVT replicated primarily early in the bursa, we found that this organ was necessary for protection using both HVT and bivalent HVT + SB-1 vaccines. Second, we measured the effects of CD8 T cells in monovalent SB-1, HVT, and bivalent SB-1+HVT vaccine treatment. Specifically, we reduced CD8 T cells to see their effect of CD8 T cells on MD incidence and vaccinal protection by injecting the chickens with a monoclonal antibody directed against chicken CD8 T cells. In this study, we found that CD8 T cells were necessary for protection induced by vaccines. Third, we identified the cytokine profiles induced by SB-1, HVT, and the bivalent vaccine to see if cytokine synergy could be one of the mechanisms to explain protective synergy. We found that SB-1 induced an innate anti-viral response typified by IFN-α, IFN-β, IL-1β, T-cell proliferation cytokine IL-21, and Th2 cytokine IL-5, while HVT suppressed TGF-β3 and TGF-β4. The early stimulation of IL-1β and IL-21 (IFN-γ-promoting cytokines) at 4 days post vaccination (DPV) by SB-1 combined with the suppression of TGF-β (IFN-γ-suppressing cytokine) at 1 day post challenge (DPC) by HVT could result in the strong induction of IFN-γ found in the bivalent vaccine at 10 DPC. The induction of IFN-γ supports the synergistic effect of cytokines by a cooperative action mechanism where multiple cytokines work together to enhance the signal. Based on these findings, we propose a model to explain bivalent SB-1 and HVT vaccine synergy, which combines the replication of vaccines, T cell response to vaccinations, and cytokine synergy between SB-1 and HVT vaccine. Our proposed mechanism provides insights on how to generate rationally designed MD vaccines.

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KEY TO ABBREVIATIONS

mAb: Monoclonal antibody

ADCC: Antibody-dependent cellular cytotoxicity

ADOL: Avian Disease and Oncology Laboratory, East Lansing, Michigan

AUP: Animal use protocol

BAFF: B-cell activating factor

ch: Chicken

ARS: Agricultural Research Service

AUP: Animal use protocol

°C: Degree Celsius

CD3: Cluster of differentiation 3 (a pan-T cell marker, part of the TCR complex)

CD4: Cluster of differentiation 4 (a co-stimulatory T cell marker)

CD8: Cluster of differentiation 8 (a co-stimulatory T cell marker)

cDNA: Complementary DNA

CEF: Chicken embryonic fibroblasts

CMIR: Cell-mediated immune response

CMV: Cytomegalovirus

CSF: Colony stimulating factor

Ct: Cycle number

CTL: Cytotoxic T lymphocytes

DEF: Duck embryonic fibroblasts

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPC: Day post challenge

DPV: Day post vaccination

FACS: Fluorescence-activated cell sorting

FASL: FAS ligand

FBS: Fetal bovine serum

FITC: Fluorescein Isothiocyanate

G: Gauge (size of needles)

GaHV-2: *Gallid herpesvirus* type 2

GaHV-3: *Gallid herpesvirus* type 3

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

gB: Glycoprotein B

H+L: Heavy+Light chains

HB: Horsfall-Bauer

HBV: Hepatitis B virus

HLA: human leukocyte antigen

HRP: Horseradish peroxidase

HVT: herpesvirus of turkeys (*Gallid herpesvirus*-3)

IACUC: institutional animal care and use committee

IFN-α: Interferon gamma

IFN-β: Interferon beta

IFN-γ: Interferon gamma

IgG: Immunoglobulin G

IL: Interleukin

IL-15: Interleukin 15

IL-21: Interleukin 21

IL-1β: Interleukin 1 beta

iNOS: inducible nitric oxide (iNOS)

IP: Intra-peritoneal

IV: Intra-venous

kDa: Kilodalton

kg: Kilogram

LM: Leibovitz's L-15 and McCoy's 5A

MD: Marek's disease

MDV: Marek's disease virus

Meq: MDV EcoQ-protein (a viral oncogene)

mg: Milligram

MHC: Major histocompatibility complex

ng: nanogram

NK cell: Natural-killer cell

OCT: Optimum cutting temperature

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PE: Phycoerythrin

PFU: Plaque-forming units

qPCR: Quantitative qPCR

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RNA: Ribonucleic acid

SPF: Specific-pathogen-free

SPRD: SpectralRed^{TM}

TCR: T-cell receptor

TGF-β2: Transforming growth factor beta 2

TGF-β3: Transforming growth factor beta 3

TGF-β4: Transforming growth factor beta 4

Th: T helper

Th1: T helper 1

Th2: T helper 2

US: United States

USA: United States of America

USD: United States dollars

USDA: United States Department of Agriculture

v: Virulent

vv: Very virulent

vv^{+}: Very virulent plus

WPC: Week post challenge

WPI: Week post injection

CHAPTER 1

Literature Review

Introduction

This chapter introduces Marek's disease (MD) and why it is important in poultry industry, the Marek's disease virus (MDV) life cycle and pathogenesis, and generations of MD vaccines. The chapter also addresses problems associated with MD vaccines including 1) MD vaccines cannot prevent chickens from shedding of pathogenic MDV so the virus can still transmit from one host to another, and 2) the vaccines cannot clear the pathogenic virus from the host allowing MDV to cohabit with vaccine strains in the same host which is probably the primary driving force for MDV evolution to higher virulence. With these problems, investigating new strategies to improve efficacy of vaccines is necessary, especially against more virulent MDVs. In addition, this chapter reviews immune responses associated with MDV infection and vaccine-induced immune responses which include both innate and adaptive immune responses. And lastly, this chapter talks about protective synergy of the bivalent MD vaccine that could potentially be used as the model to study mechanisms underlying protective synergy of MD vaccines.

Marek's disease history

Marek's disease (MD) was first described in 1907 by József Marek (Marek 1907). Initially, the disease was not differentiated from avian leukosis as the clinical signs between the two are similar. Due to the confusion with avian leucosis and the inability to transmit the disease using cell filtrates, identifying the causative agent for MD was very challenging. However, about 60 years later, an α-herpesvirus named as Marek's disease virus (MDV) was identified to be the causative agent of the disease. As the industry shifted to high-intensity rearing in the 1960s, MDV evolved to higher virulence causing large losses to poultry production. To combat these costly losses, the attenuation of the pathogenic HPRS-16 strain was developed in 1970s by Churchill and used as the first effective MD vaccine (Churchill, Chubb, and Baxendale 1969) to control MD. Therefore, the MD vaccine is considered as the first vaccine developed to control cancer. Despite their success, MD vaccines do not prevent viral infection and replication. Consequently, MDV field strains have repeatedly emerged that have become more virulent and overcome the existing vaccine. Thus, there is a need to identify and produce MD vaccines that can provide protection against more pathogenic MDV strains (Witter 2007).

Marek's disease and its importance

MDV is an oncogenic a-herpesvirus that induces polyneuritis and visceral lymphoma (Cone 1929; Walle 1929; Morrow and Fehler 2004), and mostly affects chickens (*Gallus gallus domesticus*). The disease is a highly contagious neoplastic disease that has plagued the chicken industry with economic losses in broiler meat and layer egg production estimated to be around $1-2 billion worldwide each year (Morrow and Fehler 2004). Vaccination is an effective method to prevent tumors and reduce losses in flocks from MD. Although multiple vaccines have been developed and are widely employed to prevent the disease, none of them provide sterilizing immunity to prevent the virus from infecting and being shed from birds. Therefore, despite widespread vaccination, MDV is ubiquitous and persists in the environment to infect naïve birds. This inability of current vaccines to block entry, replication, and shedding of the virus from the host facilitates further evolution of virus, resulting in more virulent viruses (Witter 1997). Consequently, MDV outbreaks are still of concern due to vaccine breaks (Read and Mackinnon 2007). Development of effective vaccines that can overcome the new more pathogenic MDV, as well as, vaccines that can prevent the transmission and protect the birds from the infection is vital to sustainably prevent infection and shedding of the virus (V. Nair 2005).

Serotypes, biology, and life cycle of pathogenic MDV

MDV and related vaccine viruses are antigenically similar and historically categorized into three different serotypes. Serotype 1 or *Gallid herpesvirus 2* is oncogenic causing MDV (e.g., Md5, JM strains), MDV serotype 2 or *Gallid herpesvirus 3* is non-oncogenic and non-pathogenic MDV (e.g., SB-1, 281MI/1 strains), and serotype 3 or *Meleagrid herpesvirus 1* is turkey herpesvirus (HVT) that is non-oncogenic and apathogenic in chicken (e.g., FC126, WTHV-1 strains). The life cycle of MDV starts when viral particles enter the lung of the host by inhalation of dander shed from previously-infected birds. Based on the current model, macrophages phagocytize the virus, which initiates viral release and replication to other cells. A unique aspect of MDV is that it highly cell associated and spread of the virus is through direct cell contact, i.e., not by free virions. In the lymphoid organs. The viral life cycle can be separated into four phases; early cytolytic, latency, late cytolytic, and transformation (Baigent and Davison 2004). The early cytolytic stage occurs after macrophages transport the viruses from the lung to the lymphoid organs such as bursa of Fabricius (bursa), spleen and thymus. The bursa is where B cells develop and mature, and is believed to be the first major target of infection in the early cytolytic stage. This stage of infection persists for about 3-6 days post infection, which leads to an acute inflammatory response, cytokine secretion from innate and infected cells, and B-cell and T cell activation. To escape from the host immune responses, the infection stage switches from an early cytolytic infection to a latent stage where virus is targeted to activated-$CD4^+$ T-cells and becomes inactive. This stage starts on 7-8 days after the infection or longer. Infected $CD4^+$ T-cells during the latency stage can be transformed, which leads to a lymphoma formation in multiple

organs of a susceptible host. Virus can also reactivate from the latent stage in susceptible hosts to propagate another late cytolytic cycle. After the late cytolytic phase, the virus moves to feather follicle where it is release to the environment in shed dander to infect another host (Baigent and Davison 2004). (Figure 1.1).

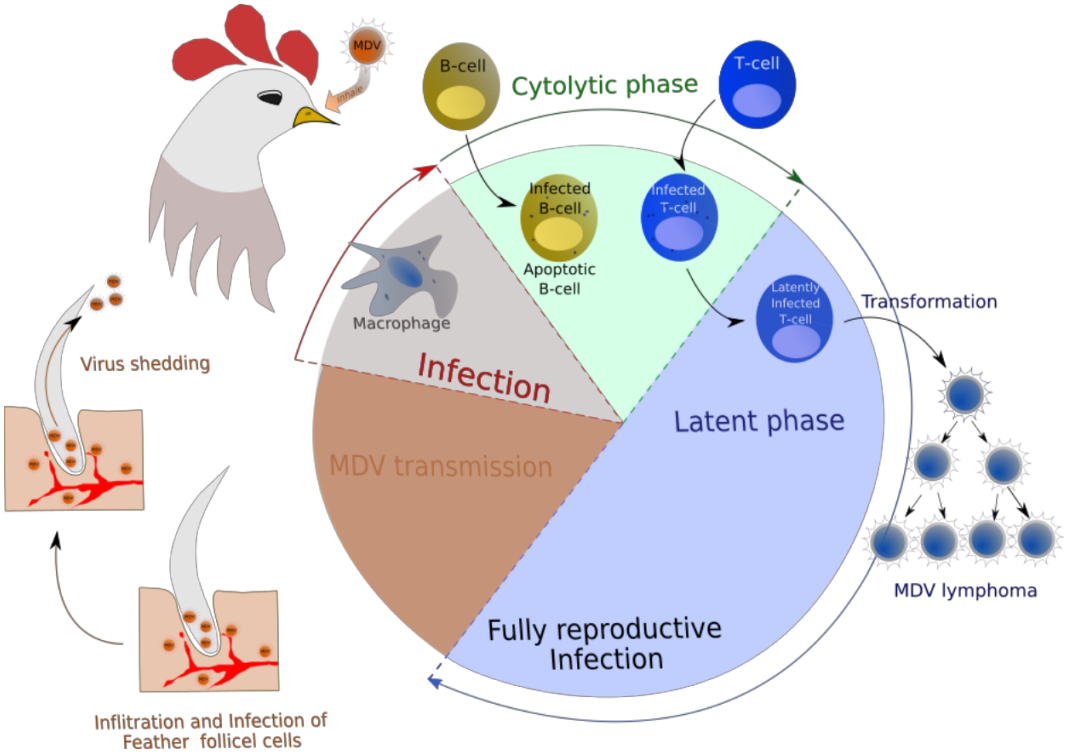

Figure 1.1. Life cycle and stages of infection of pathogenic MDV. Virus particles enter the host and go into cytolytic and latent stages, respectively. Infected CD4 T cells are transformed. Fully productive viruses can release as free particles and infect another susceptible host. Figure was illustrated based on the information in (F. Davison and Nair 2005).

Pathogenesis of MD

The Cornell model has proven to stand the test of time to describe pathogenesis of MD. The Cornell model describes the life cycle of MDV starting from the inhalation of MDV into the lung, MDV can later cause neuropathology and abnormal CD4 T cell proliferation resulting in paralysis and visceral tumors in many organs. At the early infection or early cytolytic infection, MDV antigens can be detected in the epithelial cells in lung (St. Hill, Silva, and Sharma 2004; Mohamed Faizal Abdul-Careem et al. 2009). Macrophages in the lung are considered to be the cells that transfer viral particles to subsequent lymphoid cells in thymus, bursa, and spleen (Barrow et al. 2003). It is reported that MDV produces IL-8, which is believed to be involved in the recruitment of B cells and T cells into the lung (Engel et al. 2012) and MDV can start to replicate in B cells as early as two days post infection (Butter et al. 2009).

The transmission of MDV is unique that in that it occurs via cell-to-cell contact. After establishing early infection in macrophage in the lung, virus particles are successfully transferred to B cells. The virus starts to replicate in B cells. Infected B cells can also produce viral IL-8 to recruit CD4 T cells to the site, which would promote the transmission of the virus from B cells to activated T cells. Pp38 is an early immediate viral gene that is expressed during the early lytic infection and is associated with cytolytic activity in B cells and T cells (Lupiani et al. 2002; Lee et al. 2005). Hunt and colleagues reported the reduction of the expression of MHC-I molecules in infected cells at this stage of infection (Hunt et al. 2001).

Latency is a common characteristic of herpesviruses (Grinde 2013) where the genome of the virus is integrated and remains in the cells without the production of viral

particles. In MDV, around 7 days post infection; the host immune response induces the virus to enter latency, especially in $CD4^+$ T cells. Latent MDV uses immune evasion strategies such as integrating the viral genome into the host chromosome using viral telomeric repeats to persist in the host (Morissette and Flamand 2010; Delecluse and Hammerschmidt 1993), and expressing *meq* gene to induce transformation of latently infected $CD4^+$ T cells to maintain oncogenic properties. Deletion of the *meq* gene from the MDV genome results in the infection and replication of the virus but the virus lacks the ability to induce neoplastic cells. (Lupiani et al. 2004). In addition, deletion of the *meq* gene reduces immunesuppression caused by MDV as the level of antibody was restored to the same level as in the control chickens (Li et al. 2011)

The virus is later transmitted to feather follicle epithelial cells where the virus can reactivate itself and becomes a cell-free virus before shedding into the environment through the dust.

Host immune responses to MDV

Innate immune response

The innate immune response is activated immediately after the chicken becomes infected with MDV. In most herpesvirus infections, the innate immune response plays a major role to limit viral replication and spread (Whitley 2011). Innate immunities that involve in the response against MDV include secretion of innate cytokines, activation of macrophage activities, and induction of NK cells (Boodhoo et al. 2016).

Cytokine responses

In general, cytokines that provide an anti-viral response include type I (IFN-α and IFN-β), type II, and type III interferon. Type-I interferons are secreted by monocytes, epithelial cells, plasmacytoid dendritic cells, and fibroblasts, while type-II IFN is produced by activated T-cells and NK cells. Type I and type II IFN are very well-known for their anti-viral activity (Plachý et al. 1999), while type-III interferon is mainly found in epithelial cells and has been discovered to be essential for mucosal immunity against viral infection (Reuter et al. 2013). Upon MDV infection, IFN-γ, which is essential for anti-viral activity and for induction of cell-mediated cytotoxicity, was induced (Aouatef Djeraba et al. 2002; Jarosinski et al. 2005; Xing and Schat 2000) . In addition, down-regulation of IFN-γ was observed in MDV susceptible line (Quéré et al. 2005). In addition to the IFN family, other cytokines also play important role for protection against MDV. For example, iNOS and IL-1β are up-regulated *in vivo* around one week to two weeks post infection with MDV (Xing and Schat 2000).

Macrophages

Macrophages connect the innate and adaptive immune responses. Specifically, macrophages act as phagocytic cells for innate immunity and also serve to present antigens to T cells for adaptive immunity. Upon MDV infection, macrophages have been shown to be infected by MDV *in vitro* (Chakraborty et al. 2017) and *in vivo* (Barrow et al. 2003). Macrophages isolated from the B19 chicken line (the MHC haplotype confers susceptibility to MD) have lower phagocytic activity than macrophages obtained from B21 chicken line (the MHC haplotype confers resistance to MD) (Powell et al. 1983). In

addition, MDV replication increased when macrophages were removed from splenocytes (Powell et al. 1983). On the other hand, activating more macrophages reduced MD incidence (Gupta et al. 1989). Activating inducible nitric oxide (iNOS) is one of the mechanisms that macrophages use to control MDV replication (Djeraba et al. 2000). Overall, macrophages are innate cells that provide an essential ability to control MD infection.

Natural killer cells (NK cells)

NK cells are part of the innate immune response that can produce IFN-γ, which plays a crucial role in providing an anti-viral response. NK cells recognize MHC class I on the cell surface. MDV down-regulates the expression of the major class I MHC, which would normally make MDV-infected cells targets for NK cells. But MDV also upregulates the minor class I MHC to avoid NK killing. NK cells can destroy infected cells and cancer cells by secreting perforin and granzyme. Chickens resistant to MD have higher NK cell activity compared to susceptible chickens (Garcia-Camacho et al. 2003; Sharma 2006). Additionally, enhancement of NK cell activity was observed in chickens inoculated with MDV (Sharma and Okazaki 1981).

Adaptive immune response

The adaptive immune response or acquired immune response is activated around two weeks after infection or vaccination. The special feature of adaptive immunity is it has memory, so this type of immune response is ideal for vaccinal

immunity. There are two types of adaptive immune response; humoral immune response and T cell-mediated immune response.

Humoral immune response

The humoral immune response is a soluble immune response that is responsible for antibody production from plasma B-cells. After infection, B-cells become activated and develop into plasma cells that can produce antibody to fight against infection. Antibody can provide anti-viral activity via blocking, neutralizing, opsonizing, recruiting complement factor, or inducing a cytotoxic response via antibody-dependent cellular cytotoxicity (ADCC). Unlike most of other viruses that lyse cells to release infectious viral particles, transmission of MDV is only by cell-to-cell contact. Thus, antibody cannot come in contact directly with the MDV particles. Thus, other than maternal antibodies, this type of immune response is not considered to play much of a role for MD resistance.

Cell-mediated immune response

The cell mediated immune response is believe to be an important immunity to intra-cellular pathogens (White, Suzanne Beard, and Barton 2012; Hanley and Bollard 2014). MDV is highly cell-associated, thus the cell-mediated immune response is critical for immune protection against MDV. Depleting CD4 T cells reduces MDV pathology but this may be because of the reduction of the cells that are the target for MDV infection. Morimura and colleagues demonstrated that removing CD8 T cells by antibody injection increases MDV levels in CD4 T cells indicating their anti-viral effect against MDV

(Morimura et al. 1998). Another direct evidence that shows the role of T cells in eliminating MDV infected cells was through plaque reduction assay where when infected cells were incubated with lymphocytes obtained from vaccinated birds, the numbers of plaques was reduced (Ross 1977).

Marek's disease vaccines

Vaccines for MD have been developed and used since the 1960s. They are categorized into four generations **Figure 2**. The generations of vaccines are divided based on the efficacy to overcome different virulent levels of MDV, which are mild (m), virulent (v), very virulent (vv), and very virulent plus (vv+). The first generation of MD vaccine was made from initially pathogenic serotype 1 strain HPRS16 by growing and passaging the virus in chicken kidney cells to reduce its virulence and become attenuated (Churchill and Payne 1969). This serotype 1 HPRS-16 vaccine was used for several years to protect chickens from MDV but was surpassed in the mid 1970s with the introduction of nonpathogenic serotype 3 HVT strain FC126 that can protect chickens from vMDV and did not require live cells, which made it is easier to handle, store, and distribute. HVT vaccine was licensed and first used in 1971 in the US. HVT vaccine effectively reduced MD incidence in the flocks and has been widely used since then. Later, due to the evolution of the virus, MDV became more virulent and HVT alone could no longer effectively control the disease. A combination of HVT with serotype 2 strain SB1 resulted in improved vaccine efficacy and was able to protect chickens from more virulent MDV (vvMDV). The combination of HVT and SB-1 was popularly used as a bivalent vaccine from 1983-1990's. Nonetheless, the adaptation of the virus to

surpass the protection of HVT+SB-1 bivalent vaccine led to the switching towards the attenuated serotype 1 strain CVI988 (aka Rispens), which provides protection against vv^{+}MDV pathogenic MDV strains. This attenuated serotype 1 strain is currently used as the most protective commercial vaccine available (Figure 1.2).

