Vaccine 37 (2019) 6397–6404

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

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

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ARTICLE INFO

Article history:
Received 19 July 2019
Received in revised form 30 August 2019
Accepted 2 September 2019
Available online 9 September 2019

Keywords:
Marek's disease
Marek's disease virus
Protective synergy
Bivalent vaccine
Bursa

ABSTRACT

Background: Marek's disease (MD) is a lymphoproliferative disease of chickens caused by Marek's disease virus (MDV), an oncogenic α-herpesvirus. Since 1970, MD has been controlled by widespread vaccination; however, more effective MD vaccines are needed to counter more virulent MDV strains. The bivalent vaccine combination of SB-1 and herpesvirus of turkey (HVT) strain FC126 has been widely used. Nonetheless, the mechanism(s) underlying this synergistic effect has not been investigated.
Methods: Three experiments were conducted where SB-1 or HVT were administered as monovalent or bivalent vaccines to newly hatched chickens, then challenged five days later with MDV. In Experiment 1, levels of MDV replication in PBMCs were measured over time, and tumor incidence and vaccinal protection determined. In Experiment 2, MDV and vaccine strains replication levels in lymphoid organs were measured at 1, 5, 10, and 14 days post-challenge (DPC). In Experiment 3, to verify that the bursa was necessary for HVT protection, a subset of chicks were bursectomized and these birds plus controls were similarly vaccinated and challenged, and the levels of vaccinal protection 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 correlated with the ability to inhibit tumor formation. SB-1 replication in the spleen increased from 1 to 14 DPC, while HVT replicated only in the bursa at 1 DPC. The bursa was 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 induce immune protection.

1. Introduction

Marek's disease virus (MDV) is a ubiquitous and highly oncogenic α-herpesvirus that causes Marek's disease (MD), the most common lymphoproliferative disorder affecting chickens worldwide [1]. Susceptible chickens commonly exhibit transient or long-standing paralysis, and CD4+ T cell lymphoid tumors as soon as a few weeks after MDV infection. As the disease progresses, chickens become severely ill and die due to the development of gross tumors in multiple internal organs. Consequently, MD is a major concern for the poultry industry with annual worldwide economic losses of $1–2 billion [2].

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) [1].

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

Abbreviations: ADOL, Avian Disease and Oncology Laboratory; ARS, Agricultural Research Service; AUP, animal use protocol; CEF, chicken embryonic fibroblasts; CTL, cytotoxic T lymphocyte; DPC, days post challenge; DEF, duck embryo fibroblasts; DIA, diffusely infiltrated area; FBS, fetal bovine serum; GaHV, *Gallid alphaherpesvirus*; HB, Horsfall-Bauer; HVT, herpesvirus of turkey; 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; vv, very virulent; WPC, weeks post challenge.

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https://doi.org/10.1016/j.vaccine.2019.09.003
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[3]. In susceptible birds, transformed T cells circulate to nerves and multiple organs causing neurological disorders and visceral tumors, respectively. The virus can stay latent in 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 fully infectious virions [4]. 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 widely used 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 clinical signs by preventing paralysis and tumor formation making them 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 promoted the evolution of more virulent field strains that have caused repeated MD outbreaks [5–8]. Nevertheless, MD vaccines are vital and unavoidable to achieve adequate protection from MD. As MD vaccines are typically administered *in ovo* or at hatch as 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 design and development of more effective vaccines that would prevent future outbreaks.

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 [9], and the first major example widely adopted by industry was combination of HVT and SB-1. Specifically, bivalent (SB-1 + HVT) significantly reduced mortality caused by very virulent (vv) pathotype MDV compared to the group of birds vaccinated with HVT or SB-1 alone [9–11]. Several trials performed by Witter and colleagues indicated that the protective synergism phenomenon predominantly occurred among serotype 2 and serotype 3 combinations, and some with serotype 1 and 3 (HVT + Rispens) [12] but the protective synergism is barely found between serotypes 1 and 2 [13].

Our study aims at understanding the underlying mechanisms of protective synergy with SB-1 and HVT to aid rationally-designed efforts for improved MD vaccines. In this initial study, we demonstrate the protective synergy of a bivalent (SB-1 + HVT) vaccine in the context 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.

2. Materials and methods

2.1. 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, Waltham, MA, USA), 20 μg/ml streptomycin, 200 U/ml penicillin (Sigma Aldrich, St. Louis, MO, USA), and 2 μg/ml amphotericin B (Thermo Fisher Scientific). Cells were maintained in a 37 °C incubator with 5% CO_2.

