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B cells do not play a role in vaccine-mediated immunity against Marek's disease

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ABSTRACT

Background: Marek's disease virus (MDV), a highly oncogenic α-herpesvirus, is the etiological agent of Marek's disease (MD) in chickens. The antiviral activity of vaccine-induced immunity against MD reduces the level of early cytolytic infection, production of cell-free virions in the feather follicle epithelial cells (FFE), and lymphoma formation. Despite the success of several vaccines that have greatly reduced the economic losses from MD, the mechanism of vaccine-induced immunity is poorly understood.
Methods: To provide insight into possible role of B cells in vaccine-mediated protection, we bursectomized birds on day of hatch and vaccinated them eight days later. The birds were challenged 10 days post vaccination with or without receiving adoptive lymphocytes from age-matched control birds prior to inoculation. The study also included vaccinated/challenged and non-vaccinated challenged intact birds. Flowcytometric analysis of PBMN cells were conducted twice post bursectomy to confirm B cell depletion and assess the effect of surgery on T cell population. Immunohistochemical analysis and viral genome copy number assessment in the skin samples at termination was performed to measure the replication rate of MDV in the FFE of the skin tissues of the challenged birds.
Results: The non-vaccinated/challenged birds developed typical clinical signs of MD while the vaccinated/challenged and bursectomized, vaccinated/challenged groups with or without adoptive lymphocyte transfer, were fully protected with no sign of transient paralysis, weight loss, or T cell lymphomas. Immunohistochemical analysis and viral genome copy number evaluation in the skin samples revealed that unlike the vaccinated/challenged birds a significant number of virus particles were produced in the FFE of the non-vaccinated/challenged birds at termination. In the bursectomized, vaccinated/challenged groups, only a few replicating virions were detected in the skin of birds that received adoptive lymphocytes prior to challenge.
Conclusions: The study shows that B cells do not play a critical role in MD vaccine-mediated immunity.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of domestic chickens caused by a highly contagious, cell-associated, oncogenic α-herpesvirus, Marek's disease virus (MDV) [1]. Clinical signs of MD include transient paralysis, crippling, weight loss, depression, immunosuppression, and lymphoma formation in visceral organs [2]. Initial respiratory infection begins by inhalation of enveloped cell-free virus particles shed into the environment from the skin of MDV-infected chickens via molted feathers and dander [3]. It is believed that MDV is transported from the lungs to the lymphoid organs via macrophages and dendritic cells that have either been infected or have phagocytosed the virus [4,5].

The early pathogenesis of MDV is characterized by a burst of productive/restrictive infection in B cells followed by a latency phase of infection in activated $CD4^+$ T cells that could last up to three weeks prior to reactivation and transformation of T cells [2,6]. Destruction of B and T cells during the lytic infection leads to atrophy of bursa of Fabricius and thymus and consequently, a transient immunosuppression [2]. Reactivation from latency is depicted as a second wave of cytolytic infection, induction of T cell lymphomas, and a permanent immunosuppression [7,8].

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MDV strains have been categorized into three serotypes based on pathogenicity, antigenic differences, and biological features. The oncogenic strains causing MD in susceptible chickens and their attenuated forms are classified as serotype 1. Non-pathogenic strains include serotypes 2 (e.g., SB-1) and 3 (Herpesvirus of turkey, HVT) [9–11]. The attenuated serotype 1 (CVI988/Rispens) and the non-oncogenic serotypes 2 and 3 (SB-1 and HVT, respectively) have been used as vaccines to control MD in the last several decades [12–14]. The naturally attenuated serotype 1, CVI988/Rispens, is considered the gold standard and the most effective vaccine against highly pathogenic strains of MDV [3,15,16]. It is speculated that due to antigenic similarity to pathogenic strains of MDV, the attenuated serotype 1 vaccine strains mediate better protection and induce a more effective immune response [17]. All MD vaccines share one intriguing feature. They prevent T cell lymphoma formation and neurological disorders, but not infection, replication or horizontal transmission of the virus [3,18,19].

Although MD vaccines have been quite successful in controlling MD for more than four decades, the underlying molecular mechanism of vaccine-mediated immunity is not well known. MD vaccines induce a robust innate immune response, but unlike most other vaccines, induction of an adaptive immune response is minimal [19]. It is speculated that shortly after vaccination, activated NK cells induce production of IFN-γ and destruction of MDV-infected B cells. IFN-γ in turn, inhibits MDV replication and activates macrophages leading to NO production. NO has direct inhibitory effect on viral replication and infection [20].

Despite the widespread use and success of vaccination, MD remains a major problem for the poultry industry. The continual evolution of MDV toward greater virulence is a concern that the existing vaccine might eventually fail to provide protection against newly evolved and highly pathogenic strains of MDV [21–25]. Therefore, deciphering the molecular mechanism of vaccine-mediated protection is of critical importance for developing more effective vaccines against highly virulent and newly evolved strains of MDV.

To provide insight into possible role of cellular components of adaptive immune system in vaccine-induced protection, we depleted B cells in day-old chicks by surgical removal of bursa of Fabricius on day of hatch and vaccinated them in the absence of B cells followed by challenge ten days post vaccination. The bursa of Fabricius found in avian species, is a pear shaped dorsal, diverticulum of cloaca in which B cells are developed [26]. By selective elimination of bursa of Fabricius, a primary lymphoid organ in chickens, it is possible to examine the role of associated B cells in vaccine-induce immunity.

Material and methods

Experimental chickens

The specific-pathogen-free chickens in this study were from the highly inbred MD-susceptible F1 progeny ($15I_5X7_1$) of the Avian Disease and Oncology Laboratory (ADOL) lines $15I_5$ males and 7_1 females. The $15I_5X7_1$ birds were from unvaccinated breeder hens and carried no maternal antibodies to MDV or to herpesvirus of turkey. Chicks were hatched at ADOL poultry facility and housed in modified Horsfall-Bauer isolation units for the duration of the experiment.

All animal experiments were approved and carried out in accordance to the guidelines set forth by the Avian Disease and Oncology Laboratory Institutional Animal Care and Use Committee and the Guidelines for Care and Use of Laboratory Animals published by Institute for Laboratory Animal Research (ILAR Guide) in 1996 (http://www.nap.edu/openbook.php?record_id=5140).