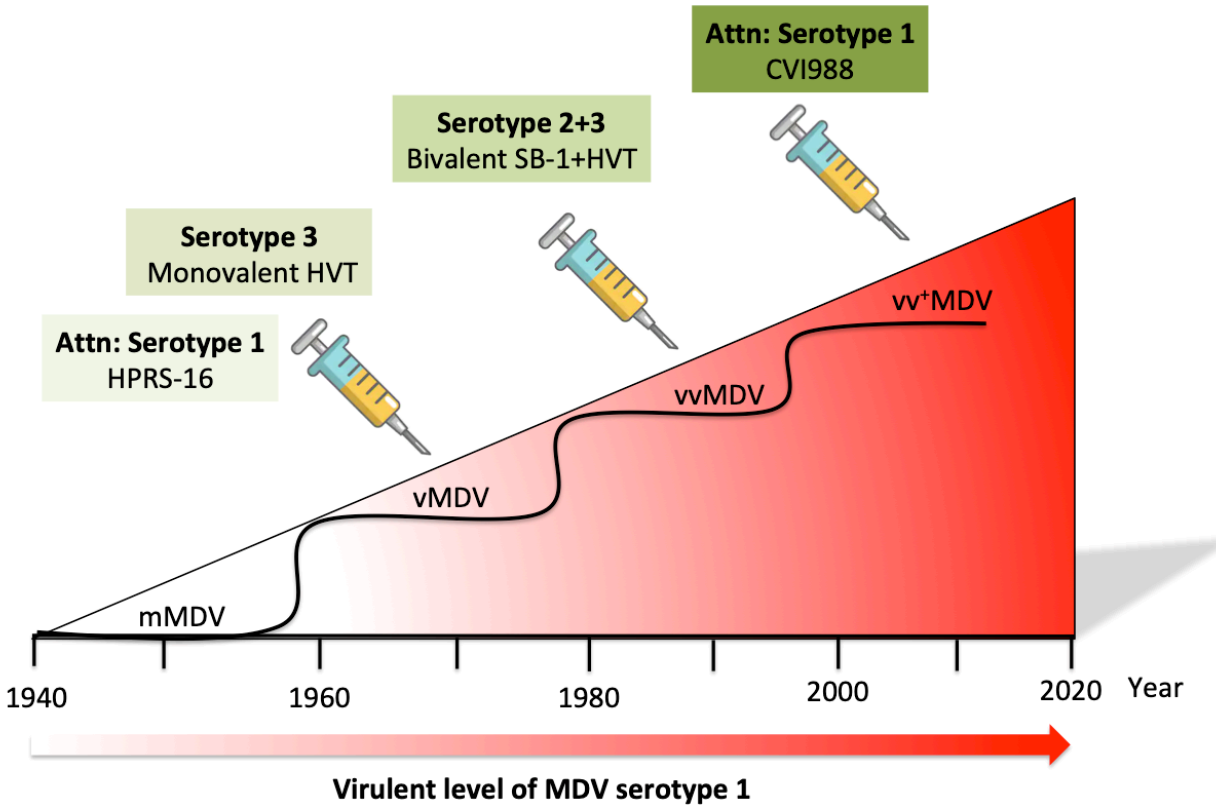

Figure 1.2. Evolution of MDV and the use of MD vaccines. Marek's disease vaccines have introduced to overcome the higher virulence of Marek's disease virus. Pictured was illustrated based on the information described by Davison and Nair, Expert Rev Vaccines (2005).

Immune induced by Marek's disease vaccines

MD vaccines have been developed from different serotypes. The immune responses induced by different vaccine serotypes are distinct. Vaccines available thus far could prevent chickens from disease by inhibiting viral replication and tumor induction. Nonetheless, virulent MDV still remains in MD-vaccinated hosts and can be shed to the environment through shed dander to infect another host. Immune response against MDV infection and immune response induced by vaccines may be similar though they have critical differences. Vaccines should be able to induce responses to protect the chickens as detected by the up regulation of the MHC class I and several cytokines including IL-6, IFNγ, and IL-18 yet these activations do not inhibit the virus from shedding (Abdul-Careem et al. 2008). Chickens vaccinated with CVI988 showed significantly higher levels of IL10 and IL18 compared to unvaccinated birds (Kano et al. 2009). Heller and Schat showed that SB-1 and HVT induce an NK cell response and the response is significantly higher when SB-1 and HVT are combined (Heller and Schat 1987). In addition activity of NK cells was enhanced in resistant and in vaccinated chickens but was suppressed in susceptible chickens (Sharma 2006). The CD8 T cell response is involved in anti-viral effects but is not involved in anti-cancer response after vaccination with attenuated CVI988 (Morimura et al. 1998). Overall, induction of cytokines and cell-mediated immune response (CMIR) through the induction of NK cells and $CD8^+$ CTL response seem to be involved in MD vaccinal protection.

Protective synergism provided by a bivalent vaccine (SB-1+HVT)

One intriguing phenomenon during the generation of serotype 3 HVT vaccine development is that a combination of this vaccine with serotype 2 SB-1 (bivalent SB-1+HVT) can enhance protection levels and increase survival rates of MDV-infected chickens compared to that of using SB-1 or HVT vaccines alone (monovalent vaccines) (Witter and Lee 1984). Moreover, this phenomenon is also found in other combinations of serotype 2 and serotype 3 strains, e.g., a combination of 281MI/1 (serotype 2) with WTHV-1 (serotype 3) provides significantly higher protection (around 56% VS 3-6% compared to each individual vaccine) (Witter 1992b). This indicates either co-operation between two different vaccines, or specific mechanisms exclusively occurring in bivalent vaccines. However, the mechanisms that underlie the protective synergism of HVT and SB-1 vaccines have never been elucidated.

CHAPTER 2

Towards a mechanistic understanding of the synergistic response induced by bivalent Marek's disease vaccines to prevent lymphomas

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Abstract

Background: Marek's disease (MD) is a lymphoproliferative disease of chickens caused by Marek's disease virus (MDV), a highly oncogenic a-herpesvirus. Since 1970, MD has been successfully controlled by widespread vaccination; however, more effective MD vaccines are needed to counter the repeated periodic emergence of more virulent MDV strains. One promising insight for MD vaccine development is based upon protective synergism, the observation that some vaccines in combination yield greater protection compared to either vaccine when administered alone. In the field, the bivalent combination of vaccine strains SB-1 and HVT strain FC126 has been widely used. Nonetheless, the mechanism(s) underlying this synergistic effect has not been investigated.

Methods: In experiment 1, SB-1 or HVT were administered as monovalent or bivalent vaccines to newly hatched chickens, then challenged five days later with pathogenic MDV (Md5 strain). To monitor MDV replication, peripheral blood mononuclear cells (PBMCs) were obtained weekly and viral titers determined by qPCR. At 1, 5, 10, and 14 days post-challenge (DPC), 8 birds per timepoint and treatment group were sacrificed to determine the replication levels of SB-1 and/or HVT in lymphoid organs (spleen, bursa, and thymus). After 8 weeks or until the birds were moribund, tumor formation was measured and the vaccinal protection determined. In experiment 2, to verify that the bursa is necessary for HVT protection, a subset of chicks were bursectomized. These birds and control birds were treated as in experiment 1 and the levels of protection for HVT, SB-1, and bivalent vaccines determined.

Results: The efficacy of bivalent SB-1+HVT surpasses that of either SB-1 or HVT monovalent vaccines in controlling the level of pathogenic MDV in PBMCs until the end of the study, and this correlates with the ability to inhibit tumor formation. In addition, SB-1 replication in the spleen increases from 1 to 14 DPC, while HVT replicates only in the bursa at 1 DPC. Finally, the presence of the bursa is necessary for immune protection induced by HVT vaccine.

Conclusion: Synergy of SB-1 and HVT vaccines is due to additive influences of the individual vaccines acting at different times and target organs. And the bursa is vital for HVT to replicate and the resulting immune protection induced by HVT vaccine.

Keywords: Marek's disease, Marek's disease virus, protective synergy, bivalent vaccine,

Abbreviations

ADOL, Avian Disease and Oncology Laboratory; ARS, Agricultural Research Service; AUP, animal use protocol; CEF, chicken embryonic fibroblasts; DPC, days post challenge; DEF, duck embryo fibroblasts; FBS, fetal bovine serum; LM, Leibovitz's L-15 and McCoy's 5A; MD, Marek's disease; MDV, Marek's disease virus; PBMCs, peripheral blood mononuclear cells; PFU, plaque forming units; SPF, specific-pathogen-free; USDA, United States Department of Agriculture; v; virulent, vv; very virulent, vv^{+}; very virulent plus; WPC, weeks post challenge.

Introduction

Marek's disease virus (MDV) is a ubiquitous and highly oncogenic a-herpesvirus that causes Marek's disease (MD), the most common lymphoproliferative disorder affecting chickens worldwide (F. D. & V. Nair 2004; Calnek 2011; Baigent and Davison 2004). Susceptible chickens commonly exhibit transient or long-standing paralysis, and predominantly CD4 T cell lymphoid tumors within a few weeks after MDV infection. As the disease progresses, chickens become severely ill and die due to the development of tumors in multiple internal organs. Consequently, MD is a major concern for the poultry industry with annual worldwide economic losses of $1-2 billion (Morrow and Fehler 2004).

MDV belongs to the genus Mardivirus, which has been grouped into three related but distinct species that can be separated serologically: serotype 1 or *Gallid alphaherpesvirus* 2 (GaHV-2) includes virulent strains of MDV, serotype 2 or GaHV-3 are naturally non-pathogenic strains, and serotype 3 is non oncogenic herpesvirus of turkey (HVT or *Meleagrid alphaherpesvirus* 1 (MeHV-1) (F. D. & V. Nair 2004).

The pathogenesis of MDV starts when feather dander containing infectious virus is inhaled into the lung. Based on the current model, resident macrophages that reside in the lung transfer the highly cell-associated virus to B cells and activated CD4 T cells (Calnek 2011). Infected and transformed T cells circulate to nerves and multiple organs causing neurological disorders and visceral tumors. The virus can stay latent in the CD4 cells during the tumor stage, or reactivate itself and release to the environment through the shedding of the dander via feather follicles, the only site of productive virions (F. D.

& V. Nair 2004; Calnek, Adldinger, and Kahn 2006). The shed virus can later spread and infect another susceptible host to complete the viral lifecycle.

Since the 1970s, control of MD has been primarily achieved through a limited number of live vaccines from all three serotypes. The most common MD vaccines include 1) serotype 3 HVT strain FC126, 2) serotype 2 strain SB-1, and 3) attenuated serotype 1 strain CVI988/Rispens. MD vaccines can effectively prevent chickens from developing MD and accompanying symptoms by preventing paralysis and tumor formation making MD vaccines the first vaccine to successfully prevent tumors.

While highly effective in preventing the induction of tumors, MD vaccines do not prevent infection and shedding of pathogenic MDV. Because vaccine viruses and pathogenic MDVs coexistence in MD-vaccinated flocks, it is likely that the widespread MD vaccination programs have resulted in the evolution of pathogenic strains with increasing virulence in the field (Atkins et al. 2013; Read et al. 2015; Gimeno 2008; V. Nair 2005). Since the first MD vaccine has been used, there are several major outbreaks of MDV because of repeated evolution of the virus to compete with MD vaccine strains. Nevertheless, MD vaccines are vital and unavoidable to achieve adequate protection from MD. As MD vaccines are typically administered in ovo or at hatch and chicks are exposed to MDV soon after hatch, MD vaccines are unlikely to function by eliciting humoral immunity. Thus, a basic understanding of how MD vaccines work is vital for the development of more effective vaccines to prevent future outbreaks caused.

One phenomenon widely employed with MD vaccines is that specific vaccine combinations of different serotypes can improve protective efficacy against MD

compared to individual vaccines. This phenomenon is called protective synergism (Witter and Lee 1984), and the first major example widely adopted by industry was combination of HVT and SB-1. For example, bivalent (SB-1 + HVT) significantly reduced mortality caused by (vv) MDV compared to the group of birds vaccinated with HVT or SB-1 alone (Calnek et al. 1983; Witter et al. 1985; Witter and Lee 1984). Several trials performed by Witter and colleagues indicated that the protective synergism phenomenon uniquely occurred among serotype 2 and serotype 3 combinations, and some with serotype 1 and 3 (HVT + Rispens) (Gimeno et al. 2012) but the protective synergism is barely found between serotype 1 and 2 (Witter 1992a).

Our study aims at understanding the underlying mechanisms of protective synergy with SB-1 and HVT. In this initial study, we demonstrate the protective synergy of a bivalent (SB-1 + HVT) vaccine in the contexts that it can control the replication of pathogenic MDV as well as tumor induction. We then characterize how the monovalent and bivalent vaccines replicate with respect to time and immune tissue distribution. Based on our finding, we also evaluate whether the bursa is required for protection provided by HVT, which may contribute to the synergy of SB-1+ HVT bivalent vaccine.

Materials and Methods

Cell culture

Chicken embryonic fibroblasts (CEF) and duck embryonic fibroblasts (DEF) were plated and cultured in 1:1 mixture of Leibovitz's L-15 and McCoy's 5A (LM) medium supplemented with 4% fetal bovine serum (FBS) from HyClone (Thermo Fisher Scientific, USA), 20 µg/ml streptomycin, 200 U/ml penicillin (Sigma Aldrich, USA), and 2 µg/ml amphotericin B (Thermo Fisher Scientific). Cells were maintained in a 37 °C incubator with 5% CO_2.

Viruses

SB-1 and HVT strain FC126 were used as viral vaccine strains. Both vaccines were propagated in CEF, while pathogenic serotype 1 MDV strain Md5 was cultured in DEF and used for challenge. The viral vaccines and pathogenic Md5 were from Avian Disease and Oncology Laboratory (ADOL) stocks. Viruses were plated on a monolayer of CEF or DEF in LM medium containing 4% FBS and later maintained in LM media containing 1% FBS. Infected cells were harvested by trypsinization and kept in freezing media containing 45% LM, 45% FBS and 10% dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, USA). The stocks of viruses were stored in liquid nitrogen until use. Viral stocks were diluted in LM medium to the desired concentration immediately prior to use *in vivo*.

Birds

Single comb white leghorn ADOL $15I_5$ x 7_1 chicks (Bacon, Hunt, and Cheng 2000) were used in all studies. The chicks came from breeder hens maintained in a specific-pathogen-free (SPF) facility. The mother hens were vaccinated with all three serotypes of vaccine including serotype 1 (CVI988), serotype 2 (SB-1), and serotype 3 (HVT strain FC126). Thus, all progeny were maternal antibody positive for all three serotypes. The ADOL Institutional Animal Care and Use Committee (IACUC) approved all bird experiments

Determination of percent protection, percent tumor formation, and replication of pathogenic MDV in peripheral blood mononuclear cells (PBMCs)

Seventeen (17) newly-hatched chicks per group were housed in negative pressure Horsfall-Bauer (HB) units and received the following treatments: 1. Mock vaccinated (negative control), 2. Mock vaccinated and challenged with MDV (positive control of MD), 3. Vaccinated with monovalent SB-1 and challenged with MDV, 4. Vaccinated with monovalent HVT strain FC126 and challenged with MDV, and 5. Vaccinated with bivalent (SB-1 + HVT) and challenged with MDV. When applicable, all vaccines were given intra-abdominally on the day of hatch with 2,000 plaque forming units (PFU) of SB-1 or HVT strain FC-126, or the combination of SB-1 and HVT FC-126 (1,000 PFU of each virus) in the bivalent vaccinated group. Five days after vaccination, chicks were challenged with 1,000 PFU MDV (Md5 strain) with the same route. 0.5 mL of blood was collected from 10 birds per group at 1 days post challenge (DPC) and on every week after challenge until the end of the study. PBMCs were isolated form blood using

Histopaque-1077 (Sigma-Aldrich, USA) as described in the manufacturer's protocol. For pathological determination, the birds were kept for up to 8 weeks of age or until moribund. The birds were examined via necropsy for signs of MD, including tumors and nerve enlargement, and percentage of protection was calculated as described below:

$$\% \text{ Protection} = \frac{(\% \text{ MD in unvaccinated control} - \% \text{ MD in vaccinated group})}{\% \text{ MD in unvaccinated group)}} \times 100$$

Quantifying the replication of viral vaccines and pathogenic MDV

Thirty-two (32) newly hatched chicks per group received the same treatment as described in Figure 2.4 (groups 2 to 5). The spleen, bursa, and thymus were collected from eight birds per group on 1, 5, 10, and 14 DPC and immediately stored in RNA*later* (Invitrogen, USA) at -80 °C. DNA was extracted from PBMCs and the collected tissues in Figure 2.4 using DNeasy blood and tissue kits (Qiagen, USA), respectively, following the manufacturer's instructions. The DNA concentration purity was measured using a NanoDrop (Thermo Fisher Scientific). The extracted DNA was diluted to 5 ng/µl and relative MDV DNA loads was determined by qPCR using an Applied Biosystems 7500 Real-Time PCR System. Primers and probes designed to detect for MDV gB gene are listed in Table 2.1. For qPCR, there was an initial incubation step at 50 °C for 2 mins, and 95°C for 10 mins, and followed by 40 cycles of amplification at 95 °C for 15 secs, 60 °C for 1 min. The expression of MDV gB gene in each virus was determined against Ct value of GAPDH using $2^{-\Delta Ct}$. The value was plot individually on the graph using Prism 8.0.0 (GraphPad Software, Inc.).

Table 2.1 Primers and probes used to detect replication of MDV, SB-1, or HVT with qPCR. GAPDH was used as the internal control

Primers	
Md5 gB TM.5	5'-CGGTGGCTTTTCTAGGTTCG-3'
Md5 gB TM.3	5'-CCAGTGGGTTCAACCGTGA-3'
SB1gB-TMF	5'-CAGTCCCACCCAACCGTAAA-3'
SB1gB-TMR	5'-GAGCATACCCGTCAAGCGTAA-3'
HVTgB-TMF	5'-CGGGCCATAAAACGGAATT-3'
HVTgB-TMR	5'-GGCAAAGTGGAAAGAGGTAACG-3'
GAPDH-TMF	5'-CAACGGTGACAGCCATTCCT-3'
GAPDH-TMR	5'-ATGGTCGTTCAGTGCAATGC-3'
Probes	
Md gB-TMP2	5' Cy3-CATTTTCGCGGCGGTTCTAGACGG-3' BHQ1
SB1-TMP	5’ Cy5-TGTGGAGTGACGAGGAA-3’ BHQ2
HVTgB-TMP2	5'-JOE-CTTGCCCACTCTAGCACGCAGCATT-3' BHQ1
GAPDH-TMP2	5’ FAM-CCTTTGATGCGGGTGCT-3’ BHQ-1

Evaluating the effect of bursectomy on MD vaccinal protection

Surgery was conducted on the day of hatch. Chicks were anesthetized using 3% isoflurane in a chamber with the flow rate of 1 l/min oxygen. A 0.5 cm incision was made between the cloaca and the base of the tail. The bursa was removed, and the incision was closed using tissue glue. Chicks were given 3 mg/kg of meloxicam SR for pain relief after surgery and 220 mg/gallon of tetracycline in water for the following five days. Chickens were observed twice a day after surgery until the end of the study. One day after surgery, 2,000 PFU of HVT, or 1,000 PFU of each SB-1 and HVT (bivalent SB-1 + HVT) were administered intra-abdominally to normal control and bursectomized birds (each group contained 17 birds). Five days after immunization, all birds including 17 birds from an unvaccinated control group were challenged using the same route with 1,000 PFU Md5 strain MDV. All chickens were kept for up to 8 weeks after challenge and examined for percent protection as in Figure 2.4.

Statistical analyses

The data were analyzed using an unpaired t-test using Prism 8.0.0 (GraphPad Software, Inc.).

Results

Validation of protective synergy between SB-1 and HVT vaccine

In order to understand the mechanism underlying protective synergy of SB-1 and HVT vaccines, our first experiment was to validate the model of protective synergy between SB-1 and HVT. After performing *in vivo* immunization with different vaccines (monovalent SB-1 or monovalent HVT, or bivalent SB-1 + HVT) and challenged with pathogenic MDV, the result shows that the bivalent vaccine provided greater protection compared to either monovalent vaccine validating protective synergy between the two vaccines (Figure. 2.1).

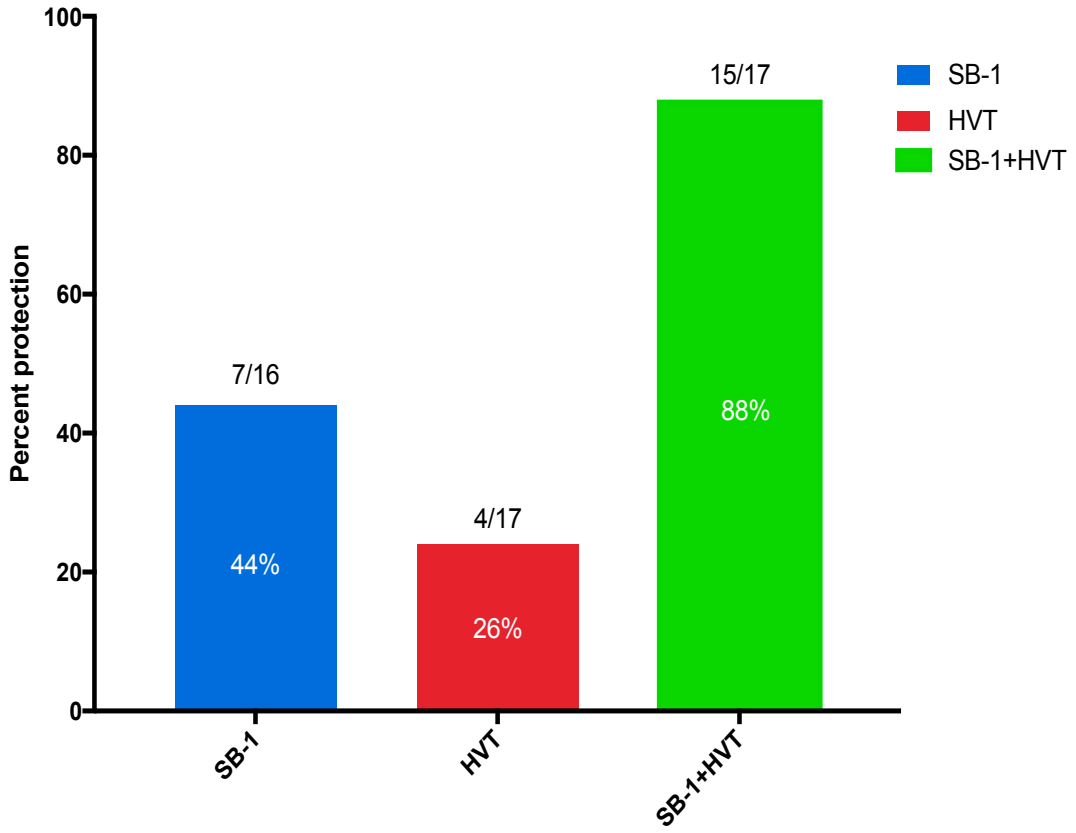

Figure 2.1. Synergistic immune protection of bivalent (SB-1+HVT) MD vaccine. Seventeen (17) chickens per group were vaccinated with 2,000 PFU monovalent SB-1, 2,000 PFU monovalent HVT, or bivalent vaccine containing 1,000 PFU SB-1 and 1,000 PFU HVT (SB-1 + HVT) at 1 day of age. At 6 days of age, all birds were challenged with 1,000 PFU MDV (Md5 strain). The chickens were kept for 8 weeks or until moribund. MD incidence was determined by scoring for nerve enlargement and tumor formation, and the percent protection of each vaccine calculated as described in the method section. The number of chickens protected out of the total challenged is given at the top of each bar graph.