2.2. Viruses

MD vaccine strains SB-1 (passage 15) and HVT strain FC126 (passage 11) were propagated in CEF. Pathogenic serotype 1 MDV strain Md5 (vv pathotype, passage 8) was cultured in DEF. All viruses 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). 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*.

2.3. Birds

Single comb white leghorn ADOL $15I_5 \times 7_1$ chicks [14] 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.

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

As briefly summarized in Table 1, in Experiment 1, seventeen (17) newly-hatched chicks per group were housed in negative pressure Horsfall-Bauer (HB) units and received the following treatments: 1. Mock (diluent only) vaccinated (negative control), 2. Mock vaccinated and challenged with MDV (positive control for 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, or the combination of SB-1 and HVT (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 and 5 days post challenge (DPC) and then weekly after challenge until the end of the study. PBMCs were isolated form blood using Histopaque-1077 (Sigma-Aldrich) 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:

Table 1
Summary of bird experiments.

Exp. No.	Samples collected	Analyses
1	PBMCs and necropsy	Replication levels of MDV (1 and 5 days post-challenge and then weekly), and disease and tumor incidence
2	Spleen, bursa, and thymus	Replication levels of MDV and MD vaccine strains (1, 5, 10, and 14 days post-challenge)
3	Necropsy	Disease incidence

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

2.5. Quantifying the replication levels of viral vaccines and pathogenic MDV

In Experiment 2, thirty-two (32) newly hatched chicks per group received the same treatment as described above for groups 2–5. 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, Carlsbad, CA, USA) at −80 °C. DNA was extracted from PBMCs and the collected tissues using DNeasy blood and tissue kits (Qiagen, Germantown, MD, USA), respectively, following the manufacturer's instructions. The DNA concentration and 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 (Foster City, CA, USA). Primers and probes (Integrated DNA Technologies, Inc., Coralville, IA, USA) designed using Primer Express Software (version 3.0.1; Thermo Fisher Scientific) to detect MDV, SB-1, or HVT gB and chicken GAPDH are listed in Table 2. 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 gB in each virus was determined relative to the Ct value of GAPDH using $2^{-\Delta Ct}$. The values were plotted individually on the graph using Prism 8.0.0 (GraphPad Software, Inc., San Diego, CA, USA).

2.6. Evaluating the effect of bursectomy on MD vaccinal protection

In Experiment 3, surgery was conducted on the day of hatch. Chicks were anesthetized using 3% isoflurane (VetOne, Boise, ID, USA) 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 closed using tissue glue. Chicks were given 3 mg/kg of meloxicam SR (Zoopharm, Windsor, CO, USA) for pain relief after surgery and 220 mg/gallon of oxytetracycline HCl (Bimeda, Oakbrook Terrace, IL, USA) 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. This experiment was replicated to provide reproducibility.

Table 2
Primers and probes used to detect replication of MDV, SB-1, or HVT relative to GAPDH with qPCR.

Primers	
Md5 gB TM.5	5′-CGG TGG CTT TTC TAG GTT CG-3′
Md5 gB TM.3	5′-CCA GTG GGT TCA ACC GTG A-3′
SB1gB-TMF	5′-CAG TCC CAC CCA ACC GTA AA-3′
SB1gB-TMR	5′-GAG CAT ACC CGT CAA GCG TAA-3′
HVTgB-TMF	5′-CGG GCC ATA AAA CGG AAT T-3′
HVTgB-TMR	5′-GGC AAA GTG GAA AGA GGT AAC G-3′
GAPDH-TMF	5′-CAA CGG TGA CAG CCA TTC CT-3′
GAPDH-TMR	5′-ATG GTC GTT CAG TGC AAT GC-3′
Probes	
Md gB-TMP2	5′ Cy3-CAT TTT CGC GGC GGT TCT AGA CGG-3′ BHQ1
SB1-TMP	5′ Cy5-TGT GGA GTG ACG AGGAA-3′ BHQ2
HVTgB-TMP2	5′-JOE-CTT GCC CAC TCT AGC ACG CAG CAT T-3′ BHQ1
GAPDH-TMP2	5′ FAM-CCT TTG ATG CGG GT GCT-3′ BHQ-1

2.7. Statistical analyses

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

3. Results

3.1. Validation of protective synergy between SB-1 and HVT vaccines

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, monovalent HVT, or bivalent SB-1 + HVT) and challenged with pathogenic MDV, the result shows that the bivalent vaccine did provide greater protection compared to either monovalent vaccine validating protective synergy between the two vaccines (Fig. 1); the MD incidence in unvaccinated controls was 100%.

