EFFECTS OF MEQ MUTATIONS ON MAREK'S DISEASE VIRUS (MDV) PATHOGENECITY AND ONCOGENICITY

by

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ABSTRACT

Marek's disease (MD) is a highly transmissible lymphoproliferative disease of chickens caused by an alphaherpesvirus, Marek's disease virus-1 (MDV-1). MD remains a constant problem to poultry production worldwide, due to the cost of vaccination and the continuous evolution of more virulent field strains. Upon analyzing MDV-1 strains of distinct pathotypes, we, and others, have found that the major oncoprotein (Meq) of MDV-1 strains has specific coding mutations that correlate with virulence level. We, and others, have previously reported differences in the transcriptional activation potential and the cellular binding profiles of the different Meq isoforms. We therefore hypothesized that these mutations could directly affect the virulence of MDV-1 strains. Using RB-1B as a well characterized genetic backbone, we constructed recombinant MDVs that harbor the *meq* genes of the vaccine strain CVI988 (399 aa form), and the very virulent plus N strain (339 aa, vv+ form). Contrary to our hypothesis, pathogenicity studies in SPF chickens revealed that there was no effect of the replacement of the *meq* genes on MD, mortality or tumor incidence. These data therefore suggest that in unvaccinated, maternal antibody-free chickens, changes in the Meq coding sequence conferred no inherent pathogenicity increase (or decrease) for the mutations examined. Experimental validation via Southern blotting showed that both copies of *meq* were restored in all the recombinant viruses upon replication in CEF *in vitro*, SPC *in vivo* or in established cell lines. Similarly, PCR and sequencing of the *meq* loci and sequencing of *meq* loci ensured

that viruses had not undergone significant mutations (duplications, deletions, etc.) during propagation in cell culture or *in vivo,* or had not been mixed during the study.

A follow up hypothesis we formulated based on the aforementioned observations was that the mutations in Meq have occurred upon selection of viruses that have the ability to overcome the innate immune responses and signaling elicited from vaccination. Vaccines are presumed to be the main drivers of MDV evolution towards higher virulence. Since, the vaccines do not elicit sterilizing immunity, but only prevent MDV-induced lymphoma formation, this provides an opportunity to the virus to continually evolve in the presence of selective pressure exerted by vaccinal immunity on the full length Meq form. To address this hypothesis, we simulated innate immune activation analogous to early innate immune responses post-vaccination, using various innate immune agonists (LPS, Poly I:C, cGAMP). Replication of the recombinant viruses in CEF showed that cGAMP treatment (inducer of STING and type I interferon expression) caused a decrease in plaque number and plaque area size for RB-1B CVI Meq, but did not affect the RB-1B parent or RB-1B N Meq viruses, suggesting that Meq from mildly virulent MDV CVI988 lacks the ability to overcome cGAS-STING anti-viral signaling. On the other hand, vv and vv+ Meq forms displayed an ability to overcome cGAS-STING anti-viral signaling. Plaque number and plaque area analysis for the RB-1B N *meq* recombinant showed that its replication was least affected by any of the agonist treatments in both CEF and spleen cells (SPC). Thus, the N strain-*meq*, seems to have overcome innate signaling triggered via either of TLR3, TLR4 or cGAS-STING pathways. Agonist-treated, virus-infected SPC co-cultured with CEF *ex vivo*, showed a profound reduction in plaque number in the order: RB-1B CVI *meq* virus (~30% for each

treatment) > RB-1B parent virus (~10% for each treatment) > RB-1B N *meq* virus (~4% for each treatment).

Our work therefore suggests that mutations in the Meq oncoprotein are not directly responsible for the observed differences in MDV virulence levels, but that these Meq mutations have likely been selected indirectly based upon the pressure exerted by the innate immune responses to vaccines and it is through this evasion that they have a role in the increased MDV virulence.

Chapter 1

INTRODUCTION

1.1 Marek's Disease (MD)

First described in 1907 as "polyneuritis" by Jozsef Marek (81) owing to its inflammation of major nerves in laying chickens, Marek's disease was named after Jozsef Marek over half a century later. Pappenheimer and colleagues showed that the causative agent of the polyneuritis described by Marek also induced lymphoid tumors in addition to lymphoid infiltration of peripheral nerves (93, 94), suggesting the term *Neurolymphomatosis gallinarum* for the disease. Changes in Marek's disease (MD) since these early studies established MD as a lymphoproliferative disease mainly of domestic chicken (*Gallus domesticus*) affecting peripheral nerves, and inducing lymphomas that lodge in the iris, gonads, spleen, heart, lung, liver and muscle.

The causative agents of MD, in terms of paralysis and lymphomas, are viruses in the recently described genus, *Mardivirus 1* (for **Mar**ek's **d**isease **virus 1, MDV-1**). MDV strains had previously been classified into three serotypes, based on common and distinct antigen expression, and these included: *Gallid herpesvirus 2* or MDV-1 which is composed of oncogenic strains and their attenuated derivatives isolated from chickens, *Gallid herpesvirus 3* or MDV-2 is composed of non-oncogenic strains isolated from chickens and *Meleagrid herpesvirus-1* or MDV-3, commonly known as herpes virus of turkeys (HVT) which is composed of non-oncogenic and apathogenic strains initially isolated from turkeys. MDV-2 (strain SB-1) and MDV-3 (strain HVT)

have been used extensively as vaccines to prevent tumors induced by MDV-1 strains (91).

1.2 Genomes of MDVs

All MDV genomes are a linear double stranded DNAs consisting of unique long (U_L) and unique short (U_S) regions flanked by inverted internal and terminal repeats (IR_L/TR_L and IR_S/TR_S) (144). The MDV U_L and U_s regions encode genes homologous to other alphaherpesviruses such as HSV-1 and VZV, while MDV-1, MDV-2 and HVT differ primarily in the repeat regions flanking the U_L region (91). The repeat-long regions of MDV-1 strains encode genes implicated in the pathogenicity and oncogenecity, such as *meq* (**M**arek's **E**coRI-**Q** encoded protein) (53), *vIL8* (75, 99), *pp38* (phosphoprotein 38 complex) (26) and *vTR* (a virus encoded telomerase RNA homolog) (Figure 1.) (35). These genes are unique to MDV-1 strains and have been the focus of our laboratory, due to their implication in pathogenicity and oncogenicity.