Viruses

A Bacterial Artificial Chromosome (BAC)-cloned very virulent (vv) strain of MDV, rMd5, was used in this experiment [27]. The CVI988/Rispens vaccine was purchased from Intervet (USA). Chickens were inoculated intraperitoneally with 2,000 plaque-forming unit (PFU) of the vaccine virus per manufacturer's instruction and challenged with 1000 PFU of rMd5. One dose of vaccine contains approximately 2,000 PFU of the vaccine virus.

Experimental design

One hundred twenty-five one-day-old chicks from line $15I_5X7_1$ were randomly distributed into 5 groups of 25 birds each in separate isolators (Table 1, A-E). Birds in group A served as the non-treated negative control. The birds in groups B and C were bursectomied on day of hatch and received 100 μg intraperitoneal (IP) injection of unlabeled anti B cells monoclonal antibody (SouthernBiotech) on day five post bursectomy to neutralize the residual B cells. The birds in groups D and E served as vaccinated/challenged and challenged only, respectively. Flowcytometric analysis of PBMN from groups A, B, and C was performed one-week post bursectomy and 24 days post challenge (41 days post-surgery) to confirm complete B cell depletion in the bursectomized birds and the effect of bursectomy on T cell populations. All birds in groups B, C, and D were vaccinated with 2000 PFU of CVI988/Rispens on day eight post hatch/bursectomy. To provide target B cells for MDV, the birds in group B received 5×10^7 lymphocytes from age-matched naïve birds on day 7 post vaccination via jugular vein injection of cell suspension in sterile PBS. Three days later, all birds in groups B, C, D, and E were challenged IP with 1000 PFU of rMd5. The birds were observed for clinical sign of MD twice per week until termination of the experiment at 57 days post challenge. The chickens from each group were euthanized by CO2 inhalation and necropsied for tissue collection at termination. Skin and other tissues were collected and stored in RNAlater (Thermo Fisher Scientific) at − 20 °C until used. Skin samples were from the dorsal cervical and capital tracts of individual birds. Skin and other tissue samples were collected for DNA analysis, genome copy number determination, and immunohistochemistry.

Genomic DNA isolation

Genomic DNA was isolated using the Gentra Puregene kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Briefly, sections of skin tissue stored in RNAlater (Thermo Fisher) were washed in PBS and blot-dried. Approximately 100 mg of blot-dried skin tissue was homogenized and mixed with 400 μl of cell lysis solution (Gentra Puregene kit, Qiagen, Hilden, Germany) with Proteinase K (100 μg/μL, final concentration). Tissue lysates were incubated overnight in a heat block shaker at 60 °C and 900 rpm. To exclude RNA contamination, the tissue lysates were incubated with RNase A for 30 min at 37 °C followed by the addition of protein precipitation solution and centrifugation. The supernatants were transferred to a clean microcentrifuge tube and 400 μl isopropanol was added. Samples were inverted several times and centrifuged again and the DNA pellet was washed in 70% ethanol and air-dried. DNA pellets were rehydrated in clean water. To remove lipid and fat contamination, all DNA solutions were mixed with chloroform:isoamyl alcohol, centrifuged, and the aqueous layer was removed for further analysis. DNA concentrations of samples were quantified using a NanoDrop 8000 (Thermo Fisher).

Table 1
Outline of experimental design: vaccination and challenge study in bursectomized chickens.

Chicken Line $15I_5X7_1$ (125 birds) 25 birds/group	Bursectomy Day of hatch	Anti B cell antibody treatment 100 μg/IP 5 dph	Flow to verify B cell depletion 7 dph	Vaccination Rispens 2000 pfu 8 dph	Adoptive lymphocyte transfer 7 dpv 5×10^7 cells	Challenge rMd5 1000 pfu 10 dpv	Flow to analyze cell population 24 dpi	Termination & sample collection: 57 dpi
A: Control No treatment	–	–	√	–	–	–	√	√
B: birds with B cell depletion	√	√	√	√	√	√	√	√
C: birds with B cell depletion	√	√	√	√	–	√	√	√
D: birds with intact B cells population	–	–	–	√	–	√	√	√
E: birds with intact B cells population	–	–	–	–	–	√	√	√

dph: days post hatch; IP: intraperitoneal, dpv: days post vaccination; dpi: days post infection.

MDV genome copy number assay

The quantitative Real-Time PCR (qPCR) for MDV genome copy number analysis was according to the previously described protocol [24,28]. Analysis of genomic DNA from each skin sample was performed in triplicate on a single 96 well plate. qPCR assays were performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers to MDV gB and chicken GAPDH were each used at 0.5 μM to amplify their respective genes. Probes to MDV gB and chicken GAPDH were used at 0.2 μM. MDV loads were shown as the copy number of MDV gB divided by the copy number of chicken's GAPDH. Statistical analysis was performed with the aid of GraphPad software (GraphPad, La Jolla, CA) using an unpaired *t*-test.

Immunohistochemistry

Samples previously flash frozen in embedding medium, Optimal Cutting Temperature (OCT) (Sakura Finetek, Torrance, CA), were sectioned on a cryotome at 5 μm and placed on slides coated with 2% 3-Aminopropyltriethoxysilane and air dried at 25 °C overnight. Subsequently, microtome sections were fixed in formal acetate fixative for 10 min at room temperature followed by 3 changes of Tris buffered saline – 5 min each. Endogenous peroxidase activity was blocked with 0.3% Hydrogen peroxide in Tris buffered saline for 20 min followed by tap and distilled water rinses. Following pretreatment standard, Avidin-Biotin complex staining steps were performed at room temperature on the DAKO Autostainer (Agilent Technologies, Carpentaria, CA). All staining steps were followed by rinses in Tris buffered saline + tween 20 (Scytek Laboratories, West Logan, UT). After blocking for non-specific protein with normal horse serum (1/30 dilution in PBS; Vector Labs, Burlingame, CA) for 30 min, sections were incubated with Avidin/Biotin blocking system for 15 min each (Vector Lab, Burlingame, CA; Sigma, St. Louis, MO). Samples were then incubated with mouse anti MDV gB monoclonal antibody [10] for 1 h in Normal Antibody Diluent (NAD) (Scytek Laboratories, West Log, UT) followed by rinsing and incubation with biotinylated horse anti-mouse IgG (H + L) prepared at 11.5 μg/ml in NAD incubated for 60 min. Samples then were incubated with R.T.U. Vector Elite Peroxidase Reagent (Vector Laboratories, Burlingame, CA) for 30 min. Reaction development utilized Vector Nova Red peroxidase chromogen incubation of 15 min followed by counterstain in Gill Hematoxylin (Thermo Fisher) for 15 s, differentiation, and dehydration, clearing and mounting with synthetic mounting medium. The working solution for the monoclonal antibody specific for MDV gB was 1:1000.

