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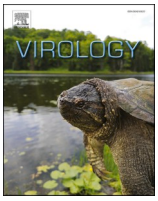

# Efficacy and tolerability of an mRNA vaccine expressing gB and pp38 antigens of Marek's disease virus in chickens

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ABSTRACT

Marek's disease is a contagious proliferative disease of chickens caused by an alphaherpesvirus called Marek's disease virus. A bivalent mRNA vaccine encoding MDV's glycoprotein-B and phosphoprotein-38 antigens was synthesized and encapsulated in lipid nanoparticles. Tumor incidence, lesion score, organ weight indices, MDV genome load and cytokine expression were used to evaluate protection and immunostimulatory effects of the tested mRNA vaccine after two challenge trials. Results from the first trial showed decreased tumor incidence and a reduction in average lesion scores in chickens that received the booster dose. The second trial demonstrated that vaccination with the higher dose of the vaccine (10 $\mu$g) significantly decreased tumor incidence, average lesion scores, bursal atrophy, and MDV load in feather tips when compared to the controls. Changes in expression of type I and II interferons suggested a possible role for these cytokines in initiation and maintenance of the vaccine-originated immune responses.

## 1. Introduction

Marek's disease (MD) is a global economic challenge for the poultry industry and it is estimated to cause annual losses of approximately $1–2 billion USD (Bertzbach et al., 2020). MD is an oncogenic and highly contagious disease in chickens caused by an alphaherpesvirus called Marek's disease virus (Boodhoo et al., 2016). MDV pathotypes are categorized as mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv + MDV) (Pathologists, 2019). Immune suppression and neoplastic T cell lymphomas appear in different visceral organs, sometimes as early as two weeks post-infection (Jarosinski et al., 2006). Infection with vMDV and vvMDV pathotypes may cause paralysis and lymphomas in susceptible chickens.

MDV can infect birds within a few days after hatching, and it is classified as airborne because it infects susceptible hosts through the respiratory tract. Marek's disease virus is shed from infected birds' feather follicle epithelium and skin (Boodhoo et al., 2016). MDV's pathogenesis consists of four phases: the early cytolytic phase, the latency phase, the late cytolytic and immunosuppressive phase, and the proliferative phase. Following inhalation, the virus is engulfed by tissue-resident phagocytic cells of the respiratory system. This infection can occur through dust or dander associated virus particles or cell-to-cell contact with the infected cells. Twenty-four hours after the initial infection, the virus arrives in the thymus, bursa of Fabricius, and spleen (Schat and Calnek, 1984). In these organs, the virus contacts its target cells, B cells, activated $CD4^+$ T cells, and occasionally $CD4^-CD8^-$ T cells or $CD8^+$ T cells for replication and cytolysis associated with the early cytolytic infection phase. This phase surges around 2–7 days post-infection (Baigent et al., 1991, 1998). The latency phase occurs around one to two weeks post-infection when MDV is sustained within lymphocytes. The latency phase leads to systemic dissemination of the virus. The late cytolytic and immunosuppressive phase takes place around two weeks post-infection when MDV gets reactivated in $CD4^+$ T cells, which is associated with immunosuppression. The last phase is the proliferative phase and begins around weeks two to three post-infection when the affected $CD4^+$ T cells grow into visceral tumors (Osterrieder et al., 2006). These pathogenesis timelines depend on the virulence of the virus and the genetic susceptibility or resistance of the host.

Since Marek's disease vaccines were introduced in 1969, vaccination has controlled and prevented this virus. While MD vaccines can prevent tumor growth and death in infected birds, they are ineffective against viral shedding and replication (Baigent et al., 2006). In general, MDV can be classified into three species: gallid alphaherpesvirus 2 (GaHV-2 or MDV serotype 1: MDV-1), gallid alphaherpesvirus 3 (GaHV-3 or MDV

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serotype 2: MDV-2), and meleagrid alphaherpesvirus 1 (MeHV-1; also known as turkey herpesvirus (HVT)) (Reddy et al., 2017).

MeHV-1 or HVT is non-oncogenic, but it can induce protection against MD. HVT vaccine is currently being used alone or in combination with other serotypes (Dunn and Gimeno, 2013). CVI-988, a GaHV-2, is another live (attenuated) vaccine made after serial cell culture passage whereby the genome of the virus has undergone random mutations during these passages leading to attenuation (Churchill et al., 1969). The virus used for MD vaccines based on GaHV-3 first originated from healthy chickens. GaHV-3 vaccines induce moderate to low protection against the disease caused by vvMDV and are inhibited by the presence of maternal antibodies (Witter and Lee, 1984). As MD vaccines do not prevent virus replication and shedding from feathers, continuous virus shedding can serve as a source of infection for non-vaccinated chickens. It might also lead to the emergence of more virulent pathotypes. Ideally, a successful vaccine can decrease or stop viral replication and shedding.

mRNA-based vaccines have recently been proven to induce immune responses against viral pathogens. Several studies are reporting the efficacy of mRNA vaccines in controlling viral pathogens in animal models (Jiang et al., 2020; Hajam et al., 2020; Pulido et al., 2009). Nelson et al. (2019), in a study on human cytomegalovirus in New Zealand White rabbits, compared three types of vaccines, including an mRNA vaccine expressing full-length gB. The results showed superior durability of the mRNA vaccine-induced antibody response compared to two other gB subunit vaccines (Nelson et al., 2019). Specifically, the mRNA vaccine-induced antibodies that was projected to be detectable after 50 weeks. In mice, a trivalent mRNA vaccine encoding the ectodomain of gC2, gD2, and gE2 of herpes simplex virus 2 (HSV-2) designed by Awasthi et al. (2022) induced potent $CD4^+$ T-follicular helper cell and germinal center B cell responses (Awasthi and Friedman, 2022). An mRNA vaccine against surface glycoproteins of HSV-1 also showed 80–100% reduction in virus vaginal shedding in the mouse model (Egan et al., 2020).

Different delivery methods such as lipid nanoparticles (LNPs), polymer-based nanomaterials, and cell-penetrating peptides have been used for the *in vivo* delivery of small interfering RNA (siRNA) or mRNA (Shim and Kwon, 2012; Liu et al., 2018; Shi et al., 2017; Xu et al., 2016). In the study described here, *in vitro*-transcribed mRNA was encapsulated in LNPs, which have been proven as a safe mRNA delivery vehicle when limited to a one to two-dose protocol (Richner et al., 2017). LNP encapsulation happens through a single-step process of microfluidic mixing devices or pipetting when an acidic fluid containing mRNA is mixed with an ethanol solution of lipids. The lipids start a condensation process and, at the same time, shape the lipid vesicles which surround mRNA molecules (Hoecke et al., 2021).

The mRNA constructs designed in the present study encoded MDV's glycoprotein B (gB) and phosphoprotein 38 (pp38) antigens. The ability of gB and pp38 antigens to induce cell-mediated immune responses against MDV has been characterized previously (Omar and Schat, 1996; Boodhoo and Behboudi, 2022a). Our group also reported an increase in the number of $CD8^+$ T cells in the feather pulps that correlated with decreased MDV genome copy numbers in the feathers of chickens treated with the Rispens-CVI988 MDV vaccine (Abdul-Careem et al., 2008). Here, we hypothesized that treating chickens with a LNP-encapsulated mRNA vaccine co-expressing gB and pp38 would induce cytokines as a correlate of an immune response, and reduce shedding of MDV from the epithelium of feather tips.

## 2. Materials and methods

### 2.1. Plasmids and cloning

The expression cassettes encoding the MDV-gB (accession number: AY129966.1) and pp38 (accession number: YP_001033989.1) genes were synthesized by Thermo Fisher Scientific gene art synthesis (Germany). Plasmids were linearized using *Eco*RI-HF (NEB, Canada) and *Kpn*I-HF (NEB, Canada). The following components were mixed in sterile microcentrifuge tubes: 1 $\mu$g plasmid DNA, 5 $\mu$l 10X NEB buffer, 10 units of each *Eco*RI and *Kpn*I. Then nuclease-free water was added to each tube to reach the total volume of 50 $\mu$l. Reaction tubes were incubated at 37 °C for 30 min followed by heat inactivation at 65 °C for 20 min.