1.3 MDV pathogenesis

Calnek and colleagues described what they termed as "the Cornell model of MDV pathogenesis" that defines four phases of MDV infection: early cytolytic replication, latency, secondary cytolytic replication, and transformation (21). The early cytolytic phase occurs from 2-7days post-infection (dpi) in chickens infected at 3 weeks of age, although this occurs for 7-14 days in chickens infected at hatch (95). Following early cytolytic infection, the host mounts an innate response that drives the establishment of latent infection by 7-10 dpi in older chickens, by 21 days in birds infected at hatch. As latently-infected CD4+ T-cells reactivate virus at peripheral sites,

a secondary cytolytic infection occurs from 18 dpi in birds infected at 3 weeks of age and from 28 days onward in birds infected at hatch. From 21-42 days, some CD4+ T-cells become transformed and lymphomas begin to appear (20, 21). The timing of the phases varies depending upon various host factors (genetic susceptibility, vaccination status, age of infection), as well as the virulence level of the challenge strain. Although these phases are recognized, they may overlap without a clear demarcation between the phases (i.e., secondary cytolytic and transformation phases).

i) **Early cytolytic infection:** Chickens become infected via horizontal transmission through the inhalation of infectious dander, resulting in the uptake of the virus by B-cells, macrophages and dendritic cells that are recruited to the respiratory tract and lungs. Infected B-cells and macrophages transmit MDV to the primary and secondary lymphoid organs (bursa of Fabricius, thymus and spleen) (7, 10). B-cells are the primary site of MDV replication. T-cells (primarily CD4+ T-cells) become activated and infected at this time, as well (8, 17, 22). As a result, semi-productive infection occurs in lymphocytes leading to production of non-enveloped intra-nuclear particles, and enveloped but not extracellular virus particles. B-cells undergo cytolytic infection and T-cells (CD4+ and CD8+) initially undergo apoptosis leading to early immunosuppression (84, 143). Host innate immune responses during this early lytic phase is mediated by macrophages and dendritic cells, with subsequent production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8), type I and type II interferons (27, 49), and soluble factors like nitric oxide (155). As these factors block stages of productive infection, the virus shifts to latency in activated CD4+ T-cells.

ii) **Latency**: MDV latent infection is characterized by integration of the viral genome into host chromosomes, primarily at telomeric sequences, providing persistence of the viral genome without production of infectious virions. CD4+ T cells are the primary target for latency (23, 68), although cases of B-cell and CD8+ T-cell latency have been reported (121). Rapid proliferation and dissemination of latently infected CD4+ T cells leads to a cell associated viremia in peripheral blood lymphocytes and disseminates the virus to various tissues, including the feather follicle epithelium (FFE).

iii) **Second cytolytic infection:** As the innate immune response to the primary lytic infection declines, latently-infected T-cells reactivate virus to infect epithelia of various visceral organs and nerves (Schwann cells, kidneys, adrenal glands, gonads, proventriculus) and the FFE. Among these tissues, complete productive replication occurs only in the FFE, resulting in the production and shed of cell free virus associated with the dander (21). This secondary cytolytic infection of various tissues, causes mononuclear cell and heterophil infiltration accompanied by inflammation. Inflammatory lesions in the nerves, particularly the sciatic plexus, results in the characteristic unilateral paralysis associated with MDV infection.

iv) **Transformation:** Latently-infected CD4+ T-cells are the primary target of MDV-mediated transformation, although not all latently-infected T-cells become transformed (119). MDV-mediated lymphomas are primarily

monoclonal in origin, although in some chickens, tumors have been found to be oligoclonal (23). Transformed CD4+ T-cells proliferate in different organs to give rise to frank lymphomas. Transformed T cells have been found primarily to consist of CD4+, MHC I^{hi}, MHC II^{hi}, IL-2R α+ (CD25), $CD28^{lo/-}$, $CD30^{hi}$, pp38-, gB-, αβ TCR+ cells (16, 17, 87). Moreover, 10-20 copies of the MDV genome are found integrated at multiple (but not consistent) sites in host chromosomes, primarily at telomeric repeat regions (28, 82, 113). MDV-transformed T cells possess a T-reg like immunophenotype (126). MDV lymphomas overexpress the CD30 antigen which promotes cell survival and induces a T-helper 2(T_{H2}) or T-regulatory (Treg-like) phenotypes (126). Lymphomas compromise the functions of the organs leading to multiple organ failure and death. Moreover, during the transformation phase, lymphomas express factors that induce a profound and permanent immune suppression. Consequently, tumor-bearing chickens often have secondary bacterial and viral infections.

1.4 The Meq Oncogene

MDV encoded genes, such as *meq* (53), viral IL-8 (vIL8) (75, 97), phosphoprotein 38 (pp38) (26) and the viral telomerase RNA (*vTR*) contribute to transformation and pathogenicity. Among these genes *meq* is the primary oncogene, as its deletion or mutation of a particular domain ablates oncogenicity (13, 78). Meq is encoded only in MDV-1 strains, is absent from the non-oncogenic strains MDV-2 and HVT, and is consistently expressed in MDV-1-induced lymphomas and tumour-derived cell lines (112). Previous studies from our and other laboratories and others have shown that mutations in the *meq* coding sequence correlate with MDV pathotype

(or virulence level) (128). Also, recent molecular evolution analysis has revealed that the *meq* gene has evolved adaptively under positive selection pressure and the evolution is believed to be very rapid, being comparable with the evolution rate of RNA viruses (92).

The role of *meq* in oncogenicity was demonstrated using a *meq* deletion mutant, generated in using very virulent (vv) strains of MDV, rMd5 and RB-1B (31, 78) in *in vivo* studies. Similarly, *in vitro* studies on Meq expressing Rat-2 (rodent fibroblast) and DF-1 (immortalized chicken embryo fibroblast)-cell lines showed morphological transformation, serum-independent growth, anchorage-independent growth, and an inhibition of apoptosis (76).

There are two copies of the *meq* gene located in the repeat regions flanking the unique long region (R_L) of MDV (144). The *meq* genes of vv and vv+MDV strains of MDV encode 339 amino acid basic leucine zipper (bZIP) Meq proteins. At the N-terminus, from amino acid positions 56-129, *meq* encodes basic and leucine zipper (bZIP) domains, a feature of *jun/fos* family oncogenes, followed by a C-terminus (positions 129-339) that encodes a proline rich activation domain, similar to WT-tumor suppressor protein (Figure 2.) (53). The CU-2 (a mildly-virulent strain of MDV), BC-1, and JM-16 (virulent strains), and CVI988 (a vaccine strain) encode larger forms of the Meq protein. These larger forms are 398-399 amino acids in length and contain five or more proline rich repeats (PRRs) in their C-terminus, whereas very virulent and very virulent plus MDVs (vv/vv+ MDVs) encode a smaller length Meq (339 aa) encoding three PRRs (69, 128).