Bursectomy

Surgical bursectomy was performed on baby chicks on day of hatch. Using a DRE Veterinary DRE-VP3 anesthesia machine (DRE Veterinary, Louisville, KY), chicks were first placed in a box and inhalation anesthesia was slowly administered at equal parts medical grade oxygen and Isofluorane (MWI Animal Health, Boise, ID). Once the chicks were anesthetized, they were held with a face mask covering their beaks and nostrils with anesthesia being administered during surgery. The surgical area was cleaned with a chlorhexidine wipe. Using a sterile scalpel, a small incision was made at the base of the tail, just above the upper lip of the vent. A pair of sterile forceps was used to carefully remove the bursa of Fabricius through the small incision over the cloaca. Once the bursa of Fabricius was removed, the incision was sealed with Vetbond surgical glue (3 M Animal Care products, St. Paul, MN). Chicks were kept warm in a portable incubator as they recovered. Each bird was given 0.06 ml subcutaneous injection of Meloxicam SR (2 mg/ml) for pain relief. Once the chicks had recovered from anesthesia, they were placed in heated isolators. The cloacal area was examined daily for 5 days to ensure proper healing. Tetracycline was administered via water for one week.

Peripheral blood mononuclear cells

At 7 and 41 days of age, three chickens from each group were bled via jugular vein (0.5–1 ml). Due to the small size of young birds, the anticoagulated blood samples from each group were pooled and 1.5–3 ml of each sample was mixed with equal volume of PBS and layered onto 3–6 ml of Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) in a 15 ml conical centrifuge tube and centrifuged at 400*g* for 30 min at room temperature. The PBMN were aspirated from the interphase, diluted with 10 ml of isotonic phosphate buffered saline solution and pelleted by centrifugation at 250*g* for 10 min. The PBMN were washed three times in PBS by resuspension of pellet and centrifugation at 250*g* for 10 min each.

Adoptive lymphocyte transfer

Three mL of anticoagulated fresh blood sample were mixed with three mL of sterile PBS and carefully layered onto 6 ml of room temperature Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) in a 15-mL conical centrifuge tube. Samples were centrifuges at 400*g* for 30 min at room temperature. The opaque interface layer was carefully transferred into a clean conical tube. The cells were washed twice by adding 5 ml of room temperature PBS and cen-

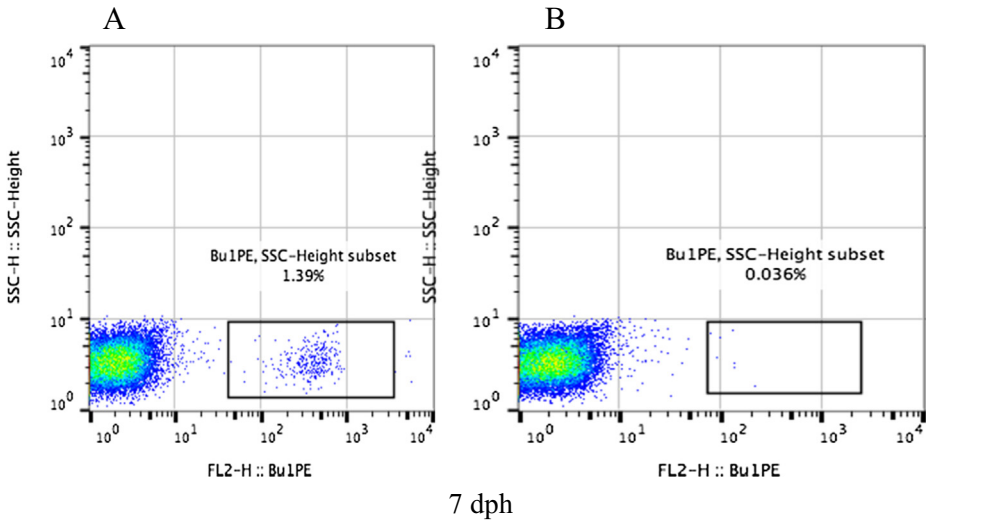


Fig. 1. The flow cytogram depicts the percentage of B cells in the tested blood samples from the control (Panel A) and the bursectomized birds (Panel B) at 7 days post-surgery. Blood samples from three birds per group were pooled, lymphocytes isolated and 100 µl of total cells at 1×10^6 cells/ml was used for cell surface antigen analysis. B cells were stained with RPE labeled anti B cells monoclonal antibody (Bu1-RPE).

trifuges at 250*g* for 10 min. After removing the final supernatant, cells were resuspended in 0.5 ml of sterile PBS and counted with a cell counter. A total of 30 ml of blood sample was processed to obtain 5×10^7 cells per bird.

Monoclonal antibodies used in flow analysis and depletion of residual B cells

The monoclonal antibody for detection of chicken B Cells (Mouse anti chicken Bu1-RPE) and $CD4^+$ T cells (Mouse anti chicken CD4-PE) were purchased from SouthernBiotech (Birmingham, AL). The monoclonal antibody for detection of $CD8\alpha^+$ T cells ($CD8\alpha^-$ FITC, 11–39) was from ThermoFisher Scientific. The unlabeled anti-chicken B cell monoclonal antibody was also purchased from SouthernBiotech.

Flowcytometry

Subpopulation of the isolated PBMN from pooled blood samples were quantified based on the expression pattern of cell surface antigens. Aliquots of 1×10^6 PBMN in 100 µl of FACS buffer were added to 96-well plate and incubated with specific monoclonal antibodies for 30 min at 4 °C. Cells were washed 4 times with 200 µl of FACS buffer. The washed cells were resuspended in 200 µl of FACS buffer and analyzed by flowcytometry. A FACScan flowcytometer from Becton Dickinson (Mountainview, CA) was used for the cell surface analysis.

Statistical analysis

Since the blood samples from three individual birds from each group were pooled due to the small size of the animals, no statistical analysis could be performed and consequently, the bar graphs represent relative changes in B and T cell populations (Figs. 2 and 4). The MDV genome copy number, however, was based on comparative analysis between individual infected and control birds. Statistical analysis for this data was performed with the aid of GraphPad software (GraphPad, La Jolla, CA) using an unpaired *t*-test.