The gel purified (Gel purification Kit; Qiagen, Canada) gB and pp38 fragments were then inserted into a PSF-T7- T7 promotor plasmid (Sigma-Aldrich, Germany). The ligated PSF-T7-gB and PSF-T7-pp38 recombinant plasmids were transformed into DH5α competent cells to generate sufficient plasmids. In brief, 3 $\mu$l of the ligation reaction was added to DH5α competent cells and was placed on ice for 30 min followed by a heat shock at 42 °C for 45 s and a cold shock on ice for 5 min. One millilitre of Luria-Bertani (LB) broth (Thermo Fisher Scientific, USA) was added to DH5α cells and tubes were incubated at 37 °C for 1 h (with shaking) for recovery. After 1 h, 100 $\mu$l of the DH5α competent cells were cultured on LB agar (Thermo Fisher Scientific, USA) plates containing kanamycin (30 $\mu$g/ml LB agar) (selection marker), and X-gal (80 $\mu$g/ml LB agar), and IPTG (50 $\mu$M) for blue/white screening of recombinant colonies. Plates were incubated in 37 °C for 18 h followed by 4 h incubation at 4 °C. Single white colonies from each plate were propagated in LB broth. Midi-prep kit (Qiagen, Germany) was used for plasmid purification.

The DNA templates include the coding sequence of full-length MDV RB1B gB or pp38 antigens flanked by untranslated regions, poly (A) at the 3′ end, Kozak sequence (GCCACC), and a V5 tag (GTCATCCGGATAGGGATTGGGAGAGGAGCCAGAGCTAAGATG). An endosomal targeting motif of chicken invariant chain (Ch-Ii; accession number: AY597053) is also added to the N-terminus of the antigen sequence (Fig. 1).

### 2.2. mRNA synthesis and purification

mRNA was synthesized using Hi-T7 RNA Polymerase (NEB, Canada). One μ g of gB/pp38 template DNA (plasmid), unmodified Ribonucleotide Solution Mix (NEB, Canada) and 10X Hi-T7 RNA polymerase reaction buffer were incubated at 50 °C with Hi-T7 RNA polymerase for 1 h in a thermocycler (Biometra, Germany). Synthesized gB and pp38 mRNAs were then purified with Trizol (Thermo Fisher Scientific, Canada) according to a previously described protocol (Gautam et al., 2016). Purified RNA was treated with a DNase I (Thermo Fisher Scientific, USA). The concentration of purified mRNA was measured using Nanodrop spectrophotometer (Thermo Fisher Scientific, Canada) and the 5′-end was capped with an enzymatic capping system that used the vaccinia virus capping enzyme (NEB, Canada). Briefly, 1 μ g RNA was suspended in 15 μ l of nuclease-free $H_2O$ and heated at 65 °C for 5 min followed by cooling on ice for 5 min. The following components were then added in this order: 2 μ l 10X capping buffer, 1 μ l 10 mM guanosine-5′-triphosphate, 1 μ l 2 mM S-adenosylmethionine, 1 μ l vaccina capping enzyme. The tube was incubated at 37 °C for 30 min and was stored at – 80 °C until needed for experiments.

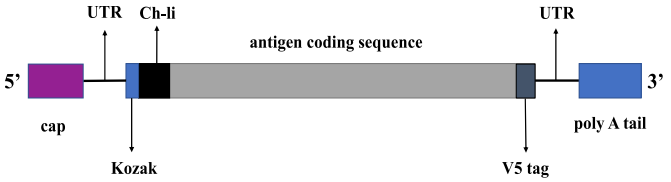


**Fig. 1.** Schematic presentation of the different units forming the mRNA vaccine expression cassette. The expression cassette included a coding sequence of full-length gB and pp38 from RB1B Marek's disease virus (antigen coding sequence) flanked by untranslated regions (UTRs), poly (A) at the 3′ end, Kozak sequence (GCCACC) and a V5 tag. An endosomal targeting motif of the chicken invariant chain (Ch-Ii) was added to the C-terminal of the antigen sequence. A 5′ cap was added enzymatically after in vitro transcription.

### 2.3. Lipid nanoparticle preparation and characterization

Lipid nanoparticle formulations were prepared as previously described (Cheng and Lee, 2016) with some modifications. Lyophilized lipids including ionizable lipid (MedKoo Biosciences, USA), distearoylphosphatidylcholine (DSPC) (Avanti Polar, USA), cholesterol (Sigma-Aldrich, USA), and PEGylated lipids (PEG) (Avanti Polar, USA) were dissolved in ethanol at a molar ratio of 50:10:38.5:1.5 (ionizable lipid: DSPC: cholesterol: PEG-lipid), respectively. The synthesized gB/pp38 mRNA was added to 50 mM acidic citrate buffer, pH 4.0 (Teknova, USA). The lipid mixture then was added to the purified gB/pp38 mRNA. The final nitrogen groups (N) to phosphate groups (P) ratio was 3:1 and the ratio of aqueous to ethanol also was 3:1. Following a gentle mixing by pipetting, the lipid:mRNA mixture was sonicated in 1.5 mL test tubes using an ultrasonic processor (Thermo Fisher Scientific, USA) at 60% pulse for 90 s (10 s ON and 5 s OFF). The test tubes containing lipid mix and mRNA were placed on ice during sonication to further cool down the mixture. The product was subsequently dialyzed against PBS (pH 7.4) in a dialysis cassette (Fisher Scientific, USA) at 4 °C for at least 18 h to allow for buffer exchange. All mRNA vaccines were prepared the day before vaccination, with immunizations following the final dialysis step to avoid possible RNA degradation due to freezing and thawing.

The physicochemical characteristics of LNPs such as particle size (DLS method) and surface charge were assessed using Zetasizer Nano ZS (Malvern Instruments Ltd. United Kingdom). The encapsulation efficiency was measured via Ribogreen dye (Thermo Fisher Scientific, USA)

### 2.4. Cell culture

Protein expression: Five hundred thousand HEK 293T cells were seeded per well in a six-well plate in complete EMEM (WISENT INC, Canada) (10% FBS+ 1% pen-strep). At 80% confluency, cells were transfected with 1 $\mu$g of mRNA encoding gB and pp38 using Lipofectin™ Transfection Reagent (Invitrogen, Canada) for 4 h in optimum media (Thermo Fisher Scientific, USA). At six-, 12- and 32-h post-transfection, cells were washed with PBS and collected following trypsin treatment for RNA extraction. *In vitro* expression of gB and pp38 was analyzed at 36-h post-transfection. Two-hundred-thousand HEK 293T cells were transfected with 5 $\mu$g of mRNA encoding gB and pp38 in 24-well plates using Lipofectin™ Transfection Reagent (Invitrogen, Canada) following the manufacturer's instructions (Cui et al., 2012).

### 2.5. Confocal microscopy

At 36-h post-transfection of HEK 293T cells with gB and pp38 mRNAs, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 45 min at room temperature (RT) in the dark. Following another wash with PBS, cells were permeabilized with 0.1% Triton X-100 buffer solution (Sigma-Aldrich, USA). Cells were blocked in PBS supplemented with 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) in PBS and incubated overnight at 4 °C with V5 monoclonal antibody (Invitrogen, Canada). Alexa Fluor™ 488 goat anti-mouse IgG (Invitrogen, Canada) was used as the secondary antibody (1 h in the dark at RT). Slides were washed twice with PBS and 4′,6-diamidino-2-phenylindole (DAPI) was used for staining nuclei (15 min in the dark at RT). Cells were sealed in with a round coverslips for fluorescence imaging using a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Germany).

### 2.6. Experimental animals

One-day-old SPF White Leghorn chickens were received from the Canadian Food Inspection Agency (CFIA, Ottawa, Canada). The chicks were randomly separated into Horsfall units and were accommodated in the Animal Isolation Unit, at the University of Guelph. Chickens had *ad libitum* access to food and water for the duration of the experiment.

### 2.7. Experimental design

#### 2.7.1. Trial 1, primary vaccination experiment

One-day-old SPF chickens (n = 120) were divided randomly into four groups. (G): G1: PBS-treated, unchallenged controls; G2: mRNA-vaccinated, challenged with MDV; G3: HVT-vaccinated, MDV-challenged controls; G4: MDV-challenged controls. On day one, chicks in G2 were inoculated with the mRNA vaccine containing 2.5 $\mu$g of gB and 2.5 $\mu$g of pp38 in 200 μ L, G1 and G4 received the diluent control (PBS) intramuscularly, and G3 were injected with one dose of HVT (herpesvirus of turkeys) vaccine (MD-Vac-CFL; Fort Dodge Animal Health, Fort Dodge, IA).

Five days after administering the vaccine, all chickens except those in G1 were challenged intra-abdominally with 250 PFU of RB1B Marek's disease virus. Following inoculation, birds were observed for several minutes, followed by additional observations before the end of the working day to ensure there were no obvious adverse effects. Throughout the period of our studies, birds were monitored at least three times a day for possible obvious signs of adverse effects.

