Efficacy and Tolerability of An mRNA Vaccine Expressing gB and pp38 Antigens of Marek’s Disease Virus in Chickens

by

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ABSTRACT

EFFICACY AND TOLERABILITY OF AN MRNA VACCINE EXPRESSING GB AND PP38 ANTIGENS OF MAREK’S DISEASE VIRUS IN CHICKENS

Fatemeh Fazel                                                     Advisor:
University of Guelph, 2023                                 Professor Shayan Sharif

Currently vaccines against Marek’s disease virus (MDV) prevent death and limit tumor growth in infected birds. However, they do not control virus shedding from the skin and feather follicle epithelial cells. The present study aimed to explore the utility of an mRNA vaccine for conferring immunity against Marek’s disease. Challenge trials were carried out to evaluate the efficacy of different doses of a MDV mRNA vaccine, the necessity of boosting, and the host responses following intramuscular injection of two doses of mRNA vaccines. Tumor incidence, lesion scores, and MDV load in feather tips at 21 days post-infection were significantly decreased after two inoculations of the vaccine compared to the MDV group. Transcriptional analysis showed upregulation of interferons, interferon-stimulated genes, and cytokines in both spleen and lungs within the first 36 hours of the immunization. The results of the present study provide evidence that mRNA vaccines can induce immunity against MD.
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<td>ADCC</td>
<td>Antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CARTs</td>
<td>Charge-altering releasable transporters</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFIA</td>
<td>Canadian Food Inspection Agency</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CNPs</td>
<td>Coated chitosan nanoparticles</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COVID-19</td>
<td>Coronavirus disease-19</td>
</tr>
<tr>
<td>CP</td>
<td>Crossing point</td>
</tr>
<tr>
<td>CPG-ODN</td>
<td>CpG oligodeoxynucleotides</td>
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<tr>
<td>CPPs</td>
<td>Cell-penetrating peptides</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T cell</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post-infection</td>
</tr>
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<td>DSPC</td>
<td>Distearoylphosphatidylcholine</td>
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<td>EBV</td>
<td>Epstein–Barr virus</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot</td>
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<td>EMCV</td>
<td>Encephalomyocarditis virus</td>
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<td>EMEM</td>
<td>Eagle's minimum essential medium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FFE</td>
<td>Feather-follicle epithelium</td>
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<td>FFU</td>
<td>Focus-forming units</td>
</tr>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FMDV</td>
<td>Foot and mouth disease virus</td>
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<td>FPV</td>
<td>Fowlpox virus</td>
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<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
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<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HF</td>
<td>High Fidelity</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td>HVT</td>
<td>Herpes virus of turkeys</td>
</tr>
<tr>
<td>IFIT5</td>
<td>Interferon-induced protein with tetratricopeptide repeats 5</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon-stimulated genes</td>
</tr>
<tr>
<td>IVT</td>
<td><em>In vitro</em> transcribed</td>
</tr>
<tr>
<td>LATs</td>
<td>Latency-associated transcripts</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LNPs</td>
<td>Lipid nanoparticles</td>
</tr>
<tr>
<td>M2e</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>MAVS</td>
<td>Mitochondrial antiviral signaling</td>
</tr>
<tr>
<td>MD</td>
<td>Marek's disease</td>
</tr>
<tr>
<td>MDA5</td>
<td>Melanoma differentiation-associated protein 5</td>
</tr>
<tr>
<td>MDCs</td>
<td>Myeloid dendritic cells</td>
</tr>
<tr>
<td>MDV</td>
<td>Marek's disease virus</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide oligomerization domain (NOD)-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>nsP</td>
<td>Non-structural protein</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate synthetase</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frames</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDCs</td>
<td>Plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque-forming unit</td>
</tr>
<tr>
<td>PKR</td>
<td>Double stranded RNA-dependent protein kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly-l-lactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly lactic-co-glycolic acid</td>
</tr>
<tr>
<td>POWV</td>
<td>Powassan virus</td>
</tr>
<tr>
<td>pp38</td>
<td>Phosphoprotein 38</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PRV</td>
<td>Pseudorabies virus</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate-specific antigen</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RL</td>
<td>Repeat long</td>
</tr>
<tr>
<td>RLRs</td>
<td>Retinoic acid-inducible gene I (RIG-I)-like receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rs</td>
<td>Repeat short</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SAR</td>
<td>Self-amplifying mRNA vaccine</td>
</tr>
<tr>
<td>SARS-CoV-2</td>
<td>Severe acute respiratory syndrome coronavirus 2</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen-free</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% tissue culture infectious dose</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/interleukin-1 receptor/resistance protein-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UL</td>
<td>Long unique</td>
</tr>
<tr>
<td>US</td>
<td>Short unique</td>
</tr>
<tr>
<td>UTRs</td>
<td>Untranslated regions</td>
</tr>
<tr>
<td>VNT</td>
<td>Virus neutralization titers</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis virus</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella zoster virus</td>
</tr>
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<td>WHO</td>
<td>World Health Organization</td>
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INTRODUCTION

Marek’s disease virus (MDV) is an oncogenic, highly contagious alphaherpesvirus in chickens [1]. MDV is classified as an airborne virus, and it infects susceptible chickens through the respiratory route. The virus is shed from the feather follicle epithelium (FFE) and skin of infected birds [2]. MDV infects birds within a few days after hatching. Virus arrives in the thymus, bursa of Fabricius and spleen twenty-four hours after inhalation [3]. Pathogenesis of MDV consists of four phases, which result in neoplastic T cell lymphomas leading to visceral organ tumors, immune suppression, neurological disorders, transient paralysis, and brain damage in infected chickens. This virus is considered a challenge in the global poultry industry and is estimated to cause economic losses of approximately $1-2 billion US dollars per year [4].

A protective vaccine against Marek’s disease has been widely used since 1969 [5]. Over the following decades, polyvalent and Rispens vaccines were introduced for greater protection [6], [7]. Current MD vaccines target three different MDV serotypes: gallid alphaherpesvirus 2 (GaHV-2), gallid alphaherpesvirus 3 (GaHV-3) and meleagrid alphaherpesvirus 1 (MeHV-1) also known as herpes virus of turkeys (HVT) [8]. GaHV-2 is a live attenuated vaccine that is made after serial cell culture passage, which causes random mutations in the genome of the virus that causes it to become attenuated [9]. The virus isolates for GaHV-3 vaccines first originated from healthy chickens. They induce moderate to low protection against vvMDV (very virulent MDV) and are affected by maternal antibodies [6]. MeHV-1 or HVT is a non-oncogenic virus, which is currently used alone or in combination with other serotypes [10].

Although MD vaccines limit tumorigenesis, prevent death, and can reduce economic consequences, they do not prevent virus replication and shedding from the feather. After
vaccination, continuous virus shedding still poses a risk to non-vaccinated chickens, which might lead to the emergence of more virulent pathotypes [11]. Hence, design and development of alternative vaccines for MD is required to address the existing issues with the current MDV vaccines.

mRNA-based vaccines have been proven to induce strong immune responses against viral pathogens in recent years. There are several studies reporting the efficacy of mRNA vaccines in controlling viral pathogens in animal models [12]–[14]. Nelson and colleagues (2019) in a study on human cytomegalovirus in New Zealand white rabbits compared three types of vaccines including an mRNA vaccine expressing full-length glycoprotein (gB). The results of this study showed superior durability of the mRNA vaccine-induced antibody response compared to two other gB subunit vaccines [15]. In mice, a trivalent mRNA vaccine encoding ectodomain of gC2, gD2, and gE2 of herpes simplex virus 2 (HSV-2) induced potent CD4+ T-follicular helper cell and germinal center B cell responses [16]. An mRNA vaccine against surface glycoproteins of HSV also showed 80 to 100% reduction in virus vaginal shedding [17].

The mRNA vaccine designed in the present study encoded the glycoprotein B (gB) and phosphoprotein 38 (pp38) of MDV. Antigenicity and the ability of gB and pp38 antigens to induce cell-mediated immune responses against MDV has been characterized previously [18], [19]. Having a bivalent mRNA construct encoding a surface glycoprotein and a phosphoprotein resulted in identical post-translation modifications, such as glycosylation similar to what is observed during natural infections. Also, translation and presentation of the antigens by host cells can better resemble viral proteins compared to the subunit protein vaccines that are exogenously produced. We speculated that co-expression of gB and pp38 might induce a robust cell-mediated immune response and reduces the virus shedding from the feather follicle epithelium. The present study
was conducted to evaluate the efficacy and tolerability of administration of *in vitro*-transcribed mRNA packaged in lipid nanoparticles through the intra-muscular route in chickens. In addition, the current work aimed to find a tolerable, yet protective dose of mRNA to induce immune response against MDV in chickens. Which cytokines were induced and potential mechanisms of protection against Marek’s disease virus were also studied. In addition, stability of the mRNA vaccine at the site of injection within the first 36 hours after inoculation was assessed.
CHAPTER 1

LITERATURE REVIEW

Marek’s disease virus

classification and genomic structure

József Marek first described Marek’s disease (MD) in 1907 after observing generalized polyneuritis in chickens. Approximately 20 years later, Pappenheimer and colleagues reported polyneuritis and visceral lymphoma as other pathological signs of MD [20], [21]. MD of chickens is a viral disease caused by a herpesvirus [22], [23]. MDV infects chickens within a few days after hatch. The virus is classified as airborne because it infects the susceptible hosts through the respiratory tract. MDV is shed from the feather follicle epithelium (FFE) and skin of MDV-infected birds. The significant production losses in poultry farms affected by this disease have necessitated the development of vaccines against MD [9], [24].

When Marek’s disease virus was first discovered, it was believed to belong to the Gammaherpesvirinae subfamily because it shares similar features with Epstein-Barr virus (EBV), such as infecting lymphocytes. In 1982, genome studies of MDV revealed that inverted repeat nucleotide sequences in its DNA are specific to Alphaherpesvirinae [25]. In recent years, molecular characterization of MDV has revealed that this virus belongs to the genus Mardivirus. MDV strains are classified into three species: GaHV-2 (MDV serotype 1: MDV-1), Gallid herpesvirus 3 (MDV serotype 2: MDV-2), and Meleagrid herpesvirus 1 (MDV serotype 3, also known as herpesvirus of turkeys). Although GaHV-3 and HVT cannot induce the development of visceral tumors, GaHV-2 can transform cells, thereby causing lymphomas in infected chickens.
The MDV genome consists of 70 open reading frames (ORF) and is 178 kb in length [26]. Similar to all alphaherpesvirus sub-family members, the MDV genome is made of long and short unique (UL and US) regions being flanked by inverted repeat sequences, repeat short (Rs) and repeat long (RL) [26].

**Virulence and pathogenesis**

GaHV-2 pathotypes are categorized as mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) [27]. Immune suppression, neurological disorders, and neoplastic T cell lymphomas appear in different visceral organs, sometimes as early as two weeks post-infection [28]. mMDV usually causes neurological signs and rarely causes lymphoma. Infection with vMDV or vvMDV strains cause paralysis and lymphoma formation in susceptible chickens. vv+MDV infection is usually associated with severe brain damage.

MDV usually infects chickens within a few hours after hatch. The virus is classified as airborne because it infects the susceptible hosts through the respiratory tract. MDV is shed from the infected birds' feather follicle epithelium (FFE) and skin.

According to the Cornell model, MDV pathogenesis consists of four phases: the early cytolytic phase, the latency phase, the late cytolytic and immunosuppressive phase, and the proliferative phase [29]. It is important to consider that the approximate day post-infection linked to each phase depends on the virulence of the virus, and more virulent viruses can act faster compared to strains of lower virulence [30]. The early cytolytic phase of the virus cycle starts with the inhalation of the virus by the host. Following inhalation, the virus is engulfed by tissue-resident phagocytic cells of the respiratory system. Twenty-four hours after the initial infection, the virus arrives in the secondary lymphoid organs such as the bursa of Fabricius, spleen, and thymus [3].
In these organs, MDV contacts its target cells, mainly B cells, and activates CD4+ T cells for virus replication and cytolysis associated with the early cytolytic infection phase. This replication surges at 3-7 days post-infection (dpi) [31], [32]. Glycoprotein B (gB) and phosphoprotein (pp) 38 antigens of MDV can be found in primary and secondary lymphoid organs in this phase [33].

Depending on virus virulence, the latency phase occurs between days 7 to 10 dpi, and MDV replicates inside lymphocytes (mainly B cells and CD4+ T cells), but the host immune system cannot detect it. While increased expression of pp14, Marek's EcoRI-Q-encoded protein (meq), and latency-associated transcripts (LATs) is characterized in this phase, expression of other genes involved in the cytolytic phase of the virus is ceased during the latency period [34]. The meq protein sustains latency by preventing late-infected CD4+ T cells from inducing apoptosis [34]. Integration of MDV DNA into the host genome happens during the latency phase [35], [36]. The latency phase results in the systemic dissemination of the virus.