Results

Bursectomy and B cell depletion

To confirm that surgical removal of bursa of Fabricius on day of hatch and anti-B cell antibody treatment of bursectomized birds on day 5 post-surgery has resulted in near complete depletion of B cells, flowcytometric analysis of pooled PBMN from three birds were performed on day 7 and day 41 post bursectomy. In addition to verifying B cells depletion, the effect of bursectomy on $CD4^+$ and $CD8^+$ T cells were also assessed.

Flowcytometric analysis of PBMN at 7 days post bursectomy

Comparative analysis of PBMN between the bursectomized and untreated control birds at 7 days post bursectomy revealed that B cells were depleted efficiently and the population was dropped to a negligible level of 0.036% of total lymphocytes in the blood sample (Fig. 1). The population of both $CD4^+$ and $CD8^+$ T cells were considerably increased in the bursectomized birds. This is likely due to an immune response to surgery (Fig. 2).

Flowcytometric analysis of PBMN at 41 days post bursectomy

To further verify the complete depletion of B cells and the effect of bursectomy and challenge virus on T cells, we compared the B cell, $CD4^+$, and $CD8^+$ T cell populations in the pooled blood samples of three treated and three control birds randomly at 41 days post bursectomy (24 days post challenge). Flowcytometric analysis showed that the population of B cells for the bursectomized birds with or without adoptive lymphocyte transfer was around 0.15% of the total lymphocytes in the tested blood sample while that of the control birds was 5.55% (Figs. 3 and 4). Comparing to the control birds, the population of B cells in the non-bursectomized vaccinated/challenged and challenged only birds were substantially lower than those of the control birds (Fig. 4). The $CD4^+$ T cell population was considerably higher in the non-bursectomized non-vaccinated challenged birds. With minor differences the population of $CD8^+$ T cells varied among the treated groups when compared to that of the control birds.

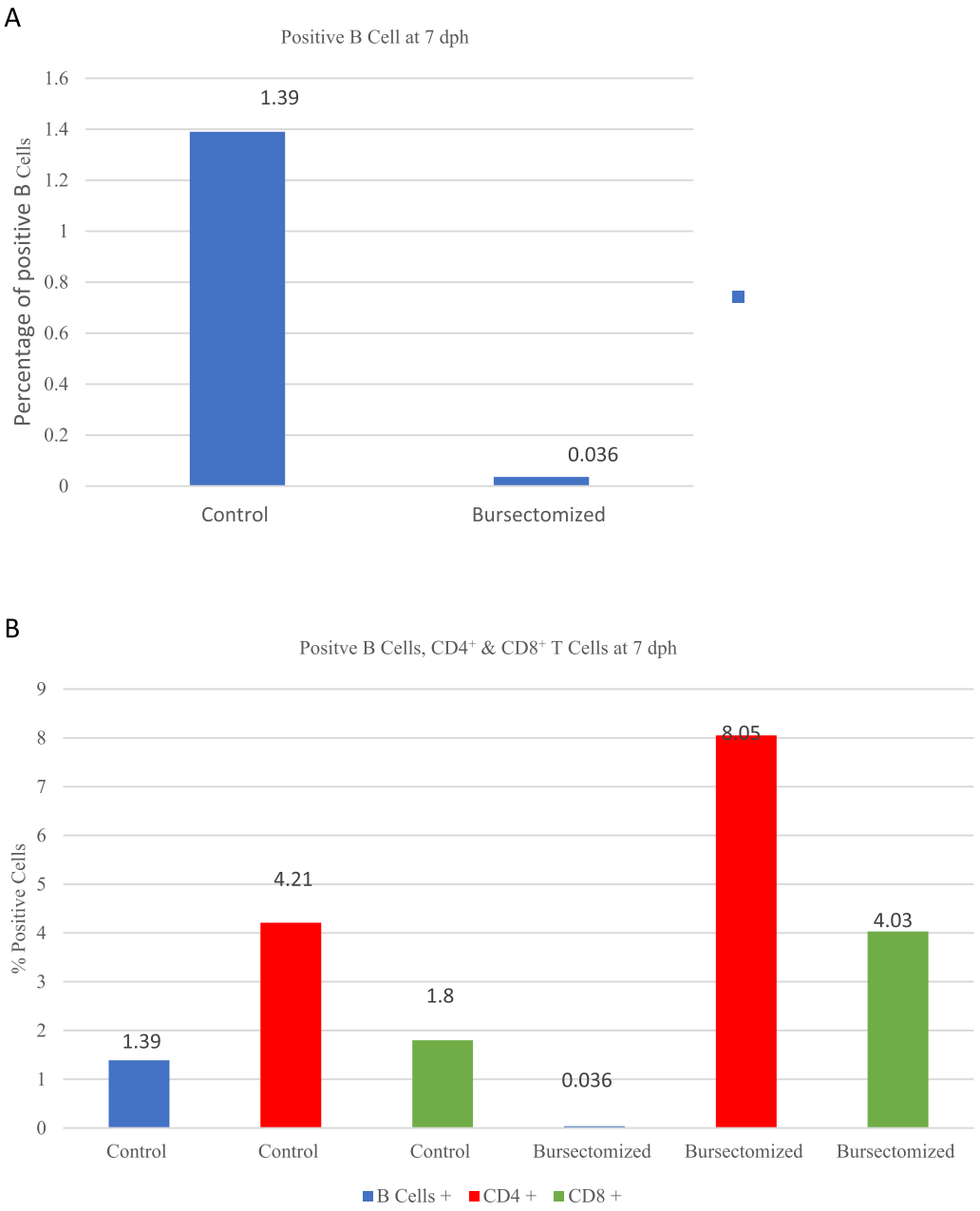


Fig. 2. Bar graph showing the percentages of B cells, $CD4^+$ T cells, and $CD8^+$ T cells in the tested blood samples at 7 days post bursectomy. Comparative analysis is made between the untreated control and the bursectomized birds. Same total blood samples were used for staining of B cells and double staining of $CD4^+$, and $CD8^+$ T cells. B cells, $CD4^+$ T cells, and $CD8^+$ T cells were stained with monoclonal antibodies Bu1-RPE, CD4-PE, and CD8α-FITC, respectively (See M and M).