On days four, 10, and 21 post-infection, six birds from each group were euthanized by $CO_2$ inhalation for collection of spleens and feather tips. Spleens and feather tips were collected aseptically in RNAlater (Thermo Fisher, Lithuania) and incubated at 4 °C for 24 h followed by storing at −20 °C until RNA extraction. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring (Table 1).

#### 2.7.2. Trial 1, prime-boost vaccination experiment

One-hundred-and-twenty of one-day-old SPF chickens were divided randomly into four groups. G1: PBS-treated, unchallenged controls; G2: mRNA-vaccinated, challenged with MDV; G3: HVT-vaccinated, MDV-challenged controls; G4: MDV-challenged controls. On day one, G2 chicks were inoculated with 2.5 $\mu$g of gB and 2.5 $\mu$g of pp38/200 $\mu$L/chick, G1 and G4 received the diluent control (PBS) and G3 were injected with one dose of HVT vaccine.

Fourteen days after the primary vaccination: G2 chicks were inoculated with the mRNA vaccine (2.5 $\mu$g of gB and 2.5 $\mu$g of pp38) and G1, G3 and G4 chicks received the diluent control (PBS). The injection volume was 200 μL/chick via the intramuscular route (iliotibialis muscle).

Five days after the second inoculation, all chickens except those in G1 were challenged intra-abdominally with 250 PFU of RB1B MDV. Following inoculation, birds were observed for several minutes, followed by additional observations every other hour before the end of the working day to ensure no obvious gross adverse effects developed. Throughout the study, birds were monitored at least three times a day for possible signs of obvious adverse effects.

On days four, 10, and 21 post-infection six birds from each group were euthanized by $CO_2$ inhalation for collection of spleens and feather tips. Spleens and feather tips were collected aseptically in RNAlater (Thermo Fisher, Lithuania) and incubated at 4 °C for 24 h and stored at −20 °C until RNA extraction. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring.

**Table 1**
Lesion score descriptions.

| Lesion score | Description |
|---|---|
| 1 | Only one organ developed tumor lesion |
| 2 | Two organs developed tumor lesion |
| 3 | Three organs developed tumor lesion |
| 4 | Four organs developed tumor lesion |
| 5 | Five organs developed tumor lesion |

#### 2.7.3. Trial 2

One-hundred-and-eighty-four of one-day-old SPF chickens were randomly assigned into seven groups: G1(high dose): mRNA (5 μg of gB and 5 μg of pp38)-mRNA (5 μg of gB and 5 μg of pp38); G2: mRNA (5 μg of gB and 5 μg of pp38)-PBS; G3: PBS-mRNA (5 μg of gB and 5 μg of pp38)-; G4 (low dose): mRNA (2.5 μg of gB and 2.5 μg of pp38)-mRNA (2.5 μg of gB and 2.5 μg of pp38); G5: HVT; G6: PBS control; G7: RB1B control (for those that are hyphenated, the first reagent is the primary treatment and the one after the hyphen is the secondary treatment).

On day one, each chick in G1 and G2 was inoculated with a relatively high dose of the mRNA vaccine (5 μg of gB and 5 μg of pp38), chicks in G3, G6 and G7 received the diluent control (PBS), G4 chicks were inoculated with the a relatively low dose of the mRNA vaccine (2.5 μg of gB and 2.5 μg of pp38), and G5 chicks were injected with one dose of HVT. On day fourteen post-primary vaccination, each chick in G1 and G3 were inoculated with a high dose of the mRNA vaccine (5 μg of gB and 5 μg of pp38), chicks in G4 were inoculated a low dose of the mRNA vaccine (2.5 μg of gB and 2.5 μg of pp38), chicks in G2, G5, G6 and G7 received the diluent control (PBS). Injection volumes were 200 μL/chick via the intramuscular route (iliotibialis muscle).

Five days after the second inoculation, all chickens except those in G6 were challenged intra-abdominally with 250 PFU of RB1B Marek's disease virus. Following inoculation, birds were observed for several minutes, followed by additional observations before the end of the working day to ensure that no obvious gross adverse effects developed. Throughout the period of our studies, birds were monitored at least three times a day for possible signs of obvious adverse effects.

On days four, 10, and 21 post-infection five birds from each group were euthanized by $CO_2$ inhalation for collection of spleens and feather tips. Spleens and feather tips were collected aseptically in RNAlater (Thermo Fisher, Lithuania) and incubated at 4 °C for 24 h and then stored at −20 °C until RNA extraction. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring, and organs weighting.

### 2.8. Virus

The very virulent MDV (vvMDV) RB1B strain used in this study was provided by Dr. K.A. Schat (Cornell University, NY, USA).

### 2.9. RNA extraction and cDNA synthesis

Trizol reagent was used for RNA extraction based on a previously described protocol (Abdul-Careem et al., 2006a). Tissue samples were homogenized in 1 mL of Trizol, and HEK 293T cells were allowed for 2 min in Trizol and then mixed in chloroform (Sigma-Aldrich, USA). The extracted RNA was precipitated in isopropanol and washed with 75% ethanol. Pellets were resuspended in 17 μL of ultra-pure distilled water (Invitrogen, USA). Extracted RNA was treated with DNase (Ambion, USA) and cDNA synthesis was performed using Superscript II (Life Technologies, USA). NanoDrop spectrophotometry at wavelengths of 260 and 280 nm was used to measure quantity and quality of the extracted DNA.

### 2.10. Genomic DNA extraction

Genomic DNA of the feather tips was extracted as described previously (Abdul-Careem et al., 2006b). Three feather tips from each chicken were chopped into small pieces. Cell lysis buffer (500 μL) containing 10 mM Tris (pH 7.5), 10 mM NaCl, 1 mM EDTA, pH 8 with 0.5% (w/v) Sarkosyl, and 100 μL of proteinase K (10 mg/mL) (Sigma-Aldrich, Germany) was added to each tube with a feather sample. Tubes were incubated overnight at 65 °C. The next day, DNA was precipitated using 25 μL of 5 M NaCl and 2.3 mL of 95% ethanol. NanoDrop spectrophotometry was used to measure quantity and quality of the extracted DNA. Extracted DNA was diluted to 50 ng/μL. Two microliters of each sample were used for quantification of MDV genome copy numbers via quantitative real-time PCR.

### 2.11. Real-time PCR for relative cytokine gene expression

Quantitative real-time polymerase chain reaction (PCR) was carried out using SYBR green dye in a LightCycler 480 II (Roche Diagnostics, Laval, Canada). The reaction plate was pre-incubated at 95 °C for 5 min followed by 40–50 cycles of 95 °C for 20 s, and 58 °C-64 °C (primer specific annealing temperatures) for 15 s, in addition to 10 s elongations at 72 °C. The melt curve was done by 10 s incubation at 95 °C. The reaction then cooled down to 65 °C for 1 min. Followed by heating to 95 °C. All primers were synthesized by Sigma-Aldrich (Canada), and their respective annealing temperatures are listed in Table 2.

β-actin was used as a housekeeping gene for all relative gene expression. Relative expression was calculated using the LightCycler© 480 software (Roche Diagnostics GmbH, Mannheim, Germany). Relative expression data represent the mean fold-change of 5–6 replicates compared to the PBS-treated control group ± standard error.

### 2.12. Real-time PCR for viral genome load measurement

Real-time PCR assay for viral genome load measurement was conducted by a LightCycler 480 II (Roche Diagnostics, Laval, Canada) using SYBR green dye, with a final reaction volume of 20 μl. Each run included a series of diluted pMeq standard samples, in addition to feather tip DNA samples. The thermal cycling consisted of an initial incubation at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 s, 58 °C for 5 s, and 72 °C for 10 s. Subsequently, a melting curve analysis was performed at 95 °C for 5 s, 65 °C for 60 s, and 95 °C for 0 s, followed by a cooling step at 5 °C for 30 s. To create the standard curve, ten dilutions of plasmid DNA (pMeq) ranging from 1 to $10^{-9}$ were utilized.

### 2.13. Statistical analysis

Tumor incidence data were analyzed with Fisher's exact test. Gene expression, lesion scores, organ weight indices, and MDV load data were analyzed with unpaired t-tests. Kruskal-Wallis followed by the Mann-Whitney test were used when data were non-parametric. $P \leq 0.05$ (*) was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software).

## Ethical approval

The protocols used in this research project were reviewed and approved by the University of Guelph Animal Care Committee and complied with the Canadian Council on Animal Care guidelines.