The late cytolytic and immunosuppressive phase occurs around 10 to 14 dpi when MDV reactivates from latency in CD4+ T cells and lyses more lymphocytes. This phase is associated with permanent immunosuppression mainly due to bursa and thymus atrophy. Reactivation time varies based on the pathotype of MDV strains.

The last phase, the proliferative phase, begins around 16 to 21 dpi when the affected CD4+ T cells transform and develop into neoplastic lesions in several organs, including nerves, eyes, skin, muscles, and visceral organs [21]. The expression of meq and pp38 genes is needed to induce and maintain the transformation phase [37]. Other than visceral tumors, transient paralysis, and encephalitis [38] are other signs of MDV infection.
Transmission of MDV

Following systemic dissemination of MDV through infected and transformed T cells, the virus arrives at the FFE, an active site for virus replication [39]. During keratinization at FFE, infected T cells become surrounded, and the virus will remain protected from degradation [40]. Feather keratinization facilitates continuous shedding of cell-free MDV from FFE. MDV shedding starts around 10 dpi [11] and continues for the rest of the chicken’s lifespan. Upon infecting a new susceptible host, the virus life cycle resumes.

To date, there is no evidence of vertical transmission for MDV, and horizontal transmission through the air appears to be the only route of infection [41]. Infection with MDV mostly happens during the first hours after hatching through inhalation of cell-free virus particles and, after 24 hours, is detectable in the lungs, spleen, thymus, and bursa.

Genetic resistance

Although MDV can infect and replicate in resistant and susceptible chicken lines, genetic background is an important factor in determining susceptibility to MDV. This genetic susceptibility is associated with the B-complex, which is the major histocompatibility complex (MHC) in chickens [42]. It is proposed that different levels of susceptibility to MD based on the B-haplotype can be related to the number of MHC class I molecules on the cell surface [43] and is associated with the various repertoires of presented peptides. For example, B21 molecules with a large central cavity for binding to peptides with various sequences could explain B21 haplotype resistance to MDV [44].
Host immune response to MDV

Both innate and adaptive immune responses are required to eliminate MDV. Evidence has proven that natural killer (NK) cells, macrophages, CD4+ T cells (αβ and γδ) [45], and cytotoxic T lymphocytes (CTLs) play roles in immunity against MDV.

Production of interferons and effector functions of macrophages and NK cells are the main components of the innate responses to MDV. The pathogen-associated molecular patterns (PAMPs) associated with MDV, including double-stranded viral DNA and unmethylated CpG DNA, are sensed by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Sensing viral nucleic acid by TLRs leads to recruitment of adaptor molecules and activation of transcription factors. Abdul-Careem et al. (2009) reported an upregulation of TLR3 and TLR7 expression following infection with MDV [46]. It has also been demonstrated that pre-treatment of chickens with TLR 4 and TLR 21 agonists could delay disease progression and reduce MDV genome copy number [47]. Production of type I interferons (IFNs)-α and β is the immediate response of the innate immune system to a viral infection. The protective effect of type I IFNs against viral infections has been shown both in vivo and in vitro. Oral administration of IFN-α has reduced MDV replication in chickens [48]. Increased expression of IFN-α in the blood of resistant chickens to MDV [49] also supports the protective role of IFN-α in MDV infection. IFN-γ is the only member of the type II IFN family and is secreted by activated T cells and NK cells. IFN-γ is considered a key player in the antiviral state, and its direct effect on the reduction of MDV has already been characterized [50]. The administration of recombinant chicken IFN-α significantly enhanced the immune response elicited by the HVT vaccine against the very virulent MDV [51].

Macrophages have also been shown to play a role in defense against MDV infection. Macrophages contribute to inhibit MDV infection through phagocytosis and nitric oxide (NO) and
pre-inflammatory cytokine secretion [52]. A previous study by Wilder et al. (1979) demonstrated elevated MDV viral titer and increased tumorigenesis following in vivo suppression of macrophage function [53]. On the other hand, in vivo activation of macrophages has been shown to decrease the incidence of MD [54].

NK cells, as one of the main arms of innate immunity against viral infection, have been shown to play a significant role in host defense against MDV through various mechanisms. Production of antiviral cytokines, such as type II interferon, killing of virally infected and tumor cells, and the secretion of granzyme B and perforin are some of the mechanisms linked to NK cell defense against MDV [55].

Adaptive immunity also plays a role in MD infection in chickens. Due to the cell-associated nature of MDV, cell-mediated immune responses appear to contribute to MDV clearance. However, the role of antibody-mediated immunity remains unclear. Several studies demonstrated the presence of antibodies against some glycoproteins (gB, gE, gI) of MDV in infected chickens [9], [56]. These antibodies may participate in antiviral immunity by blocking virus entry or promoting antibody-dependent cell-mediated cytotoxicity (ADCC) of infected cells [56]. However, their exact function in eliminating MDV is not yet fully characterized.

In addition to innate antiviral immunity and the potential role of antibody-mediated immunity, CD8+ T cells, as a main component of adaptive immunity, appear to play a major role in protection against MD. Antigen-specific T cell-mediated immune responses were reported in chickens after vaccination with recombinant fowlpox virus (rFPV) [57]. CD8-deficient chickens also showed higher MDV titers in CD4+ T cells (main MDV target cells), suggesting the key role of CD8+ T cells in antiviral defense against MDV [58]. Abdul Careem et al. (2008) reported CD4+
and CD8+ T cell infiltration in FFE of MDV-challenged chickens and accumulation of CD8+ T cells in MDV-vaccinated chickens, highlighting the involvement of cell-mediated immune responses during MDV infection and immunization [59]. Regarding the contribution of CD4+ T cell response in immunity against MDV, Boodhoo et al. (2022) identified pp38 immunodominant epitopes from pp38 and characterized specific CD4+ T cell responses to these immunodominant epitopes in both MD-susceptible and MD-resistant chickens [60]. γδ T cells are another subset of T cells reported to be involved in the immune response against MDV. An increased number of γδ T cells in the skin and spleen following MDV infection has been observed [2], [61]. Matsuyama-Kato et al. (2023) reported significant production of IFN-γ and down-regulated expression of the TGF-β gene in γδ T cells derived from blood-derived mononuclear cells, following TCRγδ stimulation [45]. Together, these findings suggest the possible participation of γδ T cells in immune responses against MDV.

**Vaccination against MDV**

Since Marek’s disease vaccines were introduced in 1969, vaccination has controlled and prevented tumor growth and death in infected birds, but they are ineffective against viral shedding and replication [62]. MD vaccines are produced from three different MDV species: GaHV-2, GaHV-3, and MeHV-1 or HVT [8]. HVT is the first vaccine that was used to induce protection against MD. A non-tumorigenic virus is still being used alone or in combination with other vaccines [10]. GaHV-2 is a live attenuated vaccine; its genome has been affected by several random mutations due to serial cell culture passages [9]. GaHV-3 vaccines are isolated from healthy chicks and can partially protect chickens against vvMDV [6]. However, MD vaccines do not prevent virus replication, and continuous virus shedding from the feathers area can serve as a
source of infection for non-vaccinated chickens. It can also lead to the emergence of more virulent pathotypes [11].

**Immune responses induced by MD vaccines**

The immune response induced by MD vaccines in chickens is affected by the virulence of MDV species used as the backbone of the vaccine, genetic background of the host, simultaneous infection with other agents, and the interval between the time of vaccination and exposure to the virus. The decreased tumor incidence and MDV load in vaccinated chickens highlight the chickens' antiviral and antitumor immunity initiation. Moreover, significant upregulation of IFN-γ is reported following MD vaccination. However, the underlying mechanisms of MD vaccine-induced immunity still need to be dissected [63]. Haq *et al.* further showed the key role of IFN-γ in the induction of an immune response initiated by the MD vaccine [51]. [64]. T cell responses are believed to be predominant in vaccine-induced immunity against MDV [65]. Abdul Careem *et al.* (2008) reported an accumulation of CD8+ T cells in MDV-vaccinated chickens (Rispens-CVI988) [59]. CD4+ T cells, γδ T cells, and NK-like cells are other cells of the immune system that have been shown to contribute to the responses induced by MD vaccines [58], [66].

**mRNA vaccines**

**The necessity of mRNA vaccines**

Traditional vaccine platforms cannot fulfill the urgent need for vaccines in special situations such as pandemics or fast-growing epidemics. For instance, among currently used influenza vaccines, most of them are manufactured based on chicken eggs or cell substrates. Normally this process is time-consuming and depends on the accessibility of adequate pathogen-free embryonated eggs. Approximately six months are needed to produce the first vaccine series.
Despite the constant need for embryonated eggs, inactivated wild-type viruses can be dangerous in case of imperfect inactivation [67]. Recombinant subunits vaccines are not often sufficiently immunogenic.

DNA-based vaccines trigger an effective immune response, but insertional mutagenesis is always a risk factor [68]. In addition, the nuclear membrane of dendritic cells, the potent antigen-presenting cells for the induction of immune responses, can resist nucleic acid translocation to the nuclei and prevent the maximum function of the DNA vaccines [69], while mRNA vaccines are designed to be delivered, and function in cytosolic translation sites.

Another feature that makes mRNA vaccines good candidates is accurate yet flexible antigen design [70], [71]. An updated mRNA sequence for each antigen can be inserted into the expression cassette, while the formulation and delivery are like the previously licensed and fully tested one. mRNA vaccine technology also requires easier stockpiling. Unformulated mRNA or low-volume libraries of plasmid can be stored for many years. In addition, mRNA in vivo half-life can be increased through the usage of different adjustments and delivery approaches such as nucleotide modification and delivery through lipid-based vehicles [72]–[75].

Finally, it must be noted that developing an mRNA vaccine does not involve virus replication and propagation, and therefore is a completely pathogen-free and non-infectious process [76].

mRNA-based therapeutics timeline/history

The first successful mRNA transfection through liposomal delivery was performed in 1989 [77], followed by a successful transfer of naked unformulated mRNA to mouse muscle cells [78]. By the early 1990s, the idea of delivering mRNA to antigen-presenting cells (APCs) to achieve
cell-mediated immunity was suggested. The induction of cell-mediated and antibody-mediated immune responses by mRNA delivery was confirmed in 1993 and 1995, respectively [79], [80].

In 2008, increased RNA translational capacity and biological stability were confirmed by incorporating the modified nucleotide pseudouridine into RNA transcripts [72]. A year later, mRNA vaccine technology was adopted in a vaccination trial to treat patients with metastatic melanomas [81]. In 2012, an influenza mRNA vaccine showed long-lasting antibody and cell-mediated immune responses to influenza A in both young and old mice. A broad, efficient, and protective immune response to the respiratory syncytial virus (RSV) was demonstrated following RSV mRNA vaccine [82], [83]. Later in the year, the first personalized neo-epitope mRNA vaccine for patients with melanomas was tested, and vaccination led to notable decreases in the number of metastases in the patients [84]. The first COVID-19 mRNA vaccine (Pfizer-BioNTech) received FDA (Food and Drug Administration) approval for emergency use in individuals 16 years of age and older in December 2020. In August 2021, this vaccine received its full approval from the FDA.

**mRNA vaccines in veterinary medicine**

Several veterinary mRNA vaccines have been studied so far. In 2009, RNA immunization against Foot-and-Mouth Disease Virus (FMDV) in swine was reported. A single immunization with 500 µg of full-length chimeric O1K/C-S8 RNAs induced both cell- and antibody-mediated immune responses [14]. Pulido et al. (2010) inoculated mice with low and high doses of FMDV transcripts encoding virus (FMDV) mutants with independent deletions of the two stem-loop structures of viral RNA. Two to three days after inoculation, viral RNA was detectable in the serum samples of the inoculated mice. Fifteen days post-inoculation, almost 60% of the animals showed a notable neutralizing antibody response to FMDV [85]. Higher RNA doses in this study did not necessarily result in higher neutralizing antibody concentrations. Further, 70% of mice in the lower
dose group showed a high concentration of neutralizing antibodies. In comparison, less than 50% of mice that received higher doses showed detectable neutralizing antibody concentrations. Upon challenge with FMDV, mice which were positive for antibodies against FMDV, and negative for the virus were considered as protected (38%). The antibody concentrations against FMDV in the protected mice were three times higher than those measured on day 15 post mRNA immunization.

In 2018, an mRNA-based vaccine encoding the prM and E genes of Powassan virus (POWV), an emerging tick-borne flavivirus, delivered in lipid nanoparticles was shown to be protective. Mice were injected with 10 µg of mRNA intramuscularly. An identical dose was used as a booster dose 28 days after the immunization. Four weeks after the booster dose, mice were challenged with two strains of POWV. All vaccinated mice survived, while 100% of the placebo group died after this lethal challenge. Furthermore, passive serum transfer from the vaccinated mice to naïve mice a day before infection protected 100% of the recipients. In addition, the vaccine-induced immune response in this study was reported to provide a cross-reactive antibody-mediated response against the Langat virus and other tick-borne flaviviruses [85].