3.2. Bivalent (SB-1 + HVT), but not mono-valent SB-1 or HVT, suppresses MDV replication at later timepoints 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 weeks post challenge (WPC) is shown in Fig. 2A. Specifically, both monovalent SB-1 or HVT had the ability to inhibit MDV replication up to 2 WPC compared to the unvacci-

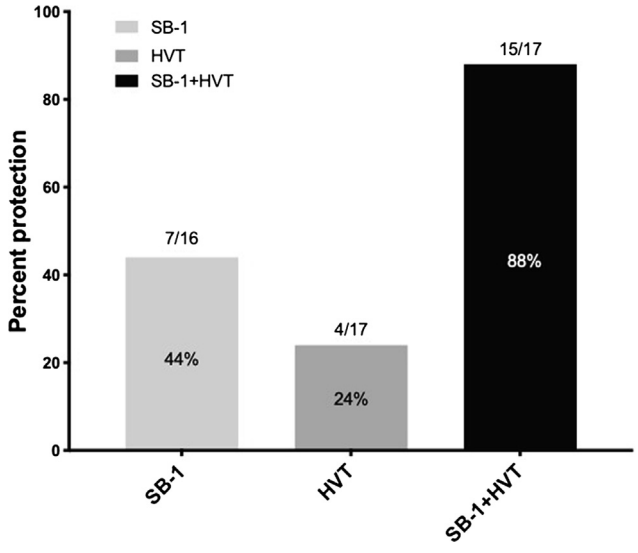

Fig. 1. Synergistic protection of bivalent (SB-1 + HVT) MD vaccine. Seventeen (17) chickens per group were vaccinated with 2,000 PFU monovalent SB-1, 2,000 PFU monovalent HVT, or bivalent vaccine containing 1,000 PFU SB-1 and 1,000 PFU HVT (SB-1 + HVT) at 1 day of age. At 6 days of age, all birds were 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.