## 3. Results

### 3.1. Physicochemical characterization of lipid nanoparticles

Data obtained from Zetasizer confirmed that lipid nanoparticles in this study were positively charged with an average surface charge of +1.72. Particles were monodispersed and had an average diameter of 180 nm (Fig. 2).

### 3.2. Translation of mRNA encoding pp38 and gB proteins in HEK 293T cells

To ensure that mRNA constructs were being translated to the desired proteins, HEK 293T cells were transfected with the mRNA vaccine. A V5-tag was incorporated into the mRNA vaccine transcripts to detect proteins after expression. Confocal microscopy results showed proper translation of *in vitro*-transcribed mRNA codes into gB and pp38 proteins. Enhanced signals in Fig. 3. A and B are indicative of marked

**Table 2**
Real-time PCR primer sequences for chicken target genes.

| Gene | Primer Sequence | Annealing temperature | Accession number/(Reference) |
|---|---|---|---|
| β-actin | F:5′-CAACACAGTGCTGTCTGGTGGTA-3′<br>R: 5′-ATCGTACTCCTGCTTGCTGATCC-3′ | 58 °C | X00182 |
| IFN-γ | F: 5′- ACACTGACAAGTCAAAGCCGCACA -3′<br>R: 5′- AGTCGTTCATCGGGAGCTTGGC -3′ | 60 °C | X99774 |
| IFN-α | F: 5′-ATCCTGCTGCTCACGCTCCTTCT-3′<br>R: 5′-GGTGTTGCTGGTGTCCAGGATG-3′ | 64 °C | Barjesteh et al. (2014) |
| IFN-β | F: 5′-GCCTCCAGCTCCTTCAGAATACG-3′<br>R: 5′-CTGGATCTGGTTGAGGAGGCTGT-3′ | 64 °C | Villanueva et al. (2011) |
| IL-2 | F: 5′-TGC AGT GTT ACC TGG GAG AAG TGGT-3′<br>R: 5′-ACT TCC GGT GTG ATT TAG ACC CGT-3′ | 60 °C | NM_204153.1 |
| IL-6 | F: 5′-CAGGACGAGATGTGCAAGAA-3′<br>R: 5′-TAGCACAGAGACTCGACGTT-3′ | 55 °C | Abdul-Careem et al. (2006a) |
| IL-10 | F: 5′-AGCAGATCAAGGAGACGTTC-3′<br>R: 5′-ATCAGCAGGTACTCCTCGAT-3′ | 65 °C | Bavananthasivam et al. (2021) |
| IL-18 | F: 5′-GAAACGTCAATAGCCAGTTGC-3′<br>R: 5′-TCCCATGCTCTTTCTCACAACA-3′ | 55 °C | Abdul-Careem et al. (2006a) |
| meq | F: 5′-GTCCCCCCTCGATCTTTCTC-3′<br>R: 5′-CGTCTGCTTCCTGCGTCTTC-3′ | 58 °C | Bavananthasivam et al. (2021) |

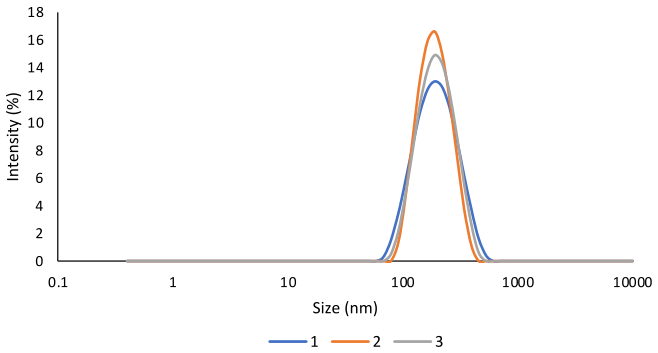


**Fig. 2.** Physical characterization of lipid nanoparticles (LNPs). The graph obtained from Zetasizer Nano ZS represents the intensity-weighted size distribution of LNPs in the nanometer range. Particles were monodispersed and had an average diameter of 180 nm. Plots number 1, 2, and 3 illustrate three measurements.

cytosolic accumulation of pp38 and gB respectively.

### 3.3. MDV-induced tumor incidence, lesion scores and MDV load in feathers

#### 3.3.1. Trial 1, primary vaccination experiment

In the first trial, a single injection of 5 μg of mRNA did not decrease tumor incidence when compared to the PBS-treated control group that had only been challenged with the RB1B MDV (93% and 94%, respectively; Fig. 4. A). Inoculation with five μg of mRNA also did not lead to a significant decrease in lesion scores when compared to the control group that received the MDV challenge only (scores of 2.1 and 2.5, respectively; Fig. 4. B).

MDV genome load (meq gene) in feather tips was decreased in the group that received a single dose of the mRNA vaccine, when assessed 10 and 21 dpi; this decrease was statistically significant at 21 dpi (Fig. 4. C).

#### 3.3.2. Trial 1, prime-boost vaccination experiment

In the booster group of the first trial, a decrease in tumor incidence (by 22%) and lesion score (by 0.9 unit) were observed when the mRNA vaccine group was compared to the RB1B MDV-challenged group (Fig. 5. A and B). However, no decrease was seen in virus genome load at 10 and 21 dpi in the mRNA vaccine group (Fig. 5. C).

#### 3.3.3. Trial 2

In the second trial, the vaccine dose was increased to 10 μg, and the booster dose (identical to the first dose) was injected 14 days later. A PBS control for the prime dose, a PBS control for the booster dose, and a low-dose control (5 μg) were also added to this trial. Among all mRNA vaccine groups, the prime-boost 10 μg group (G1) showed a 43% decrease in tumor incidence, 1.3 unit decrease in the average lesion score, and a significantly reduced MDV genome load at 21 dpi compared to the RB1B MDV-challenged control group (G7) (Fig. 6). mRNA-PBS, PBS-mRNA, and low-dose mRNA vaccine groups (G2, G3, and G4) showed 44%, 36%, and 28% decreases in tumor incidence compared to the RB1B group, respectively (Fig. 6. A). Lesion scores were also reduced by 1.8, 1.06, and 1.3 unit in G2, G3, and G4 compared to the RB1B MDV-challenged group (G7) (Fig. 6. B). The reduction in MDV genome load in feather tips in the chickens which received only one dose of the vaccine (G2 and G3) and the chickens which received the low-dose vaccine (G4) was not statistically different at any time points when compared to the RB1B MDV-challenged group (Fig. 6. C).

#### 3.3.4. Organ weight indices

At 21 dpi, chickens in MDV-infected groups showed significantly lower bursa of Fabricius (BF) to body weight (BW) ratios when compared to PBS control, mRNA and HVT group (Fig. 7. A). BF:BW ratio was significantly higher in mRNA group when compared to RB1B-MDV control. This indicates that mRNA vaccine can partially prevent bursal atrophy caused following MDV challenge. At 21 dpi, RB1B-MDV control chickens showed significantly higher spleen:BW ratio when compared to PBS control and HVT vaccine groups. However, in mRNA vaccine group chickens did not show significantly lower spleen:BW ratio when compared to RB1B-MDV control chickens (Fig. 7. B).

### 3.4. Cytokine expression at 4-, 10-, and 21-days post-viral challenge in the spleen

#### 3.4.1. Trial 1

Since Marek's disease was of less severity in the boosted group, spleen samples from this group were processed to analyze the relative expression of cytokine genes. Compared to the PBS-treated group that was unchallenged (G1), IFN-α expression was significantly higher at four dpi in the group that was primed and boosted with the mRNA vaccine. Compared to the PBS-treated group that was challenged with MDV, no significant changes were observed in IFN-α expression at any time point in the spleen (Fig. 8. A). No significant changes were seen in IFN-β expression at any time point when compared to PBS-treated groups that were or were not challenged with MDV (Fig. 8. B). IFN-γ expression was significantly upregulated in the mRNA-vaccinated group at four and 21