An mRNA vaccine against Pseudorabies virus (PRV) has recently been tested in mice [12]. The vaccine was designed based on glycoprotein D (gD) and the antibody response was monitored for eight weeks after the immunization. The highest specific IgG and neutralization antibody concentrations were detected at week four, and the concentrations of PRV-specific neutralizing antibodies were enhanced upon a booster dose at a 14 day interval. The vaccinated groups also showed significant upregulation of cytokines such as IFN-γ and interleukin (IL)-2 in blood-derived mononuclear cells. The CD4+/CD8+ T cell ratio in blood was higher in the vaccinated group when compared to the control group [12]. Immunized mice (n=15) were then challenged with PRV, and
the animals were assessed for clinical signs, weight loss, and survival for 15 days. The great majority (95%) of vaccinated mice stayed healthy, while all the control mice died.

**Potential problems associated with mRNA vaccines**

Although RNA-based vaccines are known to be better than existing vaccines in terms of fast and flexible design and development, some challenges should be noted. RNA instability, excessive inflammatory responses, dosing, and inefficiency in the *in vivo* delivery are among the problems.

Unprotected RNA is highly degradable by extracellular RNase [86]. To overcome this problem and ensure effective RNA internalization, various reagents have been engineered to facilitate mRNA delivery to the cytosol. Adding a 5’ cap and flanking with 5’ and 3’ untranslated regions stabilize the mRNA sequence and increase its half-life [87], [88]. Adding an ideal length of Poly (A) tail is recommended to enhance the stability and translation of the mRNA construct [89]. High guanine and cytosine content is another modification associated with constant mRNA concentrations *in vitro* and protein expression *in vivo* [73], [90].

Upon vaccine delivery, exogenous mRNA behaves as a pathogen-associated molecular pattern (PAMP) after recognition by various innate immune receptors. This immunostimulatory feature of exogenous RNA can be considered a double-edged sword. Adjuvants are often administered in conjunction with vaccines to initiate and enhance a pro-inflammatory state to promote the maturation of DCs, and, consequently, activate a greater adaptive immune response. This adjuvant-like characteristic of mRNA facilitates such a pathway. However, recognition of mRNA by the innate immune system can result in defective antigen presentation and incompetent immune responses [72], [91]. To address these problems, modified nucleosides such as
pseudouridine and 1-methylpseudouridine have been developed and used in the field of modified mRNA-based therapeutics [72], [92].

Another concern associated with mRNA-based vaccines and other RNA-based therapeutics is inadequate in vivo delivery. In this regard, methods such as DCs loaded ex vivo, mechanical (gun), and intranodal injections have been proposed [93]. These methods are not widely applicable because of their complexity and associated costs. Therefore, an approach that induces an efficient immune response while keeping the naked mRNA secure and undegraded is still required. One of the concerns associated with in vivo delivery is “dosing.” A suboptimum vaccine dose will lead to inadequate immune response. On the other hand, excess dosing may result in toxicity due to the liver accumulation of nanoparticles [94], in addition to excessive immune responses and/or induction of exhaustion/tolerance in the effector cells of the immune system.

**Delivery methods for mRNA vaccines**

mRNA vaccines can be delivered through various methods, such as lipid nanoparticles (LNPs), polymer-based nanomaterials [95], and cell-penetrating peptides [96]. The main role of vaccine delivery vehicles is to encapsulate the mRNA and prevent its degradation via RNase, help its delivery to DCs, and provide an appropriate level of compaction for proper interaction with the ribosomal translation machinery. In addition, surfaces of delivery materials can be designed and coated to increase stability and target DCs or other cells/organs.

**Lipid nanoparticles**

After the first successful in vivo delivery and translation of mRNA via LNPs in 2015 [97], this delivery method has been widely accepted and used. Many vaccine studies have used LNPs in humans and animals [16], [98]–[101]. Whilst the operation of this delivery approach is not
completely clear, these particles are believed to enter through endocytosis and inverted non-bilayer lipid phase fusion. Upon entrance, LNPs are directed to early and late endosomes and then are degraded enzymatically [102]. As their lipid structures are quite similar to cell membrane phospholipids, their cellular uptake is facilitated by resembling interactions with cell membranes. Formulating RNA in LNPs can be easily performed through a single-step process via microfluidic mixing devices. In these machines, an acidic solution of mRNA is mixed with an ethanol solution of lipids till the time of condensation and shaping the lipid vesicles that are surrounding and covering mRNA molecules [94]. In addition to easy manufacturing, favorable biocompatibility and biodegradability are other advantages of LNPs. A key step in developing LNPs was the introduction of a new “ionizable” cationic lipid [103]. Compared to the previous formulations, this class of LNPs is more powerful at transfecting hepatocytes with minimum charge-related toxicities. Studies proved that the liver could be specifically targeted by altering parameters such as the molar ratios of lipids (the lipid-RNA ratio) and the type of phospholipid in the particle [104]. Using LNP formulation to target specific organs is not limited to the liver. LNPs formulation can be modified to target specific tissues or even particular cells. Modified LNP can target inflammatory monocytes and APCs in some organs, such as the spleen and bone marrow [105], [106]. It is also possible to use antibody conjugates to boost receptor-mediated uptake via desired cells [107], [108].

Although this method of RNA delivery is being used widely, it should not be neglected that LNPs have some disadvantages, such as stability concerns, possible cargo leakage, and rapid clearance through the kidneys and liver [109].
Production and modifications to the mRNA template

Easy manufacturing of single-stranded mRNA molecules can be performed in most laboratories. However, special considerations must be taken into account to prevent RNase contamination. To produce in vitro-transcribed mRNA molecules, the first step is to provide the DNA template corresponding to the desired mRNA sequence. Plasmid vectors used in mRNA transcription comprise a coding sequence that UTRs (untranslated regions) surround. At the beginning of the sequence, an RNA polymerase-specific promoter borrowed from bacteriophages, mostly T7, SP6, or T3 phages, is incorporated. There is a poly-A tail (50-250 bp) on the 3’-end of the sequence.

Phage RNA polymerase plays a key role during the in vitro transcription process. The direction of RNA polymerase is from the 3’- to the 5’-end of the DNA template, and the mRNA sequence that is produced will be from 5’- to 3-end [110]. A 7-methyl guanosine cap molecule is incorporated during this transcription procedure at the 5’ extremity. This cap is necessary to mature and translate mRNA molecules [111], [112]. A poly-A tail is incorporated into the 3’-end the construct. Adding this tail to the developing mRNA molecules is an important step that improves mRNA’s stability, translation, and transport [113], [114]. A poly-A tail also has post-transcriptional influences, such as splicing [115]. An essential step in mRNA manufacturing is eliminating the DNA template, complete with the addition of the DNases enzyme. Lastly, the produced mRNA molecule passes its final purification step, with the option of combining with column-based clearance [116].

Self-amplifying mRNA vaccine (SAR)

SAR vaccines are larger than conventional mRNA vaccines and contain additional segments, including four non-structural proteins (nsP1-4) and a sub-genomic promoter borrowed
from an alphavirus genome. nsP1-4 encode replicas that are able to amplify mRNA and produce a large number of copies of the antigen [117]. This feature helps mRNA replicate several times and provides an enhanced amount of antigen compared to non-replicating mRNA and conventional protein-based or killed vaccines.

In a comparative study, BioNTech Pharmaceuticals compared synthetic mRNA and self-amplifying RNA encoding influenza hemagglutinin antigen of three distinct strains (H1N1, H3N2, and B). The results of this study showed that both vaccine types were able to induce strong immune responses. Still, similar levels of protection were generated using 1.25 μg of self-amplifying RNA compared to 80 μg mRNA (64-fold less material) [118]. Therefore, less self-amplifying mRNA is required than non-amplifying mRNA to generate similar immune responses. However, accurate dosing is one of the challenges of self-amplifying mRNA vaccines.

**Dendritic cell mRNA-based vaccines**

DCs are professional APCs that express antigen-derived peptides through MHC classes I and II to stimulate naïve or memory CD8+ and CD4+ T cells. Because of their key role in immunity, DCs are excellent candidates to be electroporated with mRNA. The basis of this technique is isolating patients’ DCs and then transfecting them with mRNA constructs encoding the desired antigens. High-voltage pulses result in small pores through which RNA molecules penetrate the cell. Then, transfected DCs are injected into the autologous person, and upon proper activation, the injected DCs stimulate antigen-specific cellular and humoral immunity. It is reported that DC electroporation leads to strong cell-mediated immune responses. This method is mostly used for cancer vaccines [119], [120].
The role of type I IFNs in mRNA vaccine outcomes

Although type I interferons can be beneficial during the initiation of the immune response, they can prevent mRNA translation [91]. Reports show positive cooperation of type I IFN in antigen presentation by DCs through the upregulation of co-stimulatory molecules [121]. Similarly, Pepini et al. reported that blockade of type I interferon receptors led to increased antigen expression and both antibody- and cell-mediated responses [122]. mRNA vaccination in knockout mice for type I IFN receptors also showed greater CD8+ T cell responses [123]. It has not yet been confirmed whether there is an overall beneficial or adverse role of type I IFNs in the context of mRNA vaccines due to the opposing mechanisms of action of these cytokines.

Uptake of LNP-formulated mRNA vaccine

In general, every cell that expresses low-density lipoprotein (LDL) receptors is proven to be able to uptake LNPs. This uptake is eased by ApoE lipoprotein circulating in serum and tissues [124], [125]. Based on this fact, most cells can pick up LNPs because they have the required endocytic mechanisms to receive them. It is proposed that direct delivery of LNPs to APCs is not necessarily required as the antigens can be produced and released by other cells, such as muscle cells, and then be taken up by APCs [126].

It is documented that intramuscular injection of mRNA vaccines results in the infiltration of leukocytes (mostly neutrophils, monocytes, myeloid dendritic cells (MDCs), and plasmacytoid dendritic cells (PDCs)) [127]. Following the delivery of mRNA to non-APCs (e.g., muscle cells), mRNA is translated into proteins via ribosomes. These proteins can be processed through proteasomes, loaded on MHC class I on the cell surface, membrane-anchored, or released to the extracellular space. APCs then move to vaccine-draining lymph nodes as the main sites of peptide
presentation to T cells. Presentation of peptides on MHC class I gives rise to CD8+ T cell activation (figure 1).

The secreted viral proteins to the outside of the cell or membrane-anchored proteins can be sensed by B cells and initiate the antibody-dependent response. Also, the uptake of secreted viral protein by APCs from the extracellular space leads to peptide (antigen) presentation through MHC class II. This pathway will trigger CD4+ T cell responses [127], [128].

In vivo imaging technologies, such as luciferase-based tracking in mice, have illustrated how mRNA vaccines are trafficked after various entry routes [97], [124], [129], [130]. Measurable antigen production was detected up to ten days after subcutaneous, intramuscular, and intradermal entry at the site of injection [97]. Conversely, following systemic administration, such as intravenous and intraperitoneal routes, translation mostly happens in the liver.

The function of innate immune system components

Innate immunity, as the first line of defense, detects exogenous mRNA via pattern recognition receptors (PRRs). Identification of RNA inside endosomes is facilitated by Toll-like receptors (TLRs). TLR 7 and 8 recognize single-stranded RNA (ssRNA), consequently triggering the MyD88 pathway that ultimately induces IFN-β release. Single-stranded RNA inside the cytosol is sensed via retinoic acid-inducible gene-I-like (RIG-I-like) receptors and nucleotide oligomerization domain (NOD)-like receptors (NLRs) that also result in the release of type I IFNs. Activation of the type I IFN pathway leads to the upregulation of genes that encode RNA-dependent protein kinase (PKR), pro-inflammatory cytokines, and oligoadenylate synthetase (OAS) receptors. To prevent mRNA degradation as a result of type I interferons function, nucleotide modification is practiced during the in vitro transcription step to make the synthetized mRNA more stable and translatable [91].
**B and T cell responses to mRNA vaccines**

The level of protection induced by most vaccines is evaluated based on the concentration of antibodies they can induce. In other words, antibody titers after immunization are considered as one of the indicators of vaccine potency. A notable feature associated with mRNA vaccines is that the manufactured antigen will be identical to the protein in the pathogen, which expands antibody specificities that react to the pathogen's antigens [118].