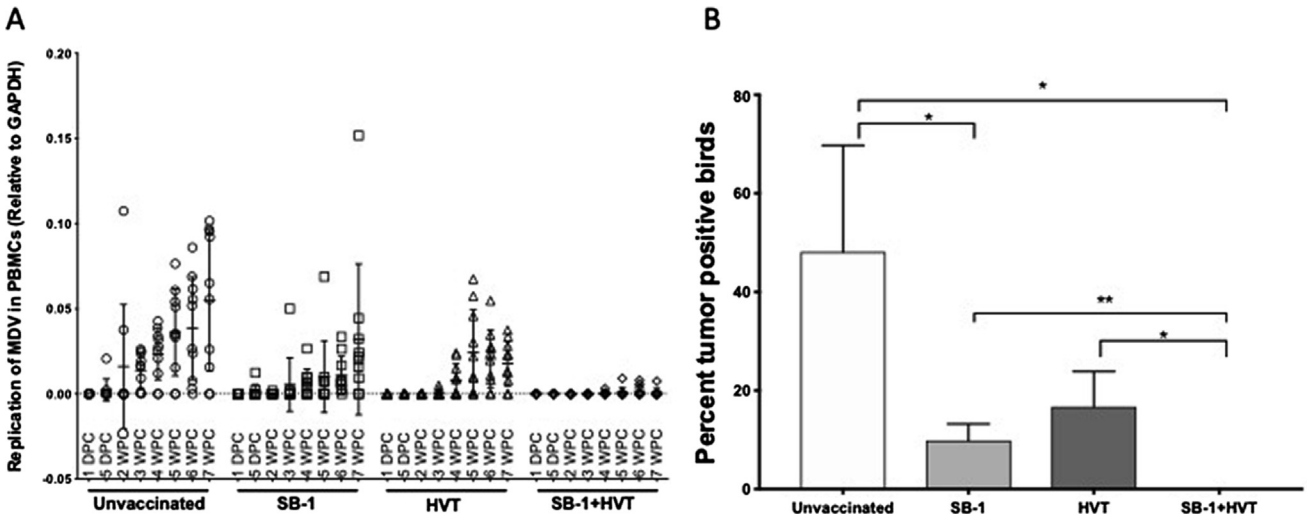

Fig. 2. Bivalent (SB-1 + HVT) can prevent MDV replication and tumor formation. Chickens were vaccinated with either SB-1, HVT, or bivalent (SB-1 + HVT) vaccine at 1 day of age and then challenged with MDV at 6 days of age. PBMC were extracted from 10 chickens on 1 and 5 DPC and then every week. Replication of MDV was determined by qPCR. A. Replication of MDV in unvaccinated, SB-1, HVT, or bivalent (SB1 + HVT) 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 tumor incidence calculated. Statistical analysis was determined using an unpair *t*-test with $^{*}P < 0.05$, $^{**}P < 0.01$.

nated 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 (Fig. 3B and C) compared to unvaccinated control (Fig. 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 (Fig. 2A) and all tissues (Fig. 3D).

Moreover, we also measured the percent of birds that developed gross tumors (Fig. 2B). Only birds vaccinated with bivalent

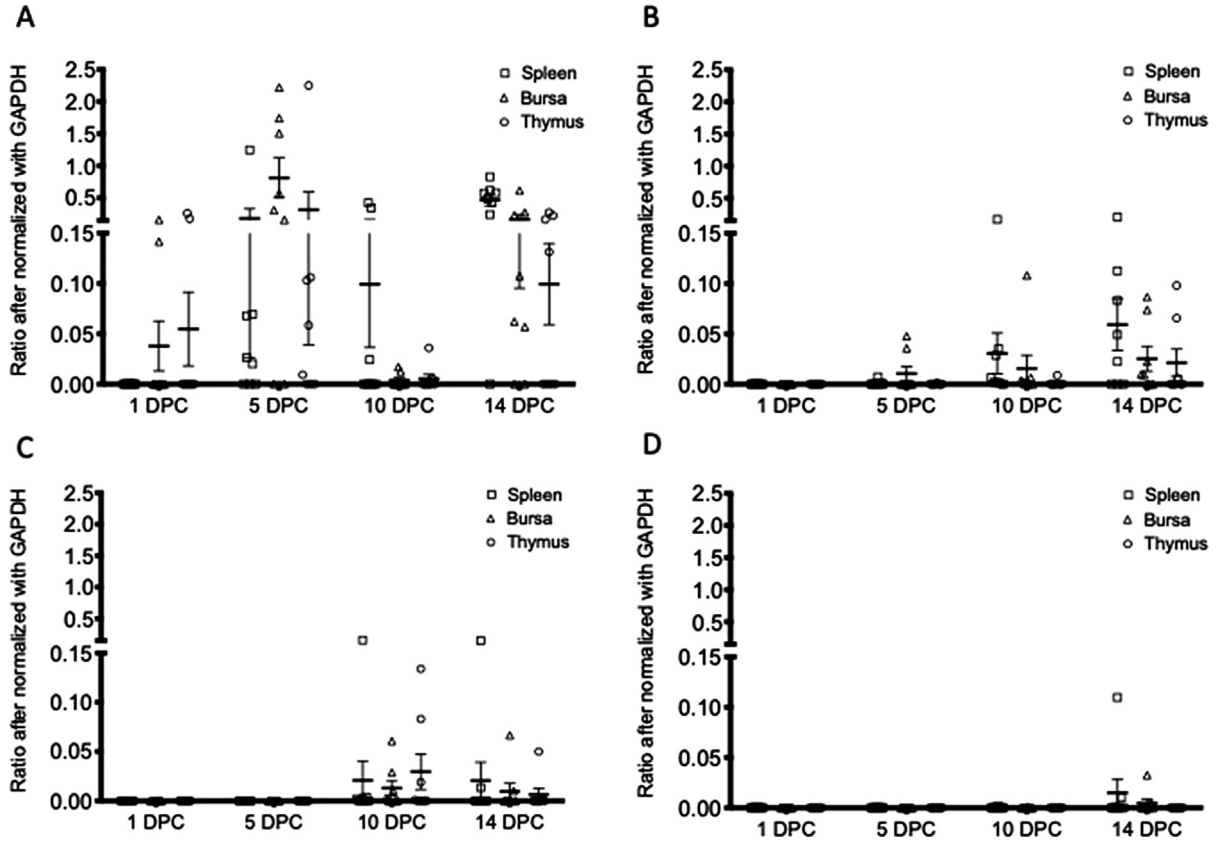

Fig. 3. Replication patterns of MDV in unvaccinated and vaccinated birds. Replication of MDV in spleen, bursa, and thymus were measured by qPCR at 1, 5, 10, and 14 DPC in unvaccinated controls (A), SB-1 vaccinated birds (B), HVT vaccinated birds (C), and bivalent (SB-1 + HVT) vaccinated birds (D). The data were analyzed using Ct cycle and normalized with Ct of GAPDH gene. Each dot represents the $2^{-\Delta Ct}$ value from an individual bird.