Immunohistochemistry

For detection of the challenge virus replication in the skin of treated birds (Groups B-E) anti-gB (MDV glycoprotein) monoclonal antibody was used. Immunohistochemical analysis of the skin samples revealed that a significant number of viruses were produced in the FFE of non-bursectomized non-vaccinated challenged birds (Fig. 5, panel B). The B cell-depleted, vaccinated, adoptive lymphocyte recipient/challenged birds exhibited far less replication of MDV and only in a few feather follicles comparing to group B (Panel C). No viral antigen was detected in the skin samples of B cell-depleted, vaccinated, challenged birds (Panel D). The non-bursectomized, vaccinated/challenged birds showed minor replication of the virus in the pulp region of only one feather follicle but not in the FFE (Panel E).

Genome copy number

To verify the replication pattern of the MDV observed in the FFE of the treated birds via immunohistochemistry, DNA samples of the skin tissues from three individual birds from each group at 57 dpi were used for determination of viral genome copy number. The high genome copy number of MDV in the skin of the non-vaccinated challenged birds supports the result of immunohistochemical analysis (Fig. 6 & Table 2). Despite minor replication of the virus in the FFE or feather pulp of other treated groups, the genome copy number was zero for all treated groups. Detection of viral DNA in the FFE depends on the skin samples taken for analysis and DNA isolation. Since the replication of the virus was minimal and only observed in a few FFE or feather pulp, it is not surprising the MDV DNA was not detected in the skin of latter groups.

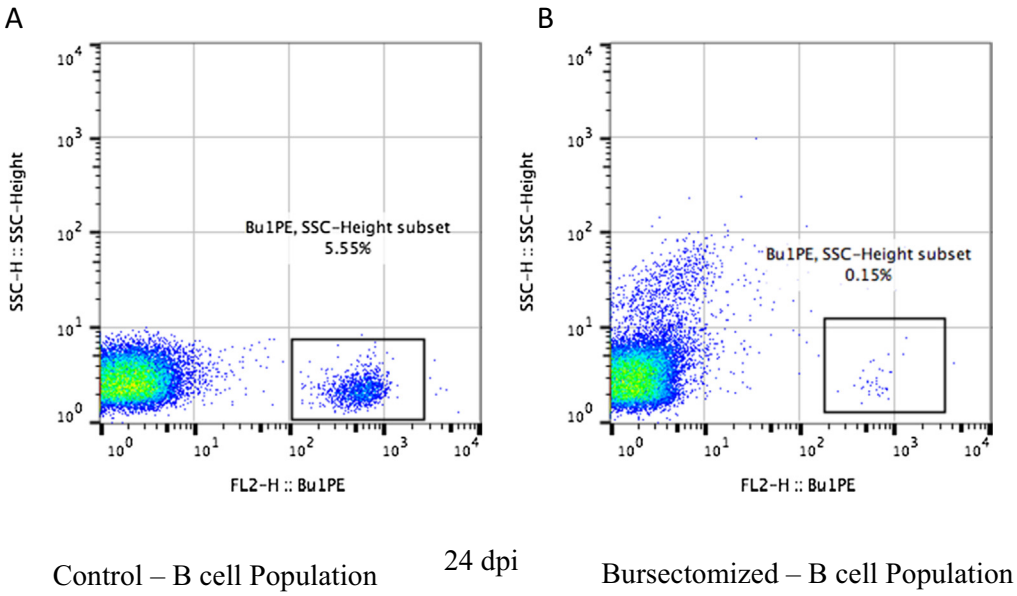
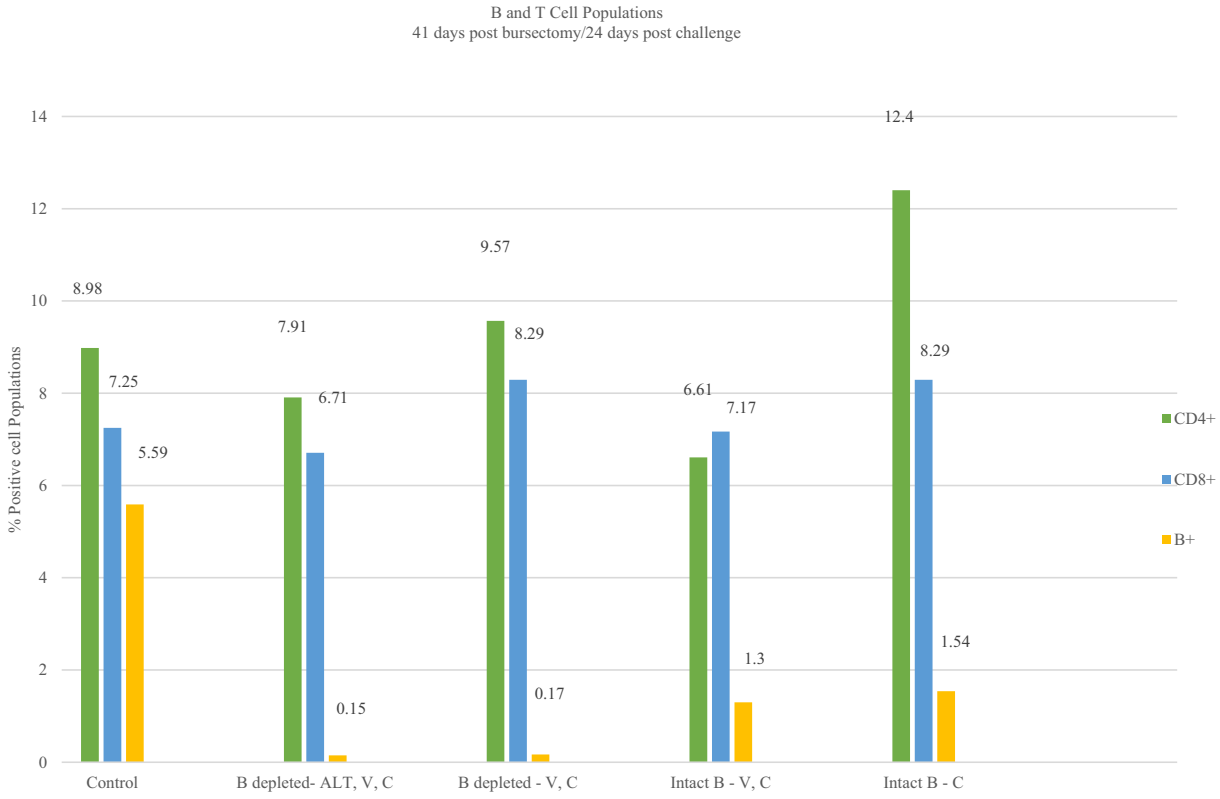


Fig. 3. The flow cytogram depicts the percentage of B cells in the tested blood samples from the control (Panel A) and bursectomized birds (Panel B) at 41 days post bursectomy (24 days post challenge). Blood samples from three birds per group were pooled, lymphocytes isolated and 100 µl of total cells at 1×10^6 cells/ml was used for cell analysis. B cells were stained with RPE labeled anti B cells monoclonal antibody (Bu1-RPE).