Bivalent (SB-1 + HVT), but not mono-valent SB-1 or HVT, suppresses MDV replication and tumor development

With the higher protection observed for bivalent vaccine compared to monovalent vaccines, we addressed whether the higher protective efficacy correlates with the level of MDV replication in PBMCs and gross tumor incidence; birds can be positive for MD with nerve enlargement only. The relative amount of viral DNA level from 1 DPC to 7 WPC is shown in Figure. 2.2A. Specifically, both monovalent SB-1 or HVT had the ability to inhibit MDV replication up to 2 WPC compared to the unvaccinated group in which pathogenic MDV started to increase as early as 5 DPC. This inhibition of MDV replication was also observed in spleen, bursa, and thymus, on 1, 5, 10, and 14 DPC (Figure. 2.3B and 2.3C) compared to unvaccinated control (Figure. 2.3A). However, in PBMCs neither monovalent SB-1 nor HVT could maintain this low level of MDV after 2 WPC. In contrast, for birds receiving bivalent vaccine, replication of MDV remained very low during the entire length of the experiment in PBMCs (Figure. 2.2A) and all tissues (Figure. 2.3D).

Moreover, we also measured the percent of birds that developed gross tumors (Figure. 2.2B). Only birds vaccinated with bivalent SB-1 or HVT did not develop any tumors while tumors were observed in all of the other groups.

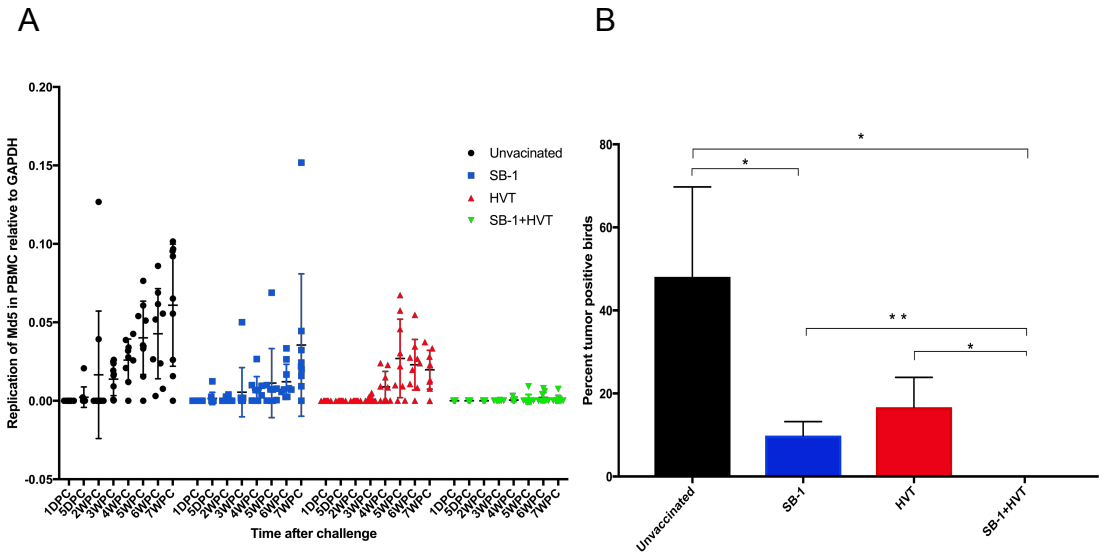

Figure 2.2. Bivalent (SB-1+HVT) can prevent MDV replication and tumor formation. Chickens were vaccinated with either SB-1, HVT, or bivalent (SB-1 + HVT) vaccine at 1 day of age and then challenged with MDV (Md5 strain) at 6 days of age. PBMC were extracted from 10 chickens on 1 day and 5 days post challenge (DPC), and every week after challenge (WPC). Replication of MDV was determined by qPCR. A. Replication of MDV in unvaccinated, SB-1, HVT, or bivalent (SB1 + HVT) is presented in black, blue, red, and green, respectively, at each time point. B. For inhibition of tumor induction, 34 chickens per group were treated as described previously and kept for up to 8 weeks and the percent tumfor incidence calculated. Statistical analysis was determined using unpair t-test with *P<0.05, **P<0.01.

Replication patterns of SB-1 and HVT vaccines differ with respect to time and tissue

To further understand the mechanism of vaccinal synergy, we monitored viral replication over time in several important lymphoid organs in the same birds described above. Results show different replication patterns for SB-1 and HVT in lymphoid tissue types at each time point (Figure. 2.4B). Specifically, SB-1 replicated well in bursa and spleen early, and increased steadily in the spleen (Figure. 2.4A). In stark contrast, HVT DNA could be detected only in the bursa at 1 DPC and no detectable virus was observed at any other time points or other tissue types through 14 DPC (Figure. 2.4B). The replication trends in term of tissue tropisms and time points of both vaccine viruses was not altered when administrated alone or in combination (Figure. 2.4A vs. 2.4C and 2.4B vs. 2.4D).

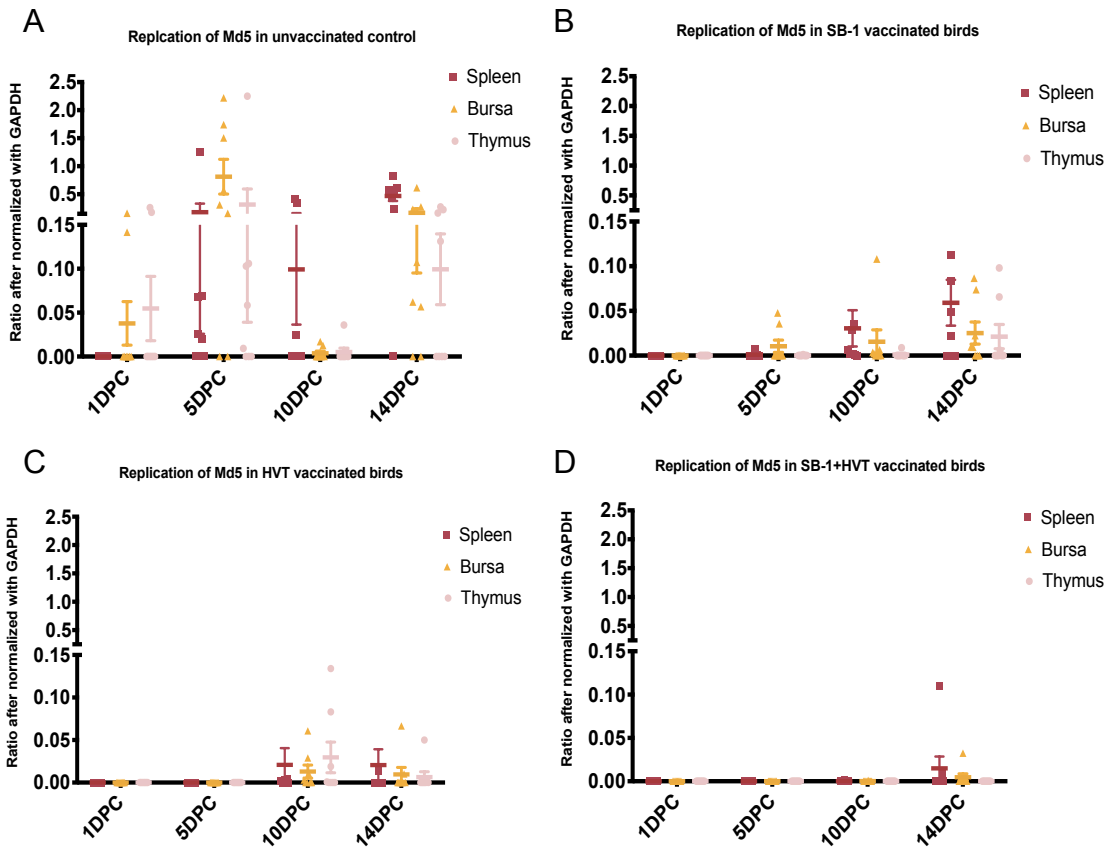

Figure 2.3. Replication pattern of MDV in unvaccinated and vaccinated birds. Replication of MDV serotype strain Md5 in spleen, bursa, and thymus were measured by qPCR at 1, 5, 10, and 14 DPC in unvaccinated control (A), SB-1 vaccinated group (B), HVT vaccinated group (C), and a bivalent (SB-1+HVT) vaccinated group (D). The data was analyzed using Ct cycle and normalized with Ct of GAPDH gene. Each dot represents the $2^{-\Delta Ct}$ value from an individual bird.

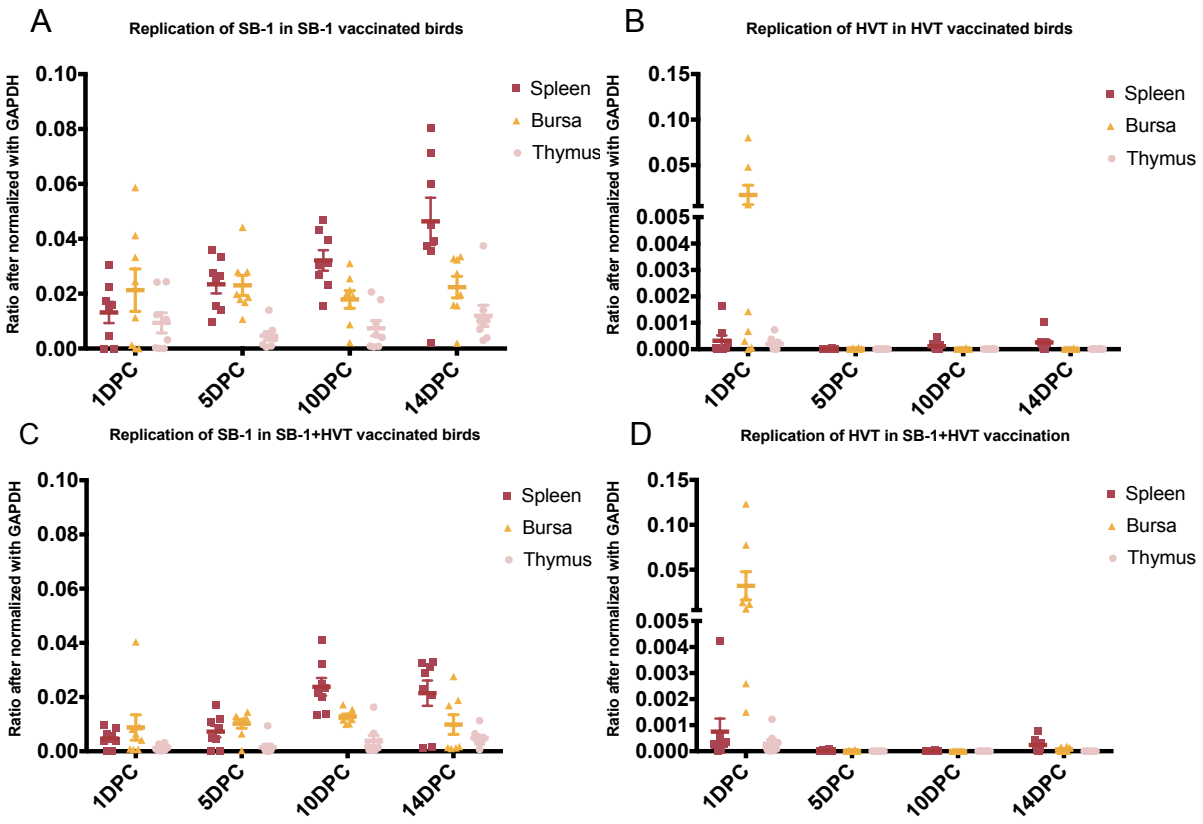

Figure 2.4. Replication patterns of SB-1 and HVT in lymphoid organs. Monovalent of SB-1 or HVT, or bivalent SB-1+HVT was administered to each group. Replication of each viral vaccine in spleen, bursa, and thymus were measured by qPCR at 1, 5, 10, and 14 DPC. A and C show replication of SB-1 in monovalent SB-1 and bivalent (SB-1+HVT) vaccinated groups, C and D show replication of HVT in monovalent HVT and bivalent SB-1+HVT vaccinated groups. The data was analyzed using Ct cycle and normalized with Ct of GAPDH gene. Each dot represents the 2-ΔCt value from an individual bird.

The bursa is necessary for protection provided by HVT vaccine

The unique replication pattern of HVT only in the bursa suggested that this organ might be necessary for HVT vaccinal protection. To confirm this hypothesis, we compared the protection of HVT or bivalent (SB-1 + HVT) vaccinated chicks with and without their bursa. Our hypothesis was confirmed in two trials. We found that the bursa is necessary for protection induced by HVT vaccine in both monovalent HVT and bivalent (SB-1 + HVT) vaccine as observed by the lower percent protection in bursectomized chickens (Figure. 2.5A and 2.5B, respectively).

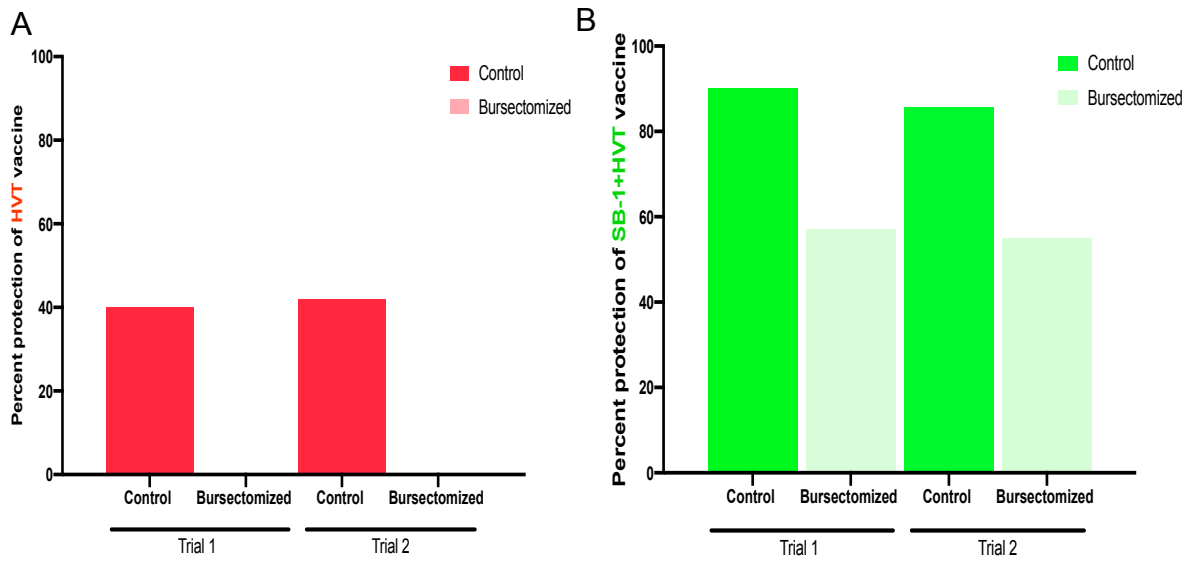

Figure 2.5. Protection of HVT and bivalent (SB-1+HVT) vaccines in normal chickens compared to bursectomized chickens. Bursectomy was performed to delete bursectomy in newly hatch chickens. Each group contained 17 chickens. Control or bursectomized chickens were immunized with HVT or SB-1+HVT and later challenged with MDV. Pathology of MD was determined and percent protection of HVT (A) or bivalent (SB-1 + HVT) (B) was calculated in control and in bursectomized chickens.

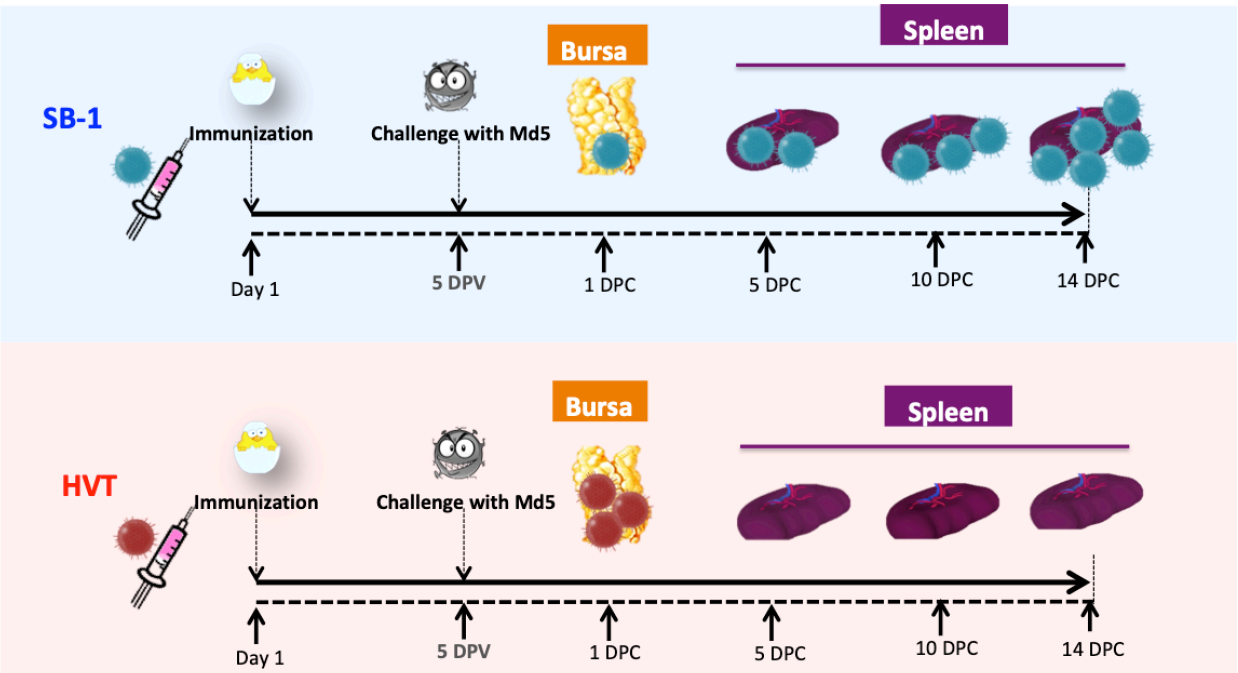

Figure 2.6. The model of protective synergy of bivalent (SB-1+HVT) vaccine. SB-1 has major replication in spleen from 1 DPC to 14 DPC, while HVT shows replication only in bursa at 1 DPC. Deletion of bursa results in the decrease of protective efficacy of HVT vaccine.

Discussion

MD vaccines have been used widely since 1970, e.g., 1 million chickens receive one or more MD vaccine each hour in the US alone. However, despite their high success in controlling tumors and other MD associated pathologies, there is a surprising lack of knowledge on how they actually protect birds against pathogenic MDV field strains. Without knowledge of the underlying mechanism of vaccinal protection, it is not possible to rationally improve MD vaccines, especially against higher virulence MDV strains that are predicted to emerge. To address this knowledge gap, we desired to get an initial understanding of vaccinal synergy using bivalent HVT + SB-1, which has repeatedly demonstrated greater protection against pathogenic MDV, especially against vvMDV strains that in laboratory conditions were not controlled by HVT only (Witter and Lee 1984; Calnek et al. 1983; Witter 1992a; Witter et al. 1985). However, there is very limited evidence that demonstrated immune patterns associated with bivalent SB-1+HVT vaccine, e.g., antibodies induced by SB-1 + HVT bivalent vaccine, provide stronger adverse effect to MDV serotype 1, which suggests that the response to bivalent vaccine is mediated by common antigens are shared between serotype 2 SB-1 and serotype 3 HVT vaccine (De Boer et al. 1986). Distinct down regulation of IL-6, IL-10, and IL-18 have been reported when vaccinated with SB-1 + HVT bivalent vaccine (Abdul-careem et al. 2007).

Pathogenic MDV are serotype 1, whereas SB-1 and HVT are apathogenic and belong to serotypes 2 and 3, respectively. The difference among the three serotypes is based on variation in gene content and transcription (Osterrieder and Vautherot 2007).

Virulent and avirulent strains co-exist and can compete in the same host (Witter, Sharma, and Offenbecker 2006). Thus, the efficacy of the monovalent SB-1 and HVT vaccine to limit MDV soon after challenge might be simply due to the competition between the vaccine strains and the pathogenic strain to replicate in the limited number of cells and tissues initially, especially since we administered the vaccine strains first.

Nevertheless, the failure of SB-1 or HVT monovalent vaccines to limit MDV replication beyond 2 WPC suggests that the competitive replication of vaccines could not overcome the ability of pathogenic MDV to start to replicate after challenge for two weeks. It also suggests that the immunity induced by either vaccine alone was inadequate to induce a long lasting anti-viral response while the immunity stimulated by both vaccines was sufficient to induce anti-viral immunity to prevent the replication of pathogenic MDV in PBMCs until the end of the study.

In our study, not only could the bivalent vaccine induce an effective anti-viral response, the anti-tumor response also seemed to be stimulated by SB-1 + HVT. Bivalent vaccine efficiently suppressed tumor development while both monovalent SB-1 or HVT vaccines were less efficient in inducing an anti-tumor response. A prior study indicated that SB-1 alone provided protection against a non-virus producing transplantable tumor JMV through a T cell dependent mechanism (K. A. Schat and Calnek 1978). Yet, anti-tumor immunity against JMV was not found in HVT vaccinated birds (Powell and Rennie 1980).