SB-1 + HVT did not develop any tumors while tumors were observed in all of the other groups.

3.3. 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 (Fig. 4). Specifically, SB-1 replicated well in bursa and spleen early, and increased steadily in the spleen (Fig. 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 (Fig. 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 (Fig. 4A vs. 4C and 4B vs. 4D).

3.4. The bursa is necessary for protection provided by HVT

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 was necessary for protection induced by HVT in both monovalent HVT and bivalent (SB-1 + HVT) vaccine trials as observed by the lower percent protection in bursectomized chickens (Fig. 5).

4. Discussion

MD vaccines have been used widely since 1970, e.g., currently 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 as most have been empirically developed, often through blind serial *in vitro* cell passages. 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 [9–11,13]. 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 [15]. Distinct down regulation of IL-6, IL-10, and IL-18 have been reported when vaccinated with SB-1 + HVT bivalent vaccine [16].

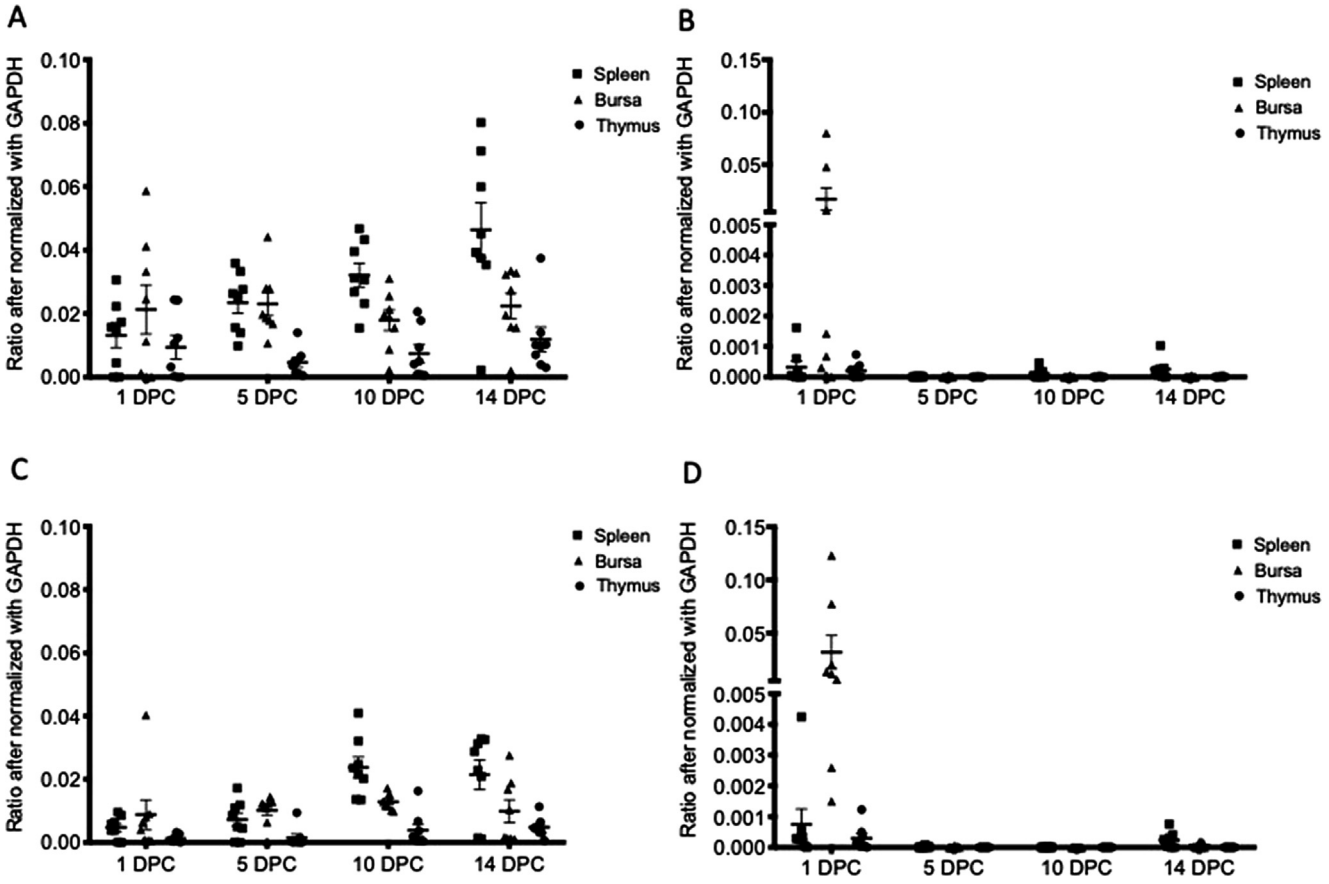

Fig. 4. Replication patterns of SB-1 and HVT in lymphoid organs. Monovalent of SB-1 or HVT, or bivalent SB-1 + HVT was administered to each group of birds. Replication of each viral vaccine in spleen, bursa, and thymus were measured by qPCR at 1, 5, 10, and 14 DPC. A and C show replication of SB-1 in monovalent SB-1 and bivalent (SB-1 + HVT) vaccinated groups, B and D show replication of HVT in monovalent HVT and bivalent SB-1 + HVT vaccinated groups. The data were analyzed using Ct cycle and normalized with Ct of GAPDH gene. Each dot represents the $2^{-\Delta Ct}$ value from an individual bird.

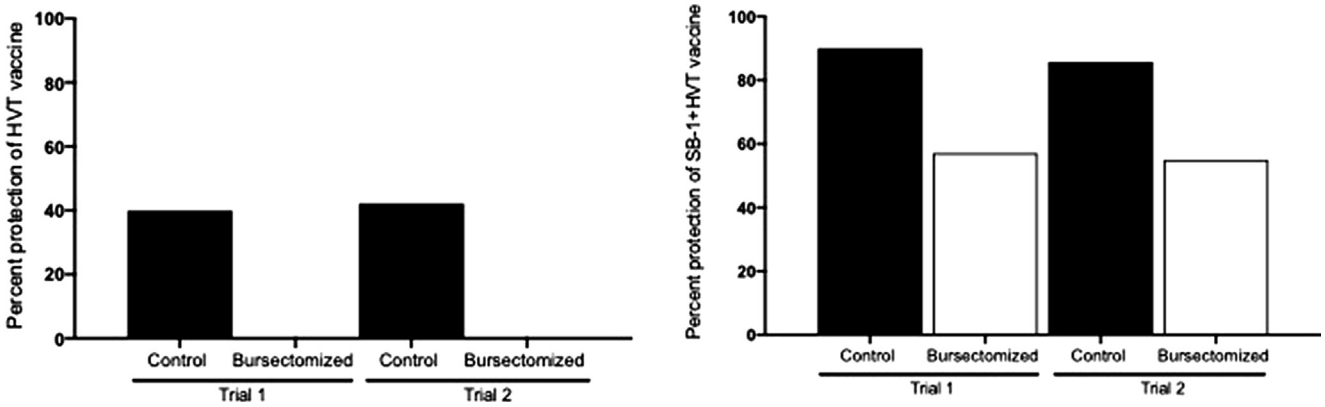