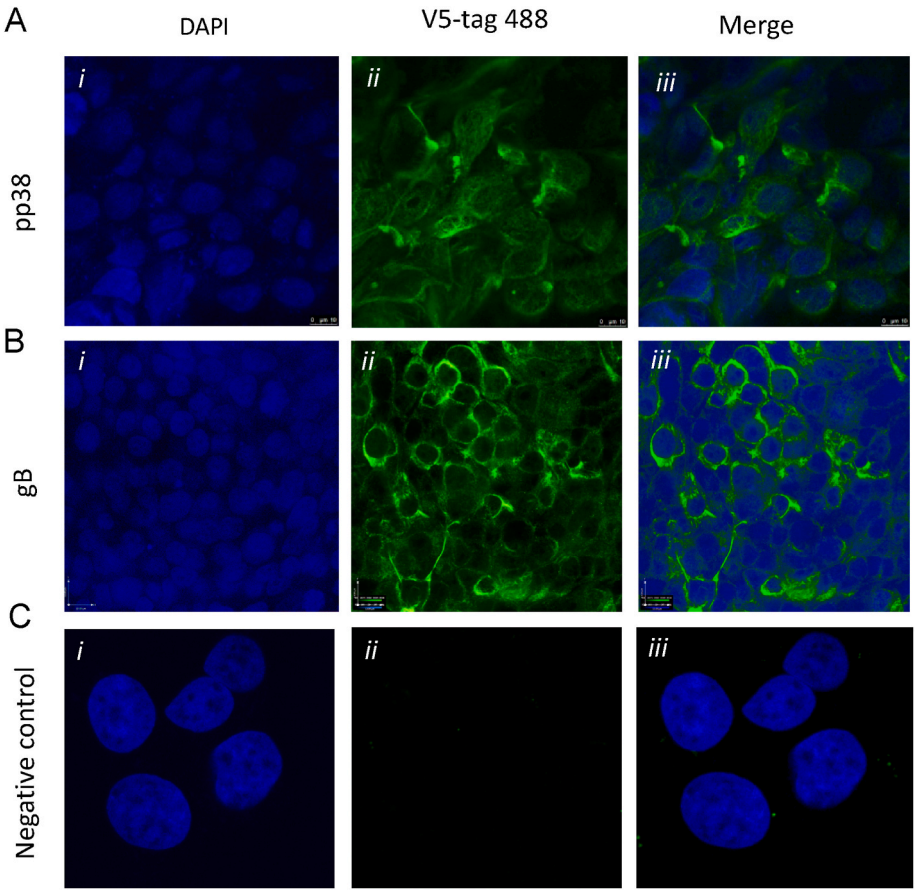


**Fig. 3.** gB and pp38 mRNA cell delivery and protein expression in HEK 293T cells. At 36-h post-transfection, HEK 293T cells were fixed with 4% paraformaldehyde (45 min at RT in the dark). Following another wash (PBS), cells were permeabilized (0.1% Triton X-100 buffer solution). Cells blocked in 5% BSA in PBS and incubated overnight at 4 °C with V5 monoclonal antibody. Alexa Fluor™ 488 goat anti-mouse was used as the secondary antibody, and DAPI was used for nuclei staining. Non-transfected but stained HEK 293T cells were used as a negative control. Cells were analyzed using a Leica SP5 laser scanning confocal microscope. Data present immunofluorescence images of cultured HEK 293T cells stained with DAPI (*i*), target proteins (pp38 and gB) stained with fluorescent-conjugated goat anti-mouse secondary antibody (*ii*), and combined channels (merge) *(iii)*.

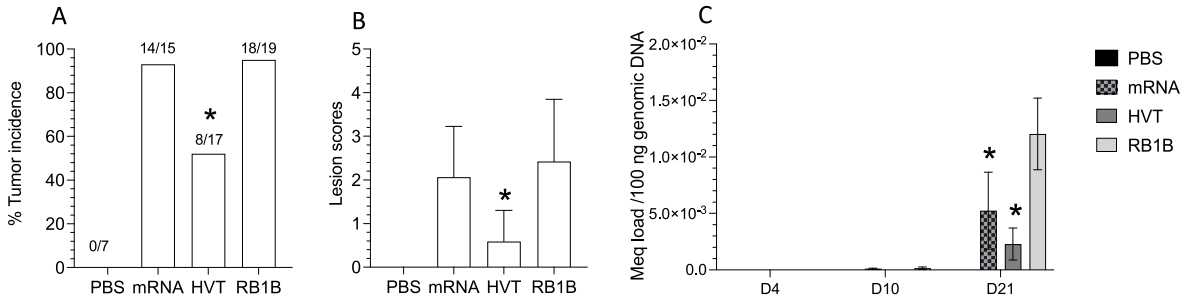


**Fig. 4.** Tumor incidence, lesion score, and meq gene load in the primary vaccination experiment, trial 1. Percentage of tumor incidence (**a**) average lesion scores (**b**) and the meq gene load in 100 ng of genomic DNA from feathers (**c**). Tumor incidence and average lesion scores were assessed during necropsy at 21 dpi. RB1B MDV genome (meq gene) levels in 100 ng of genomic DNA were measured in feather at four, 10, and 21 dpi. Tumor incidence data were analyzed using Fisher's exact test. Lesion scores and MDV genome load data were compared using an unpaired *t*-test. Data represent the mean of 5–6 biological replicates (chickens) compared to the RB1B MDV-challenged controls (*) ± standard error. $p \leq 0.05$ was considered statistically significant.

dpi compared to the PBS-treated and unchallenged controls (Fig. 8. C).

IL-10 expression was not significantly different when the mRNA-vaccinated group was compared to the PBS-treated and MDV-challenged controls (Fig. 8. D). The expression of IL-6 and IL-18 were not statistically significantly different from controls (Fig. 8. E and F).

At four dpi, IL-2 gene expression was approximately six times higher in the mRNA-vaccinated group when compared to the PBS-treated unchallenged group (Fig. 8. G).

### 3.4.2. *Trial 2*

The prime-boost high-dose mRNA vaccine group showed significantly higher expression of IFN-α at 21 dpi when compared to PBS-treated plus MDV-challenged and PBS-treated but unchallenged control groups (Fig. 9. A). IFN-β expression was also significantly higher in the boosted high-dose group at 21 dpi when compared to the PBS-treated unchallenged group (Fig. 9. B).

All mRNA-vaccinated groups showed a significant upregulation of IFN-γ at four and 21dpi when compared to the PBS-treated unchallenged

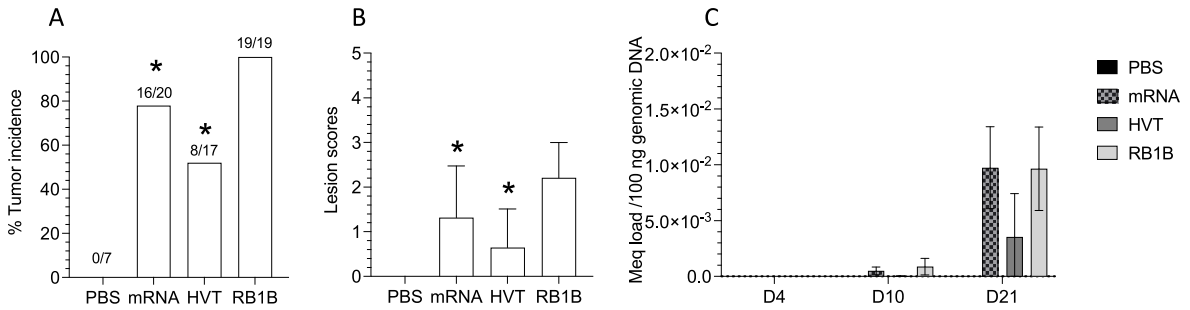


**Fig. 5.** Tumor incidence, lesion score, and meq gene load in prime-boost vaccination experiment, trial 1. Percentage of tumor incidence (**a**) average lesion scores (**b**) and the meq gene load in 100 ng of genomic DNA from the feather (**c**). Tumor incidence and average lesion scores were assessed during necropsy at 21 dpi. RB1B MDV genome (meq gene) levels in 100 ng of genomic DNA were measured in feather at four, 10, and 21 dpi. Tumor incidence data are analyzed using Fisher's exact test. Lesion scores and meq gene data were compared using an unpaired *t*-test. Data represent the mean of 5–6 biological replicates (chickens) compared to the RB1B MDV-challenged controls (*) ± standard error. $p \leq 0.05$ was considered statistically significant.

group (Fig. 9. C). Elevated expression of IL-10 was recorded at 10 and 21 dpi in all vaccinated groups compared to the PBS-treated unchallenged group (Fig. 9. D). At days four and 21 post-infection, IL-6 expression was elevated in all mRNA-vaccinated groups compared to PBS-treated unchallenged controls (Fig. 9. E). Among all vaccinated groups, those that were primed and boosted with the high-dose mRNA vaccine had the highest number of IL-18 transcripts at 10 dpi (Fig. 9. F). At four dpi, IL-2 genes were significantly upregulated in all mRNA-vaccinated groups when compared to the PBS-treated unchallenged control group (Fig. 9. G).