Mutations in the PRR of Meq proteins correlate with the relative virulence level (pathotype) of the MDV strain encoding it, where the tandem proline repeat

sequence PPPP have second position mutations PPPP->P(Q or A)PP in the case of vv+ MDVs. Although a higher number of PRRs is associated with lower transcriptional potential, point mutations in PRRs in Meqs of higher virulent strains were found to have enhanced transcriptional potential (128).

By virtue of its bZIP domain, Meq forms homodimers with itself and heterodimers with cellular bZIP proteins such as c-Jun, ATF-1/2/3, CREB, Fra-2 (70, 72), and also Jun-B, Fos and NFIL3 in *in vitro* studies (65, 72, 107). As a heterodimer with c-Jun, Meq binds to AP-1 and AP-1 like DNA motifs, primarily in the promoter regions of target genes (104). Meq also binds to cell cycle regulatory proteins CDK2, p53, Retinoblastoma (Rb), chaperone HSP70, and chromatin scaffold protein, the C-terminal binding protein 1 (CtBP-1) (13, 15, 60, 65, 77, 163). Interaction of Meq with these cellular proteins is via various domains and motifs. Meq binds to the C-terminal tetramerization domain of tumor suppressor p53 through its bZIP domain (15), causing decreased transcription, and thereby decreased cell cycle arrest and decreased apoptosis. Similarly, Meq interacts with the Rb protein, ostensibly via the LXCXE motif located at the end of bZIP domain (65), and to transcriptional repressor and chromatin remodeling enzyme scaffold protein CtBP via a PLDLS (pro-leu-asp-leu-ser) motifs in its amino terminus. Mutation of this PLDLS to the similar sequence AVEFT abrogated tumor formation *in vivo*, indicating that this CtBP-interaction is essential for transformation.

Meq is primarily nuclear and localizes in the nucleoplasm, nucleolus, and Cajal or coiled bodies (6, 74). Nuclear and nucleolar localization of Meq is related to two basic regions present in the amino terminus of Meq designated as basic region-1 (BR-1) and basic region-2 (BR-2) which are rich in arginine and lysine amino acids. BR-1

(aa 30-35) acts as nuclear localization signal, whereas BR-2 (aa 62-78) acts as both a nuclear and nucleolar localization signal.

In Meq-expressing DF-1 cells, Meq heterodimerized with and co-localized with c-Jun, resulting in upregulated expression of anti-apoptotic genes like JTAP, JAC, Bcl-2 and HB-EGF and downregulated expression of pro-apoptotic genes Fas and DAP5 (70). Moreover, when either of Meq or c-Jun were knocked down via RNA-interference in these cells, the expression of JAC, JTAP-1 and HB-EGF was downregulated (70). These data suggest a role for Meq in combination with c-Jun in the transformation of chicken cells, at least in *in vitro*.

In the case of chicken lymphoblastoid cell line derived from the BC-1 strain-induced spleen lymphoma called MSB-1 (89), Meq-c-Jun heterodimers bind to AP-1 motifs (DNA sequences called MERE-I for Meq response elements I), whereas Meq-Meq homodimers bind to ACACA motifs (called MERE-II elements) (103).

AP-1 like motifs (MERE-I) are present in the *meq* promoter, while ACACAs motifs (MERE-II) are present at the MDV origin of replication (Ori) which functions as a bidirectional promoter for pp38, pp24 and pp14 genes. Interestingly, binding of Meq-c-Jun heterodimers to AP-1 leads to transcriptional activation, and binding of Meq-Meq homodimers to ACACAs leads primarily to transcriptional repression, suggesting a regulatory role of Meq in controlling the latency and MDV genome replication (72, 104, 145). Furthermore, AP-1 motifs, which are also the components of the IL-2 promoter, can recruit Meq-Jun heterodimers, suggesting a role of the heterodimerization in T-cell proliferation (24, 72).

Transcription of the *meq* locus not only results in an unspliced Meq gene product, but also at least two other splice variants, Meq/vIL8 and Meq/vIL8Δexon 3

(6, 31, 96, 99, 100). These splice variant-derived proteins have been detected in MDV-transformed cell lines, tumors and during latency and transformation phases of MDV infection in chickens, suggesting a putative role in tumorigenesis (31, 99). The full length Meq protein is expressed throughout the course of infection, but the splice variants are only expressed during latent and transforming infection phases (96).

1.5 Evolution of Virulence

MD was first described as a disease of central and peripheral nerve inflammation ((38, 81), which by the late 1920's was found also to be associated with visceral lymphomas along with neurological signs (93). With the advent of large scale poultry production during the 1950's, MD became primarily associated with neoplastic disease, causing mortality up to 30% (11, 38). Introduction of HPRS-16att (attenuated form of HPRS-16, an MDV-1 strain) in 1969 was the first vaccine against MD (against any cancer causing viral disease) and led to control of the disease (90). In 1970, HVT (FC-126 strain) was introduced as a vaccine against virulent strains of MDV (90, 102, 150) and became the most successful vaccine in decreasing the losses from MD by up to 99% (88).

More virulent strains of MDV, such as Md5 and RB-1B, emerged in the early 1980's, and these overcame the protective effects of HVT, and caused disease even in genetically resistant chickens (151). This need for a better vaccine led to the introduction of a bivalent vaccine composed of HVT and SB-1 (an MDV-2). MDV field strains of greater virulence evolved in the early 1990's, causing losses in bivalent-vaccinated chickens (121). Consequently, an attenuated MDV-1 strain called CVI988 (Rispens strain), already in use in the Netherlands since 1972, was licensed

for use in the US, and has been in use since then, often in combination with HVT (109, 110).

A major limitation of MDV vaccines is their inability to offer sterilizing immunity. As a result, vaccinated chickens are able to shed oncogenic field strains into the environment. Therefore, MD vaccines could actually be driving the evolution of MDV by selecting field strains that are fit for replication and growth in immunized chickens (39, 88).