Fig. 5. Protection of HVT and bivalent (SB-1 + HVT) vaccines in normal chickens compared to bursectomized chickens. Bursectomy was performed to remove the bursa in newly hatch chickens. Each group contained 17 chickens. Control or bursectomized chickens were immunized with HVT or SB-1 + HVT and later challenged with pathogenic MDV. Pathology of MD was determined and percent protection of HVT or bivalent (SB-1 + HVT) was calculated in control and in bursectomized birds.

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 [17]. Virulent and avirulent strains coexist and can compete in the same host [18]. 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 [19] Yet, anti-tumor immunity against JMV was not found in HVT vaccinated birds [20].

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) [21]. 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 [19]. 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 tumors were 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 replication patterns in the bursa and spleen appeared to be similar to those 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* (2). In addition, synergism can occur even when only 80 PFU of SB-1 is added into HVT vaccine [22,23]. 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 where slightly lower replication of SB-1 was observed when it was combined with HVT [24]. However, the reduction did not influence the synergistic outcome. In our study, the lower replication of SB-1 from the bivalent vaccine, compared with monovalent SB-1, is most likely due to the fact that the dose of SB-1 in the bivalent vaccine was half that compared to the monovalent vaccine. 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 of 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 broader immune cell types in different time frame resulting in broader 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.