## 4. Discussion

The present study evaluated the tolerability of *in vitro*-transcribed mRNA packaged in lipid nanoparticles and administered through the intra-muscular route to chickens. In addition, the current research aimed to find a protective dose of gB and pp38 mRNA product packaged in lipid nanoparticles for layer hens. We also evaluated the necessity of boosting the primary mRNA injection. Our results showed that two immunizations with 10 μg of mRNA vaccine encoding gB and pp38 antigens lowered or postponed tumor incidence, lesion scores, and MDV load in the FFE.

Finding the optimum dose for a new vaccine platform is one of the critical steps in each vaccine trial. Research showed that different species have different tolerability to high doses of mRNA vaccines. A recent work by Tahtinen et al. (2022) showed that mice and humans react differently to equal relative doses of mRNA vaccine, and mice could tolerate 1000 times higher relative mRNA doses (on a per weight basis) (Tahtinen et al., 2022). Tolerable and/or toxic doses of *in vitro*-transcribed mRNA for chickens are yet to be defined, the two doses studied in the present study were calculated using body weight and based on the doses that were tested in mouse and chicken (Jiang et al., 2020; Hajam et al., 2020).

Physicochemical characteristics of the formulated LNPs play a major role in their *in vivo* behavior and cellular uptake of mRNAs. Size and surface charge are considered to be the most important factors affecting the delivery of mRNA. It has been reported that LNPs between 100 and 200 nm showed better cellular uptake than smaller or larger sizes (Kulkarni and Feng, 2013). The cationic lipid surrounding the negatively charged mRNA should have a neutral to slightly positive surface charge at a physiological pH of 7.4. As the surface of the bilayer phospholipid membrane of cells is negatively charged, a slight positive surface charge within the tolerable range approved by regulatory agencies such as the World Health Organization (WHO) (World Health Organization, 2021). Can improve internalization of LNPs. Physicochemical characterization of LNPs in this study showed monodispersed particles with an average size of 180 nm and a slightly positive surface charge (Fig. 2).

To ensure proper translation of *in vitro*-transcribed mRNA into target proteins, a V5-tag was incorporated into the mRNA vaccine transcripts to detect proteins after expression. Confocal microscopy images showed enhanced fluorescent signals indicating marked cytosolic accumulation of gB and pp38 in HEK 293T cells (Fig. 3).

Another goal of the present study was to assess the tolerability of the mRNA vaccine following IM injection. To achieve that, chickens were monitored every other hour on the days of vaccination, followed by every 8 h assessment until the last day of the experiment. Food and water intake, activity, appearance (mostly feathers) were observed during the assessment. Based on these criteria, no gross adverse effects or mortality were observed after vaccination with 10 μg of mRNA packaged in lipid nanoparticles.

gB and pp38 were selected as the target antigens in this study because of their direct involvement in the virus life cycle and their previously proven ability to induce cell-mediated immune responses against MDV (Omar and Schat, 1996). gB is one of the main surface glycoproteins of MDV which forms a heterodimer with other surface glycoproteins and helps in viral attachment to host cells (Omar and Schat, 1996). Nazerian et al. (1992) reported protective immunity against RB1B MDV challenge and significantly decreased viremia of $B^2B^{15}$ chickens after vaccination with recombinant fowlpox virus (rFPV) expressing gB (rFPV-gB) (Nazerian et al., 1992). Omar et al. (1996) also found gB-specific cell mediated immune responses in $B^{19}B^{19}$ chickens immunized with rFPV-gB (Omar and Schat, 1996).

mRNA vaccines expressing gB or other glycoproteins of herpesviruses have already been developed and assessed for their immunogenicity. Nelson et al. (2019) in a study on New Zealand White rabbits, compared three types of vaccines, including an mRNA vaccine expressing full-length gB against human cytomegalovirus. The results of the above study showed superior durability of the mRNA vaccine-induced antibody response compared to two other gB subunit vaccines (Nelson et al., 2019). The study by Nelson et al. also showed superior durability of the immune response induced by the mRNA vaccine that was projected to stay detectable after 50 weeks. Other than durability of the immune response, the mRNA vaccine in this study led to an enhanced breadth of peptide-binding responses compared to other subunit protein vaccines. In mice, A trivalent mRNA vaccine encoding the ectodomain of gC2, gD2, and gE2 of herpes simplex virus 2 (HSV-2) designed by Awasthi et al. (2022) induced potent $CD4^+$ T-follicular helper cell and germinal center B cell responses (Awasthi and Friedman, 2022). An mRNA vaccine against surface glycoproteins of HSV also showed 80–100% reduction in vaginal shedding of HSV-1 (Egan et al., 2020).

Pp38, the second antigen encoded by our mRNA vaccine, is a highly immunogenic protein which is expressed in the cytolytic stage of infection of B and T cells. Similar to gB, T cell-mediated immune responses against pp38 antigen has been reported (Omar and Schat, 1996). Boodhoo et al. identified pp38 immunodominant epitopes and

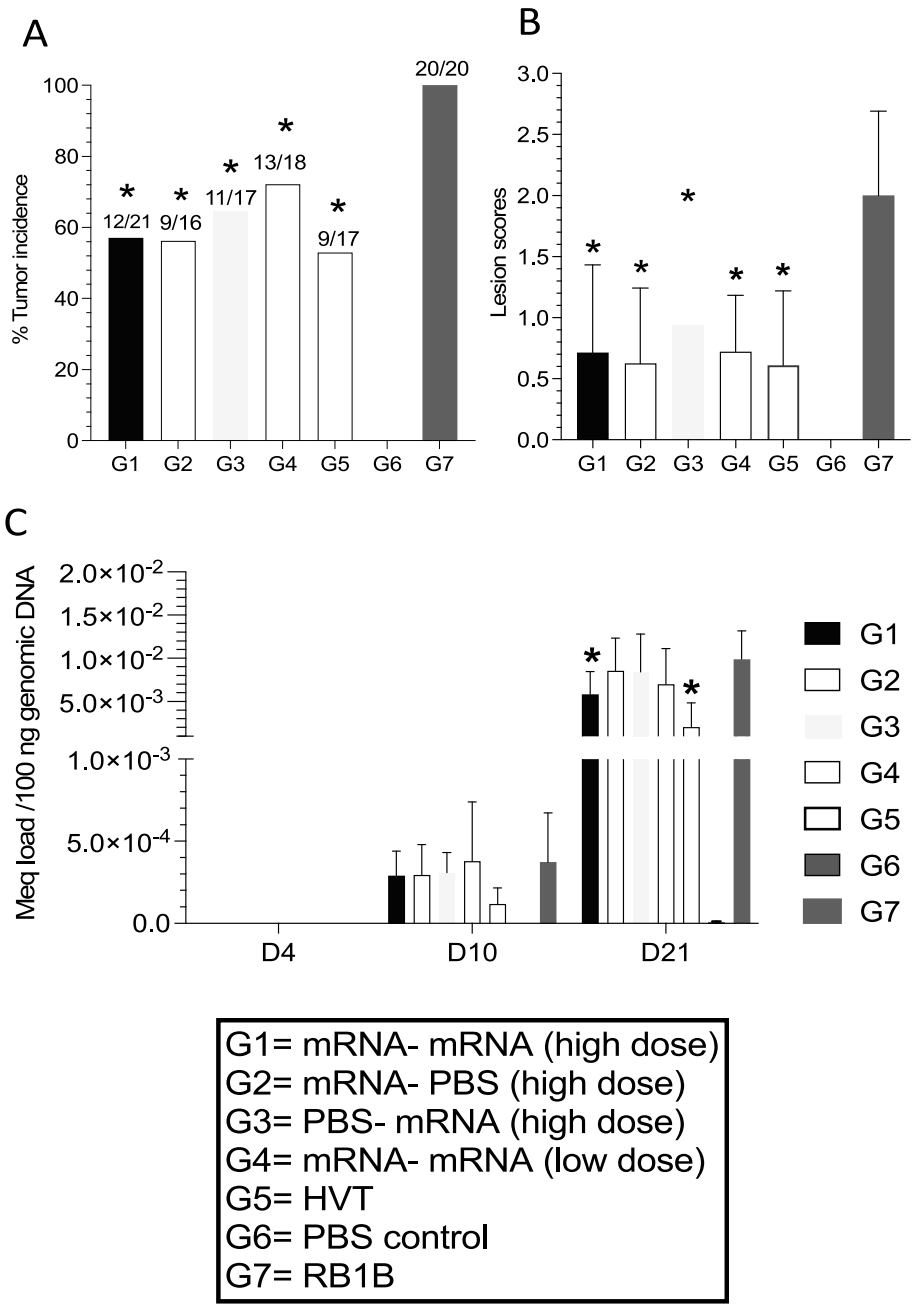


**Fig. 6.** Tumor incidence, lesion score and meq gene load, trial 2. Percentage of tumor incidence (**a**) average lesion scores (**b**) and meq gene load in 100 ng of genomic DNA from the feather (**c**). Tumor incidence and average lesion scores were assessed during necropsy at 21 dpi. RB1B MDV genome (meq gene) copy numbers in 100 ng of genomic DNA were measured in feathers at four, 10, and 21 dpi. Tumor incidence data are analyzed using Fisher's exact test. Lesion scores and MDV genome data were compared using an unpaired *t*-test. Data represent the mean of 5–6 biological replicates (chickens) compared to the RB1B MDV-challenged controls (*) ± standard error. $p \leq 0.05$ was considered statistically significant.

characterized $CD4^+$ T cell-specific responses to these immunodominant epitopes in MD-susceptible and MD-resistant chickens (Boodhoo and Behboudi, 2022a).