Although B cells can receive LNP-encapsulated mRNAs and produce the encoded antigen(s), this function is reported to be conducted more efficiently by monocytes and DCs probably because of higher expression of LDL receptor [128]. B cells typically sense the intact antigen released or expressed by other cells. Consequential interactions between antigens and cognate B cell receptors at the vaccine-draining lymph nodes result in B cell selection and expansion and conversion to antibody-generating cells [131]. It is reported that B cells at the vaccine-draining lymph nodes represent dynamic phenotypes with more germinal center formation resulting in more plasmablast production [130], [132]. Increased T follicular helper cells and activated germinal center B cells after immunization with mRNA were also shown in a murine model [133].

mRNA vaccines can also induce CD8+ T cell responses. A modified mRNA/LNP vaccine against CMV harboring CMV glycoproteins (gB) and the pentameric complex (PC) has been reported to induce potent T cell responses in mice and nonhuman primates [99]. Similarly, SARS-CoV-2 mRNA vaccines developed by CureVac, Moderna, and BioNTech were able to induce both CD4+ and CD8+ T cell responses [134]–[136]. Vaccination with modified mRNA encoding hemagglutinin (HA), and modified mRNA encoding a variant of clade B HIV-1 R3A Env (envelope), induced antigen-specific CD4+, CD8+ and Tfh cell responses [132], [133], [137].
Modified mRNA-LNP-based vaccines have been shown to increase the activity of germinal centers (GCs) [133]. GCs are the key place for B lymphocyte proliferation, affinity maturation, isotype class switching, and differentiation into plasma and memory cells [137].

Figure 1. Schematic representation of intracellular pathways used to process LNP-encapsulated mRNA after cellular uptake.

Upon arrival to the intercellular space, LDL receptors uptake LNPs through ApoE lipoprotein. The LNPs then stay in the endosome (a) where, due to a decrease in pH, the cationic lipid charges and this helps disrupt the endosomal membrane. Then the mRNA molecules can traffic to ribosomes that translate the mRNAs into viral antigens (b). Viral antigens are degraded via proteasomes and MHC class I molecules present epitopes to CD8+ T cells in non-APC cells (e). Viral antigens can also be internalized by APCs

Figure 1. Schematic representation of intracellular pathways used to process LNP-encapsulated mRNA after cellular uptake.
and then be processed in the lysosomes of APCs and presented by MHC class II molecules to CD4+ T cells (f). (Microsoft PowerPoint was used to draw the figure.)
EXPERIMENTAL APPROACH

Objective 1
Efficacy and tolerability of mRNA vaccine expressing gB and pp38 antigens of Marek’s disease virus in chickens.

Key steps involved:

- Immunization of SPF (specific pathogen-free) chickens with one or two doses (14 days apart) of low-dose and high-dose mRNA vaccine
- Infection of vaccinated chickens with 250 plaque-forming unit (PFU) of RB1B-MDV five days after mRNA immunization
- Harvesting spleen and feather tip samples at four, 10, and 21 dpi
- Recording tumor and lesion scores at 21 dpi
- Measurement of viral genome load in feather tip samples using PCR
- Determine the relative expression of type I and II interferons, and IL-2, IL-6, IL-10, IL-18 cytokines via real-time PCR

Objective 2
Mechanisms involved in immune responses modulated by a Marek’s disease virus messenger RNA vaccine encoding gB and pp38 antigens in chickens.

Key steps involved:

- Immunization of SPF chickens with one or two doses (14 days apart) of low-dose and high-dose mRNA vaccine
• Harvesting spleen, lung, and muscle samples six, 18, 24 and 36 hpi
• Determining the relative expression of type I and II interferons, antiviral ISGs and proinflammatory cytokines (IL-1β, IL-2) via real-time PCR
CHAPTER 2

EFFICACY AND TOLERABILITY OF AN mRNA VACCINES 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 Marek’s disease virus (MDV). Currently available vaccines can prevent death and limit tumor growth in infected birds. However, they do not control virus shedding from the skin and feather follicle epithelial cells (FFE). The objective of the present study was to explore the utility of an mRNA vaccine for conferring immunity against MD. Two challenge trials were carried out to evaluate and compare the efficacy of different doses of an MDV mRNA vaccine. In addition, the necessity of a booster dose of the mRNA vaccine was assessed. Trial 1 consisted of two groups (primed versus boosted). In the primed group, chicks only received one dose of the mRNA vaccine (5 μg), while in the boosted group, chicks received a second identical dose after 14 days. In the second trial, the vaccine dose was increased to 10 μg. In addition, two more control groups were added to the study to compare the effect of the booster dose in chickens of the same age (to exclude the possible effects of 14 days age difference). 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 boosting chickens with the higher dose of the vaccine (10 μg) 14 days after the first immunization significantly decreased tumor incidence, average lesion scores, and MDV load in feather tips at 21 days post-infection (dpi) when compared to the untreated controls. Changes in expression of type I and II interferons at four, 10, and 21 dpi suggested a possible role for these cytokines in initiating immune responses in vaccinated chickens.
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 [4]. MD is an oncogenic and highly contagious disease in chickens caused by an alphaherpesvirus called Marek’s disease virus (MDV) [55]. MDV pathotypes are categorized as mild (mMDV), virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv+MDV) [27]. Immune suppression and neoplastic T cell lymphomas appear in different visceral organs, sometimes as early as two weeks post-infection [28]. Infection with vMDV and vvMDV pathotypes may cause paralysis and lymphomas in susceptible chickens.

MDV infects 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 (FFE) and skin [55]. 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 cell-free virus particles or cell-to-cell contact with epithelial cells. Twenty-four hours after the initial infection, the virus arrives in the thymus, bursa of Fabricius, and spleen [3]. 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 (dpi) [31], [32]. 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 [21]. 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 [138]. In general, MD vaccines are one or a combination of the following viruses: gallid alphaherpesvirus 2 (GaHV-2), gallid alphaherpesvirus 3 (GaHV-3), and meleagrid alphaherpesvirus 1 (MeHV-1; also known as herpes virus of turkeys (HVT)) [8]. CVI-988, a GaHV-2, is a 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 [9]. The virus for GaHV-3 vaccines first originated from healthy chickens. They induce moderate to low protection against the disease caused by vvMDV and are inhibited by the presence of maternal antibodies [6]. MeHV-1 or HVT is a non-oncogenic virus currently used alone or in combination with other serotypes [10]. 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 [12]–[14]. Nelson and colleagues (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 [15]. 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 [16]. An mRNA vaccine against surface glycoproteins of HSV also showed 80 to 100% reduction in virus vaginal shedding [17].

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 [95], [139]–[141]. 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 [142]. 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 [94].

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 [57], [60]. Our group also reported an increase in the number of CD8+ T cells in the feather pulp that correlated with decreased MDV genome copy numbers in feather tips of chickens treated with the Rispens-CVI988 MDV vaccine [143]. 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.
Materials and Methods

Plasmids and cloning

The expression cassettes encoding the MDV-gB and pp38 genes were synthesized by Thermo Fisher Scientific gene art synthesis (Germany). Plasmids were linearized using EcoRI-HF (NEB, Canada) and KpnI-HF (NEB, Canada). The target fragment was then inserted into a PSF-T7- T7 PROMOTER 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. The plasmids were isolated using a kit (Qiagen, Germany).

The DNA template included the coding sequence of full-length MDV RB1B gB and pp38 antigens flanked by untranslated regions, poly (A) at the 3´ end, Kozak consensus sequence ((gcc)gccRccAUGG), and a V5 tag (GTCATCCGGATAGGGATTGGGAGAGGAGCCAGAGCTAAGTG). An endosomal targeting motif of chicken invariant chain (Ch-Ii; accession number: AY597053) was added to the C-terminal of the antigen sequence.

mRNA synthesis and purification

mRNA was synthesized using Hi-T7 RNA Polymerase (NEB, Canada). One μg of gB/pp38 template DNA (plasmid), unmodified Ribonucleotide Solution Mix (NEB, Canada) and 10X Hi-T7 RNA polymerase reaction buffer were incubated at 50 ºC with Hi-T7 RNA polymerase for one hour 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 [144]. Purified RNA was treated with a DNase (Ambion, 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 μg RNA was suspended in 15 μl of nuclease-free H₂O and heated at
65°C for five minutes followed by cooling on ice for five minutes. The following components
were then added in this order: 2 μl 10X capping buffer, 1 μl 10 mM guanosine-5’-triphosphate, 1
μl 2 mM S-adenosylmethionine, 1 μl vaccina capping enzyme. The tube was incubated at 37°C
for 30 minutes and was stored at –80 °C until needed for experiments.

**Lipid nanoparticle preparation and characterization**

Lipid nanoparticle formulations were prepared as previously described [145] 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 seconds (10 sec ON and 5 sec
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 hr 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)

**Cell culture**

(i) DF1 cells and HEK 293T cells were used for *in vitro* assessment of gB/pp38 mRNA stability. DF1 cells were maintained in complete eagle’s minimum essential medium (EMEM) (10% FBS+ 1% pen-strep) while HEK 293T cells were cultured in complete Dulbecco’s modified eagle medium (DMEM) (10% FBS+ 1% pen-strep).

(ii) mRNA stability and protein expression: Five hundred thousand cells were seeded per well in a six-well plate in complete EMEM (10% FBS+ 1% pen-strep). At 80% confluency, cells were transfected with 1μg of mRNA encoding gB and pp38 using Lipofectin™ Transfection Reagent (Invitrogen, Canada) for four hours in optimum media. At six-, 12- and 32-hours 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-hours post-transfection. Two-hundred-thousand HEK 293T cells were transfected with 5μg of mRNA encoding gB and pp38 in 24-well plates using Lipofectin™ Transfection Reagent (Invitrogen, Canada) following the manufacturer’s instructions [146].

**Confocal microscopy**

At 36-hours post-transfection of HEK 293T cells with the mRNA vaccine, the cells were washed two times with PBS and fixed with 4% paraformaldehyde (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. Cells were blocked in 5% bovine serum albumin (BSA) 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, and DAPI
(4′,6-diamidino-2-phenylindole) was used for staining nuclei. Cells were analyzed using a Leica SP5 laser scanning confocal microscope (Leica Microsystems, Germany).

**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. 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.

**Experimental Design**

*Trial 1, Primary vaccination experiment*

One-hundred-and-twenty day-old SPF chickens 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 μg of gB and 2.5 μg of pp38, G1 and G4 received the diluent control (PBS), 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 hours and stored at -20°C until RNA extraction. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring.

**Trial 1, Prime-boost vaccination experiment**

One-hundred-and-twenty 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 μg of gB + 2.5 μg of pp38 per 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 μg of gB and 2.5 μg 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 Marek’s disease virus. 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 hours and stored...
at -20°C until RNA extraction. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring.

**Trial 2**

One-hundred-and-eighty-four day-old SPF chickens were randomly assigned into seven groups: G1: mRNA-mRNA (high dose); G2: mRNA-PBS; G3: PBS-mRNA; G4: mRNA-mRNA (low dose); 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 hours 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.

**Virus**

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

**RNA extraction and cDNA synthesis**

Trizol reagent (Thermo Fisher Scientific, Canada) was used for RNA extraction based on a previously described protocol [147]. Tissue samples were homogenized in 1 mL of Trizol, and HEK 293T cells were allowed for two 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 (Thermo Fisher Scientific, USA) at wavelengths of 260 and 280 nm was used to measure quantity and quality of the extracted RNA.

**Genomic DNA extraction**

Genomic DNA of the feather tips was extracted as described previously [148]. 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) 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 RNA. 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.

**Real-Time PCR**

Quantitative real-time polymerase chain reaction 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 five minutes followed by 40 to 50 cycles of 95°C for 20 sec, and 58 °C–64 °C (primer specific annealing temperatures) for 15 sec, in addition to 10 sec elongations at 72 °C. The melt curve was done by 10 sec incubation at 95 °C. The reaction then cooled down to 65 °C for one minute. Followed by heating to 95 °C. All primers were synthesized by Sigma-Aldrich (Canada), and their respective annealing temperatures are listed in table 1.

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.

**Statistical analysis**

Tumor incidence data were analyzed with Fisher’s exact test. Gene expression, lesion scores, 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 ≤ 0.05 (*) was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software).
Results

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 (figure 6).

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 figure 7 panels b and c are indicative of marked cytosolic accumulation of gB and pp38. Expression of gB and pp38 proteins was also confirmed separately (data not shown).

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

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; Figure 8. 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; Figure 8. 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 (Figure 8. c).
**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 (Figure 9. a and b). However, no decrease was seen in virus genome load at 10 and 21 dpi in the mRNA vaccine group (Figure 9. c).

**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) (Figure 10). 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 (Figure 10. 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) (Figure 10. 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 (Figure 10. c).

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

**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 (Figure 11. 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 (Figure 11. b). IFN-γ expression was significantly upregulated in the mRNA-vaccinated group at four and 21 dpi compared to the PBS-treated and unchallenged controls (Figure 11. c).

IL-10 expression was not significantly different when the mRNA-vaccinated group was compared to the PBS-treated and MDV-challenged controls (Figure 11. d). The expression of IL-6 and IL-18 were not statistically significantly different from controls (Figure 11. 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 (Figure 11. g).

**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 (Figure 12. 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 (Figure 12. b).