Payne and colleagues proposed a two-step hypothesis where MD vaccine protection is firstly induced through anti-viral effects and later by anti-tumor effects via cytotoxic T lymphocytes (CTL). Another model of MD vaccine protection proposed by

Schat and co-workers is a single step where MD vaccines provide only anti-viral immunity but not an anti-tumor response via cytotoxic T cells [18]. In our case, the lack of tumor development found in the bivalent vaccinated birds cannot rule out the fact that this vaccine can inhibit MDV replication in the first place, which would reduce the chance of CD4 cells becoming transformed later. The bivalent vaccine could work additively to firstly provide a competitive exclusion effect against MDV and later potentially provide an early anti-viral response to limit MDV replication, and subsequently decrease tumor induction as no MDV was detected and tumor ware not observed after this vaccination. This explanation fits well with the Schat model. There is also the possibility that bivalent vaccine could also provide both anti-viral immunity and anti-tumor response induced by SB-1 to enhance protection against MD. Determining which types of protection is generated and why one vaccine is not sufficient to control both MDV replication and tumor induction requires further experimentation and clarification.

MDV is a highly cell-associated virus that infects B cells and T cells and ultimately transforms CD4 T cells. Understanding the replication patterns of SB-1 and HVT vaccines and whether their replication patterns are similar to or different from that of pathogenic MDV could shed light on how the two vaccines work together to enhance protective response. SB-1 replicates in bursa and spleen appeared to be similar to replication patterns of pathogenic MDV. Thus, prevention of MDV replication by SB-1 could happen at the earliest step due to the fact that SB-1 and pathogenic MDV share the same tissue tropisms and time frame of replication.

Our study clearly showed that SB-1 and HVT replicate differently in terms of cell types and time after infection. In addition, the replication patterns of SB-1 and HVT vaccine are not altered by the presence of the other MD vaccine virus suggesting that the two viruses replicate independently and are not influenced by the presence of the other virus. This hypothesis is supported by a previous report that showed varying the dosage of SB-1 in bivalent vaccines does not influence viremia levels of HVT *in vivo* (Witter and Lee 1984). In addition, synergism can occur even when only 80 PFU of SB-1 is added into HVT vaccine (Bublot and Sharma 2004; Witter 2006). Although, we observed a slightly lower replication level of SB-1 in the bivalent vaccine when compared to SB-1 alone in all timepoints, the finding agreed with previous observation by Witter in 1994. He demonstrated slightly lower replication of SB-1 when it was combined with HVT (Witter, Bacon, and Calvert 1994). However, the reduction did not influence the synergistic outcome. In our study, the lower replication of SB-1 in bivalent compared to the monovalent due to the half dosage of vaccine given; despite this, synergism still occurred, proving that synergism was not simply due to the increase in replication of each vaccine but rather the cell types or time points or immune response being activated. Different replicative tropisms and time of the two vaccines could potentially increase the chance of vaccines to occupy more cells that are also targets for MDV infection, and may also induce boarder immune cell types in different time frame resulting in boarder and stronger immune response. With the different replication patterns, it was likely that SB-1 and HVT do not use the same strategy to induce vaccinal immunity. This suggests that MD vaccine synergy may be more accurately

defined as “additivity” of the two vaccines. Studies are currently underway to determine if this might occur due to the cytokines being induced.

The bursa is an important organ for the MDV life cycle (K. A. Schat, Calnek, and Fabricant 1981). B cells in the bursa are initially infected with MDV during the early cytolytic phase (Shek et al. 1983; Calnek et al. 1984; T. F. Davison, Ross, and Baigent 1998). From our study, HVT appears to replicate very early in the bursa and the presence of this organ greatly influences the level of protection induced by HVT-containing vaccines, which is in agreement with similar prior studies showing that bursectomy greatly decrease protective efficacy of HVT against MDV. The humoral immune response induced through bursa cells was proposed to be essential for MD resistance induced by HVT [19, 20]. Schat et al. also showed that the cytolytic infection of oncogenic MDV and HVT could be disturbed by embryonic bursectomy but not during the MDV latent phase, implying that the bursa was essential at the early stage of infection of MDV and HVT (Schat et al. 1981). Nonetheless, embryonic bursectomy did not interfere with SB-1 infection (Schat, Calnek, and Fabricant 1981), which may not need the bursa during its cycle. Further study of why the bursa is essential and which cell types in the bursa are responsible for HVT-induced immunity will give insights of how HVT works and why this immunity alone is not sufficient to provide protection against MDV itself.

In conclusion, SB-1 prefers to replicate in the spleen while the bursa was essential for immunity generated by HVT. The replication patterns and kinetics of MD vaccines SB-1 and HVT are different and are unaffected by the presence of the other vaccine, which suggests that the vaccines act additively either through replication

inhibition against MDV or the broadening of immune cells and types of immune response being activated. HVT induced bursa immunity combined with the protection obtained from SB-1 could synergize the protection against pathogenic MDV (Figure 2.6). The data of SB-1 induced immunity could give us more detail on the mechanism of protective synergy through the additive effect of the bivalent vaccine.

Meeting

Parts of the results from this study were presented in;

Supawadee Umthong, Hans Cheng, and John Dunn. Mechanistic insights on Marek's disease vaccinal synergy between SB-1 and HVT. The 12th International Symposium on Marek's disease and Avian Herpesviruses 2018 (Oral Presentation)

Supawadee Umthong, Hans Cheng, and John Dunn. Determining the underlying protective mechanisms of bivalent Marek's disease vaccine to prevent tumor induction. AACR Tumor Immunology and Immunotherapy 2018 (Poster B89)

Authors contribution

All authors designed the study. SU performed *in vitro* and *in vivo* experiments, collected data, and analyzed the results. JD measured and scored MD pathology during necropsy for vaccinal protection studies, and also helped to performed and monitored the condition of chickens during and after bursectomy. All authors interpreted the data and reviewed on the manuscript.

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CHAPTER 3

Depletion of CD8αβ^{+} T cells in chickens demonstrates their involvement in protective immunity towards Marek's disease with respect to tumor incidence and vaccinal protection

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Abstract

Marek's disease (MD) is a lymphoproliferative disorder in chickens caused by Marek's disease virus (MDV), a highly oncogenic a-herpesvirus. As MDV is an intracellular pathogen that infects and transforms CD4 T cells, the host cel-mediated immune response is considered to be vital for control of MD. In addition, cellular immunity through the cytotoxic CTL response is responsible for anti-viral and anti-tumor effects in other oncogenic viral diseases. In this study, we address the role of CD8 T cells in vaccinal protection by widely-used MD vaccines SB-1, HVT, and SB-1+HVT. We hypothesized that depletion of CD8 T cells would reduce the protective efficacy of MD vaccines. We found that anti-CD8 injection into chickens reduced the CD8 T cell population by 50-80%. More importantly, depletion of CD8 T cells increased MD pathology, tumor induction, and reduced vaccinal protection to MD. Our finding supports the important role of CD8 T cells for MD protection and protection by SB-1, HVT, and SB-1+HVT vaccines.

Introduction

The cytotoxic T-lymphocyte (CTL) response generated from activated $CD8^+$ T cells is an effective immune response that combats infectious pathogens (Wong and Pamer 2003). For example, studies have provided evidence that the CD8+ T cell response is associated with controlling 1) infections by intra-cellular bacteria such as *Mycobacterium tuberculosis* (Woodworth and Behar 2012; Libero, Flesch, and Kaufmann 2007; Flynn et al. 2006; Stover et al. 1991), *Salmonella* typhimurium (Mittrücker, Köhler, and Kaufmann 2002; Lo et al. 1999), *Listeria monocytogenes* (Lenz, Butz, and Bevan 2002), 2) infections by intra-cellular viruses such as herpes simplex virus type 1 (T. Liu et al. 2000; Heath et al. 2002; Coles et al. 2002), hepatitis B virus (Tsui et al. 2006; Guidotti et al. 1996), respiratory syncytial virus (Cannon, Openshaw, and Askonas 1988; Kulkarni et al. 1993), and 3) intra-cellular infection by protozoa such as *Plasmodium* (Weiss et al. 1988; Romero et al. 1989; Rodrigues et al. 1991; Khusmith, Sedegah, and Hoffman 1994), *Toxoplasma gondii* (Khan, Ely, and Kasper 1994), and *Trypanosoma cruzi* (Tarleton 1990). The $CD8^+$ CTL response functions by recognizing foreign antigens presented by the MHC class-I of antigen presenting cells, then destroying the infected cells by releasing perforin and granzyme to induce apoptosis. $CD8^+$ T cells also produce and secrete cytokines such as IFNγ that directly inhibit viral replication, induce maturation of macrophages, and activate the natural killer (NK) cell response. CD8-effector cells can also secrete chemokines to further prolong the CD8 T cell response. In herpesvirus infections, activation of the CD8+ CTL response is generally developed against viral glycoproteins, which are part of the viral envelop (J C Mester and Rouse 1991; Joseph C Mester et al. 1990; Karel A. Schat and

Xing 2000; Markowski-Grimsrud and Schat 2002).

Marek's disease (MD) is a CD4 T cell lymphoma in chickens caused by the infection of an α-herpesvirus called Marek's disease virus (MDV). MDV is a highly cell-associated virus and the spread of infection within the host is via cell-to-cell contact (Richerioux et al. 2012). Although maternal antibody has been shown to reduce the severity of MD (Calnek 1982; F. Davison and Kaiser 2004), the cell-mediated immune response is believed to be the more important than the humoral immune response as MDV generally has a minimal chance to be exposed to the host's soluble antibody. CD8 CTL have been reported to response to glycoproteins of MDV (Markowski-Grimsrud and Schat 2002). Omar reported in 1997 that SB-1 vaccine could induce a CD8+ CTL response (Omar and Schat 1997). These emphasize the importance of CD8 CTL response in protection against MDV and in vaccine induce protective immunity.

Several vaccines have been developed from attenuated serotype 1 (CVI988), serotype 2 (SB-1), and serotype 3 (HVT strain FC126) viruses to protect chickens from MD. SB-1 and HVT strain FC126 are common vaccines used commercially. These two vaccines can be used as monovalent vaccines SB-1 or HVT or in combination as bivalent vaccine (SB-1+HVT), which demonstrates greater protection compared to either monovalent SB-1 or monovalent HVT. However, none of MD vaccines thus far can clear the pathogenic MDV from the host or can prevent the shedding of pathogenic MDV.

In this study, we investigate the effect of CD8 T cells in MD protection and in vaccinal protection against MDV especially in that of bivalent SB-1+HVT vaccine. Our hypothesis was that bivalent SB-1 and HVT vaccine would induce a stronger CD8 T cell

response than monovalent SB-1 or HVT. We monitored CD8 T cell populations after immunization in normal VS depleted CD8 T cell chickens. We also measured the protection, tumor incidence, and neuro-pathology after vaccination in control compared to in CD8 depleted chickens. From this study, we provided evidence that CD8 T cells are essential for MD protection and prevention of tumor induction in unvaccinated and vaccinated chickens.

Materials and Methods

LC4 hybridoma culture

LC-4 hybridoma cells which secrete anti-chicken CD8 antibody (Kondo et al. 1990a; Kondo et al. 1990b), were used as a source to produce the antibody for *in vivo* CD8 depletion. The hybridoma cells were initially maintained in Dulbecco's Modified Eagle's Media (DMEM) with 20% Fetal Bovine Serum (FBS) supply (Thermo Fisher Scientific, USA) in a 37°C incubator with 5% CO_2 for three days. Later, the cells were passed and maintained in the lower concentration of FBS at 10% FBS for three days, then in protein-free hybridoma medium (PFHMII) supplied with 0.2% chemically defined (CD) lipid concentrate (Life Technologies, USA). The CELLine 1000 bioreactor flask for high-density suspension cells was used (Wheaton, USA) to grow the cells for large-scale antibody production for up to three months.

Purification and characterization of anti-chicken CD8 mAb

The LC-4 hybridoma culture supernatant was collected every 3-4 days depending on the density of the cells. Proteins in the culture supernatant were precipitated by the gentle addition of saturated ammonium sulfate solution (Thermo Fisher Scientific, USA) until the solution became turbid. The solution was centrifuged at 3,000g for 30 minutes at 4°C. The pellet was resuspended in a small volume of phosphate buffer saline (PBS) and dialyzed against PBS overnight at 4°C using Slide-A-Lyzer 10K dialysis cassettes (Thermo Fisher Scientific, USA). The resulting antibody was measured for concentration using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, USA) and further analyzed by Coomassie staining and western blot. For the latter, the blot was probed

with goat anti-mouse IgG HRP, washed, and visualized using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, USA).

Characterization of anti-CD8 mAb binding activity

Anti-chicken CD8 antibody was tested for its specific capacity to bind to chicken CD8 T cells by immunohistochemistry and flow cytometry. For Immmunohistochemistry, splenic and thymic samples from $15I_5x7_1$ chickens were cut and embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, USA) and kept at -80 °C until use. The tissue then cut using a cryostat microtome HM505 Microm and fixed on tissue slides with acetone. The fixed tissues were stained with 1:100 of anti-chicken CD8 mAb and immunohistochemistry staining was performed using VECTASTAIN Elite ABC HRP mouse IgG kit and ImmPACT DAB peroxidase (HRP) substrate (Vector laboratory, CA, USA) kit. The stained sections were observed under a microscope. For flow cytometry, peripheral blood mononuclear cells (PBMCs) were purified from whole blood of one of the chickens above using Histoplaque-1077 (Sigma Aldrich, USA). The PBMCs were stained with 1:100 of anti-chicken CD8 mAb, washed, and stained with Alexa Fluor 488 goat anti-mouse IgG (H+L) (Life Technologies, USA), and detect using flow cytometry.

Optimization of route and dosage for CD8 depletion in vivo

The protocol for antibody depletion was optimized to compare routes of injection between intra-peritoneal (IP) vs. intra-venous (IV)) and dosage of injections (1 or 3 mg of anti-chicken CD8 mAb). Newly hatched $15I_5 \times 7_1$ antibody positive chickens were divided into five groups each with 5 birds per group: 1) control (no injection), 2) 1 mg

anti-chicken CD8 mAb by IP, 3) 3 mg anti-chicken CD8 mAb by IP, 4) 1 mg anti-chicken CD8 mAb by IV, and 5) 3 mg anti-chicken CD8 mAb by IV. Blood was collected at 1 day post challenge (DPC), and 1, 2, 3 and 4 week post challenge (WPC). PBMCs were stained for $CD8\alpha\beta^+$ T cells using mouse anti-chicken CD8α-FITC and mouse anti-chicken CD8β-PE (Southern BioTech, USA) and analyzed by flow cytometry.

Determination of $CD3^+CD4^+$ T cells and $CD8\alpha\beta^+$ T cells in control and CD8-depleted chickens after vaccination

Spleens collected from five birds/group from unvaccinated, SB-1 vaccinated, HVT vaccinated, and bivalent vaccinated groups in both chickens and CD8-depleted chickens were homogenized and stained for $CD3^+CD4^+$ and $CD8\alpha\beta^+$ T cell populations. Mouse anti-chicken CD3 Alexa Fluor 647, CD4 PE, CD8α FITC, CD8β-SPRD monoclonal antibodies from Southern Biotech, USA were used for multi-color flow cytometry detection.

Measurement of vaccinal protection in control and CD8-depleted chickens

The efficacy of vaccines to protect chickens from MDV was tested *in vivo*. Newly hatched chickens were divided into ten groups as described in Table 3.1. These groups are: 1) unvaccinated and unchallenged control, 2) CD8 depleted-unvaccinated and unchallenged control, 3) unvaccinated-Md5 challenged control, 4) SB-1 vaccinated-Md5 challenged, 5) HVT vaccinated-Md5 challenged, 6) SB-1+HVT vaccinated-Md5 challenged, 7) CD8 depleted-unvaccinated-Md5 challenged control, 8) CD8 depleted-SB-1 vaccinated-Md5 challenged, 9) CD8 depleted-HVT vaccinated-Md5 challenge, and

10) CD8 depleted-SB-1+HVT vaccinated-Md5 challenged. Chickens were vaccinated with 2,000 PFU of monovalent SB-1 or 2,000 PFU of monovalent HVT, or bivalent SB-1+HVT (1,000 PFU each) on the first day of age. For CD8 depletion, 1 mg (100 µL) of anti-chicken CD8 mAb was administered by IV injection (jugular vein) using a BD ultra-fine insulin syringe 31G needle on the days 2, 3, and 4 of age. Chickens were then challenged with 1,000 PFU of pathogenic MDV (strain Md5). Anti chicken CD8 mAb was injected weekly with the same dose and same route after challenge until the end of the study (eight weeks). Percent protection of the vaccine was calculated based on the formula below;

$$\% \text{ Protection} = \frac{(\% \text{ MD in unvaccinated control} - \% \text{ MD in vaccinated group})}{\% \text{ MD in unvaccinated group)}} \times 100$$

In addition, percent tumor induction was measured based on the number of chickens that develop tumor in each vaccination compared to the control group.

Table 3.1. Experimental scheme to measure the effect of CD8 T cell on protection of SB-1, HVT, and SB-1+HVT vaccine

Group	Vaccine	CD8 depletion	Challenge (day 6)	Number of chickens
1	Unvaccinated	-	-	17
2	Unvaccinated	Yes	-	17
3	Unvaccinated	No	1,000 PFU of Md5	17
4	SB-1	No		17
5	HVT	No		17
6	SB-1+HVT	No		17
7	Unvaccinated	Yes	1,000 PFU of Md5	17
8	SB-1	Yes		17
9	HVT	Yes		17
10	SB-1+HVT	Yes		17
Total number of chickens				170

Results

Production of anti-chicken CD8 mAb by culturing LC-4 hybridoma cells

To avoid using animals, anti-chicken CD8 mAb was produced by culturing LC-4 hybridoma cells in CELLine 1000 bioreactor flasks, which was able to yield up to 100 mg of the antibody per week. We checked the purity of the antibody by Coomassie staining as shown in Figure 3.1A. The heavy chain (50 kDa) and the light chain (25 kDa) of antibody was confirmed by Western blot as shown in Figure 3.1B.

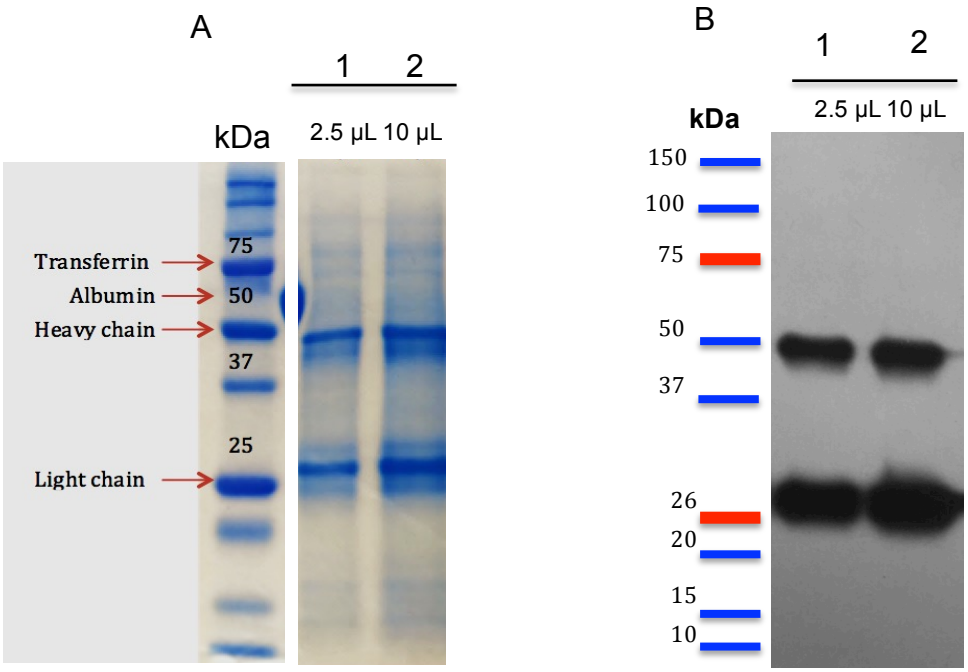

Figure 3.1. Production of anti-chicken CD8 mAb by LC-4 hybridoma culture. Lane 1: 2.5 µL loading of culture supernatant, lane 2: 10 µL loading of culture supernatant. The purity of the protein is shown by Coomassie staining (A). Heavy chain at 50 kDa and light chain at 25 kDa of IgG of the antibody were detected by Western blotting (B).

Binding activity of anti-CD8 mAb to chicken CD8 T cells

Prior to our *in vivo* studies, the binding activity of anti-CD8 mAb was confirmed by immunohistochemistry staining for CD8 T cells in spleen and thymus, and by flow cytometry staining for CD8 T cells in chicken PBMCs. The results confirmed that the anti-chicken CD8 mAb specifically bound CD8 T cells in the spleen (Figure 3.2B) and in thymus (Figure 3.2D) compared to the no antibody staining controls (everything except anti-chicken CD8 mAB) in Figure 3.2A and 3.2C, respectively. Anti-chicken CD8 mAb also shows the binding activity to CD8 T cells in total PBMCs obtained from blood (Figure 3.2E).