V = vaccinated; C = challenged; ALT = Adoptive Lymphocyte Transfer

Fig. 4. Bar graph showing the percentages of B cells, $CD4^+$ T cells, and $CD8^+$ T cells in the tested blood samples at 41 days post bursectomy (24 days post challenge). Comparative analysis was made among birds from all five groups including the untreated control, bursectomized birds with adoptive lymphocyte transfer that were vaccinated/challenged, bursectomized, vaccinated/challenged, un-bursectomized, vaccinated/challenged, and un-bursectomized, un-vaccinated/challenged. Same total blood samples were used for staining of B cells and double staining of $CD4^+$, and $CD8^+$ T cells. B cells, $CD4^+$ T cells, and $CD8^+$ T cells were stained with monoclonal antibodies Bu1-RPE, CD4-PE, and CD8α-FITC, respectively.

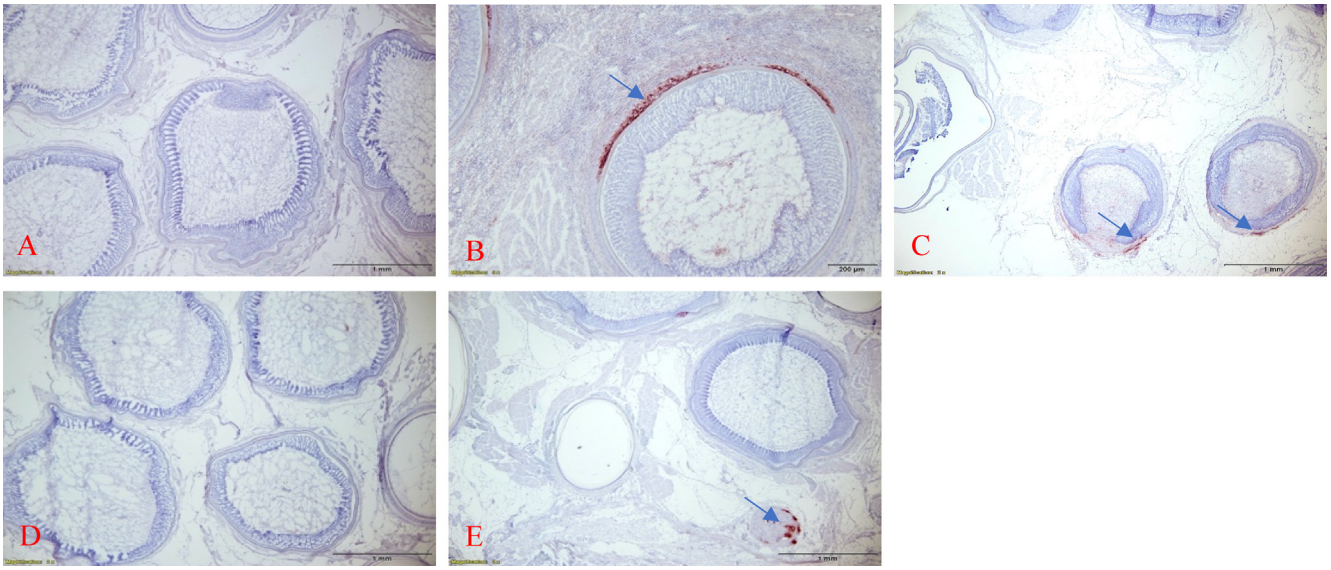


Fig. 5. Immunohistochemical analysis of MDV antigen in the skin samples of all 5 groups. Anti-gB monoclonal antibody was used for detection of virus particles in the skin tissues of challenged groups. Panel A depicts skin sample from a control bird with no MDV antigen detected. Panel B is showing significant viral replication in the FFE of a bird from un-bursectomized, un-vaccinated/challenged group (Arrow). Panel C represents the skin sample from B cell-depleted, vaccinated, adoptive lymphocyte received, and challenged bird with minor MDV antigen detection in the FFE (Arrows). Panel D is depicting skin sample from a bursectomized, vaccinated/challenged bird that exhibits no sign of viral antigen. Panel E is the skin sample from a vaccinated/challenged bird with intact bursa of Fabricius with minor replication of MDV in the pulp region of one feather shaft (Arrow). Immunohistochemistry was performed on the skin tissues of three individual birds per group.