All mRNA-vaccinated groups showed a significant upregulation of IFN-γ at four and 21dpi when compared to the PBS-treated unchallenged group (Figure 12. c). Elevated expression of IL-10 was recorded at 10 and 21 dpi in all vaccinated groups compared to the PBS-treated unchallenged group (Figure 12. d). At days four and 21 post-infection, IL-6 expression was elevated in all mRNA-vaccinated groups compared to PBS-treated unchallenged controls (Figure
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 (Figure 12. f). At four dpi, IL-2 genes were significantly upregulated in all mRNA-vaccinated groups when compared to the PBS-treated unchallenged control group (Figure 12. g).

**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 1,000 times higher relative mRNA doses (on a per weight basis) [149]. 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 [12], [13].

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 100-200 nm showed better cellular uptake than smaller or larger sizes [150]. 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 WHO [151]) 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 (figure 6).

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 (figure 7).

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 eight hours 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 [57]. 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
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) [152]. Omar et al. (1996) also found gB-specific cell mediated immune responses in $B^{19}B^{19}$ chickens immunized with rFPV-gB [57].

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 [15]. 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 [16]. An mRNA vaccine against surface glycoproteins of HSV also showed 80 to 100% reduction in vaginal shedding of HSV-2 [17].

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 [57]. Boodhoo et al. identified pp38 immunodominant epitopes and characterized CD4+ T cell-specific responses to these immunodominant epitopes in MD-susceptible and MD-resistant chickens [60].
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) 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 [153]. 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 [48]. Increased expression of IFN-α in the blood of resistant chickens to MD [49] 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 21 dpi. The pivotal role of IFN-γ in an immune response against MDV has been proven previously [51], [154]. 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 [60]. 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 [63].
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 [155]. In chickens, co-stimulation with CpG-ODN (CpG-oligodeoxynucleotide) and polyI:C (TLR ligands) synergistically increased the expression of IFN-γ and IL-10 and led to a more robust Th1-biased immune response in chicken monocytes [156]. 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 [149]. 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 [156].

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 (HPV), and SARS-CoV-2 vaccinated humans [157]–[159]. Dennis et al. compared the protective immunity induced by a single vaccination and a boosted vaccination with a recombinant Leucocytozoon caulleryi subunit vaccine in chickens. Comparison of the antibody titers between the two groups at 15 and 21 dpi showed two-fold higher antibody concentrations in the chickens that received booster vaccination [160].

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.

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 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.
Figures

Figure 2. 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 consensus sequence ((gcc)gccRccAUGG), 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.
Figure 3. Schematic presentation of primary vaccination experiment in trial I.

On day one, chickens were injected with 5 \( \mu \text{g} \) of mRNA. On day five, chickens, except the PBS-treated control group, were challenged intra-abdominally with 250 plaque-forming units of RB1B Marek’s disease virus. On days four, 10, and 21 post-infection, six birds from each group were euthanized for collection of spleens and feather tips. On day 21 post-challenge, all the remaining birds were euthanized for tumor and lesion scoring.
Figure 4. Schematic presentation of prime-boost vaccination experiment in trial 1.

On day one, chickens were injected with 5 μg of mRNA. Two weeks later, mRNA group received the booster dose (5μg). On day 19, chickens, except the PBS-treated control group, were challenged intra-abdominally with 250 plaque-forming units of RB1B Marek’s disease virus. On days four, 10, and 21 post-infection, six birds from each group were euthanized for collection of spleens and feather tips. On day 21 post-challenge, all the remaining birds were euthanized for tumor and lesion scoring.
Figure 5. Schematic presentation of the high-dose prime-boost vaccination experiment in trial 2.

On day one, chickens were injected with 10 μg of mRNA. Two weeks later one of the groups that had been primed with the mRNA vaccine received a booster dose (10 μg) and the other group received PBS.

On day 19, chickens, except the PBS-treated control group, were challenged with 250 PFU RB1B Marek’s disease virus (intra-abdominally). On days four, 10, and 21 post-infection, six birds from each group were euthanized to collect spleens and feather tips. On day 21 post-challenge all the remaining birds were euthanized for tumor and lesion scoring.
Figure 6. 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.
Figure 7. gB and pp38 mRNA cell delivery and protein expression in HEK 293T cells.

At 36-hour 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 (a and d), target proteins stained with fluorescent-conjugated goat anti-mouse secondary antibody (b and e), and combined channels (merge) (c and f).
Figure 8. Tumor incidence, lesion score, and meq gene load in the primary vaccination experiment in 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 ≤ 0.05  was considered statistically significant.
Figure 9. Tumor incidence, lesion score, and meq gene load in prime-boost vaccination experiment in 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 ≤ 0.05 was considered statistically significant.
Figure 10. Tumor incidence, lesion score and meq gene load in the 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 ≤ 0.05 was considered statistically significant.
Figure 11. Relative expression of genes in spleens at four-, 10-, and 21-days post-viral challenge for prime-boost vaccination experiment in 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≤0.05 was considered statistically significant). ß-actin was used as a reference gene for all relative expressions.
Figure 12. Relative expression of genes in spleens at four-, 10-, and 21-days post-viral challenge for 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≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
### Table 1. Real-time PCR primer sequences for chicken target genes

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<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession number/Reference</th>
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<tr>
<td>β-actin</td>
<td>F: 5’-CAACACAGTGCTGTCTGGTGAT-3’</td>
<td>X00182</td>
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<td></td>
<td>R: 5’-ATCGTACTCCTGCTTGATG-3’</td>
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<tr>
<td>IFN-γ</td>
<td>F: 5’-ACACTGACAAGTCAAAGCCGCACA-3’</td>
<td>X99774</td>
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<td></td>
<td>R: 5’-AGTCGTTCATCGGGAGCTTG-3’</td>
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<td></td>
<td>R: 5’-GGTGTTGCTGGTGTCGAGATG-3’</td>
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<td>F: 5’-GCCTCCAGCTCTCCAATACG-3’</td>
<td>[162]</td>
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<td></td>
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<td></td>
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<td>meq</td>
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<td>[163]</td>
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CHAPTER 3

A MAREK’S DISEASE VIRUS MESSENGER RNA-BASED VACCINE MODULATES LOCAL AND SYSTEMIC IMMUNE RESPONSES

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Abstract

Marek’s disease (MD) caused by Marek’s disease virus, is a contagious lymphoproliferative disease in chickens that can be controlled by vaccination. The immune responses induced by the current vaccines limit tumor growth and death but not virus replication, shedding, and transmission. To overcome these features of the current vaccines against MD, the present study initially aimed to evaluate host responses following intramuscular injection of two messenger-RNA vaccines encoding glycoprotein B (gB) and phosphoprotein38 (pp38) proteins of the MD virus within the first 36 hours of the vaccination. The vaccine was injected in low and high doses using prime and prime-boost strategies. Expression of type I and II interferons (IFNs), a panel of interferon-stimulated genes, and two cytokines, interleukin (IL)-1β and IL-2, which are the key players in antiviral responses, were measured in spleens and lungs after vaccination. Transcriptional analysis of the above genes showed significant increases in the expression of melanoma differentiation-associated protein 5 (MDA5), myeloid differentiation primary response 88 (Myd88), IFN-α, IFN-β, IFN-γ, interferon regulatory factor 7, 2’, 5’-oligoadenylatesynthase (OAS), dsRNA-dependent protein kinase, interferon-induced protein with tetratricopeptide repeats 5, myxoma-resistance protein 1 (Mx1), and IL-2 in both spleens and lungs within the first 36 hours of the immunization. The booster injection significantly increased all measured genes in the lungs. In contrast, only five genes (IFN-γ, MDA5, MyD88, Mx1, and OAS) showed statistically significant upregulation in the spleens after the booster injection.

Introduction

Marek’s disease virus (MDV) is an oncogenic, highly contagious alpha herpesvirus in chickens [1]. This virus is considered a major global challenge in the poultry industry and is estimated to cause annual losses of approximately $1-2 billion USD [4].
A protective vaccine against Marek’s disease has been widely used since 1969 [5]. Due to the evolution of field strains of the virus with higher virulence, the original vaccine was no longer highly protective. Over the following decades, polyvalent and Rispens vaccines were introduced for greater protection [6], [7]. Although MD vaccines protect against tumorigenesis and death and can reduce economic consequences, they do not prevent virus replication and shedding from infected chickens. After vaccination, continuous virus shedding still poses a risk to non-vaccinated chickens, which might lead to the emergence of more virulent pathotypes. Ideally, a successful vaccine is one that decreases or stops viral replication and shedding.

The MDV genome encodes several glycoproteins. gB is one of the main surface glycoproteins of the MDV. gB forms heterodimer with other surface glycoproteins and helps in viral attachment to host cells [57]. Among MDV proteins, pp38 is one of the early proteins expressed in cytolytic infection of B and T cells and is involved in maintaining the transformed state. pp38 is also expressed in tumor cells [164]. The antigenic potential of both gB and pp38 to initiate cytolytic T cell immune response has been characterized previously [57], [165]. Boodhoo et al. reported a direct association between the magnitude of the T cell immune response to pp38 antigen and resistance to the MDV [60].

In recent years, mRNA-based vaccines have been shown to induce immune responses against viral pathogens. Several studies reported the efficacy of mRNA vaccines in controlling viral pathogens in animal models [12]–[14]. Nelson and colleagues compared mRNA vaccine expressing full-length gB with the vaccines expressing subunit proteins in New Zealand White rabbits infected with human cytomegalovirus, demonstrating superior durability of the antibody response originating from the full-length gB mRNA vaccine [15]. The study by Nelson et al. also showed superior durability of the immune response induced by the full-length gB mRNA vaccine
that was projected to stay detectable after 50 weeks. Other than durability, the full-length gB mRNA vaccine in the study led to an enhanced breadth of peptide-binding responses compared to the subunit protein vaccines. A trivalent mRNA vaccine encoding the ectodomain of gC2, gD2, and gE2 of herpes simplex virus 2 (HSV-2) induced potent CD4+ T-follicular helper cell and germinal center B cell responses in mice [16]. An mRNA vaccine against surface glycoproteins of HSV also showed an 80 to 100% reduction in virus vaginal shedding [17].

To our knowledge, no mRNA vaccine for MDV based on gB and pp38 has been developed so far, and the construction and efficacy of such vaccines need to be examined. Different delivery methods, such as lipid nanoparticles (LNPs), polymers-based nanomaterial, and cell-penetrating peptides, have been used for siRNA or mRNA in vivo migration [95], [139]–[141]. LNP encapsulation happens through a single-step process of microfluidic mixing devices or pipetting when an acidic fluid of mRNA is mixed with an ethanol solution of lipids. Lipids start a condensation process and, at the same time, shape the lipid vesicles which surround mRNA molecules [94]. In addition to easy manufacturing, biocompatibility and biodegradability are other advantages of LNPs. Therefore, in this study, we used LNPs to encapsulate mRNA constructs [142].

The present study explored the immune response induced by our mRNA-constructed vaccine and the mechanisms of protection that might be involved in protecting against Marek’s disease virus using prime and prime-boost vaccination strategies. The immunostimulatory effects following two doses of mRNA administrations and gB and pp38 expression have been studied. The spleen, a major immunological organ in chickens, and lungs as an indicator organ of vaccine-induced mucosal immunity were chosen to evaluate the expression of genes associated with anti-
viral immune responses. In addition, the stability of the mRNA vaccine at the site of injection in the first 36 hours of injection was assessed.

**Materials and Methods**

*In vitro* mRNA synthesis, lipid nanoparticle preparation and characterization, and expression of desired antigen are described in chapter 2.

**Cell culture**

*In vitro* stability of gB/pp38 mRNA was assessed using DF1 cells. DF1 cells were maintained in EMEM complete medium (10% FBS+ 1% pen-strep). Five hundred thousand cells were seeded per well in a 6-well plate in EMEM complete medium (10% FBS+ 1% pen-strep). When 80% confluent, cells were transfected with 1μg mRNA encoding gB and pp38 using Lipofectin™ Transfection Reagent (Invitrogen, Canada) for 4 hours in optimum media. At 6-, 12- and 32-hour post-transfection, cells were washed with PBS, treated with trypsin, and collected following for RNA extraction.

**Experimental animals**

One-day-old specific-pathogen-free (SPF) White Leghorn chickens were received from the Canadian Food Inspection Agency (CFIA, Ottawa, Canada). The chicks were randomly grouped in Horsfall units and were sheltered in the Animal Isolation Unit, at the University of Guelph. Chickens had ad libitum access to food and water over the period of the experiment. The protocols used in this research project were reviewed and approved by the University of Guelph Animal Care Committee.
Experimental design

One-hundred-and-twenty day-old SPF chicks were delivered to the Animal Isolation Unit and divided into three groups (n = 30 chicks). The next day, all chicks were treated as follows: Group 1: 200 μl/chick diluent control (PBS); G2: inoculated intramuscularly (IM) (iliotibialis muscle) with 2 μg gB + 2 μg pp38 per chick of mRNA packaged in LNPs (low dose group); and G3: inoculated intramuscularly (iliotibialis muscle) with 4 μg gB + 4 μg pp38 per chick of mRNA packaged in LNPs (high dose group). The vaccinated groups (G2: inoculated intramuscularly with 2 μg gB + 2 μg pp38 per chick of mRNA packaged in LNPs; and G3: inoculated intramuscularly with 4 μg gB + 4 μg pp38 per chick of mRNA packaged in LNPs) were boosted 14 days later. The control birds (G1) were injected with 200 μl/chick of PBS. Following injections, birds were observed for several minutes, followed by an additional observation before the end of the working day to ensure that no gross adverse effect was developing. Throughout our studies, birds were monitored at least three times a day for possible signs of adverse events. On 6, 12, 24, and 36 hours post-prime and boost injection, six birds from each group were euthanized by CO₂ inhalation for spleen, lung, and muscle (muscle tissue was sampled only from prime groups) sample collection.