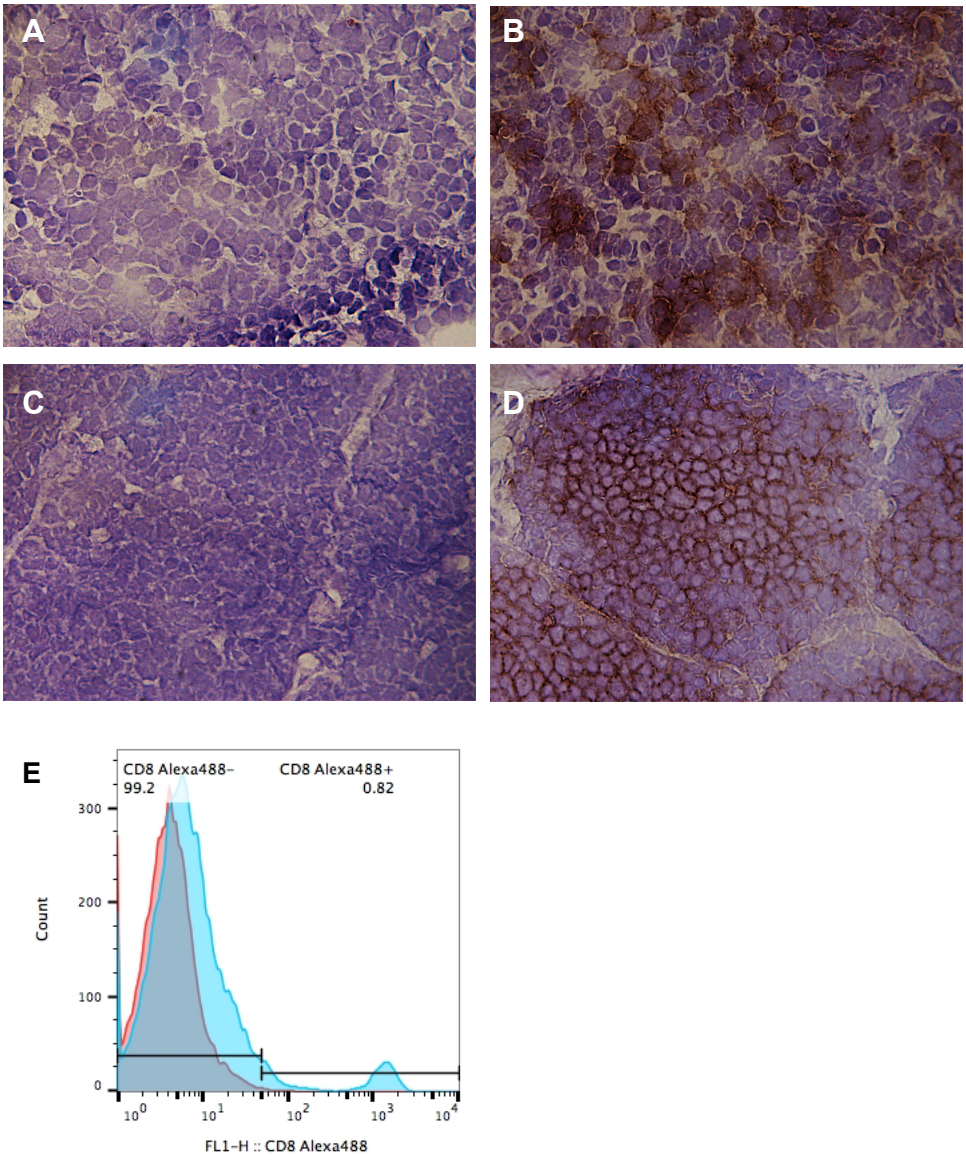

Figure 3.2. Binding activity of anti-CD8 mAb. The binding was observed in chicken CD8 T cells in spleen (A and B), and thymus (C and D), using Immunohistochemistry; control (A and C), with anti-CD8 mAb (B and D). The binding of anti-CD8 mAb also confirm in chicken PBMCs by flow cytometry (E).

IV injection is the better route of injection to deplete chicken CD8 T cells using anti-chicken CD8 mAb

After confirming the anti-CD8 mAb-binding efficacy, the antibody was then used to inject chickens to deplete the CD8 T cell population. We tested the protocol to deplete CD8 T cells by varying the dose and route of injection. Our results indicated that 1 mg injection through IV was a feasible and effective way to reduce the level of CD8 T cells, and the reduction remained up to four weeks post injection (WPI) as showed in Figure 3.3.

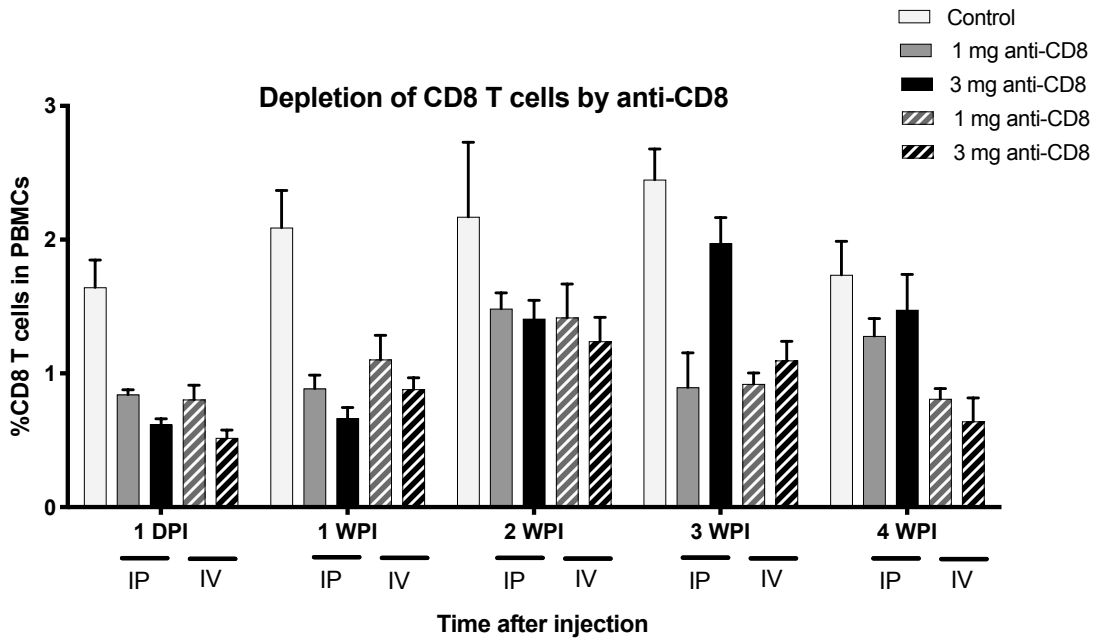

Figure 3.3. Depletion of CD8 T cells antibody injection. PBMC were extract from whole blood and flow cytometry was used to determine percent of CD8 T cells in PBMC. Each bar of each color represents percent of CD8 T cells in whole blood in control, 1 mg anti-CD8 IP, 3 mg anti-CD8 IP, 1 mg anti-CD8 IV, and 3 mg anti-CD8 IV injections, respectively.

Levels of $CD3^+CD4^+$ and $CD8\alpha\beta^+$ T cells in normal and CD8 depleted chickens after vaccinations

We measured levels of $CD3^+CD4^+$ cells to confirm that the anti-chicken CD8 mAb injected specifically binds only to $CD8\alpha\beta^+$ T cells but not CD4 T cells in live birds. Total splenocytes were collected and stained for those T cell markers. The level of CD4 and CD8αβ were monitored 1-day post challenge (DPC) to 7 weeks post challenge (WPC). The results show that levels of $CD3^+CD4^+$ T cells were not changed by the injection of anti-chicken CD8 mAb in all vaccinations (Figure 3.4A). Injection of anti-chicken CD8 mAb decreased the level of $CD8\alpha\beta^+$ T cells 2-4 times compared to control without antibody injection and this happened in all treatments including unvaccinated control, SB-1, HVT, and bivalent SB-1+HVT vaccination. However, the levels of $CD8\alpha\beta^+$ T cells can be depleted only up to four weeks post infection as the levels were about the same as control after 4 WPC (Figure 3.4B).

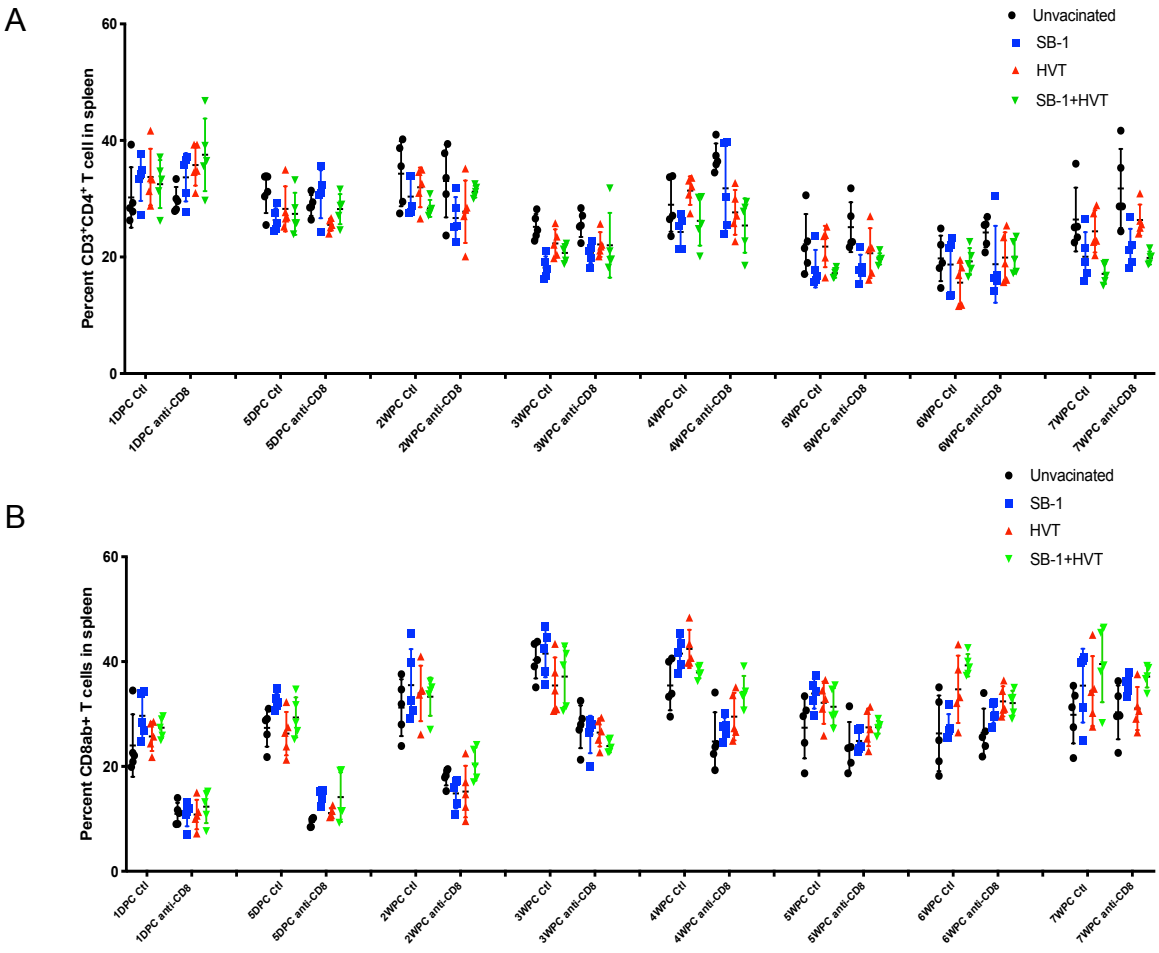

Figure 3.4. Levels of $CD3^+CD4^+$ and $CD8\alpha\beta^+$ T cells after anti-chicken CD8 mAb injection Levels of $CD3^+CD4^+$ (A) and $CD8\alpha\beta^+$ T cells (B) in control and in CD8 depleted groups in all types of vaccination observed by flow cytometry.

CD8+ T cells play an important role for MD resistant and vaccinal protection

In this experiment, we investigated whether the CD8 T cell response contributes to MD resistance, in general, and vaccinal protection induced by SB-1, HVT, and SB-1+HVT vaccines. The results show protection in the SB-1, HVT, or SB-1+HVT vaccinated groups in both with and without CD8-T cell depletion (Figure 3.5A). As shown, depletion of CD8 cells reduces the protective level of SB-1 and HVT monovalent vaccines as well as that of bivalent vaccine (Figure 3.5A). In addition, depletion of CD8 T cells increases tumor incidence in unvaccinated and the monovalent vaccinated group but none of the chickens in bivalent vaccinated group developed tumors in both control CD8 or depleted CD8 groups (Figure 3.5B). For MD pathology observed from nerve lesion scores, higher sciatic lesions were observed when CD8 T cells were depleted in all treatments including the unvaccinated-challenged control and all vaccinations (Figure 3.5C). Interestingly, brachial and vagal lesions remained the same in the unvaccinated group when CD8 cells were depleted but the lesions were increased in vaccinated groups especially in those with HVT and SB-1+HVT vaccination (Figure 3.5D and 3.5E). These results indicate that CD8 T cells are involved in protection against MD infection and also involved in vaccinal protection by SB-1 and HVT vaccines. Moreover, our results show that depletion of CD8 T cells increases nerve lesions after vaccinal protection by HVT and bivalent (SB-1+HVT).

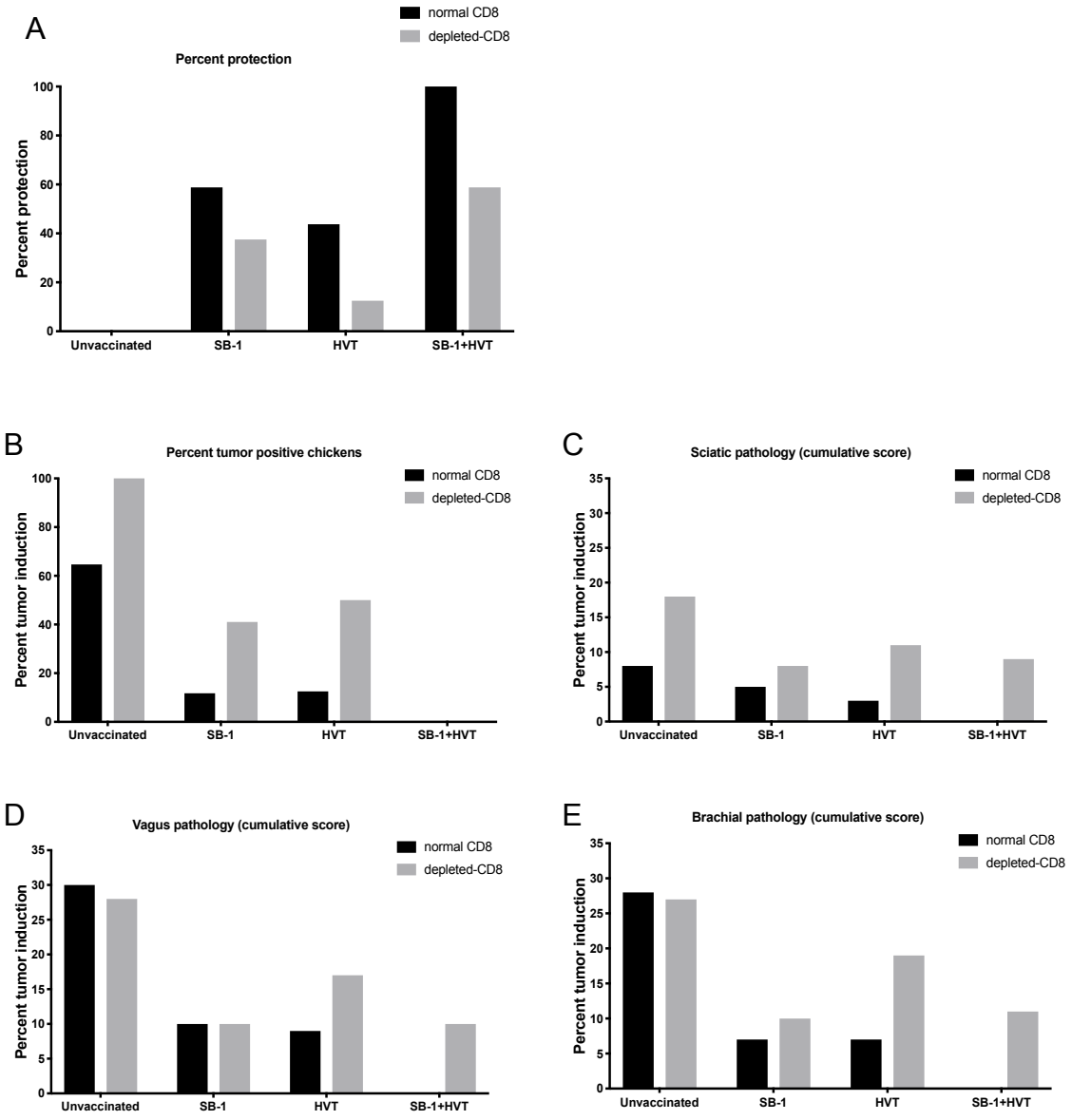

Figure 3.5. Effect of CD8 T cells on MD pathology and protection of vaccines in chickens immunized with SB-1, HVT, or SB-1+HVT vaccines in control chickens VS CD8-depleted chickens. Percent protection and percent tumor development were measured between vaccinated groups compared to unvaccinated control. Both measurements were determined during necropsy.

Discussion

The CD8+ CTL response is considered as an effective immune response against intracellular pathogens and cancer. Given the cell-associated and oncogenic nature of MDV, the CD8 T cell response should be necessary for MD protection. Prior studies on MD vaccines showed that CD8 T cells were induced upon vaccinations and the induction of CD8 T cells correlated with vaccinal protection (Morimura et al. 1999; Morimura et al. 1998; Schat 1987). In this study, we investigated whether the CD8 T cell response was involved in vaccinal protection and the response levels correlated with level of protection.

First, in order to minimize the use of animals, we produced anti-chicken CD8 mAb by large-scale culturing of LC-4 hybridoma cells *in vitro* instead of producing antibody in mice through the ascites induction method (Yokoyama 2001). LC-4 hybridoma cells secreted monoclonal antibody against chicken CD8 T cells and antibody produced bound specifically to chicken CD8 T cells in spleen, thymus, and also in PBMCs as proven by immunohistochemistry and flow cytometry. The optimization of route of injection indicated that IV injection is more effective than IP injection. 1 mg via IV injection was the procedure to use to deplete CD8 T cells.

We measured the levels of $CD3^+CD4^+$ and $CD8\alpha\beta^+$ T cells. In all vaccinated groups, the levels of CD4 T cells were not different in control vs. CD8 depleted birds ensuring that anti-CD8 injection did not interfere with the level of $CD4^+$ T cells. This result agrees with the results of Kondo in 1990 (Kondo et al. 1990a). However, we observed that the level of CD4 T cells are slightly higher in

CD8 T cells depleted group compared to the normal chickens, in unvaccinated-challenged group, after four weeks post challenge. This could be due to the higher lymphoma induction in the CD8 depleted group implying that CD8 T cells are involved in controlling lymphoma development in MDV infected chickens. Antibody injection depleted CD8 T cells up to around 4 WPC as observed by the reduction of $CD8^+$ T cells after challenge to around 4 WPC. This could be explained in several ways. First, the chickens became bigger and the injection of 1 mg of antibody after 4WPC was not enough to be able to deplete the levels of $CD8^+$ T cells. Second, all chickens were challenged and some were vaccinated. These may cause the strong induction of CD8 T cells to the level that the antibody injection could not reduce.

Reduction of percent protection in unvaccinated group, monovalent SB-1 vaccinated group, and bivalent vaccinated group was observed in CD8-depleted chickens indicating that CD8 T cells are involved in MD resistance from both infection and vaccination. Higher pathological scores and percent tumor induction in the unvaccinated group and monovalent vaccinated group were observed when CD8 T cells were depleted. However, we did not observe higher tumor incidence in SB-1+HVT vaccinated group. This suggests that SB-1+HVT vaccine can induce a strong anti-tumor response and the response likely requires fewer CD8 T cells. Rather, depletion of CD8 T cells plays a role in the anti-viral response in bivalent vaccination as shown by the reduction of protection after CD8 T cells were depleted. CD8 T cell depletion also affected levels of vagus nerve lesions in HVT and SB-1+HVT vaccinated groups.

CHAPTER 4

Induction of anti-viral cytokines, suppression of TNF-β, and activation of IFN-γ are associated with protective synergy of Marek's disease SB-1 and HVT bivalent vaccine

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Abstract

Enhancement of the immune response through the cooperation of two or more cytokines has been term “cytokine synergy.” In Marek’s disease (MD), a lymphoproliferative disease of chickens caused by the oncogenic Marek’s disease virus (MDV), enhancement of vaccinal protection has been observed with MD vaccines SB-1 and herpesvirus of turkey (HVT). Hence, we hypothesized that cytokines specifically induced by SB-1 or HVT vaccination are likely to account for the protective synergy of the SB-1 + HVT bivalent vaccine. To address this, we surveyed over time cytokine gene expression levels in chicks that were vaccinated at hatch with one or both vaccines and challenged with MDV at 5 days of age. Our results show early activation of IFN type-I (IFN-α and IFN-β) was detected in response to SB-1-vaccination. Moreover, SB-1 alone also induced a broad range of other cytokines including pro-inflammatory cytokines (IL-1β), Th2 cytokines (IL-5), and T cell proliferation cytokines (IL-21) at 4 days post-vaccination (DPV). In HVT vaccinated birds, there was significant suppression of transforming growth factor, beta three (TGF-β3) and beta four (TGF-β4). Significantly, we detected the up-regulation of IFN-β and IFN-γ at 10 days post challenge (DPC) in the bivalent vaccinated group. This study suggests that early induction of innate anti-viral cytokines by SB-1, suppression of TGF-β by HVT, and induction TFN type-I and II is important to provide an effective response against MDV and provides the foundation for future rationally-designed MD vaccines.

Introduction

Cell signaling through cytokines is one of the host's immunological means to fight infection or to respond to vaccination. Cytokines secreted by infected cells or immune cells such as macrophages, B cells, CD4 T helper cells (Th), CD8 T cells, and natural killer (NK) cells, orchestrate particular host immune responses. Thus, specific cytokine patterns that respond to infection or vaccination in specific organs at specific times are critical in determining the final immunological outcome. Cytokine immunotherapy is one strategy to treat patients with infection or disease as described in the review by Steinke et al in 2008. Specifically, cytokine treatment has shown to be a promising and effective way to improve chronic infections (Hübel, Dale, and Liles 2002; Finter 1994), autoimmune disease (Moudgil and Choubey 2011), and cancer (Waldmann 2018; Mocellin et al. 2010; Golomb et al. 1986). Moreover, cytokine profiling can be used as a potential marker for disease diagnosis (Elmarakby et al. 2010) or prognosis (Rotstein 2014; Bozkurt 2000).