1.6 MDV Pathotypes

Witter and colleagues have classified MDV strains based on the criteria to induce lesions in vaccinated birds as (149):

i) Classical or mild MDVs (mMDVs): classical MDV strains isolated before the early 1960s, like HPRS-41 and CU-2.

ii) Virulent MDVs (vMDVs): strains isolated in the early 1960s to the mid-1970s, like JM-16 and GA.

iii) Very virulent MDVs(vvMDV)s: strains isolated in the late 1970s and early 1980's from HVT- vaccinated flocks, like Md5 and RB1B.

iv) Very virulent plus MDVs (vv+MDVs): strains isolated in the early to mid-1990s, which induced lesions in bivalently-vaccinated chickens. These cause high levels of early cytolytic infection, early mortality, stunting, and increased neurological signs (39, 88).

1.7 Innate Immune Responses to MDV Infection

MDV infects chickens and elicits both innate and acquired immune responses are elicited. In the early cytolytic phase, virus replicates in B- and T- lymphocytes and

establishes latency due to host pro-inflammatory and interferon responses. MDV early lytic replication serves to activate T lymphocytes, making them more permissive to infection by MDV. Viral interleukin 8 (vIL8) is thought to act as a chemo-attractant cytokine to attract activated T-cells towards infected B-cells and cause infection of these activated T-cells (122) . Certain immune responses are elicited after infection that mainly involve cytokines and soluble mediators (iNOS, interferon- γ), macrophages and natural killer (NK) cells.

Innate immune responses during lytic infection of B- and T- cells, as well as establishment of latency and transformation of CD4+ T-cells, involve deregulated expression of interleukins, interferons and soluble mediators (120). Studies with recombinant chicken interferons (rChIFN-α and rChIFN-γ) have shown that both affect MDV replication (156). Similarly, production of IFNs has been supported by various studies after infection of chickens with MDV, in different tissues such as lung (129), brain and serum (54). Upregulation of IFN-γ and increased NK cell activity are found to be the first immune responses to MDV infection. It was proposed that the early upregulation of IFN-γ plays a vital role during early immune response to MDV infection (122). IFN-γ stimulates the expression of IL-8 receptors on T-cells (160) that leads to attraction of activated T-cells to virus infected B-cells via production of vIL8. Besides, IFN-γ also stimulates production of IL-1β and inducible nitric oxide (iNOS) by avian macrophages (158). IL-1β can also upregulate expression of IL-2, which is an essential cytokine for proliferation of T-cells (9).

A delayed onset of latency due to MDV was observed during the infection of one-day old chickens compared to older chickens. Since, the one-day old chickens are immunologically immature, it was suggested that immune responses should be vital

for the establishment of latency (19). Two soluble factors were found in conditioned medium (CM) from concanavalin (Con)-A stimulated spleen cells that could maintain MDV latency in spleen cell culture from MDV infected chickens between 8 and 25 dpi (18). These factors were latency maintaining factor (LMF) and IFN-γ. However, the mechanisms for latency maintenance in the presence of CM and IFNs were not determined. Since, the addition of rChIFN-α and rChIFN-γ to cell cultures can stimulate the production of NO by macrophages (158), NO may have a role in controlling MDV latency in infected cells.

Besides cytokines and soluble factors, the other components of the innate immune response include the macrophages and NK cells which are the main effector cells of the innate immunity.

The importance of macrophages in the MD immunity has been highlighted by several in vitro and in vivo studies. Macrophages have phagocytic, antimicrobial and tumoricidal activities, and serve as antigen presenting cells, connecting the innate and adaptive immune responses (106). Macrophages have been linked to transport MDV from lungs to lymphoid organs (21), including the bursa of Fabricius (10). The role of macrophages in virus distribution was also supported from the report that macrophages were infiltrated in the bursa of Fabricius during early stages of infection (3). *In vitro* studies showed that macrophages are refractory to infection with MDV, as no productive replication was observed even though the virus was internalized (41, 147). However, *in vivo* studies in MDV-infected chickens showed cytolytic infection of macrophages at 4-6 dpi (62). Macrophages isolated from MDV-infected birds inhibit MDV replication in DEFs and CKCs (61, 67), and inhibition of macrophage activity increased viral titre and tumor incidence *in vivo* (41, 62). This inhibitory effect

is mediated by by nitric oxide (NO) (30, 156), which is produced by the enzymatic action of inducible nitric oxide synthase (iNOS). Upregulated expression of iNOS during MDV infections has been observed in both *in vitro* and *in vivo* studies (1, 2, 49, 155).

NK cells induce rapid cell death in the virus-infected cells as well as tumor cell via a serine protease (granzyme) and a pore forming protein (perforin) (133). NK cells are functionally and morphologically defined in chickens, but not fully characterized yet. Several studies have suggested the importance of NK cells in MD immunity. MDV vaccinated or infected chickens showed increased NK cell like activity (37, 44, 130), while the genetically resistant chicken lines showed early and persistent NK cell activity compared to those from a susceptible line (37). Similarly, *in vitro* studies in lymphoblastoid cell lines showed NK cell-mediated cytotoxicity (105). In addition, mRNA expression levels of perforin, granzyme-A, and NK-lysin genes were found to be upregulated at 4 and 7 dpi in infected birds compared to control (116).

1.8 Innate Signaling during MDV Infection

Innate immune receptors such as TLRs, RLRs and cytoplasmic sensors detect pathogen associated molecular patterns (PAMPs) such as bacterial and fungal cell wall constituents, double stranded (ds) RNA or DNA, single stranded, uncapped (ss) RNA, and unmethylated CpG DNA and activate intracellular signaling pathways that are responsible for production of pro-inflammatory cytokines and typeI interferons (IFNs). TLRs – 1, 2, 3, 4, 5, 7, 9, 15 and 21 have been identified and characterized in chickens (12, 14, 36, 52, 63). How MDV DNA and other viral PAMPs are sensed in the chicken has not been identified, as yet. In mammals, antiviral responses primarily involve TLRs 3, 7, 8 and 9, RLRs RIG-I, MDA5, LGP2, and dsDNA sensors IFNγ-

inducible protein 16 (IFI16), dead box protein 41 (DDX41), DNA-activator of interferon (DAI), and leucine-rich repeat Flightless-interacting protein 1 (LRRFIP1). Of these, homologs to TLR 3, 7, 21, RLRs MDA5 and LGP2, and dsDNA sensors DDX41 and LRRFIP1 have been identified in chickens.