In this study, a homologous challenge was used as the gB and pp38 sequences of GaHV-2 were used and the challenge virus was RB1B. However, heterologous immunity is relatively common within closely related species but can also be seen with unrelated agents. Indeed, two of the vaccines used against Marek's disease are SB-1 and HVT, which as mentioned, belong to different species compared to the virulent strains of MDV. While both SB-1 and HVT/FC-126 have a pp38 molecule, the pp38 molecule encoded by SB-1 strains has low sequence identity and the HVT/FC-126 has a truncated pp38 when compared to GaHV-2/MDV-1 serotypes. On the other hand, viruses within the GaHV-2/MDV-1 encode a pp38 that share greater than 98% sequence identity. Furthermore, viruses within the GaHV-2/MDV-1, GaHV-3/MDV-2 and MDV-3 (MeHV-1) encode a gB that shares greater than 99%, 80% and 50% sequence identity respectively (Boodhoo and Behboudi, 2022b). Based on sequence identity, since the gB and pp38 were synthesized based on the CVI988 vaccine virus strains, it is expected that chickens vaccinated with the LNP-mRNAgB/pp38 will display heterologous immunity, whereby chickens are protected against MD marked by reduced tumor lesions, but they are probably not from primary infection.

Significantly elevated expression of IFN-α at 21 dpi (compared to both the MDV and PBS groups) and IFN-β (compared to the PBS group)

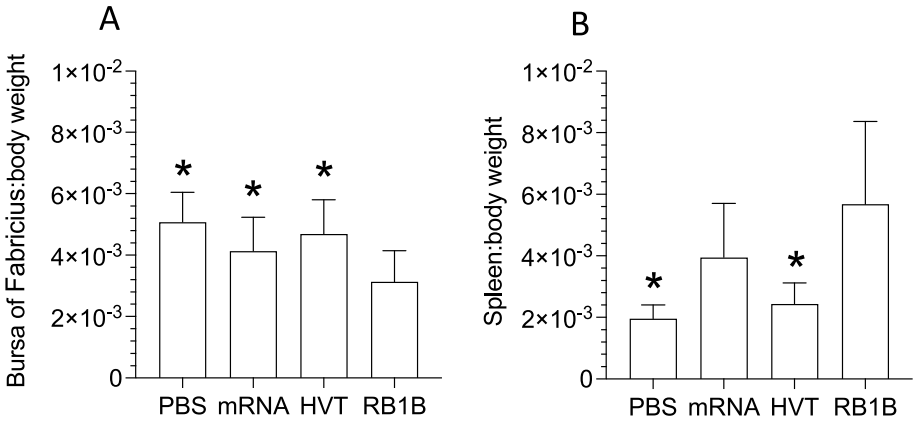


**Fig. 7.** Organ weight indices, trial 2. Bursa of Fabricius:BW **(a)** and spleen:BW **(b)** ratios were calculated at 21 days post-IM administration of PBS, mRNA (high-dose, 2 injections), and the HVT vaccine. PBS and MDV-RB1B (untreated, MDV-infected) groups used as control groups. Data were compared using an unpaired *t*-test. Data represent the mean of 9–10 biological replicates (chickens) compared to the RB1B MDV-challenged controls (*) ± standard error. $p \leq 0.05$ was considered statistically significant.

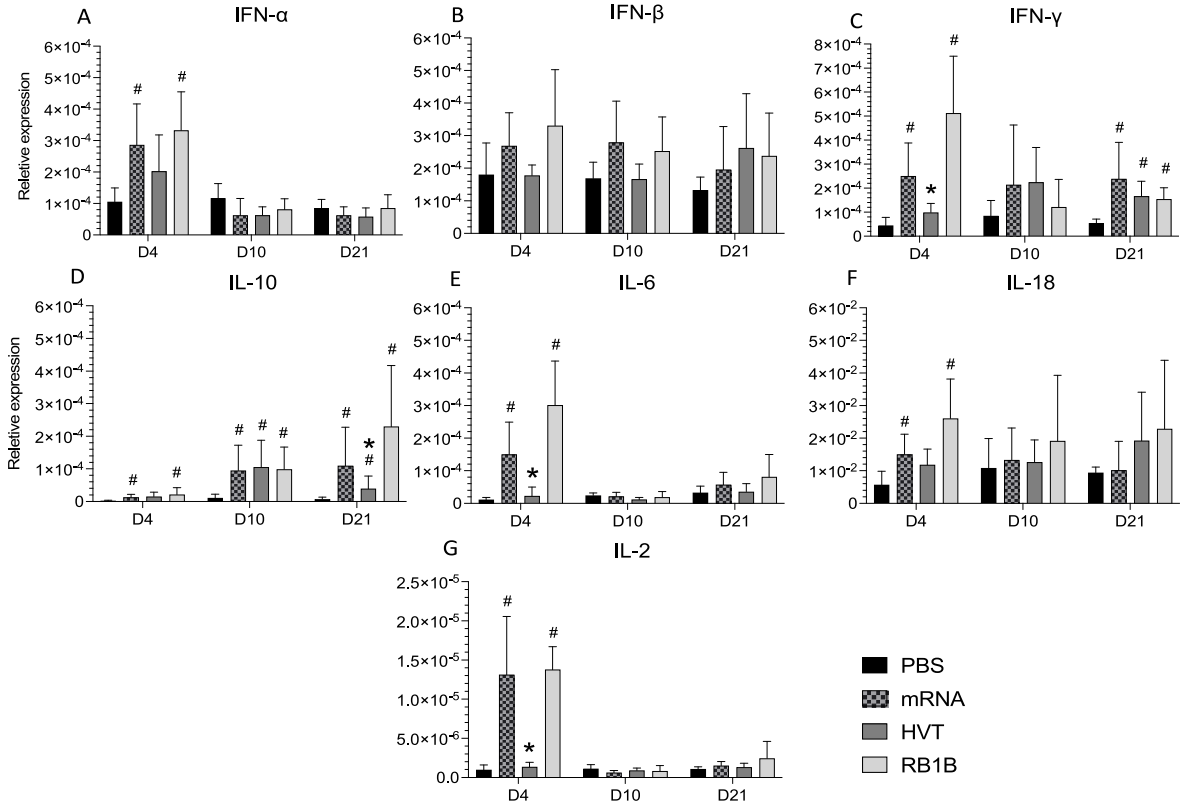


**Fig. 8.** Relative expression of genes in spleens at four-, 10-, and 21-days post-viral challenge, trial 1. One $cm^2$ of splenic tissue was excised and processed for RNA extraction and cDNA synthesis at four-, 10-, and 21 days post-IM administration of PBS, mRNA or the HVT vaccine. Graphs compare the relative expression of IFN-α (**a**), IFN-β (**d**), IFN-γ (**c**), IL-10 (**d**), IL-6 (**e**), IL-18 (**f**), and IL-2 (**g**) in spleens. Relative expression data represent the mean fold-change of 5–6 biological replicates (chickens) compared to the RB1B MDV-challenged control group (*) and PBS-treated group (#) ± standard errors. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test ($p \leq 0.05$ was considered statistically significant). β-actin was used as a reference gene for all relative expressions.

in the high-dose group of the second trial might have contributed to the initiation of the vaccine-induced immune response against MDV. Also, it is proven that MDV downregulates the expression of type I interferons at the early and late cytolytic stages (Sun et al., 2019). Comparing the MDV genome load and type I interferon expression level in this study shows an inverse relationship between MDV genome load and type I IFN expression. The reason for such observation might be lower replicating MDV in the mRNA vaccinated group (high-dose) and consequently less inhibition of type I IFN expression. The protective effect of type I interferons against viral infections is shown both *in vivo* and in vitro. Oral administration of IFN-α has reduced MDV replication in chickens (Jarosinski et al., 2001). Increased expression of IFN-α in the blood of resistant chickens to MD (Quéré et al., 2005) also supports the protective role of IFN-α in MDV infection.