Cytokine synergy is the concept describing the enhancement of specific cytokine functions when more than one cytokine are working together. Bartee and McFadden described the concept of cytokine synergy in anti-viral responses in that two or many cytokines can work together to promote anti-viral effect via 1) cooperative action; when two different cytokines provide the same type of anti-viral response and the response is more strongly induced when the two cytokines are together, 2) independent action; when two different cytokines provide different types of anti-viral response maybe from different pathways and both

induced responses provide a stronger anti-viral effect together, or 3) cooperative induction; when two different cytokines work together to generate special signal that cannot be obtained by one cytokine to induce a specific anti-viral response (Bartee and McFadden 2013) (Figure 4.1).

Many studies have demonstrated the effect of cytokine synergy in anti-viral responses. For example, the combination of IFN type-I or type II with tumor necrosis factor (TNF) restricts replication of varicella zoster virus (Mestan et al. 1988) and severe acute respiratory syncytia virus (Scagnolari et al. 2007).

Information regarding cytokine responses to vaccination has been studied for several diseases. However, the patterns of cytokine response to Marek's disease (MD) vaccines have not been fully explored. MD is a contagious disease in chickens caused by the infection of an α-herpesvirus called Marek's disease virus (MDV). Infected chickens develop several symptoms including neurological symptoms, oncogenic symptoms, and immunosuppression. Control of MD since the 1970s has been through widespread vaccination. A popular MD vaccine is the combination of MDV serotype 2 strain SB-1 and herpesvirus of turkey (HVT) strain FC126, which show greater ability to protect chickens from MD compared to either vaccine alone (Witter and Lee 1984). The ability of bivalent SB-1+HVT vaccine to induce better protection is called protective synergy of the MD bivalent vaccine. However, the mechanism underlying the protective synergy of the two vaccine strains has never been well explored, especially with respect to the cytokine response.

In this study, we hypothesized that cytokines specifically induced by SB-1 or HVT vaccination are likely to account for the protective synergy of the SB-1 + HVT bivalent vaccine. Experimentally, we profiled cytokines in chicks that were vaccinated by at hatch with one or both vaccines and challenged with MDV at 5 days of age. These cytokines included those in the IFN family (IFN-α, IFN-β, and IFN-γ), pro-inflammatory cytokines (IL-1β, IL-6, and IL-17A), IL-10 family cytokines (IL-19, IL-22, and IL-26), Th1 cytokines (IL-12 and IL-23), T cell proliferation cytokines (IL-2, IL-15, and IL-21), TGF family cytokines (TGF-β2, TGF-β3, and TGF-β4), Th2 cytokine (IL-5), CSF family cytokines (CSF-1 and CSF-2) and other cytokines involving in anti-tumor response (BAFF and FASL).

In our study, we found that SB-1 could induce anti-viral cytokines as well as cytokines that activate IFN-γ while HVT suppresses cytokines that inhibit IFN-γ production. Thus, we believe that SB-1 promotes cytokine pathways that induce IFN-γ expression and HVT inhibits cytokines that block IFN-γ activation. The cooperative action of SB-1 and HVT vaccines in promoting IFN-γ induction resulted in strong induction of IFN-γ by bivalent SB-1+HVT vaccine.

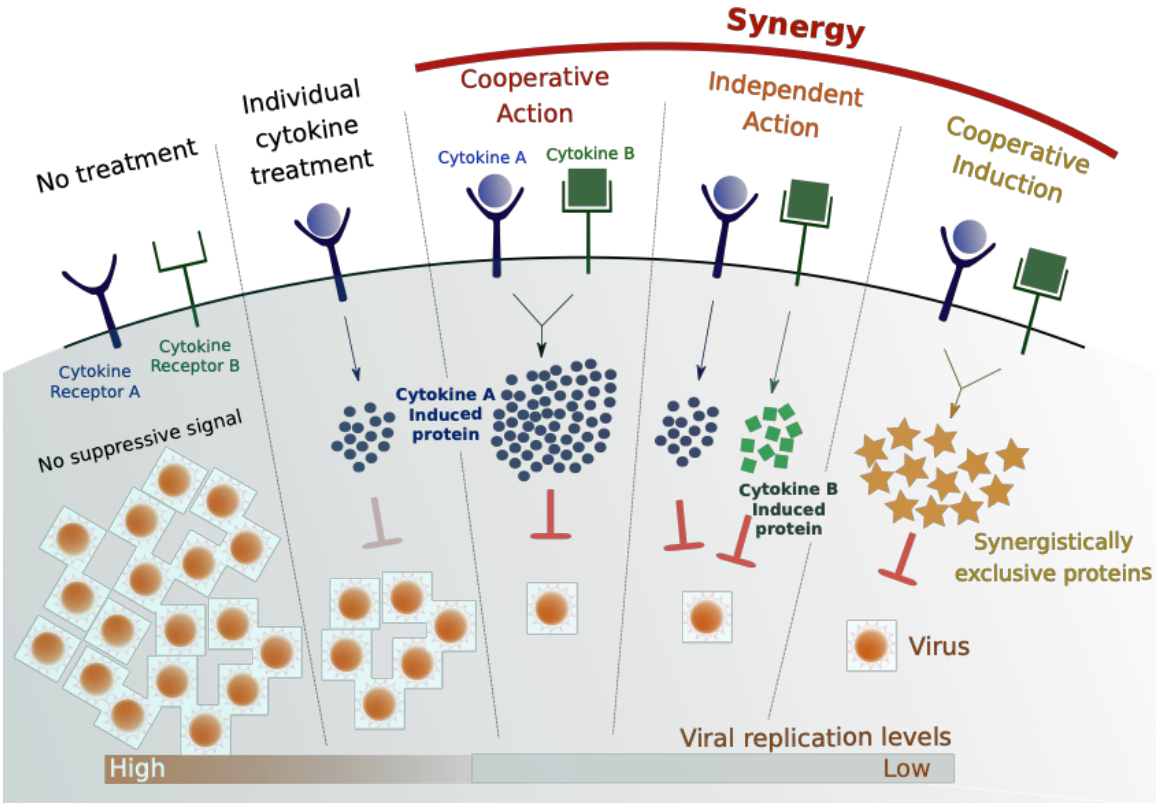

Figure 4.1. Mechanisms of cytokine synergy for anti-viral response. Two or more than two cytokines can work together to provide a synergistic effect for an anti-viral response through three or more distinct mechanisms including 1) synergy by cooperative action; when two cytokines that provide the same anti-viral signal are induced together to provide a stronger anti-viral response, 2) synergy by independent action; when different cytokines provide different signals to control viral replication, and 3) synergy by cooperative induction; when two different cytokines provide a special signal that cannot be obtained from one cytokine alone and this signal helps to control viral replication.

Materials and methods

Viruses

Chicken embryonic fibroblasts (CEF) and duck embryonic fibroblasts (DEF) were used to grow vaccine viruses and challenge virus. Cells were cultured in 1:1 mixture of Leibovitz's L-15 and McCoy's 5A (LM) medium supplemented with 4% fetal bovine serum (FBS) from HyClone (Thermo Fisher Scientific, USA), 20 µg/ml streptomycin, 200 U/ml penicillin (Sigma Aldrich, USA), and 2 µg/ml amphotericin B (Thermo Fisher Scientific, USA). Cells were maintained in a 37 °C incubator with 5% CO_2. Pathogenic MDV serotype 1 strain Md5 was grown in DEF until plaques were observed, while serotype 2 MDV strain SB-1 and HVT stain FC126 were cultured in CEF. Infected cells were harvested by trypsinization and the viruses were stored in liquid nitrogen. Plaque titers were determined before use for *in vivo* experiments.

Plaque reduction test

20 µL of serum from individual bird (10 birds per group) that were vaccinated with HVT, and bivalent (SB-1+HVT) was diluted 1:10 (total volume is 200 µL) with LM media and incubated with 200 µL of 1:100 dilution of 2 $x10^3$ MDV in DEF cells at 37 °C for 1 hour, and then plated onto a monolayer of CEF cells. The culture was maintained in 37°C with 5% CO_2 with LM medium containing 1% FBS for five days. Plaque numbers were counted and the data were plotted using GraphPad Prism software version 8.0.0.

In vivo experiment and tissue collection

The birds used were USDA, ARS Avian Disease & Oncology Laboratory (ADOL) line $15I_5x7_1$ white leghorn chickens, a F_1 hybrid cross of MD susceptible line $15I_5$ males and line 7_1 females (Bacon, Hunt, and Cheng 2000). Chicks were from maternal antibody positive (ab+) hens, which had been vaccinated with 1,000 plaque forming units (pfu) each of HVT and SB-1 at hatch followed by CVI988/Rispens at 25 weeks of age for exposure to all three serotypes. A single hatch of chicks was separated into four groups of 20 chicks per group: 1) unvaccinated, 2) SB-1 vaccinated, 3) HVT vaccinated, and 4) bivalent (HVT and SB-1) vaccinated. All vaccines were administered intra-peritoneally (IP) at hatch with 2,000 PFU for monovalent SB-1 or HVT, or 1,000 PFU of each type for the bivalent vaccine. Five days later, the birds were challenged using the same route with 1,000 PFU pathogenic MDV strain Md5. Spleens were collected from five chickens per group on 4 days post vaccination (DPV) (before challenged), and 1, 5, and 10 days post challenge (DPC), and stored in RNA*later*TM stabilizer solution (Thermo Fisher Scientific, USA) at -80°C until use.

RNA extraction

RNA extraction was performed using Qiagen RNeasy kit (Qiagen, USA). The concentration of each sample was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific) and diluted to 100 ng/µL. The integrity of RNAs was determined by visualization on a 1% agarose gel. 1 µg RNA of each sample was later converted into cDNA using the High-Capacity

cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). cDNA was used for qPCR amplification to detect cytokine expression levels.

Primers and qPCR

Key chicken cytokines were determined by qPCR using previously published primers (see **Table 4.1**). The primers were ordered from Integrated DNA Technologies, Inc. (Coralville, IA). qPCR was performed using EVAGREEN dye from Biotium, Inc. (Fremont, CA) with 40 cycles at 95 °C for 15 secs and 60 °C for 1 min. Cycle number (Ct) was used for the data analysis.

Data analysis

For each cytokine, the Ct values were normalized to the Ct values of the 28S gene using the $2^{(-\Delta Ct)}$ method, where ΔCt is the difference between Ct of cytokine gene and Ct of 28S gene. Statistical significant was determined using an unpaired-t test comparing vaccinated and unvaccinated groups at each time point.

Results

Serum of HVT and bivalent vaccinated chickens reduces MDV plaque number

Significant reduction of MDV plaques was observed when MDV infected CEF cells were incubated with serum from chickens that were vaccinated with HVT or bivalent SB-1+HVT **(Figure 4.2)**. In addition, bivalent-vaccinated serum had the highest ability to decrease the number of MDV plaques followed by HVT-vaccinated serum. This result pointed out that soluble substance in the serum of bivalent (SB-1+HVT)-vaccinated birds provides effector functions to either destroy MDV or to diminish the replication of MDV.

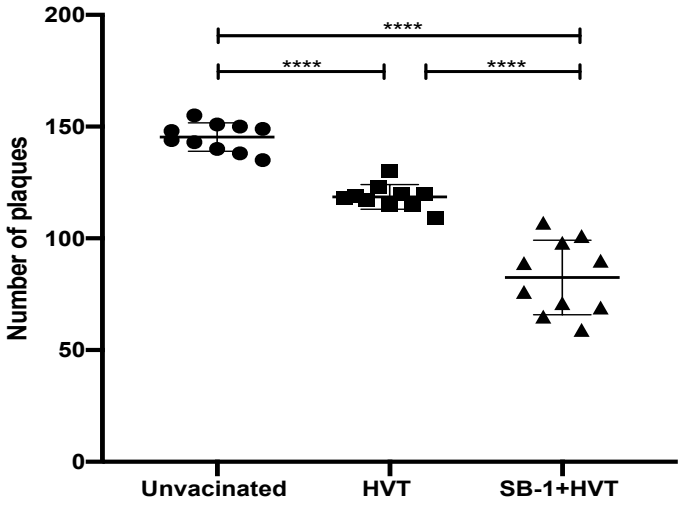

Figure 4.2. Reduction of MDV plaque numbers by serum from HVT and bivalent (SB-1+HVT) vaccinated chickens. MDV infected DEF cells were incubated with serum collected from HVT or SB-1+HVT vaccinated chickens and plated on DEF monolayer and maintained for five days. Numbers of plaques were counted. Individual plot represents plaque number observed when treated with serum from individual chicken. **** = $p < 0.0001$.

SB-1 induces anti-viral cytokines at early time point 4 DPV in spleen

Cytokines that were induced by SB-1 vaccination ($p<0.05$) include type 1 IFN (IFN-α and IFN-β), pro-inflammatory cytokine (IL-1β), T cell proliferation cytokine (IL-21), and Th2 cytokine (IL-5) at the earliest time point of detection (4 DPV) before the chickens were challenged with MDV (Figures 4.3A, 4.3B, 4.3D, 4.3G, and 4.3H, respectively). At this time point, chickens were not exposed to pathogenic MDV. So, the cytokine responses were solely the responses generated upon vaccination. We did not detect significant up-regulation or down-regulation of any cytokines in HVT or bivalent SB-1+HVT vaccination at this time point (Supplementary Figure 4.3.S1 - 4.3.S9). However, bivalent SB-1+HVT vaccine provided the same up-regulation trends of IFN-α and IFN-β as found in SB-1 vaccine, yet the expression levels were not significant compared to the unvaccinated group ($p = 0.11$ for IFN-α and $p = 0.30$ for IFN-β).

HVT suppresses TGF-β family cytokines

At 1 DPC, there was no significant up-regulation of any cytokines in SB-1 and SB-1+HVT relative to unvaccinated group. However, TGF-β3 and TGF-β4 were significantly decreased ($p=0.026$ for TGF-β3 and $p=0.014$ for TGF-β4) (Figure 4.3E and 4.3F) upon HVT vaccination at this time point (1 DPC). Nevertheless, we did not see significant down regulation of TGF-β3 and TGF-β4 in the bivalent (SB-1+HVT) vaccinations ($p = 0.49$ for TGF-β3, and $p = 0.38$ for TGF-β4).

Bivalent SB-1+HVT vaccine activates IFN-β and IFN-γ response

We detected a significant induction of IFN-β and IFN-γ at 10 DPC (Figure 4.3D and 4.3C) in bivalent SB-1+HVT vaccinated group. At this time point, all vaccinated groups were vaccinated for 15 days (10 DPC).

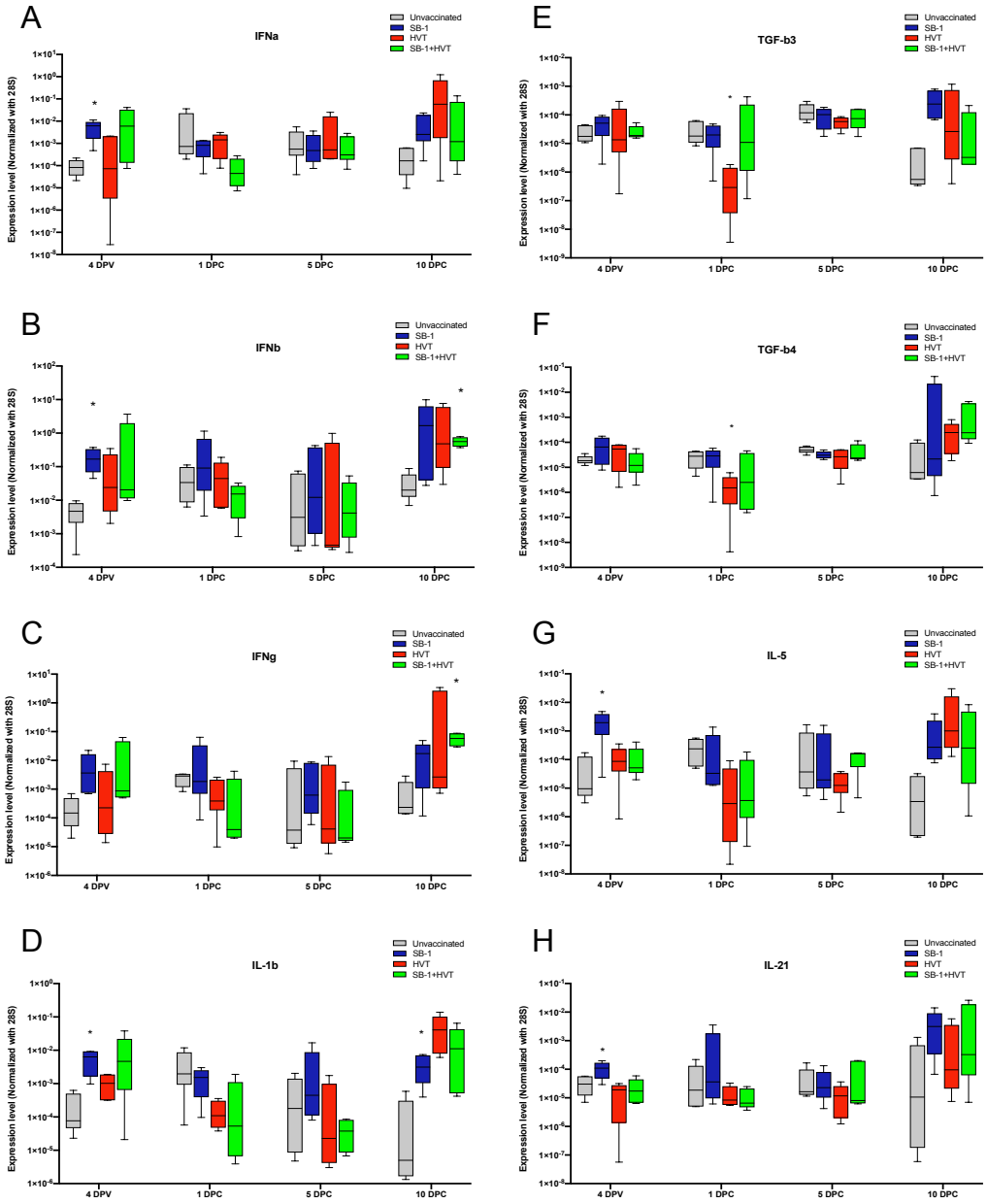

Figure 4.3. Significant up and down regulation of cytokines upon vaccinations. Chickens were vaccinated with different vaccine and cytokine profiles were determined up on vaccinations. Cytokines that show some significant up or down regulation compared to the control group include IFN-α (A), IFN-β (B), IFN-γ (C), IL-1β (D), TGF-β3 (E), TGF-β4 (F), IL-5 (G), and IL-21 (H), respectively. * = $p < 0.05$.

Discussion

Our previous data demonstrated protective synergy of the SB-1 and HVT bivalent vaccine. The bivalent vaccine showed better ability to control replication of pathogenic very virulent (vv) MDV strain Md5 in PBMCs and exhibited superior activity in controlling tumor induction compared to that obtained from either SB-1 or HVT monovalent vaccine. However, there was no study that compared the cytokine profiles between both the monovalent vaccines with the bivalent vaccine.

Since MDV is a highly cell-associated virus, neutralizing antibody is not an effective mechanism to respond to MDV. Rather, in our study, the reduction of MDV plaque numbers could be due to the effect of soluble substances in the serum of HVT and bivalent SB-1+HVT vaccinated chickens. In this case, it suggested that a soluble substance in serum could suppress MDV replication or could provide some negative impact on MDV replication. This effect could be a result of cytokine rather than antibody; for MDV antibodies may not be able to neutralize and opsonize the virus directly. Based on all these data, investigating the cytokine induction by SB-1 and HVT and bivalent vaccine would shed light on how SB-1 and HVT work to provide better protection via the aspect of cytokine responses.

We tested cytokine inductions in spleen in chickens after vaccinated with SB-1, HVT, or bivalent (SB-1+HVT), compared to the control group using qRT-PCR at 4DPV (before challenge), 1, 5, and 10 DPC (after challenge). Multiple cytokines including IFN-α, IFN-β, IL-1β, IL-21, and IL-5 were significantly up-

regulated by monovalent SB-1 vaccination at 4 DPV. Although, we found increasing trends of these cytokines after bivalent vaccination group, the results were not significantly higher compared to the unvaccinated control. Still, there could be a partial effect due to the SB-1 component in the bivalent vaccine that induced these cytokines to be higher than the unvaccinated group but not achieve significance level at $p < 0.05$.

The information about the cytokines that are activated after SB-1 immunization is limited. The induction of IFNα and IFN-β is very interesting because IFN-α and IFN-β are type-I IFN cytokines that provide an innate anti-viral response. These cytokines can be induced after infection or vaccination by virus. Xu and colleagues demonstrated that line 6 chickens, which are resistant to very virulent plus (vv+) MDV, demonstrated significant induction of IFN-α and IFN-β cytokines when challenged with the vv+ MDV strain while the levels of these cytokines were lower in the susceptible line 7 chickens (Xu et al. 2012). In addition to type-I IFN, in our study, SB-1 also promoted IL-1β at an early stage before challenging with MDV (4DPV). IL-1β acts as a pro-inflammatory cytokine responsible during acute infection to activate further immune cells such as macrophages and T lymphocytes to produce other cytokines and chemokines, in order to respond to infection (Wigley and Kaiser 2005). Chickens vaccinated with CVI988 serotype 1 MD vaccine also showed up-regulation of IL-1β around 5 days post inoculation (D. Wang, Sun, and Heidari 2018). Induction of IL-1β was detected when chickens were challenged with virulent MDV strain JM16 (Xing and Schat 2000). The induction of IL-1β at 4 DPV in our study gave further

information that IL-1β is not only a response to pathogenic MDV but also to the SB-1 MD vaccine. In addition to type-I IFN and IL-1β that were induced by SB-1, IL-21 was also up-regulated at this early time point in the SB-1-vaccinated group. Chicken IL-21 is considered as type-I cytokine that is produced by $CD4^+$ T cells, NK cells, and $CD8^+$ T cells. IL-21 is an immunomodulator cytokine that regulates the differentiation and effector function of many immune cells such as T-cells, B-cells, NK cells, and dendritic cells. IL-21 can be used as an anti-cancer agent due to its ability to enhance the CD8 CTL and NK cell responses (Leonard and Wan 2016). Moreover, IL-21 production leads to the expansion of CD8+ T cells and secretion of IFN-γ (Fröhlich et al. 2009) and is required for chronic viral infection (Elsaesser, Sauer, and Brooks 2009). Additionally, IL-21 restricted the proliferation of regulatory T cells and is involved in the response to LCMV infection (Schmitz et al. 2013). SB-1 vaccination also induced the Th2 cytokine IL-5. However, the knowledge on the IL-5 and MDV is limited. A few studies reported the up-regulation of IL-5 cytokine after infectious bursal disease virus infection (IBDV) (S. Wang et al. 2014; H. Liu et al. 2010). All in all, effects on several cytokines by SB-1 at early time point before challenge could contribute to the MD protection induced by SB-1.