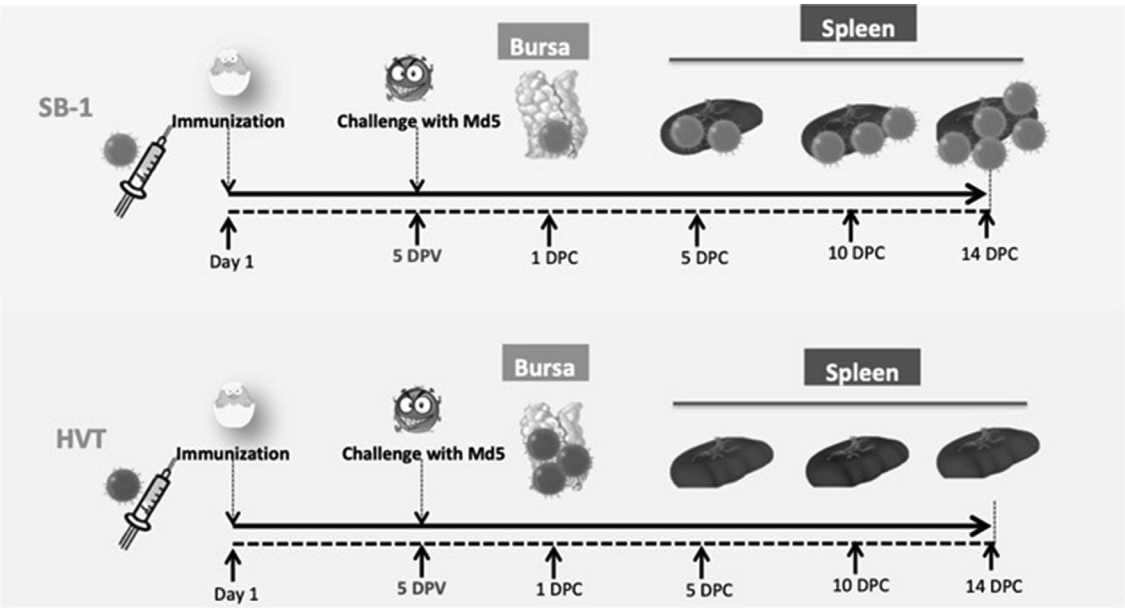

Fig. 6. The model of protective synergy of bivalent (SB-1 + HVT) vaccine. SB-1 replicates predominantly in the spleen from 1 DPC to 14 DPC, while HVT shows replication restricted to bursa at 1 DPC and the bursa is necessary for HVT-induced protection.

It is interesting to note that virulent MDV replication levels were highest in lymphoid organs compared to PBMCs, and were substantially higher compared to those of either MD vaccine strain. This observation might be accounted for by two possible explanations. First, immature cells may be more prone to infection compare to mature and circulating cells. Second and more likely, infected cells tend to home to lymphoid tissues and proliferate resulting in expansion.

The bursa is an important organ for the MDV life cycle [25]. B cells in the bursa are initially infected with MDV during the early cytolytic phase [26–28]. 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 agrees 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 [29,30]. 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 [25]. Nonetheless, embryonic bursectomy did not interfere with SB-1 infection [25], which may not need the bursa during its cycle.

Recently Bertzbach and co-workers [31] demonstrated using a bioengineered chicken that lacked mature B cells showed these cells are not necessary for MD. Furthermore, MDV could still replicate in the bursa but spread to the thymus and spleen was delayed. This suggests that the bursa, as an organ itself, may be necessary for the protective effects induced by HVT. Specifically, the bursa possesses a diffusely infiltrated area (DIA) where T cells localize [32] and respond to antigens [33]. In short, further studies on why the bursa is essential and which cell types in the bursa are responsible for HVT-induced immunity are needed to fully elucidate the mechanism(s) of MD vaccinal protection and/or synergy.

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 (see our model in Fig. 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.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Laurie Molitor and Lonnie Milam for their technical assistance, and Henry Hunt and Jerry Dodgson for their constructive input and comments. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

Funding

Partial support for this project was provided by the Development and Promotion of Science and Technology Talents Project (DPST), Thailand.

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