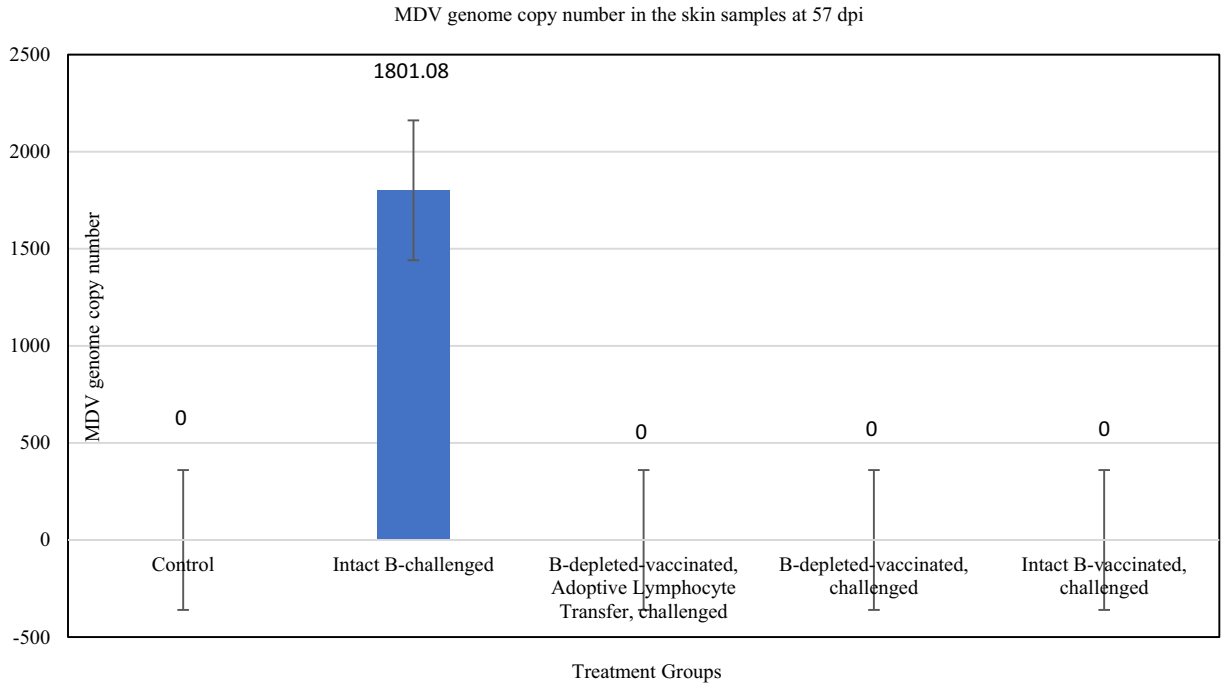


Fig. 6. The genome copy number of MDV was based on the DNA samples isolated from skin tissues of treated birds at termination (57 dpi). Despite detection of minor replication of the virus in the skin of couple of treated groups, the genome of MDV was only detected in the skin samples of non-vaccinated/challenged birds. DNA samples were isolated from three individual birds of each group and genome copy number analysis was performed in duplicates for each sample.

Vaccine protection in the absence of B cells

Vaccination of the bursectomized birds with or without receiving adoptive lymphocytes provided 100% protection without any clinical signs of MD. The bursectomized, vaccinated, and challenged birds looked normal like the age-matched control birds and did not show sign of transient paralysis, weight loss, or depression post challenge. At termination, these birds did not display nerve enlargement or tumor development and weighed as much as the control birds. The birds with intact B cells that were vaccinated and challenged, also were protected without any clinical sign of disease. The non-vaccinated/challenged group, however, exhibited transient paralysis around 11 days post infection and weight loss, nerve enlargement, and tumor development at termination (Table 3).

Table 2
MDV genome copy number at termination (Skin samples).

Group	Genome Copy Number*
Control	0
Intact B Cells - Challenge	1801.08
B cell-depleted – adoptive lymphocyte transfer, vaccinated, challenged	0
B cell-Depleted – vaccinated, challenged	0
Intact B Cells – vaccinated, challenged	0

3 samples/virus group tested in duplicate.
*Genome copy number:
(MDV Genome Copy Number/GAPDH Copy Number) * 1000.

Discussion

Marek's disease a prevalent contagious lymphoproliferative disease of domestic chickens is capable of causing enormous economic losses to poultry industry estimated at more than one billion US dollars per year [29]. The clinical sign of MD includes transient paralysis, weight loss, blindness, immunosuppression and T cell lymphomas [30].

MD has been controlled by immunization since late 1960s [12,31,32]. Despite intense vaccination, however, emergence of hypervirulent field strains of MDV have been observed for the last several decades causing great economical losses to poultry industry worldwide [21,33,34]. MD vaccines prevent tumor formation but not superinfection or replication and dissemination of the virus particles from feathers [25]. Although MD vaccines contribute to a significant reduction in viral replication in FFE, prevention of transient paralysis, immunosuppression, and interference with the infection process of pathogenic strains of MDV [35,36], the underlying mechanism of MD vaccine-induced immunity is not well understood.

A recent study by Hao et al. [37] shows that vaccination by CVI988 induces a significant expansion of γδ T cells and CD8α$^+$ T cells in spleen, lung, and blood tissues. The CD4$^+$ T cell population, however, was not affected by the vaccination. A booster immunization with CVI988 induced expansion of CD8$^+$ T cells but not γδ T cells. The authors conclude that vaccination of chickens with CVI988 results in generation of memory CD8$^+$ T cells but not γδ T cells.

It is believed that latently infected CD4$^+$ T cells migrate though the blood stream and establish lymphomas in visceral organs, peripheral nerves, and skin [2]. Fully infectious cell-free enveloped mature virus particles are assembled in the FFE cells of the skin and disseminated into environment to infect contact birds [3]. Studies have shown that infection with virulent strains of MDV induces immunological responses within the skin that include infiltration of CD4$^+$ and CD8$^+$ T cells and up regulation of cytokine gene expression [38]. These host responses, however, are not adequate to prevent viral assembly and replication in the FFE and eventual shedding of the virus particles [39,40].

Because of the highly cell-associated nature of MDV, it is assumed that antibody-mediated humoral immunity plays a minimal role and control of MDV infection is likely mediated by cytotoxic CD8$^+$ T cells and cytokines producing CD4$^+$ T cells [41]. However, detection of antibodies against MDV-encoded glycoproteins (e.g., gB, gE, and gI) have been reported in MDV-infected chickens [42,43]. Anti-gB antibodies are believed to play an important role in controlling MDV by blocking the attachment and entry of virus or destruction of infected cells by antibody dependent cell-mediated cytotoxicity [41]. The role of humoral immunity in controlling of MDV infection is also confirmed by the presence of maternal antibodies that hinder the development of clinical signs and tumor development associated with MDV infection [41].

Earlier studies have shown that sensitized splenocytes of vaccinated chickens inhibits plaque formation in MDV-infected leukocytes or kidney cells [44]. Splenocytes isolated 7 days post vaccination were also shown to kill MD lymphoblastoid cell lines but not antigenically unrelated target cells [45]. Inhibition of plaque formation and killing of target cells were both T cell dependent. Morimura et al. [46] also have demonstrated that antibody-mediated depletion of CD8$^+$ T cells results in a significant increase in MDV titer and a decrease in survival of vaccinated birds post challenge [46,47]. A recent investigation by Umthong et al. [48] provided further evidence that depletion of CD8+ T cells increased tumor incidence and MD pathogenicity and reduced vaccine-mediated protection. Despite the supportive evidence for the role of humoral and T cell-mediated immune responses in viral infection, MDV has evolved and developed sophisticated mechanisms of suppression and evasion of cellular and soluble effectors of the innate and adaptive immune system.