All samples were stored in RNAlater (Thermo Fisher, Lithuania) at 4 °C for 24 hours and in -20°C until RNA extraction.

RNA extraction and cDNA synthesis

Trizol reagent (Thermo Fisher Scientific, Canada) was used for RNA extraction based on a previously described protocol [147]. Briefly, tissue samples were homogenized in 1 mL of Trizol, and DF1 cells and HEK 293T cells were allowed for 2 min in Trizol, then mixed in chloroform (Sigma-Aldrich, USA). Isopropanol and 75% ethanol were used to precipitate and wash the extracted RNA, respectively. Pellets were resuspended in ultra-pure distilled water (Invitrogen,
USA). Extracted RNA was treated with DNase enzyme (Ambion, USA), and cDNA synthesis was performed using Superscript II (Life Technologies, USA). To measure the quantity and quality of the extracted RNA, Nanodrop spectrophotometry at 260 and 280 nm wavelength (Thermo Fisher Scientific, USA) was practiced.

**Real-Time PCR**

Quantitative real-time polymerase chain reaction was performed using SYBR green dye in a LightCycler 480 II (Roche Diagnostics, Canada). Briefly, the plate was pre-incubated at 95 °C for 5 mins followed by 40 to 50 cycles of 95°C for 20 sec, and 58 °C–64 °C (primer specific annealing temperatures) for 15 sec, in addition to 10 sec elongations at 72 °C. Melt curve was done by 10 sec 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 listed in table 1.

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 control group ± standard error.

**Statistical analysis**

Gene expression data were analyzed with Kruskal-Wallis followed by the Mann-Whitney test. p ≤ 0.05 (*) was considered statistically significant. Statistical analysis was performed using GraphPad Prism version 9 (GraphPad Software).
Results

Detection of mRNA molecules encoding gB and pp38 in DF1 cells

DF1 cells were transfected with 1µg of in vitro-transcribed mRNA (gB, pp38) at three time points (6h, 12h, 32h). At 12h, the absolute quantity of both gB and pp38 was higher than at 6h and 32h (Figure 13).

Detection of mRNA molecules encoding gB and pp38 at the site of injection

In vivo stability of mRNA molecules encoding gB and pp38 proteins was analyzed at the injection site (iliotibial muscle) at four-time points post-administration. At each time-point, the absolute amount of mRNA decreased by 3-4 logs for gB and 2.5 logs for pp38 compared to the initial measurement at the first time-point (six hpi). In the low-dose group, the pp38 mRNA was not detectable at 24 hpi and 36 hpi time points (Figure 14).

Expression of cytokines and interferon-stimulated genes (ISGs) genes after administration of mRNA molecules encoding MDV gB and pp38 proteins

Spleen

To analyze mRNA-driven changes following mRNA vaccination in chickens, tissue samples were collected at six-, 18-, 24-, and 36-hours post-vaccine administration, and relative expression of a panel of interferons, interferon regulatory genes, and two proinflammatory cytokines (IL-β and IL-2) was measured.

Increased expression of MDA5 and MyD88 was detected in the spleen, lungs, and injection site of the birds six hours after receiving either a low-dose or high-dose of the vaccine. MDA5 expression remained significantly high until 36 hours post-stimulation in the spleen and lungs of the high-dose group (Figure 15. a and c). The high-dose group also showed significantly elevated MyD88 expression in the site of injection, spleen, and lungs at six hpi (Figure 15. d, e and f).
Vaccination significantly increased the expression of IFN-α in both low- and high-dose groups at six hpi. A single injection of a low-dose vaccine could significantly increase IFN-α expression in the spleen at six hpi, which decreased to half and one-third after 18 and 24 hours, respectively. The high dose of vaccine led to four times higher IFN-α expression at six hpi compared to 18 hpi in the spleen (Figure 16. a). IFN-α expression at the six-hour time-point following the booster dose was two-fold higher than that at 18h time-point. Boosting the low-dose group led to almost five times and eight times higher expression of IFN-α at six hpi compared to 18h and 24h time-points, respectively (Figure 16. b).

The highest number of IFN-β transcripts was observed six hours after prime vaccination, when it was five times and 10 times higher in the low- and high-dose groups compared to the PBS-treated controls, respectively. IFN-β expression stayed significantly high until 18 hpi in the high-dose group after both prime and booster doses (Figure 16. c and d).

One dose of vaccine did not alter IRF7 expression (Figure 16. e), while at six hours after the booster injection, IRF7 expression had increased significantly (Figure 16. f).

OAS expression peaked at 18h following a single injection with low-dose mRNA and it remained high until 24 hpi (Figure 17. a). Upon the booster administration, OAS expression elevated early (2.5-fold and 4.5-fold higher in low-dose and high-dose groups, respectively, compared to the PBS-treated control group), and it stayed significantly high until 24 hpi in the high-dose group (Figure 17. b). PKR expression increased significantly by six hours after the first and second injection in both low- and high-dose groups (Figure 17. c and d). PKR expression in the high-dose group was significantly higher than in the low-dose group at six hours after booster injection (Figure 17. d).
A high dose of the vaccine resulted in elevated IFIT5 expression that remained significantly high until 24 hpi in the prime group (Figure 17. e). Booster injection also increased IFIT5 expression at six, 24, and 36 hpi time-points in both low- and high-dose groups (Figure 17. f).

A single injection of low-dose mRNA did not significantly increase Mx1 expression, whereas increasing the dose significantly elevated its expression at six hpi (Figure 18. a). Following boosting, Mx1 expression was highest at 6 hpi and remained significantly high at 24 and 36 hpi time-points in both low-dose and high-dose groups (Figure 18. b).

One dose of vaccine led to a two-fold increase (low-dose) and a three-fold increase (high-dose) in IL-1β expression, starting at six hpi and lasting until 24 hpi (Figure 18. c). The second injection of mRNA also increased IL-1β expression by three-fold at six hpi; this transient increase was no longer apparent at later time points (Figure 18. d).

A single mRNA administration sharply increased the expression of IFN-γ in both low- (10-fold increase) and high-dose (20-fold increase) at six hpi in the spleen. This expression dropped later but was still significantly higher until 24 hpi in the high-dose group (Figure 19. a).

At 18 hpi, IL-2 expression was increased by three times (low-dose) and five times (high-dose) (Figure 19. c), and the second injection of high-dose mRNA resulted in a significantly increased expression of IL-2 at 18 hpi and 36 hpi (Figure 19. d).

Lungs

While a single dose of the mRNA vaccine did not alter IFN-α expression, boosting with the high dose of vaccine led to an over two-fold increase at 18 hpi (Figure 20. a and b). A high dose of the vaccine also increased IFN-β expression by two- and three-fold after a single injection in the high-dose group at 18 hpi and 24 hpi, respectively. The elevated expression of IFN-β in the lungs was significant at 18 and 36 hpi after the booster injection (Figure 20. c and d). One dose of
the vaccine did not significantly alter IRF7 expression, but the booster dose increased the expression of IRF7, which was significant in the low-dose group at six hpi (Figure 20. e and f).

Single-dose and double-dose groups showed significantly higher OAS expression until 36 hpi and 24 hpi respectively. At the earliest time-point (six hpi), a five-fold increase in the prime group and a 10-fold increase in the booster group were detected (Figure 21. a and b). OAS expression in the high-dose group was significantly higher than the low-dose group at six and 24 hours after prime dose injection.

PKR expression was elevated following a single high dose of vaccine at six, 24, and 36 hpi. After injection of the high-dose vaccine, PKR expression remained significantly high until 36 hpi in the booster groups (Figure 21. c and d).

Following a high-dose injection in the prime group, IFIT5 expression increased by six times, four times, and three times at six hpi, 18 hpi, and 24 hpi respectively (Figure 21. e). Boosting the chicks with another dose of mRNA led to 10 times increase at six hpi and a five times increase at 18 hpi which dropped at 24 hpi, but it was still significantly higher when compared to PBS-treated controls (Figure 21. f).

Mx1 expression peaked 18 hours post-prime injection and stayed significantly high until 36 hpi in the high-dose group. The booster dose resulted in an earlier increase in Mx1 expression compared to the prime dose. Following the booster injection, Mx1 expression increased by around 20-fold at six hpi and 18 hpi. Elevated Mx1 expression had dropped to around four-fold at 24 hpi in the booster group (Figure 22. a and b). IL-1β expression was increased 18 hpi in the prime group, and it stayed high until 36 hpi in the high-dose group.

IFN-γ expression in the lungs increased at six hpi and remained significantly high until 36 hpi after a single injection with the high-dose vaccine (Figure 23. a). The second injection of
mRNA resulted in a significant increase of IFN-γ expression in the lungs at six, 18 and 36 hpi at both low- and high-dose groups (Figure 23. b).

IL-2 expression in the lungs peaked at 18 hpi, and after a transient drop, it increased at 36 hpi again (Figure 23. c). The booster injection did not statistically significantly increase IL-2 production (Figure 23. d).

Changes in gene expression following booster injection were different in the spleen and lungs. Among 13 gene profiles analyzed in the spleen, only five genes (IFN-γ, MDA5, MyD88, Mx1, and OAS) showed significantly elevated expression after the booster dose compared with the same time point after primary vaccination. In the lungs, all 13 genes showed a significant increase in all or at least one time-point after the booster dose (IFN-α, IFN-β, IFN-γ, MyD88, OAS, PKR, TRIF, IL-2 genes showed upregulated expression in all four time-points) (data not shown).

**Discussion**

This study aimed to evaluate the induction of immunological genes upon IM injection of two doses of an mRNA vaccine encoding gB and pp38 proteins of MDV. Expression of type I and II interferons, a panel of ISGs, and two inflammatory cytokines, as key players in response to mRNA vaccination, was measured in spleens and lungs.

The *in vitro* stability of synthesized mRNA packaged in LNPs at four time points after injection was first assessed. At each time-point post-injection, the amount of mRNA molecules decreased by approximately 3.5 and 2.5 logs for gB and pp38 constructs, respectively. Although both gB and pp38 mRNAs were constructed using the same protocol, the molecules' size and nucleotide composition might have impacted the speed of degradation *in vivo*. A higher G:C content of the mRNA molecules can increase mRNA stability and its half-life inside the cell [166].
The G:C content of the mRNA encoding gB and pp38 proteins in this study was 42.7% and 51.9%, respectively. It may explain the slower degradation pace of pp38 compared to gB at the injection site (2.5 logs decrease in each time-point for pp38 compared to 3.5 logs decrease for gB). Although the major structures involved in mRNA stability, such as UTRs, 5’ cap, and polyA tail, were incorporated in the *in vitro*-synthesized mRNA molecules used in this study, we did not use modified nucleotides. By modifying the nucleotide composition of mRNA constructs, the half-life of mRNA in the cytosol increases, which will impact the efficiency of protein production from these constructs.

How mRNA distributes in the body upon injection is an important question that has been partially answered in other studies using different animal models. Generally speaking, the injection site, lymph nodes, and spleen are among the organs and tissues listed with the highest concentrations of the administered mRNA [98]. Bahl *et al.* compared the $T_{\text{max}}$ (time of the maximum concentration) and $T\frac{1}{2}$ (required time for mRNA to be reduced to half) in different organs upon IM injection. The maximum concentration of mRNA in the muscle, spleen, and lung were recorded at two hpi. Injected mRNA dilutes to half its original amount in the muscle within 18 hrs, while the lung and spleen showed mRNA dilution at 16 and 25 hrs, respectively. This agrees with the mRNA degradation time in the muscles observed in the present study.

To assess the immunostimulatory effects of the mRNA vaccine, we examined the spleen and lungs and monitored changes in the expression of type I and II interferons, a panel of ISGs, and two cytokines that are key players in antiviral response. Chickens lack draining lymph nodes, and their spleens are considered major secondary lymphoid tissues. The lungs are the primary site of infection upon inhalation of MDV particles and represent the initial site of the host response to this virus. Upon administration, mRNA is sensed by endosomal TLRs (TLR7 and 8) and
cytoplasmic sensors (MDA5) [167]. TLR binding to its ligands activates MyD88, followed by RNA recognition by MDA5 compartments which consequently activates mitochondrial antiviral signaling protein (MAVS). Activation of these two adaptor proteins induces a signaling cascade resulting in the expression of genes encoding antiviral response elements, including type I IFNs [168]. MDA5 also plays a role in the phosphorylation of IRF7 through its caspase-recruitment domains (CARDs) [169], resulting in increased expression of IFN-α and IFN-β.