In chickens, antiviral response has been found to occur through activation of interferon regulatory factors (IRFs) and induced expression of Type I IFNs occurs via detection of viral nucleic acids by TLRs -3, 7, and 21 (42, 63) and ds DNA sensors. TLRs 3 and 7, and their downstream target genes IL-8 and IL-1β, were highly expressed in lung tissues of chicken infected with a vv+ MDV via the aerosol route (1). *In vitro* infection of CEFs with RB-1B and HVT also showed upregulation of IRF1 and IRF3/7 in micro-array analysis (55, 83). Similarly, chicken whole genome microarray analysis of infected and control birds of susceptible lines showed that several innate immune function genes were upregulated including TLR3 and TLR15, in both spleens and thymii of infected birds at both 3 and 4 dpi (132). Both *in vitro* and *in vivo* studies have shown reduced viral replication upon treatment with IFN- α (48, 71, 146).

Different agonists or ligands that are recognized by specific TLRs (Poly I:C by TLR3, LPS by TLR4, ssRNA by TLR7/8 and oligodinucleotides, ODN by TLR21) were found to induce type I IFN and IL-6 in freshly prepared chicken spleen cells (124). Chicken TLR3 ectopically expressed in human 293 cells strongly responded to Poly I:C suggesting that Poly I:C recognizes TLR3 in chickens similar to in mammal TLR3. Moreover, IFN-α and IFN-β readily induced expression of TLR3 (56).

1.8.1 LPS agonist and innate signaling

LPS (lipopolysaccharide) is a main bacterial surface glycolipid and is sensed by LPS-binding protein (LPB). LPB transfers LPS monomers to CD14 (a co-receptor to MD-2 and TLR4) and CD14 delivers the LPS to myeloid differentiation protein (MD-2). MD-2 ultimately transfers it to TLR4 (59), which triggers TLR4 signaling pathway to elicit anti-viral response in mammals. Antiviral responses due to LPS in avian species are rare. TLR4 signaling in chicken is unique compared to mammals (58). LPS activates chicken TLR4 and co-receptors to induce production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-18 and soluble mediator NO via iNOS activation (29, 33, 43). Besides, LPS also upregulates the expression of IFN-gamma via this pathway (117, 138). LPS, therefore, can stimulate immune responses leading to protection against bacteria and viruses. It was indeed reported that LPS (as TLR4 agonist) delayed the onset of disease in RB-1B-infected chickens and reduced MDV genome copy number in the infected spleens (98). Moreover, increased levels of IFN-γ and iNOS mRNA were observed between 3 to 15 dpi, and 6 to 15 dpi, respectively in MDV infected chickens (155). Furthermore, the inhibitory effects of IFN-γ and NO on MDV replication have been confirmed both *in vitro* and *in vivo* (156).

1.8.2 Poly I:C agonist and innate signaling

Poly I:C (poly-inosine – poly-cytosine) is a synthetic analog of dsRNA. dsRNA is produced by most viruses at some point during their replication (47) . In the case of DNA viruses, ds RNA appears to accumulates as a result of overlapping convergent transcription, such that viral transcripts fail to terminate at discrete sites at the ends of genes and thereby complementary mRNAs are produced from genes transcribed in opposing directions. Such complementary transcripts have been

detected in HSV (46, 148). Poly I:C as an dsRNA analog can follow different signaling depending on the location, extracellular or intracellular. The extracellular dsRNA (viral genome or genomic intermediate) produced from dead infected cells, gets endocytosed and detected by TLR3 on the endosomal membrane (5). In this case, TLR3 uses adaptor protein TRIF (TIR domain containing adaptor protein inducing IFN-β) that recruits protein kinase IKK to activate the transcription factor Nf-KB and protein kinase TBK1/IKK-ε to activate transcription factors IRF3 and IRF7 (157).

In addition, intracellular ds RNA produced by viruses replicating in the cytoplasm is recognized by cytosolic sensors such as ds RNA dependent protein kinases (PKR), as well as retinoic inducible gene-1 (RIG-I), and melanoma differentiation gene 5 (MDA5), and Laboratory of Genetics and Physiology Group 2 (LGP2), collectively known as RIG-like helicases (RLH) (159). Upon binding uncapped RNAs, RLHs deploy mitochondrial membrane bound adaptor protein, the mitochondrial antiviral signaling protein (MAVS), also known as IPS-1 or Cardiff, that recruits several members of TRAF family proteins, that ultimately activate the same protein kinases (TBK-1) and transcription factors as TLR3 (57, 125). Finally, the activated transcription factors drive the expression of type I interferon genes and interferon stimulated genes which are important for immune antiviral defense.

Rapid induction of IFN-α and IFN-β via TLR3 signaling has been observed in chickens, upon treatment with Poly I:C (56). Moreover, upregulation of melanoma differentiation associated gene-5 (MDA-5) after infection with MDV has been observed (34). Also, the same study found a differential expression of IRF-3/7 and IFN-β with resistant lines having higher expression compared to susceptible lines.

1.8.3 cGAMP agonist and innate signaling

c2'-3' Cyclic GMP-AMP (cGAMP) is a downstream molecule in cGAS-STING DNA sensing pathway that is generated due to conversion of ATP and GTP by cGAS (cyclic 5'-2' guanosine monophosphate, 5'-3' adenosine monophosphate synthase). cGAS is an innate DNA sensor which recognizes dsDNA or RNA: DNA hybrids in the cytoplasm (80, 139, 161), either directly or via interactions with DDX41, IFI16 or DAI. The generated cGAMP then binds to the Stimulator of IFN Genes (STING) which recruits and activates TANK-binding kinase (TBK-1) and IFN regulatory factor (IRF3/7) to induce the expression of type I IFNs and to a lesser extent, pro-inflammatory cytokines.

Two oncogenes of DNA tumor viruses, E7 of human papilloma virus and E1A of adenovirus, were reported to block cGAS-STING signaling pathway by binding to STING (66) . cGAS via cGAMP was reported to inhibit replication of DNA viruses, including HSV-1 (73, 123). Interestingly, functional and structural similarities are found between oligoadenylate synthase (OAS) proteins and cGAS (45). Both are nucleotidyl transferases that are activated by double-stranded nucleic acids in the cytosol and produce cyclic linked unique secondary messengers. OAS and cGAS act through distinct mechanisms as anti-viral proteins. While OAS activates enzyme RNAse L which degrades host and viral RNA, cGAMP activates downstream signaling pathway to induce type I IFN gene expression.