In our study, flow cytometric analysis of PBMN of the bursectomized birds at 7 days post-surgical removal of bursa of Fabricius was effective in depletion of circulating B cells. Combination of bursectomy and IP injection of anti B cells monoclonal antibody reduced the circulating B cell population to an insignificant level of 0.036% of the total lymphocytes used in flow analysis (Fig. 1). The population of CD4$^+$ and CD8$^+$ T cells were almost doubled in the bursectomized birds when compared to those of the age-matched control birds (Fig. 2). This initial increase in T cell populations could be due to an immune response to surgery as the birds were not exposed to any viruses or stimuli prior to this date. Depletion of B cells leading to an increase in the percentage of other lymphocytes including T cells in the fixed number of blood cells analyzed by flow cytometry, could also be an alternative explanation for the increase in the T cell populations.

Table 3
Protection efficacy of Rispens vaccine in B cell depleted birds with and without adoptive lymphocyte transfer.

Chicken Line 15I5 × 7_1 (Ab-)	Bursectomy (Day of age)	Anti-B cells antibody treatment (5dph)	Vaccination (3 days post antibody treatment)	Adoptive lymphocyte transfer (7 dpv)	Challenge rMd5 (10dpv)	MD Incidence	Protection Efficacy
Control	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B cells depleted birds	√	√	Rispens	√	√	None	100%
B cells depleted birds	√	√	Rispens		√	None	100%
Intact B-cell birds			Rispens		√	None	100%
Intact B-cell birds					√	100%	NA

Ab-: maternal antibody negative; dph: days post hatch; dpv: days post vaccination.
Vaccine used: Rispens. See Table 1 and Experimental Design for more details.

At 41 days post bursectomy, the B cell population in the control birds was 5.55% of the circulating lymphocytes while those of the bursectomized birds were around 0.15% (Fig. 3). The percentages of circulating $CD4^+$ and $CD8^+$ T cells varied among the treated groups when compared with those of the control birds. MDV transforms $CD4^+$ T cells around three weeks post challenge that become the foci of tumor formation in the visceral organs. Therefore, it is not surprising to see a substantial increase in the population of $CD4^+$ T cells in non-vaccinated challenged birds. The vaccinated/challenged birds with intact bursa of Fabricius had lower circulating $CD4^+$ T cell population when compared to untreated control birds. It is well documented that vaccination induces a significant reduction in $CD4^+$ T cells [49,50]. The percentage of $CD4^+$ T cells in the bursectomized, vaccinated/challenged birds that did not receive adoptive lymphocytes prior to challenge, was almost at the level of that of the control birds. There was, however, a slight reduction in the population of circulating $CD4^+$ T cells in the bursectomized, vaccinated/challenged birds that received adoptive lymphocytes prior to challenge. This is likely due to the presence of B cells in the transferred lymphocytes that acted as the initial target cells for the vaccine virus that leads to activation and consequent infection and destruction of $CD4^+$ T cells. There was also slight increase in the population of CD8+ T cells in B cell depleted, vaccinated/challenged birds that did not receive adoptive lymphocytes and birds with intact bursa of Fabricius that were challenged without vaccination. It is safe to speculate that the increase in these two groups of birds is due to T cell-mediated immune response to challenge virus infection (Fig. 4).

The immunohistochemical analysis of the skin samples at termination revealed that MDV replicated in the FFE of the non-vaccinated challenged birds at much higher level than those of the vaccinated challenged birds (Fig. 5). Of the bursectomized, vaccinated/challenged birds, only minor replication of the oncogenic virus was observed in the pulp region of a few feather follicles of the birds that received adoptive lymphocytes. Assessment of MDV genome copy number in the skin of treated birds verifies the results obtained from the immunohistochemical studies of viral replication in the FFE (Fig. 6 and Table 2). The minor differences in replication of MDV in the FFE cells of the skin observed between the bursectomized, vaccinated, and challenged birds with or without adoptive lymphocytes transfer, refutes the long-held dogma that the presence of the B cells acting as the initial target cells for MDV is an essential step in the activation and consequent infection of a larger number of $CD4^+$ T cells that transfer the virus to FFE, the only anatomical site within chickens where infectious, enveloped, and cell-free virus particles are produced and shed into environment via feathers and dander that become the source of reinfection of the contact birds. In a recent study by Bertzbach et al. [51] chickens with Ig heavy chain J gene segment knockout (JH-KO) lacking mature peripheral B cells were infected with a very virulent strain of MDV to evaluate the role of B cells in viral replication and T cell transformation. The data clearly shows that viral load in the blood samples of the infected birds lacking mature B cells was no different than the birds with normal B cell population infected with the same virus. Additionally, MDV-induced tumor incidence was comparable between the JH-KO and wild type birds. The study concludes that peripheral blood B cells are dispensable for MDV pathogenesis.

Vaccination of the bursectomized birds with or without receiving lymphocytes from the age-matched naïve birds provided 100% protection against a very virulent strain of MDV (Table 3). The vaccinated/challenged birds with intact bursa of Fabricius were also protected without any clinical sign of MD. All the non-vaccinated/challenged birds, however, developed typical clinical sign of MD including weight loss, depression, and lymphomas in the visceral organs.

The adoptive lymphocyte transfer did not have an effect on the outcome of vaccine-mediated protection or MDV pathogenesis.

In summary, our data demonstrate that B cells do not play an essential role in vaccine-mediated immunity against oncogenic MDV strains. In our future studies, we will concentrate on the role of T cells in vaccine-induced protection. If data provide evidence that the adaptive immune system does not play a critical role in the initial steps of vaccine-mediated protection, then the innate immune system and its cellular components would be the logical candidates for providing protection post vaccination. This observation will pave the road in construction of effective recombinant vaccines harboring chicken cytokines/chemokines that will have direct immunomodulatory effects on the cells of the innate immune system that would be the likely candidates providing protection in vaccine-mediated immunity.

CRediT authorship contribution statement

Mohammad Heidari: Conceptualization, Supervision, Writing – original draft. **Huanmin Zhang:** Writing-review and editing. **Cari Hearn:** Methodology (flow cytometry). **Lakshmi Sunkara:** Data curation, Writing – review & editing.

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.

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