The results obtained from the injection site, spleen, and lungs showed elevated expression of MDA5 and MyD88 at early vaccination time points. This agrees with the findings reported by Edwards et al. in the mouse model (C57BL/6 mouse) and human cell lines [167]. Type I interferons can consequently induce ISGs after binding to their receptors. Myxoma-resistance protein (Mx), dsRNA-dependent protein kinase (PKR), 2’, 5’-oligoadenylatesynthase (OAS), and interferon-induced protein with tetratricopeptide repeats 5 (IFIT5) are some of the ISGs highly associated with the antiviral response. Kato and colleagues transfected DCs with RNAs extracted from vesicular stomatitis virus and encephalomyocarditis virus and measured the production of IFN-α. Their result showed impaired production of IFN-α in MDA5−/− mice, highlighting the role of MDA5 in sensing exogenous RNA and induction of IFN-α expression [169].

IL-1β is another pro-inflammatory cytokine produced following non-self RNA recognition by PRRs in the cytosol. Increased IL-1β expression after the mRNA administration in our study agrees with the results reported by others [149], [167]. Upregulated expression of IL-1β can help in the maturation of DCs [170], resulting in increased antigen presentation and stronger adaptive immune responses after viral challenges. In addition, IL-1 (mostly IL-1β) has a major role in balancing the inflammatory response following mRNA injection. Tahtinen et al. (2022) showed that mice and humans reacted differently to equal relative doses of mRNA vaccine. IL-1β
expression following injection of two different ionizable lipids (MC3, SM-102) broadly used for LNPs formation was also analyzed [149]. It has been shown that empty LNPs made by MC3 lipids could not stimulate IL-1β production, while LNPs made by SM-102 (used in the Covid-19 vaccine designed by Moderna) were potent inducers of IL-1β [149]. As the LNPs in the present study were made with MC3, the elevated expression of IL-1β after vaccine administration is likely to be initiated by the mRNA molecules and not by the LNPs.

IFN-γ is the sole member of type II IFN, secreted by activated T cells and NK cells. IFN-γ is considered a key player in the antiviral state, and its direct effect on the reduction of MDV has already been characterized [51]. The administration of recombinant chicken interferon-gamma significantly boosted the immunity elicited by the HVT vaccine against virulent MDV. As IFN-γ is mainly secreted by NK cells and activated T cells, elevated expression of IFN-γ in the lungs in this study can indicate activation CD4+ Th1 cells, CD8+ cytotoxic T cells, and NK cells following mRNA immunization of chickens.

IL-2 is another cytokine that is characterized to have a key role in T cell activation, NK cell stimulation, and B cell expansion. IL-2 reduces HSV-1 replication and pathogenicity in mice, linked to effector CD4+ and CD8+ T lymphocytes. Ghiasi et al. also observed increased virulence of HSV-1 after IL-2 depletion in mice [171] In chickens, IL-2 also acts in NK cell activation, lymphocyte proliferation, and defense against intracellular pathogens [172], [173]. Susta et al. (2015) reported reduced systemic viral load linked to increased expression of IL-2 during infection with a highly virulent strain of Newcastle disease virus in chickens [174]. To analyze possible T cell activation following mRNA vaccination, we measured IL-2 expression in the spleen and lungs. Significantly elevated expression of IL-2 was demonstrated in both organs at 18 hpi following a
single injection of the mRNA vaccine. IL-2 expression remained high until 36 hpi after booster injection in the spleen. Increased IL-2 expression was also reported following mRNA immunization against the Omicron variant of SARS-CoV-2 [175]. Similarly, Jiang and colleagues measured the IL-2 and IFN-γ expression in blood after immunization with a gD mRNA vaccine and reported a two-fold increase in IL-2 and IFN-γ expression in the vaccinated mice [12].

Comparison of gene expression following immunization with low versus high doses of the vaccine showed that for most of the genes and at most of the time points, the relative gene expression was higher in the high-dose group. However, these differences were only significant at one or two time-points for a few genes. One of the possible reasons for such an observation can be the activation of the intracellular inhibitory mechanisms involved in the degradation and dilution of excessive non-self mRNA, such as exonucleolytic degradation, when the vaccine was injected at the higher dose. Finding the toxic and tolerable margin of non-self mRNA in chickens is one area that needs further dissection.

Changes in gene expression after the booster dose were monitored to understand the necessity of the booster injection. Spleens and lungs showed different patterns of gene expression after the second vaccination. Among 13 gene profiles analyzed in the spleen only 5 genes (IFN-γ, MDA5, MyD88, Mx1, and OAS) showed significantly elevated expression after booster dose when compared with the same time point after prime injection. All 13 genes showed significant increases in all or at least one time-point in the lungs after the booster dose (IFN-α, IFN-β, IFN-γ, MyD88, OAS, PKR, TRIF, IL-2 genes showed upregulated expression in all four time-points). The reason why different organs showed different gene expression patterns after the second dose might be the diverse cell composition in each of the organs after the primary immunization. Migration of various subsets of innate or adaptive leukocytes from the spleen to other organs (such
as the lung, GI system, etc. [176] might have affected the transcriptional patterns in the spleens and lungs. For instance, the expression of genes involved in effector cell activation, such as IL-2, was significantly increased at all time points in the lungs after the booster injection, which might have happened due to the recruitment of activated NK cells and T cells to the lungs.

Aside from this, mRNA cellular uptake might have differed in the spleen and lungs following the second injection. Cellular uptake of mRNA is affected by the number of extracellular proteins, such as ApoE, which is responsible for lipid trafficking and facilitating LNP uptake [177]. Although the liver is the primary organ that synthesizes ApoE, it can be secreted from lung cells. For instance, alveolar macrophages are one of the producers of ApoE in lungs [178]. Alveolar macrophages are called free avian respiratory macrophages (FARM) in birds [179]. Although, the number of FARMs are much lower than the number of alveolar macrophages in mammals [179], activated alveolar macrophages in the lungs following the first injection might have contributed to the higher ApoE secretion and, higher LNPs uptake in the lungs upon booster administration.

Further investigations are needed as to whether this applies to our studies. Mammalian studies characterized the pathways involved in ApoE secretion by bronchoalveolar macrophages and consequently increased secretion of IL-1B by alveolar macrophages of the lungs [178]. As ApoE level has not been measured in the present study, we speculate that three times higher IL-1β expression in the high-dose group after booster injection might have happened due to increased ApoE secretion by bronchoalveolar macrophages.

The robust expression of IFNs, ISGs, and proinflammatory cytokines after the booster injection in the lungs as the primary site of injection might have played a role in lower tumor incidence, lesion scores, and viral load recorded in the booster groups as discussed in the second chapter of this thesis.
In the second chapter of this thesis, the vaccine-induced protection against MDV after two immunizations with high-dose of mRNA and its relationship with type I and II interferons was discussed. Significantly upregulated IFN-α expression at 21 dpi (compared to the both MDV-challenged but unvaccinated and PBS-treated groups), and IFN-β (compared to the PBS-treated group) was seen. Type I interferons are proven to be downregulated by MDV [153]. Results from the challenge trials showed anticorrelation between MDV genome load and type I IFN expression. It can be due to lower replicating MDV in mRNA vaccinated group (high-dose) and as a result less inhibition of type I IFN expression by the virus. The MDV trial in this study also showed higher expression of type II interferon in chickens immunized with two high-doses mRNA vaccine when compared to the PBS-treated controls at four and 21 dpi. The central role of IFN-γ in immune response against MDV has been discussed previously [51][154]. The pp38-specific T cell response was measured using chicken IFN-γ ELISPOT assay. The highest frequency of IFN-γ producing T cells was recorded in MDV-vaccinated and challenged chickens [60]. In the present study, activated T cells might be the main source of IFN-γ after two high-doses mRNA vaccinations.

Together, our results showed that a bivalent mRNA vaccine encoding gB and pp38 proteins could induce cytokine gene expression, at early time points, followed by significantly higher expression of IL-2 and IFN-γ that play a crucial role in the effective induction of adaptive immune response and potent anti-tumor response against MDV. To further investigate the antiviral response and anti-tumor function of this vaccine, additional in vivo and in vitro studies are needed. Antigen-specific T cell response following viral challenge in vaccinated chickens could potentially be a good indicator of efficacy of this mRNA vaccine. In addition, more efficient and extended antigen presentation time by modifying mRNA constructs and targeting antigen-presenting cells to maximize T cell cytotoxicity can be considered for future studies.
Figure 13. *In vitro* stability of mRNA vaccine.

*In vitro* stability of gB-RB1B (left) and pp38-RB1B (right) mRNA molecules were analyzed within 6 to 32 hrs post-transfection in DF1 cells. Five hundred thousand cells were seeded per well in a 6-well plate in EMEM complete medium (10% FBS+ 1% pen-strep). Cells were transfected with 1μg mRNA encoding gB and pp38 using Lipofectin for four hours in the optimum medium. At six-, 12- and 32 hours post-transfection, cells were washed with PBS and collected following trypsin treatment for RNA extraction.
Figure 14. *In vivo* stability of mRNA vaccine.

*In vivo* stability of gB-RB1B (*left* panel) and pp38-RB1B (*right* panel) mRNA molecules were analyzed within six to 36 hours post-IM administration at the injection site. One cm² muscle sample from the injection site (Iliotibial muscle) was excised and processed for RNA extraction and cDNA synthesis at six-, 18-, 24-, and 36-hrs post-IM administration of PBS (C-), low-dose mRNA (L) and high-dose mRNA (H). Positive control (C+) is DNA extracted from a lung tissue sample from an infected chicken with RB1B MDV at 21 dpi. Red arrows show a decrease in the absolute amount of injected mRNA at each time point.
Figure 15. Relative expression of MDA5 and MyD88 genes at 6-, 18-, 24-, and 36-hrs post-vaccine administration in muscle, spleen, and lungs.

One cm² muscle sample from the injection site (iliotibial muscle), in addition to the spleen and lungs, was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of PBS, low-dose mRNA (L) and high-dose mRNA (H). **Panel A.** Relative expression of MDA5 in the spleen (a), muscle (b), and lungs (c). **Panel B.** Relative expression of MyD88 in the spleen (d), muscle (e), and lungs (f). Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 16. Relative expression of IFN-α, IFN-β, and IRF7 in the spleen at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration.
One cm$^2$ tissue from the spleen was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of IFN-α (a and b), IFN-β (c and d), and IRF7 (e and f) in the spleen. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 17. Relative expression of OAS, PKR, and IFIT5 in the spleen at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the spleen.
One cm² tissue from the spleen was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of OAS (a and b), PKR (c and d), and IFIT5 (e and f) in the spleen. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 18. Relative expression of Mx1, IL-1β, and TRIF at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the spleen.
One cm$^2$ tissue from the spleen was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of Mx1(a and b), IL-1$\beta$ (c and d), and TRIF (e and f) in the spleen. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). $\beta$-actin was used as a reference gene for all relative expressions.
Figure 19. Relative expression of IFN-γ and IL-2 at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the spleen.

One cm² tissue from the spleen was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of IFN-γ (a and b), and IL-2 (c and d) in the spleen. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data
were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 20. Relative expression of IFN-α, IFN-β, and IRF7 in the lungs at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration.

One cm² tissue from the lungs was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS
administration was used as control. Graphs compare the relative expression of IFN-α (a and b), IFN-β (c and d), and IRF7 (e and f) in the lungs. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 21. Relative expression of OAS, PKR, and IFIT5 in the spleen at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the lungs.
One cm$^2$ tissue from the lungs was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of OAS (a and b), PKR (c and d), and IFIT5 (e and f) in the lungs. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis followed by the Mann-Whitney test (p≤0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 22. Relative expression of Mx1, IL-1β, and TRIF at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the lungs.
One cm² tissue from the lungs was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of Mx1 (a and b), IL-1β (c and d), and TRIF (e and f) in the lungs. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p<0.05 was considered statistically significant). β-actin was used as a reference gene for all relative expressions.
Figure 23. Relative expression of IFN-γ and IL-2 at 6-, 18-, 24-, and 36-hrs post-IM vaccine administration in the lungs.