1.9 Hypothesis and Specific Objectives

1.9.1 Hypothesis

Previous work from our lab has shown that changes in the Meq coding sequence correlates to the virulence level of the strains of MDV (128). Generally, it

was found that the longer form of Meq is encoded in low or mild strains (as seen in CVI 988, BC-1, CU-2, JM-102, *etc.,*), whereas the shorter form is a feature of vMDV, vvMDV or vv+ MDVs. Moreover, the Meqs of vv or vv+MDVs contained targeted second position disruptions of proline rich repeats (PRRs) and these disruptions correlate with the virulence level of the strains. A change in the second position proline of the PRR to glutamine or alanine (PPPP -> PQPP or PPPP -> PAPP) was found in the case of vv+MDVs (as in the N strain), but either no change in this proline was observed for the vv strains (as in RB-1B), or only one of the tandem repeats was affected (as in Md5) (128).

In MDV field strains from China and Australia, larger forms of Meq were found to be encoded (398-399 aa), however these also had targeted second-site proline-> alanine/glutamine insertions, suggesting that these mutations more strongly correlate with virulence than the number of PRR alone (108, 162).

Based on these observations, we hypothesized that the mutations in Meq are instrumental to the virulence and pathogenicity of MDVs. In previous work, we sought to address this hypothesis, using recombinant MDVs selected *in vivo* (64). In this case, the Meq-coding regions from JM16 (a vMDV), RB-1B (a vvMDV) and the N strain (a vv+MDV) were co-transfected with Md5ΔMeq DNA into CEF, passaged once and used to infect chickens at hatch. After two such experiments, one stable recombinant virus (rMd5-1137) was isolated that had recombined the Meq region of RB-1B into both copies of rMd5ΔMeq-transfection-inoculated chickens, however no double-recombined N strain recombinants were isolated. These data suggested that the expression of Meq provided a selective advantage to the replication of MDV, but that

the mutations in the vv+MDV form did not provide a competitive advantage to the vvMDV (RB-1B) form.

In addition, we also hypothesized that the mutations in Meq may have been occurring based on the ability of the viruses to overcome the innate immune responses and signaling. One of the widely held hypotheses in the MD research community is that the vaccines are the main drivers of MDV evolution towards higher virulence. Attenuated oncogenic MDV-1 (CVI988 Rispens), non-oncogenic MDV-2 (SB-1), HVT or a combination of these vaccines can prevent MDV-induced lymphomas (109, 118, 150) and have largely been successful. However, MD vaccines only prevent tumor formation, but not pathogenic MDV-1 replication, providing an opportunity for the virus to continually evolve in the presence of selective pressure exerted by vaccinal immunity.

As chickens are vaccinated *in ovo* (HVT, HVT/SB1) or at hatch (CVI988), and then placed in a contaminated environment where they are immediately challenged, there is not time for the newly-hatched chick to develop an adaptive immune response to the vaccine. Therefore, a main driver may be the exposure of challenge viruses to the innate responses elicited by MD vaccines, as opposed to CTL or antibody responses. Consequently, we hypothesize that the ability of vv+ MDVs to overcome the vaccinal immunity may depend upon the ability of vv+ MDV Meq to somehow affect innate sensing or signaling. In other words, Meq mutations may be responsible for altering and modulating the vaccine induced innate immune activation.

1.9.2 Specific Objectives

i. To construct and characterize recombinant MDVs (mutants) having representative Meq isoforms inserted into the backbone of the pRB-1B-BAC (a very virulent strain of MDV).

ii. To determine if these changes in Meq coding sequence elicited changes in pathogenicity.

iii. To determine if these changes in Meq coding sequence elicit changes in immune evasion.

Chapter 2

MATERIALS AND METHODS

2.1 Cells and Viruses

2.1.1 Cells

Secondary chicken embryo fibroblasts (CEF) were prepared from 10-day-old specific pathogen free (SPF) embryos (Sunrise Farms, Inc. Catskill, NY) for propagation of the viruses. Secondary CEF were propagated in M199 medium supplemented with 3% filtered calf serum, L-Glutamine and antibiotics (all reagents from Gibco, Carlsbad, CA) and maintained at 37°C in 5% CO_2 humidified chamber.

2.1.2 Construction of BAC viruses

Recombinant MDVs (specifically CVI988 and MK/N meq-containing pRB1B) were constructed in Dr. Benedikt Kaufer's laboratory using a two-step, Red-mediated recombination technology (143). Using RB-1B as a well characterized genetic backbone, the different forms of *meq* were inserted to provide a method to assess the effects of meq mutations on the pathogenicity and virulence. CVI988 (399 aa form of Meq)- and MK (N strain, 339 aa form of Meq)- expressing mutants were constructed using RB-1BΔIRL, a BAC-based virus having one copy of the repeats flanking the unique long deleted (32) (Figure 3.). These were constructed by collaborators Dr. Benedikt Kaufer (Freie Universitäet, Berlin, Germany) and Dr. Shiro Murata (Hokkaido University, Hokkaido, Japan).

2.1.3 Propagation and generation of virus stocks

The BAC-based infectious clone viruses used in our study were obtained as agar-stab cultures from our collaborators. These cultures were grown in LB broth with chloramphenicol (@25 μg/ml), and the viral BAC DNA was extracted using a modified-Qiagen midi-prep procedure. For the modified procedure, the QF elution buffer was heated to 65^0C prior to use. Purified DNAs were resuspended in 1X TE, pH 7.5 and stored at 4^0C. To generate infectious virus, viral BAC DNAs (2 μg) were transfected into secondary chicken embryo fibroblasts (CEF) using Lipofectamine 2000 (Invitrogen). Virus infected CEF were passaged twice to generate high-titer virus stocks. MDV-infected CEF stocks were frozen in 90% FBS/10% DMSO using control-rate freezing chambers and transferred to liquid nitrogen for storage after three days at -80˚C. Virus titer determination was performed for each CEF-infected virus stock.