In the booster group of the first trial, IFN-γ expression was significantly higher in the mRNA vaccine groups when compared to the PBS control at four and 21 dpi. Similarly, in the second trial, all mRNA vaccine groups showed significantly higher IFN-γ expression at four and

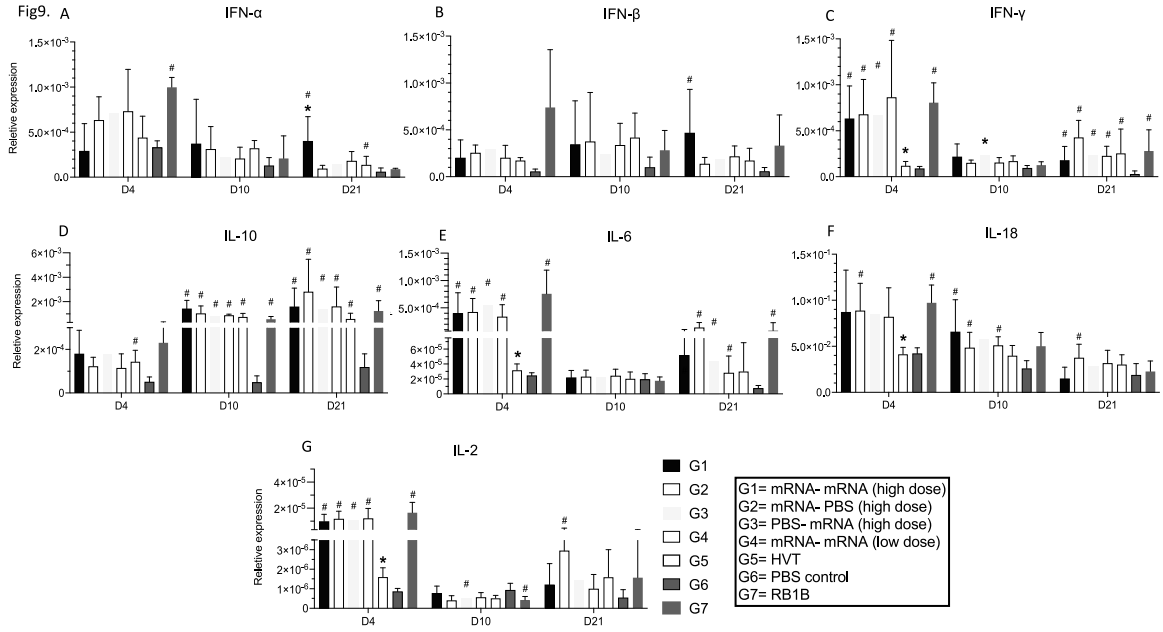


**Fig. 9.** Relative expression of genes in spleens at four-, 10-, and 21-days post-viral challenge, trial 2. One $cm^2$ of tissue from spleens was excised and processed for RNA extraction and cDNA synthesis at four-, 10-, and 21 days post-IM administration of PBS, mRNA, and HVT vaccine. Graphs compare the relative expression of IFN-α (**a**), IFN-β (**d**), IFN-γ (**c**), IL-10 (**d**), IL-6 (**e**), IL-18 (**f**), and IL-2 (**g**). Relative expression data represent the mean fold-change of 5–6 biological replicates compared to the RB1B MDV-challenged control group (*) and PBS-treated group (#) ± standard errors. Data were analyzed with Kruskal-Wallis followed by the Mann-Whitney test ($p \leq 0.05$ was considered statistically significant). β-actin was used as a reference gene for all relative expressions.

21 dpi. The pivotal role of IFN-γ in an immune response against MDV has been proven previously (Haq et al., 2011, 2015). Boodhoo et al. (2022) reported antigen-specific T cell response induced by pp38 immunodominant peptides using chicken IFN-γ ELISPOT (enzyme-linked immunosorbent spot) assay. The highest frequency of IFN-γ-producing cells is observed in MDV-vaccinated and then challenged chickens (Boodhoo and Behboudi, 2022a). As IFN-γ is mainly secreted by natural killer (NK) cells and activated T cells, decreased tumor incidence in the first trial (booster group) and the second trial might be because of induction of NK and T cell responses.

The second trial showed a higher expression of IFN-γ and IL-10 in vaccinated groups. Although IL-10 has been characterized to have an inverse effect in protection against MDV (Abdul-Careem et al., 2007). In humans, increased expression of IL-10 has been proven to be involved in anti-tumor responses through the modulation of IFN-γ. Specifically, IL-10 can upregulate IFN-γ and granzyme secretion from tumor-infiltrating $CD8^+$ T cells (Mumm et al., 2011). In chickens, co-stimulation with CpG-ODN (CpG-oligodeoxynucleotide) and polyI:C (C ligands) synergistically increased the expression of IFN-γ and IL-10 and led to a more robust Th1-biased immune response in chicken monocytes (He et al., 2012). Another reason for the elevated expression of IL-10 in the mRNA groups of the second trial might be a response to a higher dose of exogenous mRNA. Tahtinen et al. also reported a high expression of IL-10 in blood-derived mononuclear cells following immunization with unmodified mRNA in humans (Tahtinen et al., 2022). As the mRNA molecules used in this study were also unmodified, upregulation of IL-10 might be a regulatory response to balance the proinflammatory feature of exogenous mRNA to control exaggerated inflammation. The result from a chicken study showed the involvement of IL-10 after stimulation with CpG-ODN and polyI:C (TLR ligands) in chickens, which agrees with our results (He et al., 2012).

Here, we sought to determine the importance of the booster dose following the initial mRNA vaccine inoculation. As mentioned earlier, the mRNA molecules used in this study did not have any modifications in their nucleotides. Unmodified mRNA is at a higher risk of degradation as it is considered a non-self-molecule following cellular uptake. We speculated that boosting with an identical dose after 14 days might increase the antigen load and, consequently, more robust immune responses. Although tumor incidence and lesion scores results did not show statistically significant differences between the double-dose and single-dose mRNA vaccine groups, the boosted high-dose group showed significantly lower MDV load in feather tips. The reason why no significant differences were seen between the prime and booster groups of the second trial might be the selected dose (10 μg). This dose might be protective even when injected once, but to prove this, a comprehensive study comparing the magnitude of the immune responses following single-dose and double-dose vaccination should be conducted. The discussion of the necessity and importance of second, third, or fourth booster doses for mRNA vaccines is ongoing. Still, there are reports highlighting lower infection rates and higher protection, and higher cellular response against varicella-zoster virus (VZV), human papillomaviruses virus (USD), and SARS-CoV-2 vaccinated humans (Leung et al., 2004; Bar-On et al., 2021; Gilca et al., 2015).

We also assessed the impact of two mRNA vaccination (high dose) on the spleen and BF by measuring the organ weight indices. These indices can indicate any changes occurring in these lymphoid organs. In the case of MDV infection, bursal atrophy and spleen enlargement are commonly observed (Berthault et al., 2018). At 21 days post-infection, significantly higher BF:BW ratio suggests prevention of bursal atrophy and B cells depletion by mRNA vaccination. Spleen:BW ratios at 21 dpi did not show significant difference when compared to RB1B-MDV control which may be attributed to the infiltration of lymphocytes and/or tumor formation.

Although the mRNA vaccine tested in this study was able to induce immune response leading to lowered tumor incidence and lesion scores caused by MDV and decreased virus load in the FFE, it did not outperform HVT vaccine which is a live attenuated vaccine. Further modifications of the vaccine and different dosing may improve protection conferred by this vaccine against Marek's disease.

## 5. Conclusion

In conclusion, our results showed that two immunizations with 10 μg of a mRNA vaccine encoding gB and pp38 antigens lowered or postponed tumor incidence and lesion scores and bursal atrophy caused by MDV and virus load in the FFE. Future studies should focus on elucidating the underlying mechanisms of protection conferred by these mRNA vaccines and how their efficacy can be improved.

## Data Availability

Data are available on reasonable request.

## Funding sources

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## CRediT authorship contribution statement

**Fatemeh Fazel:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ayumi Matsuyama-Kato:** Investigation, Writing – review & editing. **Mohammadali Alizadeh:** Investigation, Writing – review & editing. **Nitish Boodhoo:** Methodology, Writing – review & editing. **Shayan Sharif:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

## Declaration of competing interest

Authors declare no financial conflict of interest.

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