One cm² tissue from the lungs was excised and processed for RNA extraction and cDNA synthesis at 6-, 18-, 24-, and 36-hrs post-IM administration of a low-dose mRNA (L) and high-dose mRNA (H). PBS administration was used as control. Graphs compare the relative expression of IFN-γ (a and b), and IL-2 (c and d) in the lungs. Relative expression data represent the mean fold-change of 5-6 biological replicates (chickens) compared to the PBS-treated control group (*) and low-dose group (#) ± standard error. Data were analyzed with the Kruskal-Wallis test followed by the Mann-Whitney test (p≤0.05 was considered statistically).
Table 2. Real-time PCR primer sequences for chicken target genes

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<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession number/Reference</th>
</tr>
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| β-actin | F: 5’-CAACACAGTGCTGTCTGGTGTA-3’  
R: 5’-ATCGTACTCTGGTGTGATCC-3’ | X00182                    |
| IFN-γ   | F: 5’-ACACTGACAAGTCAAAGGCGACA-3’  
R: 5’-AGTCGTTTCATCGGGAGCTTGCC-3’ | X99774                    |
| IFN-α   | F: 5’-ATCTCTGGCTGCTCAGCTCCTCCTCT-3’  
R: 5’-GGTGTTGCTGGTGTCCTCCAGGATG-3’ | [161]                     |
| IFN-β   | F: 5’-GCCTCCAGCTCTCTCAGAATACG-3’  
R: 5’-CTGGATCTCTGGTGAGGAGCTGT-3’ | [162]                     |
| IL-2    | F: 5’-TGC AGT GTT ACC TGG GAG AAG TGG T-3’  
R: 5’-ACT TCC GGT GTG ATT TAG ACC CGT-3’ | NM_204153.1               |
| IL-1β   | F: 5’-GTGAGGGCTCAATGGCGTCTGTA-3’  
R: 5’-TGTCAGGCGGTAGAAGATGAAG-3’ | [180]                     |
| MDA-5   | F: 5’-GCAAAACCAGCACTGAATGGG-3’  
R: 5’-CGTAAATGCGTTCCACTAACGG-3’ | [161]                     |
| MyD88   | F: 5’-AGCGTGGAGGAGGACTGCAAGAAG-3’  
R: 5’-CCGATCAAACACACACACGCCTCAG-3’ | [182]                     |
| OAS     | F: 5’-AGAACTGCGAAGAAGACTTTTCGT-3’  
R: 5’-GCTTCAACATCTCTCTGTACC-3’ | [181]                     |
| PKR     | F: 5’-TGTCAGGCGGTAGAAGATGAAG-3’  
R: 5’-GAGCACATCCGCAGGTAAGGAG-3’ | [181]                     |
| Mx1     | F: 5’-GGACTTCTGCAACAGAATTG-3’  
F: 5’-TCCCACAAGATTCATCGTACG-3’ | [180]                     |
| IFIT5   | F: 5’-CAGAATTATGCGGCGCTATGC-3’  
R: 5’-TGCAAGTAAAGCCAAAAAGATAAGTGT-3’ | [180]                     |
| IRF7    | F: 5’-CTCCCTCTCTCCAAAAAGCTG-3’  
R: 5’-CTGCGAGGCGGAAGGAGGAAATG-3’ | [180]                     |
<p>| TRIF    | F: 5’-GCTGACCAAGAACTTCTGTGC-3’ | [183]                     |</p>
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<th>Primer Information</th>
<th>Literature</th>
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<td>[184]</td>
</tr>
<tr>
<td>pp38</td>
<td></td>
<td>F: 5'-AAGGGTGATGGGAAGCGATAG-3’&lt;br&gt;R: 5'-GCATAACGACCTTCGTCAAGATG-3’</td>
<td>[184]</td>
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CHAPTER 4

GENERAL DISCUSSION

MD is a viral disease of chickens caused by an alphaherpesvirus [22], [23]. Neoplastic T cell lymphomas leading to visceral organ tumors, immune suppression, neurological disorders, transient paralysis, and brain damage are signs of MDV infection. MDV infects chickens within a few days after hatch. Production losses due to this disease have necessitated the rapid development of vaccines against this virus [9], [24]. Marek’s disease is being partially controlled by vaccination. Although current MD vaccines partially protect against tumorigenesis and death and can reduce economic consequences, they do not prevent virus replication and shedding from infected chickens. After vaccination, continuous virus shedding still poses a risk to non-vaccinated chickens, which might lead to the emergence of more virulent pathotypes [11]. Hence, the design and development of alternative vaccines for MD are required to address the limitations of the current MDV vaccines.

In recent years, mRNA-based vaccines have been shown to induce immune responses and immunity against viral pathogens. Different animal models have been used to study the efficacy of mRNA vaccines [12]–[14]. In mice, a trivalent mRNA vaccine encoding the ectodomain of gC2, gD2, and gE2 of herpes simplex virus 2 (HSV-2) was shown to induce potent CD4+ T-follicular helper cell and germinal center B cell responses [16]. An mRNA vaccine against surface glycoproteins of HSV also showed 80 to 100% reduction in virus vaginal shedding [17]. Comparing three types of vaccines, including an mRNA vaccine expressing full-length glycoprotein (gB) against human cytomegalovirus in rabbits, showed superior durability of the mRNA vaccine-induced antibody response compared to two gB subunit vaccines [15].
In the present study, the objective was to explore the utility of mRNA vaccines for conferring immunity against MD and assess whether these vaccines can reduce virus shedding from infected birds. In addition, the necessity of boosting the mRNA vaccine was assessed. The mRNA vaccine designed in the present study encoded glycoprotein B (gB) and phosphoprotein 38 (pp38) proteins of MDV. The ability of gB and pp38 antigens of MDV to induce cell-mediated immune responses against MDV has been characterized previously, albeit using a traditional vaccine technology [57], [60].

Two challenge trials were carried out to evaluate and compare the efficacy of different doses of an MDV mRNA vaccine (chapter 2). Trial I consisted of two groups (prime only and prime-boosted). In the prime group, chicks only received one dose of the mRNA vaccine (5 μg), while in the booster group, chicks were boosted with an identical dose after 14 days. The first trial showed decreased tumor incidence and average lesion scores in the chickens that received the booster dose. In the second trial, the vaccine dose was increased to 10 μg. In addition, two more control groups were added to the study to compare the effect of the booster dose in chickens of the same age (to exclude the possible effects of 14 days age difference). Boosting chickens with the second dose of the vaccine (10 μg) 14 days after the first immunization significantly decreased tumor incidence, average lesion scores, and MDV load in feather tips at 21 days post-infection (dpi) when compared to the group that was unimmunized but challenged with RB1B MDV. Changes in cytokine expression at four, 10, and 21 dpi suggested a possible role of type I and II interferons (IFN) in initiating immunity in vaccinated chickens.

The second trial demonstrated a higher expression of IFN-γ and IL-10 in vaccinated groups. An increase in co-expression of IL-10 and IFN-γ is shown to correlate with a stronger anti-tumor response in humans. IL-10 can upregulate IFN-γ and granzyme secretion from human tumor-
infiltrating CD8+ T cells [155]. In chickens, co-stimulation with CpG-ODN and polyI:C (TLR ligands) synergistically increases the expression of IFN-γ and IL-10 and leads to a more robust Th1-biased immune response in monocytes [156]. In our study, the elevated expression of IL-10 in the mRNA groups of the second trial could be a response to the higher dose of exogenous mRNA [149] that led to the elevation of IFN-γ expression.

Subsequently, we sought to determine the importance of the booster dose following the initial mRNA vaccine inoculation. 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 any 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 due to 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 immune responses following single-dose and double-dose vaccination should be conducted.

After observing decreased tumor incidence, lesion scores, and lower MDV genome load in mRNA-vaccinated groups, we investigated changes in the key cytokines and ISGs involved in antiviral response to MDV. The goal was to elucidate possible mechanisms contributing to vaccine-induced host response following administering low and high doses of messenger-RNA vaccines, 5 µg and 10 µg, respectively. Expression of type I and II interferons (IFN), a panel of interferon-stimulated genes (ISGs), and two cytokines that are the key players in antiviral responses were measured in the spleen, lungs, and muscle within the first 36 hours of the
vaccination. This study revealed a significant increase in the expression of MDA5, Myd88, IFN-α, IFN-β, IFN-γ, IRF7, OAS, PKR, IFIT5, Mx1, IL-2 in both spleen and lungs within the first 36 hours of immunization. Booster injection led to a significant increase in expression of all genes in the lung. In contrast, only five genes (IFN-γ, MDA5, MyD88, Mx1, and OAS) showed significant upregulation in the spleen after the booster injection. Differences in gene expression between low- and high-dose groups following vaccination were detected as significant only for a few genes (IFN-α, IFN-γ, OAS, PKR, IFIT5, MDA5 in lungs, and IFN-α, Mx1, PKR, IFIT5, IL-2 in spleen). Different organs showed different gene expression patterns after the second dose, perhaps because of each organ’s diverse cell composition after the prime injection. Migration of leukocyte subsets from the spleen to other organs (such as the lungs and gastrointestinal tract) might have affected the transcriptional patterns of gene expression in the spleen and lungs. Aside from this, mRNA cellular uptake might have differed in the spleen and lungs following the second injection. The cellular uptake of mRNA is affected by the number of extracellular proteins such as ApoE [177]. Although the liver is the primary organ which synthesizes ApoE, it can be secreted by alveolar macrophages in the lung [178]. Alveolar macrophages are called free avian respiratory macrophages (FARM) in birds. Although, the number of FARMs are much lower than number of alveolar macrophages in mammals (mice showed 20 times more alveolar macrophages than chickens) [179], activated alveolar macrophages in the lungs following the first injection might have contributed to the higher ApoE secretion and, consequently, higher LNPs uptake in the lungs upon booster administration. Studies in mammals have characterized the pathways involved in ApoE secretion by bronchoalveolar macrophages, which increased the secretion of IL-1B [178]. In our study, a higher B expression in the high-dose group after booster injection might have happened due to increased ApoE secretion by bronchoalveolar macrophages. Further studies are
needed to characterize the alveolar macrophage subsets phenotypically and functionally in our studies.

The *in vivo* stability of synthesized mRNA was also assessed at four-time points post-IM injection (6, 18, 24, and 36 hpi). At each time-point, the amount of mRNA molecules decreased by approximately 3.5 and 2.5 logs for gB and pp38 molecules, respectively. Although both mRNAs were constructed using the same protocol, the size of molecules, and nucleotide composition might have impacted the speed of degradation *in vivo*. Higher G:C content of the mRNA molecule can increase mRNA stability and its half-life inside the cell [166]. The G:C content of the mRNA encoding gB and pp38 proteins in this study was 42.7% and 51.9%, respectively. This may explain the slower degradation pace of pp38 compared to the gB at the injection site.

There were some limitations in different aspects of our experiments. For instance, the natural route of infection for MDV is respiratory, while we challenged the chicks through the intra-abdominal route. The reason why intra-abdominal injection was used for the viral challenge was to ensure that an equal and precise amount of virus (250 PFU) was delivered to each chicken. An aerosol-based MDV infection model [185] can partially address this shortcoming, but still, the amount of virus delivered cannot be assured.

Also, the cytokines involved in the immune response were only assessed at the transcript level. This was done mainly due to the limited availability and specificity of reagents/antibodies for chicken experiments. Investigating antigen-specific T cell-mediated response could further help to analyze the vaccine-induced immune response. Re-stimulating splenocytes with gB and pp38 synthetic peptides and assessing the expansion of effector T cells could elucidate the
underlying mechanisms and cells involved in reducing MDV genome load, lesion scores, and tumor incidence in vaccinated groups.

Gross pathology was used to evaluate the effects of the vaccine on reducing tumor incidence and lesion scores. We could evaluate tumor formation by scoring them through histopathology.

To assess prevention of infection or inhibition of virus shedding from feathers, MDV genome load (meq gene) was measured in FFE. An alternative way to measure the viral load in FFE or skin is histochemistry staining and comparing the tissues obtained from different vaccine/challenge groups. In addition, virus titration in chicken kidney cells infected with equal dilutions of skin lysate from different vaccine/challenge groups could have enabled us to compare virus shedding from FFE.

Studies have shown higher translation efficiency in mRNA vaccines built with modified nucleotides. The nucleotides used in our vaccines were unmodified. Replacing uridine with pseudouridine during the \textit{in vitro} RNA transcription might enhance the translation efficiency of the mRNA construct, increase its resistance to degradation, and consequently improve antigen presentation and the magnitude of vaccine-induced immune responses. Functional \textit{in vitro}-synthesized mRNA is transcribed from a plasmid DNA (pDNA). This pDNA is eliminated during the DNase I digestion step. In addition to pDNA, the sample might include shorter transcripts due to premature termination throughout the elongation step. mRNA vaccines can be further purified by adding a chromatographic purification step to remove all unwanted transcripts [91]. In this study, any type of chromatography, such as size exclusion chromatography (SEC) or ion pair reverse-phase chromatography (IPC) were not applied to eliminate shorter or longer RNA
transcripts. Adding this step to the mRNA purification process may increase protein expression and enhance antigen presentation.

In conclusion, our results showed that two immunizations with 10 μg of mRNA vaccine encoding gB and pp38 antigens could lower or postpone tumor incidence and lesion scores 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. Potential future steps for this research work can be focused on two main areas, vaccine preparation steps and assessment of antigen-specific T cell responses. Modifying mRNA constructs to achieve maximum in vivo stability and exploring antigen-specific T cell responses would be the next steps for this work.
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