2.2 Growth properties of viruses

2.2.1 qPCR for MDV genome copy number

In order to assess the virus replication in CEF (cell culture) as well as in spleen cells (*in vivo*), quantitative PCR (qPCR) or real time PCR analysis was performed. Real-time PCR has been a widely used tool for accurate quantification of both DNA and RNA in the field of virology (79).The UL27 (gB) gene located in the UL region of the MDV genome and chicken ovotransferrin gene were selected to measure the number of MDV and chicken genome copies respectively (51, 111). DNA was extracted from homogenized spleen tissue cells using the standard proteinase K method for digestion followed by phenol-chloroform extraction (114). qPCR was performed using AB 7500 Fast Real-Time PCR System (Life technologies) in a

reaction containing 50 ng of DNA template, forward and reverse primers (0.4μM), Maxima SYBR Green/ROX qPCR Master Mix (Life technologies), and nuclease-free water. Standard curves were generated based on the Ct values for dilutions of plasmids from 2 x 10^7 to 20 copies for both MDV UL27 and chicken ovotransferrin genes. The previously described formula was used to determine total copies for each of the MDV and chicken genomes (50): Total copy = [(pg of input)(1 pmole/340 pg)(1/template size in bp)(1 mole/1 x 10^{12}pmole)]/(6.02 x 10^{23} copies/mole). MDV and chicken genome copy numbers were measured by correlating the Ct values of the samples to their respective standard curves. A ratio was generated by dividing the number of MDV genomes with the number of chicken genomes for each sample and was multiplied by 10,000 in order to obtain the number of MDV genomes per 10,000 chicken cells.

2.2.2 Cell culture virus growth curves

For cell culture virus growth curve determination, DNA samples were extracted from parent- and recombinant virus-infected CEF at 24, 48, 72, 96, 120 and 144 hrs post-infection. An estimated 100 PFU of each virus-infected CEF culture was used to infect secondary CEF adjusted to a total of 1×10^6 cells, in triplicate (total of (18) 60 mm dishes per virus). Prior to harvesting the final time point (144 hrs), plaques were counted to determine the initial number of PFU plated. DNA extraction was performed using the standard proteinase K digestion followed by phenol-chloroform extraction method (114). Viral copy numbers were determined using MDV gB-specific primers with chicken ovotransferrin gene-specific primers for normalization (127).

2.2.3 *In vivo* replication

Evaluation of the *in vivo* replication of the parent and recombinant viruses was performed using homogenized spleen cell sample DNA at VR1 (7 day), VR2 (14 day), VR3 (21 day), and VR4 (28 days) time points. For virus re-isolation, spleens were pooled from three chickens per treatment for each of the time points. Spleen cells were homogenized using glass Tenbroeck tissue grinders. Homogenized spleen cells (SPC) were filtered through cheese-cloth, washed, and re-suspended in M199 medium. DNA was extracted via proteinase K method and viral copy numbers were determined as mentioned above.

2.3 *In vivo* study of recombinant MDVs

2.3.1 Compliance of research with Agriculture Animal Care and Use Committee (AACUC), and University Biosafety Committee (UBC) requirements

For the research involving the deliberate introduction of recombinant MDVs into susceptible hosts, the protocols were approved by the AACUC (protocol #22-09-30-14R, a three-year protocol) and UBC (protocol #13-021, a three-year protocol). The research described below was performed in May-July of 2015, corresponding to the terms of these approved protocols.

2.3.2 Isolator bird study

The isolator bird study was carried out to examine if the the mutations in Meq (Meq isoforms) directly affected the pathogenicity and oncogenicity of MDVs in specific pathogen free (SPF) chickens. This study was conducted using glove port isolators in the Allen Laboratory of the University of Delaware. This study was a part of a multi-project study examining the effects of mutations in the Meq coding

sequence in RB-1B based recombinants, two CVI988 BAC-based recombinants, and rMd5ΔMeq and rMd5 viruses. For the focus of the thesis, only the pertinent groups will be described.

For each treatment group, (28) one-day-old, SPF white leghorn chickens were inoculated intra-abdominally with a target dose of 1000 PFU (infected CEF) of recombinant viruses: pRB1BΔIRL CVI988 Meq, pRB1BΔIRL N Meq and parental virus pRB1B. In addition, 25 mock-infected chickens were used as a common negative control group for all of the viruses tested. Virus re-isolation was performed from PBMC and spleen cells from (3) chickens per treatment group at 7, 14, 21 and 28 days post-infection. Briefly, chickens were bled via cardiac puncture, immediately euthanized via cervical dislocation, and then spleens were asceptically removed. PBMC were isolated from pooled whole blood via histopaque 1119 (Sigma) centrifugation, and spleen cells were homogenized using Tenbroeck tissue grinders, filtered through sterile cheese cloth, washed, and resuspended in M199 complete medium, as described (64).

The remaining birds were monitored daily for MD signs and mortality for a total of 6 weeks. For the entire period of study, chickens were provided *ad libitum* access to commercial starter feed and water. Birds showing signs of MD (unilateral paralysis, torticollis, ataxia, inability to move to food and water) were removed, euthanized via cervical dislocation and scored for MD lesions. Tumor samples were collected for cell population analysis and cell line establishment. At the termination of the experiment at 6 weeks post-infection, all remaining birds were euthanized and scored for lesions.

2.4 Confirmation of input viruses

2.4.1 PCR amplification and sequencing for Meq loci

PCR of the DNA samples from VR2 spleen cells, using *meq*-specific primers was performed, for the confirmation of the viruses inoculated into the birds. The primers used were: Meq locus forward, 5'-GTA AAG AGA TGT CTC AGG AGCCA-3', and Meq locus reverse, 5'-GGA GGC CAT CGG GAG ATT ATC-3'. The conditions for amplification were an initial denaturation for 5 min at 94˚C, followed by 35 cycles of 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 2.5 min. PCR amplicons were separated on 0.8% agarose, 1X TBE (Tris-borate-EDTA) gels containing 0.5 µg/ml ethidium bromide and were visualized and imaged using a Gel-Doc video camera (Protein Simple, Inc.). In addition, the amplified *meq* genes from the VR2 spleen cells DNA for each of the viruses were cloned using the Topo-TA cloning kit (Invitrogen), and positive clones were sequenced at Delaware Biotechnology Institute (DBI) at the University of Delaware.

2.4.2 Southern blotting

To confirm the structure and stability of the BAC-derived recombinant viruses, Southern blotting was performed. For assessing the structure of BAC-based recombinants, 1µg of BAC DNA of each of the original constructs of RB-1B (parent), CVI988-Meq, and N strain-Meq viruses were used. For MDV-infected cell DNAs and transformed cell line DNAs (UD39, UD40), 10 µg of the respective DNA was used. These DNAs were digested with restriction enzyme *Sca* I cell at 37^0C, overnight. The digested DNA fragments were separated using a 0.6% agarose gel in 1X TBE and run at a constant voltage of 20 V, until the bromophenol blue loading dye reached ~1 cm from the bottom of the gel.