In our study, significant down-regulation of TGF-β3 and TGF-β4 was detected in monovalent HVT vaccinated group on 1 DPC, which we believe to contribute to MD protection. TGF-β is involved in tumor microenvironment and plays a role in the progression of tumors because its functions in cell proliferation and migration (Pickup, Novitskiy, and Moses 2013). Several studies link the

effect of TGF-β to tumor progression. Over expression of this cytokine provided poor prognosis in human breast cancer (Gold and Arrick 1992) and lung carcinoma (Hasegawa et al. 2001). In chickens, TGF-β^+ Treg cells have been identified (Gurung et al. 2017). These cells secret TGF-β and are associated with pathogenesis of MDV by suppression of host immune responses and induction of T cell lymphomas (Gurung et al. 2017). Zhou and colleagues showed that super-infection of MDV and avian leucosis virus subgroup J (ALV-J) induce the expression of TGF-β cytokines which promoted severe cytopathy in CEF cells (Zhou et al. 2018). In addition to the effect of TGF-β in promoting tumor development, TGF-β1 was found to suppress the expression of IFN-γ in $CD4^+$ T cells in mice by interfering with Stat4 and T-bet signals that promote the Th1 response (Lin et al. 2014) . Trotta and colleagues reported the inhibition of IFN-γ production though the regulation of SMAD3 protein by TGF-β. In our study, TGF-β3 and TGF-β4 were down regulated by HVT, indicating that HVT could be involved in IFN-γ induction by suppressing the TGF-β family cytokines.

Several studies showed that IFN-γ is one of the keys in the response to MD infection and MD vaccines (Xing and Schat 2002; Haq et al. 2011; Abdul-careem et al. 2007; Haq et al. 2010). Genetically resistant line B21/B21 chickens show induction of the IFN-γ gene while susceptible line chickens show the suppression of this gene after infection with the vvMDV strain RB1B (Quéré et al. 2005). Moreover, immunization with HVT induces IFN-γ (Haq et al. 2011). In our study, induction of IFN-γ was detected in the bivalent vaccinated group

suggesting that stimulation of IFN-γ by bivalent vaccine could be involved in the protection of this vaccine.

In table 4.2, we summarize all cytokines whose levels were changed upon each vaccination compared to the unvaccinated group. SB-1 vaccine stimulates type-I IFN cytokines, the pro-inflammatory cytokine (IL-1β) and the T cell proliferation cytokine (IL-21). Interestingly, IL-1β and IL-21 are two cytokines that promote IFN-γ expression. Moreover, down-regulation of TGF-β3 and TGF-β4 upon HVT vaccination after challenge may also help to prevent the reduction IFN-γ by TGF-β signal.

Based on our cytokine data, the model of cytokine synergy through cooperative action (refer to synergy through cooperative action in Figure 4.1) can be applied to explain protective synergy of the bivalent (SB-1+HVT), which is 1) SB-1 induces an early anti-viral response (Type-I IFN), and IL-1β, and IL-21 resulting in the induction of the IFN-γ cytokine, 2) HVT suppresses TGF-β resulting the ability to maintain IFN-γ levels. The effect of SB-1 and HVT vaccine together helps to keep the level of IFN-γ high. The higher level of IFN-γ found after bivalent vaccination provides higher ability to reduce MDV replication (Figure 4.4).

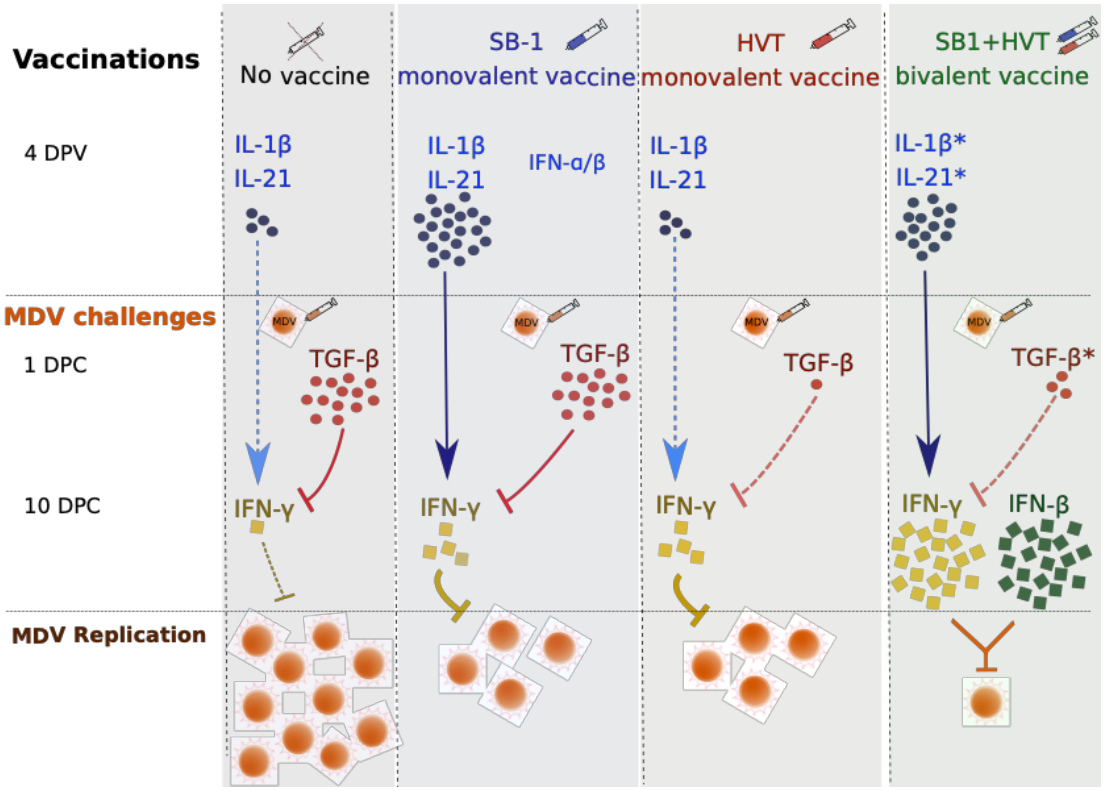

Figure 4.4. Model summary of cytokine synergy of a bivalent (SB-1+HVT) vaccine. SB-1 and HVT may work together to provide synergistic effect through the cooperative induction by 1) SB-1 induces IL-1β and IL-21, (cytokines that activate IFN-γ expression) and 2) HVT suppresses TGF-β signal, which is a cytokine that negatively regulates IFN-γ level. Thus, contributions of both SB-1 and HVT together would provide better activating IFN-γ as detected at 10 DPC in bivalent vaccine. In addition, induction of IFN-β was also found in the bivalent vaccine at 10 DPC. The effect of both IFN-β as well as IFN-γ may help promoting stronger anti-viral response.

Table 4.1. Cytokine of interests and primer sequences

Gene	Primer sequence (5' to 3')	Reference
IFNα	Forward: GACAGCCAACGCCAAAGC	(Ewald et al. 2011)
	Reverse: GTCGCTGCTGTCCAAGCATT	
IL-26	Forward: AATGCCTGTCTTCCGTGTG	(Truong et al. 2016)
	Reverse: TCATTGATGGCCTTGTAGACC	
IL-2	Forward: TTCTGGGACCACTGTATGCTCTT	(H. Liu et al. 2010)
	Reverse: TACCGACAAAGTGAGAATCAATCAG	
IL-5	Forward: TGGCAGGAATGTGCAGACGC	(H. Liu et al. 2010)
	Reverse: TGGCAAGGGCAGTGTATGCTG	
TGF-β3	Forward: ATGATGCTACCCCCACATCG	(Rengaraj et al. 2012)
	Reverse: CAGGTTCCGGAAGCAGTAGT	
FASL	Forward: CACTTAACAGGAAACCCCACAC	(Berndt et al. 2007)
	Reverse: TTGATCACAAGGCCCTGGT	
IFNβ	Forward: GGAATTCCATATGTGCAACCATCTTCGTC	(Qu et al. 2013)
	Reverse: CCGGAATTCTCACTGGGTGTTGAGAC	
CSF2	Forward: CACCCCGCAGGTTCCTGATAA	(Garceau et al. 2010)
	Reverse: GTCTTTCTCCTCTGGGAGCAC	
IL-12	Forward: CGAAGTGAAGGAGTTCCCAGAT	(H. Liu et al. 2010)
	Reverse: GACCGTATCATTTGCCCATTG	
IL-15	Forward: TAGGAAGCATGATGTACGGAACAT	(Kaiser, Underwood, and Davison 2003)
	Reverse: TTTTTGCTGTTGTGGAATTCAACT	
TGF-β4	Forward: CGGCCGACGATGAGTGGCTC	(Brisbin et al. 2010)
	Reverse: CGGGGCCCATCTCACAGGGA	
IFNγ	Forward: AAGTCAAAGCCGCACATCAAAC	(Xu et al. 2012)
	Reverse: CTGGATTCTCAAGTCGTTCATCG	
IL-19	Forward: AGCCGGGAACACGATCCTCCACTT	(Kim et al. 2009)
	Reverse: TGCAGAGAGTGTGGGTGGGACAGG	
IL-23	Forward: TGGCTGTGCCTAGGAGTAGCA	(Happel et al. 2005)
	Reverse: TTCATCCTCTTCTTCTCTTAGTAGATTCATA	
IL-21	Forward: AAAAGATGTGGTGAAAGATAAGGATGT	(Rothwell et al. 2012)
	Reverse: GCTGTGAGCAGGCATCCA	
IL-6	Forward: GAACGTCGAGTCTCTGTGCTAC	(Kim et al. 2009)
	Reverse: CACCATCTGCCGGATCGT	
CSF1	Forward: GCGACTCTGTCTGCTACGTG	(Garceau et al. 2010)
	Reverse: CGAAGGTCTCCTTGTTCTGC	
BAFF	Forward: TGATTGCAG ACAGTGACACACCGA	(Yeramilli and Knight 2010)
	Reverse: AGGTACCCGGTTTCTTTGACCACT	
IL-1β	Forward: TGGGCATCAAGGGCTACA	(Hong et al. 2006)
	Reverse: TCGGGTTGGTTGGTGATG	

IL-22	Forward: TGTTGTTGCTGTTTCCCTCTTC	(Kim et al. 2012)
	Reverse: CACCCCTGTCCCTTTTGGA	
IL-17A	Forward: ATGGGAAGGTGATACGGC	(Zhang et al. 2013)
	Reverse: GATGGGCACGGAGTTGA	
TGF-β2	Forward: TGCACTGCTATCTCCTGAG	(Saxena et al. 2007)
	Reverse: ATTTTGTAAACTTCTTTGGCG	
28S	Forward: GGCGAAGCCAGAGGAAACT	(Kogut et al. 2013)
	Reverse: GACGACCGATTGCACGTC	

Table 4.2. Summary of cytokine profiles upon SB-1, HVT, and bivalent (SB-1+HVT)

Time	SB-1	HVT	SB-1+HVT
4 DPV	Up-regulation IFN-α, IFN-β, IL-1β, IL-21, IL-5	NS	NS
1 DPC	NS	Down-regulation TGF-β3, TGF- β4	NS
5 DPC	NS	NS	NS
10 DPC	Up-regulation IL-1β	NS	Up-regulation IFN-β, IFN-γ

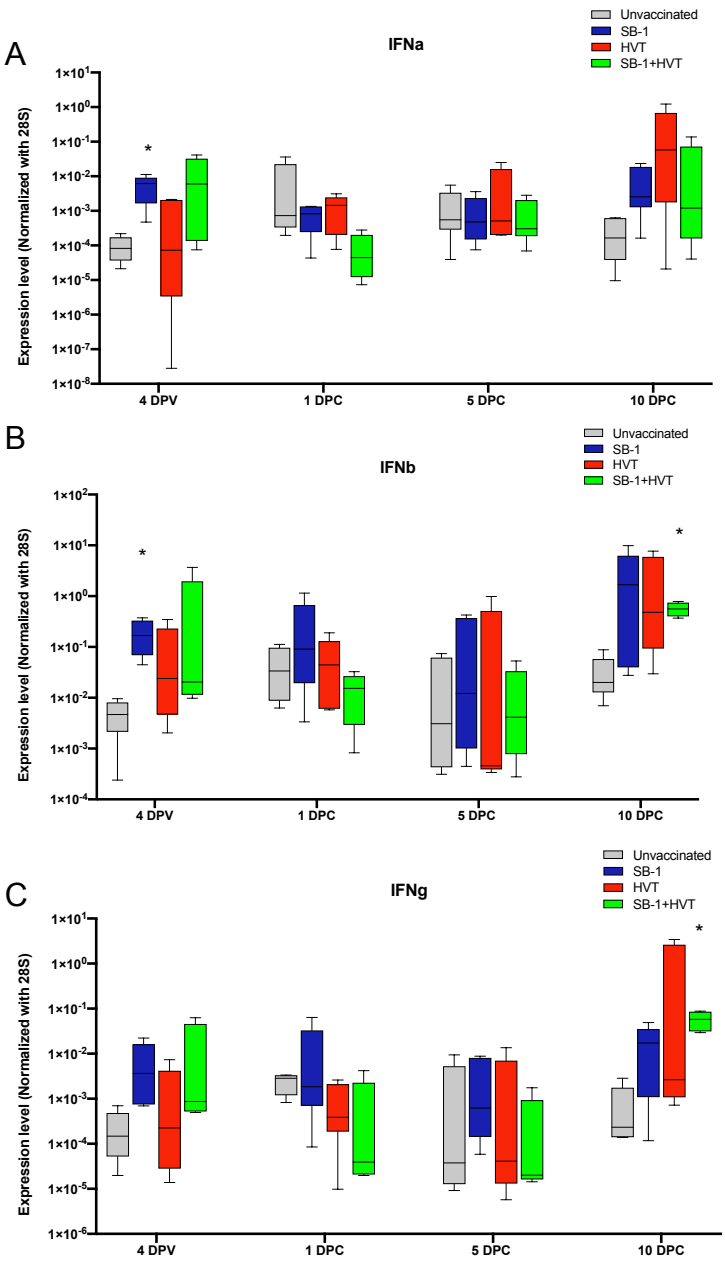

Figure 4.3 S1. Expression of IFN family cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression levels of IFNα, IFNβ, and IFNγ are shown in A, B, C, respectively.

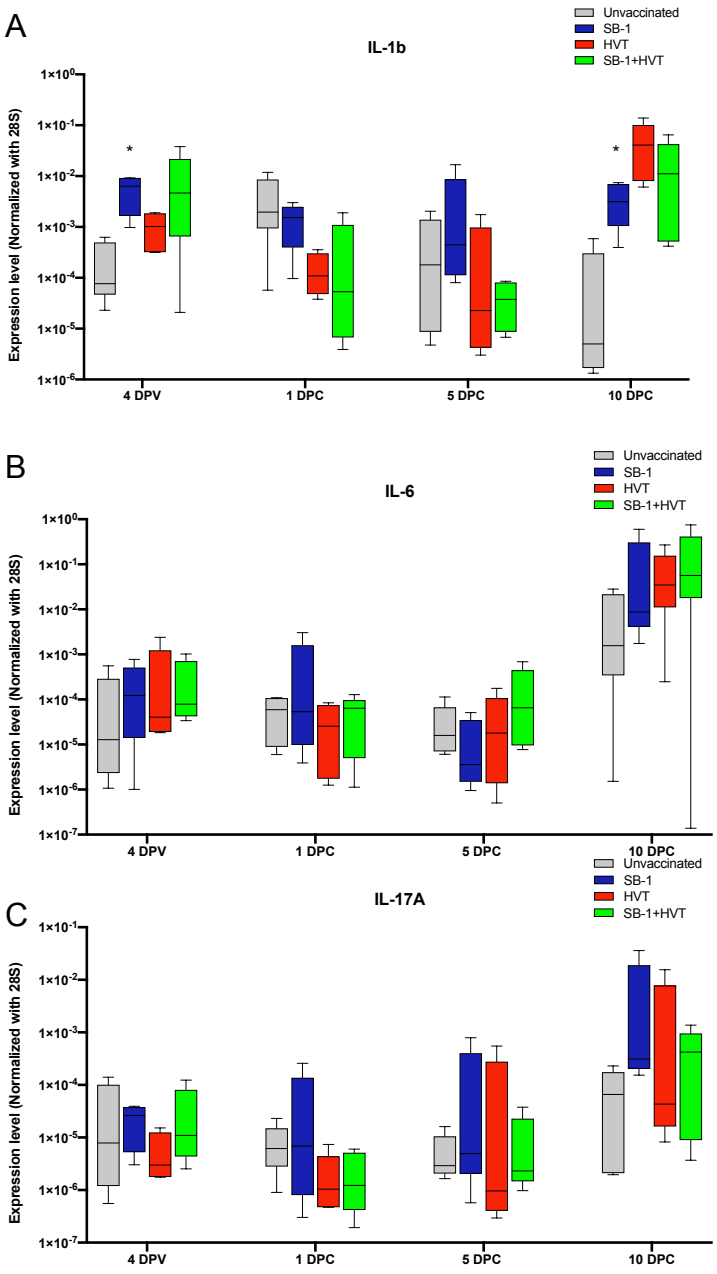

Figure 4.3 S2. Expression of pro-inflammatory cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression levels of IL-1β, IL-6, and IL-17A are shown in A, B, C, respectively.

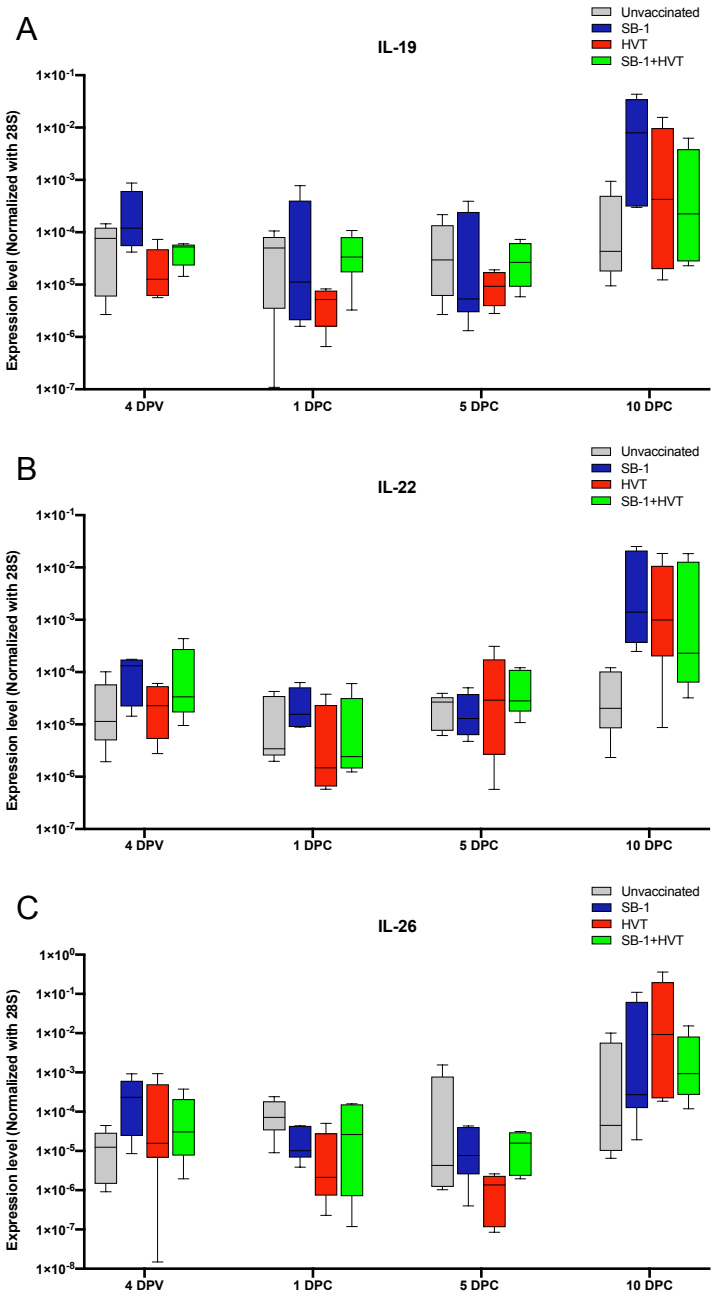

Figure 4.3 S3. Expression of IL-10 family cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression levels of IL-19, IL-22, and IL-26 are shown in A, B, C, respectively.

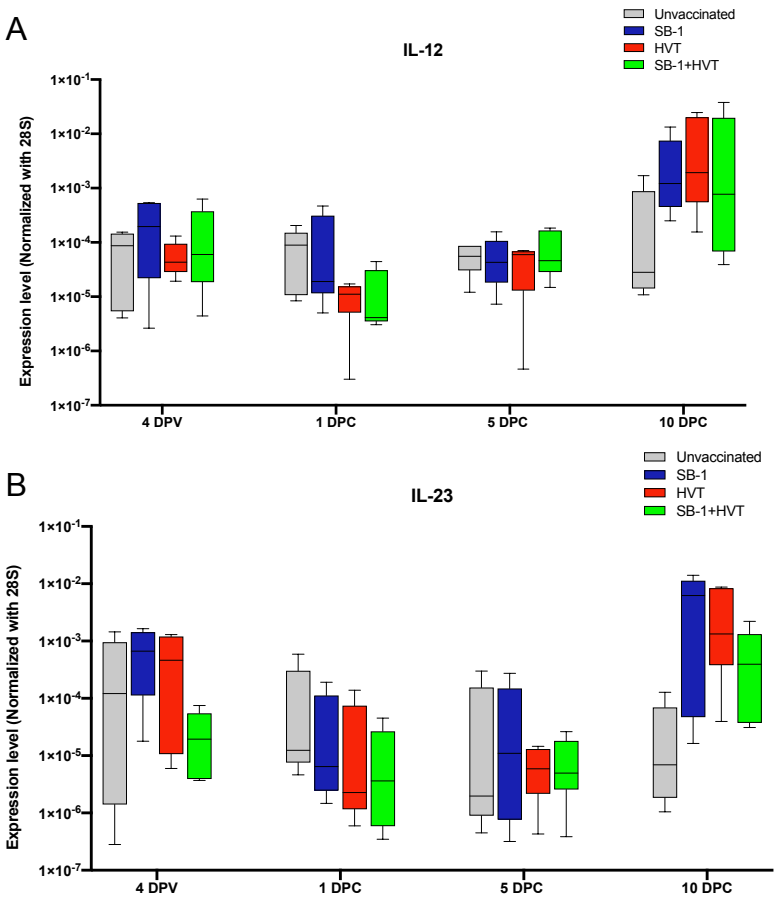

Figure 4.3 S4. Expression of Th1 family cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression levels of IL-12 and IL-23 are shown in A and B, respectively.

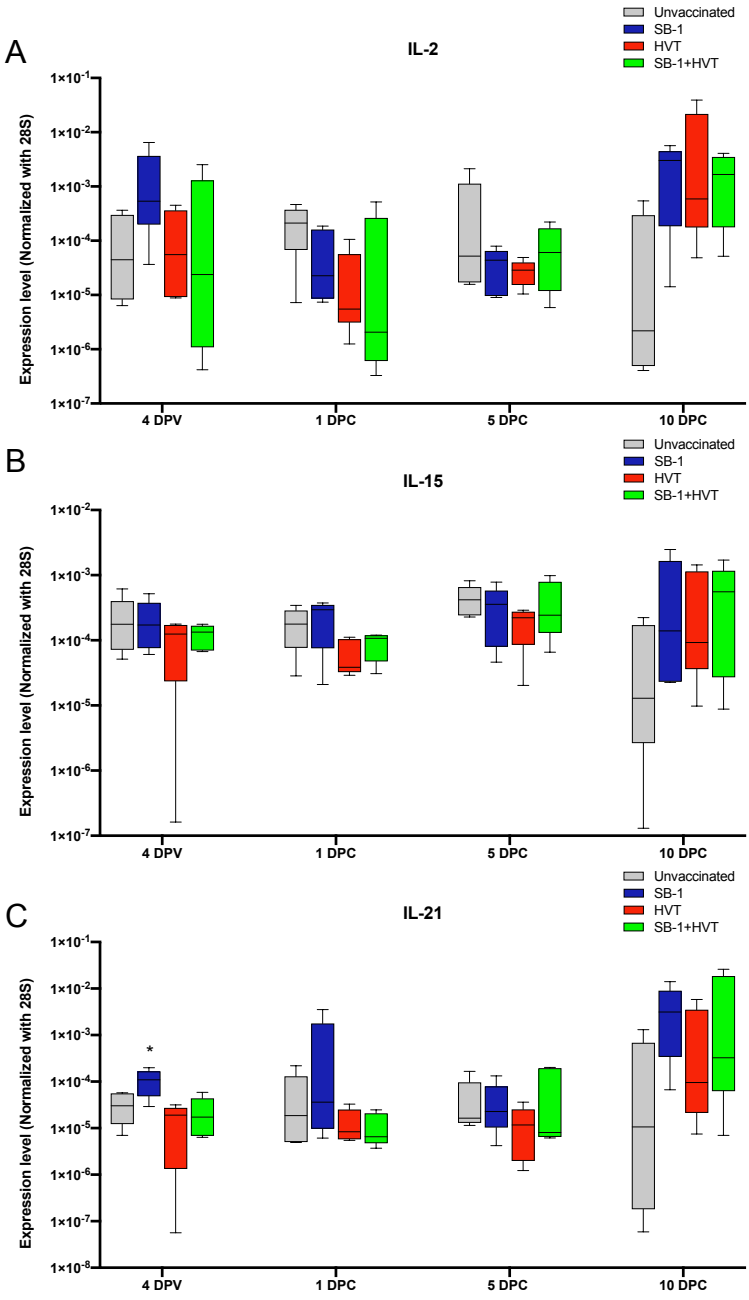

Figure 4.3.S5. Expressions of T cell proliferation cytokines Upon vaccinations with SB-1, HVT, and bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. Expression levels of IL-2, IL-15, and IL-21 are shown in A, B, and C, respectively.

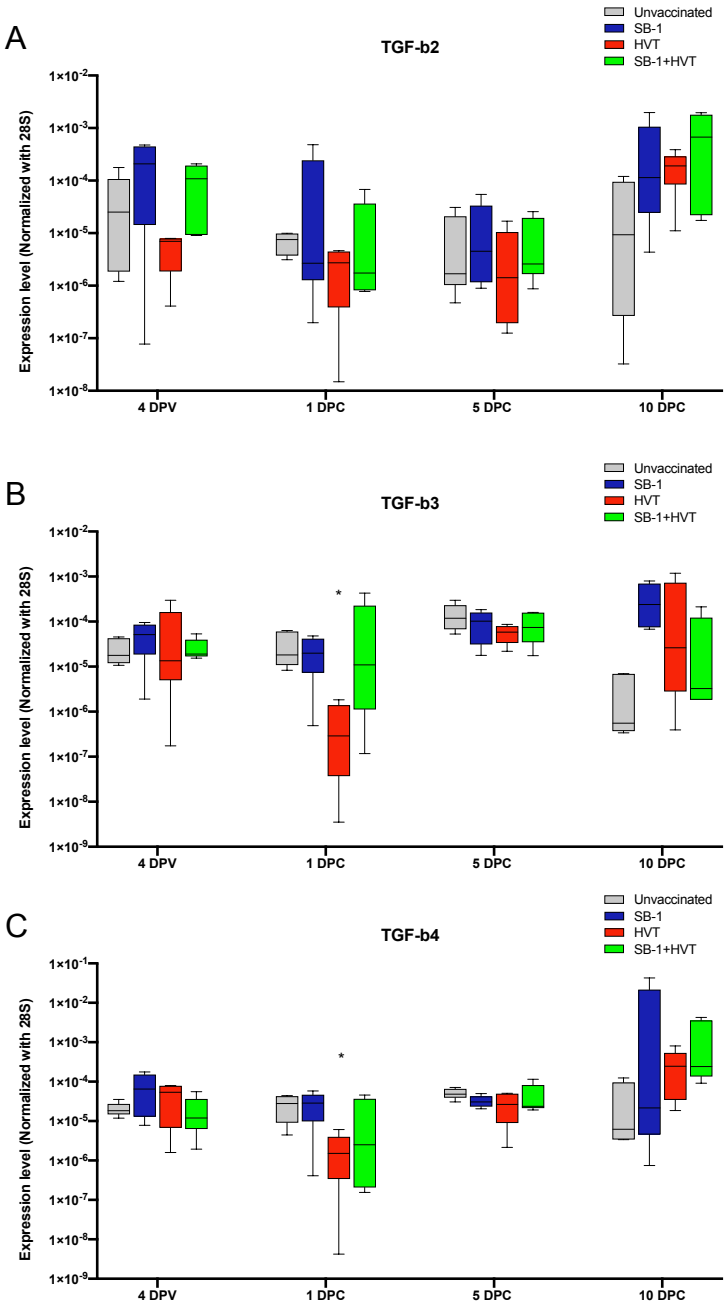

Figure 4.3 S6. Expression of TGF family cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. Expression levels of TGF-β2, TGF-β3, and TGF-β4 are shown in A, B, and C, respectively.

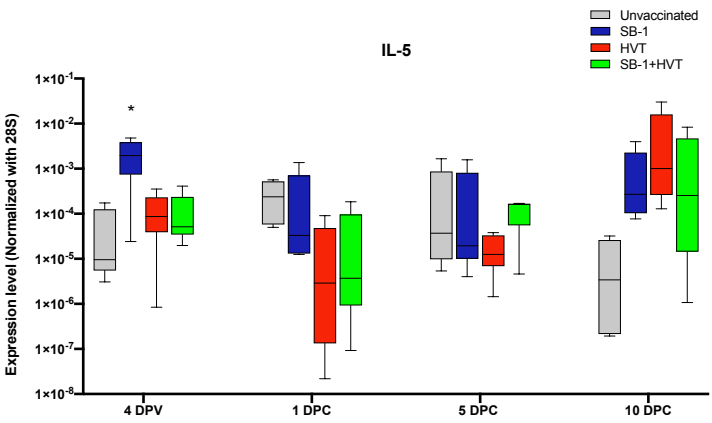

Figure 4.3 S7. Expression of Th2 cytokine (IL-5) Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC. the expression level was measured calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene.

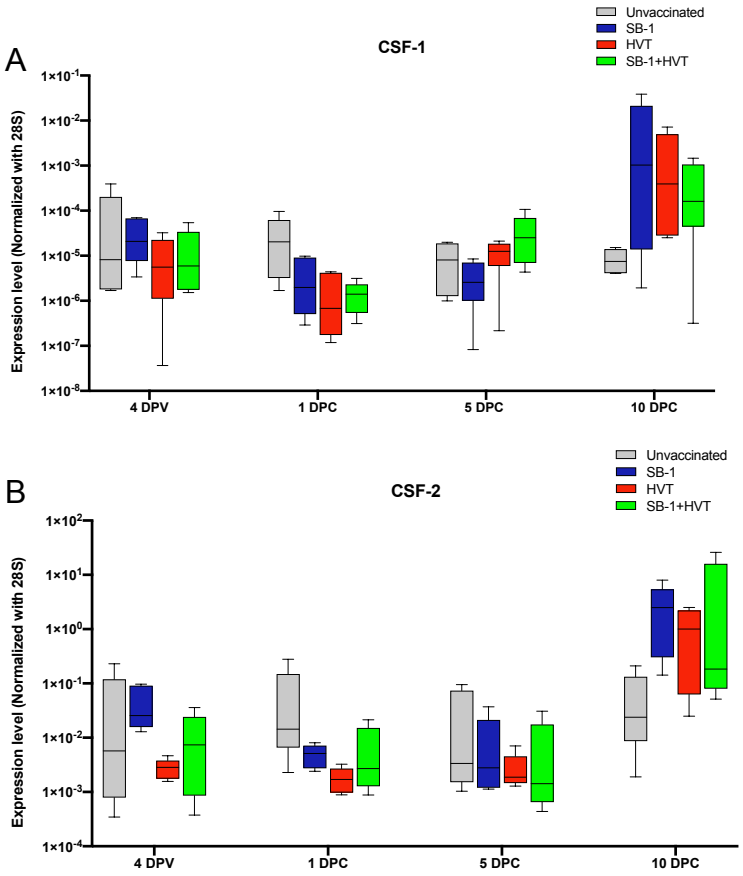

Figure 4.3 S8. Expressions of CSF family cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression of CSF-1 is shown in A, and the expression of CSF-2 is shown in B.

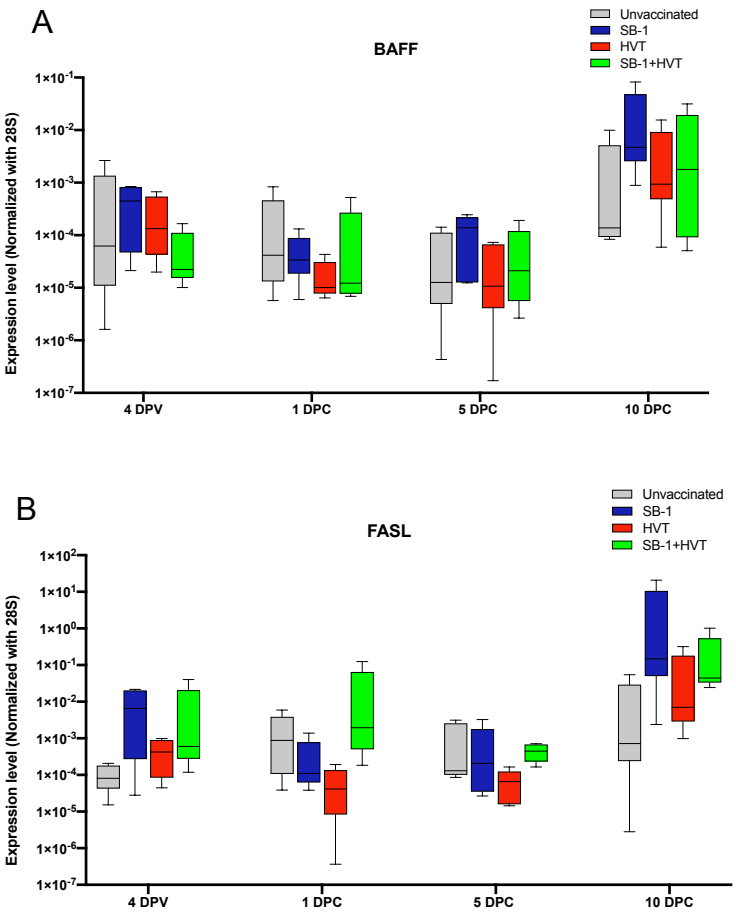

Figure 4.3 S9. Expression of BAFF and FASL cytokines Upon vaccinations with SB-1, HVT, and SB-1+HVT bivalent vaccine at 4DPV, 1, 5, and 10 DPC, the expression level was measured and calculated from $2^{-\Delta Ct}$; where ΔCt is the difference of cycle number of cytokine of interest and cycle number of 28S gene. The expression of BAFF is shown in A, and the expression of FASL is shown in B.

CHAPTER 5

Summary

Model of mechanisms underlying protective synergy of bivalent vaccine

Marek's disease (MD) is a contagious disease in chickens caused by the infection of the α-herpesvirus called Marek's disease virus. Infected chickens develop neurological symptoms, oncogenic symptoms, and immunosuppression. The disease is controlled by mass vaccination with MD vaccines. One of the problems of MD vaccines is that they do not induce sterile immunity to clear the virus from host. This allows the pathogenic strain to co-evolve with vaccine strains inside the same host, which is believed to be one of the factors driving MDV to gain higher virulence levels. Future generations of vaccines are needed to overcome vaccine outbreak. The combination of MD vaccines between serotype 2 strain SB-1 and herpesvirus of turkeys (HVT) serotype 3 strain FC126 is a common MD vaccine. Combination of the two viruses shows greater ability to protect chickens from vvMDV compared to thep protection of vaccine developed from either SB-1 or HVT alone. The ability of bivalent SB-1+HVT vaccine to induce better protection is called protective synergy.

As the bivalent SB-1+HVT shows the greater MD protection, our study used a bivalent (SB-1+HVT) model to study mechanisms of effective vaccine protection to MD. We identified mechanisms that can explain how SB-1 and HVT work together to provide synergistic effects to control MDV and to protect the chickens from MD.

We used several approaches to investigate how the bivalent vaccine works and we were able to identify three potential mechanisms that may promote protective synergy of the bivalent (SB-1+HVT) vaccine.

First, we investigated the replication patterns of SB-1 and HVT vaccines to see which organs and at which time points SB-1 and HVT replicate in the host. We found that SB-1 replicates in spleen from 1DPC to 14 DPC, while HVT replicates only in bursa at 1DPC. In addition, we found that vvMDV strain Md5 also replicates in bursa at early time points and also replicates in spleen until 14 DPC. With the overlapping of tissue tropisms between MDV and SB-1+HVT vaccine, we propose the first mechanism to explain the protective synergy of the bivalent (SB-1+HVT) in that the replication of SB-1+HVT is similarly in diverse cell types as that of Md5. So, when we challenge the chickens with MDV later on day 5 after vaccination, MDV has fewer target cells in which to replicate leading to lower replication of MDV, which, in long term, increases protection over SB-1 or HVT alone Figure 5.1 (B).

Second, since MDV is a highly cell-associated virus, the CTL response via CD8 T cells is critical for MD infection. We tested the effect of CD8 T cells in MD protection in the unvaccinated group, SB-1, HVT, and bivalent (SB-1+HVT) vaccinations by depleting the level of CD8 T cells in chickens using anti-chicken CD8 mAb injection. We found that CD8 T cells are essential for protection against MDV. CD8 T cell depletion results in higher MD pathology, tumor induction, and lower protection, in unvaccinated and also in all types of vaccination. Hence, we propose the second mechanism that MD protection is

CD8 dependent. And the ability to induce CD8 T cells would be one of the mechanisms that is vital for protection generated by bivalent and monovalent vaccines Figure 5.1 (C).

Third, we screened the expression profiles of cytokines after vaccination with different vaccines to investigate if cytokine synergy would be one of the mechanisms to explain protective synergy of the bivalent vaccine (SB-1+HVT). We discovered that SB-1 could induce type-I IFN and more importantly SB-1 vaccine induces IL-1β and IL-21. These two cytokines have ability to promote IFN-γ expression. In addition, we found that HVT suppresses the TGF-β signal, which is a signal that inhibits IFN-γ expression. Thus, the additive effect between SB-1 and HVT helps to increase the level of IFN-γ, which is found to be significantly upregulated in the bivalent (SB-1+HVT) trial at 10 DPC. And the induction of higher IFN-γ signal in the bivalent group may result in higher anti-viral activity and higher T cell response to control MDV Figure 5.1 (A).

Further work

Our studies; we were able to explain some mechanisms underlying the protective synergy of a bivalent (SB-1+HVT) vaccine. However, there are some gaps that have to be addressed at the cellular level. Firstly, investigating what types of cells in which SB-1 and HVT replicate would be essential. We know that SB-1 replicates in bursa and spleen, while HVT replicates in bursa. It may imply that SB-1 should be found in B cells and T cells in spleen and HVT should be found in B cells in bursa. However, investigating and confirming which cell types

in spleen and bursa are really target cells of SB-1 and HVT replication would shed more light on patterns of immunological induction and pathways involved in the protection. Secondly, for the effect of CD8 T cells on MD protection, our result is based on the partial depletion level of CD8 T cells. It would be nicer if we could apply a system to completely remove CD8 T cells from the chickens or have a chicken model that does not have CD8 T cells to test the effect of CD8 T cells. In that way, we can truly confirm the effect of CD8 T cells on MD protection or vaccinal protection. In addition, adoptive transfer of CD8 T cells from vaccinate-protected birds to unvaccinated birds would help to answer whether CD8 T cells induced by vaccination are associated in MD protection. Third, for cytokine synergy, the proposed model is based on the up-regulation and down-regulation of cytokines detected by qRT-PCR. The expression levels of the proposed cytokine synergy model could be further confirmed at the protein expression level using Western blotting. Furthermore, validating the cytokine synergy results could also be potentially done *in vivo* by injection of cytokines such IL-1β and IL-21 into the host together with HVT vaccine to see if the protection of HVT is improved compared to regular HVT vaccine.

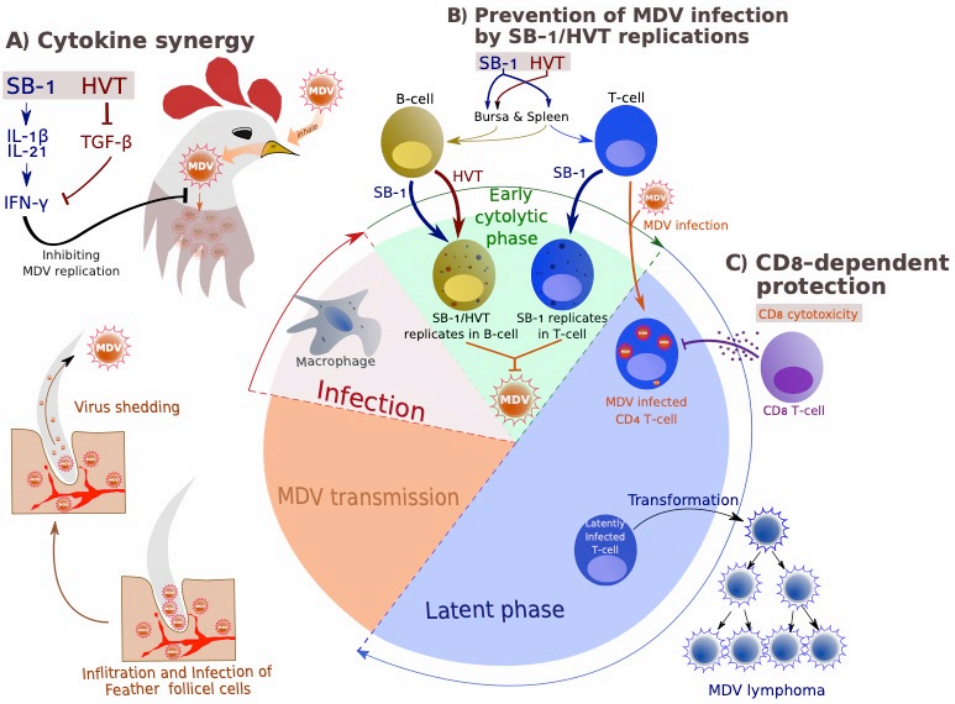

Figure 5.1 Mechanisms underlying protective synergy of a bivalent SB-1+HVT vaccine. Three distinct mechanisms that potentially contribute to protective synergy of a bivalent (SB-1+HVT) vaccine include A) cytokine synergy; cytokines induced by SB-1 (IL-1β and IL-21) promote IFN-γ expression and this together with the ability of HVT to suppress the cytokines that inhibit IFN-γ signal (TGF-β family), increase the expression IFN-γ (the anti-viral cytokine). B) Prevention of MDV replication by the replication of SB-1 and HVT; SB-1 and HVT demonstrates replication in different organ and time point. This leads to higher chance to block MDV replication as it shows the overlaps in organs and time point of infection compared to HVT and HVT vaccine. C) CD8 dependent protection; depletion of CD8 T cells indicate negative effects on MD pathology, tumor induction, and protection in unvaccinated, and vaccinated groups indicating that CD8 T cells are essential for MD protection.

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