Southern blotting was carried out essentially as described (114). After separation, DNA fragments were nicked in-gel using a Stratalinker UV cross-linker at 800 mJoules to facilitate transfer of DNA (Stratagene). The gel was denatured using 0.5 N NaOH, 1.5 M NaCl for 45 min followed by neutralization of gel with (2) washes of 1 M Tris, pH 8 + 1.5 M NaCl of 15-20 min each. DNAs were then transferred to a positive charged nylon membrane (Hybond-N+, Millipore, Bedford, MA) via capillary transfer using sodium chloride/sodium citrate buffer (SSC) (114), and fragments were immobilized via UV crosslinking.

DNA fragments were probed with a pp38 region PCR product probe (spanning the IR_L/TR_L common region) that was labelled directly via crosslinking with alkaline phosphatase. Crosslinking was done using Amersham Alk-Phos direct labeling reagents following the manufacturer's instructions (GE Healthcare). Pre-hybridization of blots, probe hybridization, and washes were performed according to the manufacturer instructions. After washing, CDP-Star (GE Healthcare) light-emitting substrate was added to the blot and was imaged using a Fluoro-Chem Q digital chemiluminescent CCD (Protein SImple, Inc.). Images were acquired, background substracted and transferred for labeling of lanes and band sizes.

2.5 MDCC cell line establishment

Lymphomas were harvested from the different affected visceral organs of culled chickens exhibiting signs of MD, and at necropsy. The tumors were sectioned into small pieces in sterile medium (incomplete M199 with antibiotics/antimycotics) and were homogenized using Tenbroeck glass tissue grinders, filtered through cheese cloth, and resuspended in M199 medium. Lymphocytes were purified by density gradient centrifugation (700 *x g* for 30 minutes) using histopaque M1119 (Sigma), and

washed twice with media, and resususpended in Iscove's ITS medium [Iscove's modified Dulbecco medium supplemented with 20% FBS, 10% chicken serum, 1% nonessential amino acids, 1% ITS supplement (insulin, transferring, selenium), 4mM glutamine, 2mM sodium pyruvate, 2 μM β-mercaptoethanol, 1X PSN, 0.5 X fungizone]. Cells were plated in 6-well dishes at different dilutions, monitored daily, and split 1:3 or 1:5 every 4 to 7 days until established. Stocks were frozen in 10% DMSO/90% FBS at various passages.

2.6 Flow cytometry

In order to evaluate changes in lymphocyte/macrophage or monocyte populations in the recombinant MDV-induced tumors, tumor cell suspensions were stained for flow cytometric analysis. Cells suspensions were fixed with 1% paraformaldehyde for 1 hr at room temperature, washed with 1XPBS, and stored in antibody diluent at at 4˚C prior to staining. Cells were stained with antibodies listed in Table 2. Anti-chicken CD3, CD4, CD8, CD 28, BU-1, IgM, KUL01, TCR1, TCR2, TCR3, MHC-I, MHC-II, CD44 and CD45 monoclonal antibodies (mAbs) were purchased commercially (Southern Biotechnology Associates). The mAb to chicken CD25 were provided by Dr. Hyun Lillehoj, USDA-ARS, Beltsville, MD (141). The mAb to chicken CD30 (AV37) was provided by Dr. Shane Burgess (CALS, University of Arizona) (16). All antibodies were diluted according to manufacturer's recommendation (1:10 for AV37, 1:50, and 1:100 for commercial antibodies) in 1X PBS, pH 7.4 + 3% goat serum, 1% BSA + 0.1% NaN3. For cell staining, ~1 x 10^6 cells in 50 μl were incubated with primary antibody for 1 hr at room temperature. Cells were washed twice with 4 mls of wash buffer (1X PBS, pH 7.4 +1% BSA + 0.1% NaN3) and pelleted at 1,500 rpm for 6 min at 4˚C. Cells were then incubated

with goat anti-mouse IgG FITC conjugate (1:100 in diluent) for 30 min. Cells were washed twice, as above, and finally resuspended in 250 µls of wash buffer and stored at 4˚C covered with parafilm and foil until analysis. Acquisition of cells was performed using a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) and CellQuest Software. 10,000 ungated events were collected for each acquisition. Cells were gated by light scattering (FSC vs SSC) and secondary antibody only-stained samples were used for gating positive fluorescence.

2.7 Effect of Meq mutations on innate immune evasion assays

A follow-up of our study was that Meq mutations may have been selected through innate immune activation elicited by vaccines (HVT, HVT+SB1). These mutations, therefore, may mediate greater resistance to innate signaling. To address this hypothesis, we simulated innate immune activation analogous to early innate immune responses post-vaccination, using various innate immune agonists. These agonists include LPS, Poly I:C, cGAMP which activate TLR4, TLR3 and MDA5, and STING receptors, respectively.

2.7.1 Plaque count and area analysis

Plaque count and plaque area determination was done after treating virus infected CEFs and the SPCs with different agonists. For plaque analysis in CEF, viruses having different Meq mutations (RB-1B CVI 988, RB-1B parent and RB-1B N strain) were allowed to replicate for 24 hours in CEF. After 24 hours, infected CEF monolayers were treated with agonists: LPS (5 µg/dish), Poly I:C (100 ng/dish), and cGAMP (100 ng/dish), and control (medium only). All treatments were in M199 medium with 1% calf serum, 1X PSN and 1X fungizone. Viruses were allowed to

replicate via incubation at 37^0C for 5 days after treatment and monolayers were fixed with cold 95% ethanol at -20^0C for at least 2 hours. Monolayers were rehydrated using 5 mls of 1X PBS and then stained overnight using 1:2000 anti-pp38 (H.19 monoclonal antibody). Stained monolayers were washed two times using 1X PBS with 1% BSA and 0.1% sodium azide. After the second wash, the PBS was removed, and secondary staining was performed using 1:200 goat-anti-M Alexa 488 antibody, for 2 hours. After two more washes, as described above, the monolayers were covered with 3 mls of 10% glycerol in 1X PBS and 6 nM DAPI for nuclei visualization. Plaques were then counted and areas were scanned using Nikon NIS Elements software.

For observing the innate signaling effects on MDV replication in lymphocytes, SPCs harvested from the VR3 (day 21) birds infected with recombinant viruses harboring different *meq* genes, were adjusted to 2 x 10^7 cells before treatment with agonists, and incubated for 2 hours with treatment at 37^0C. Treatment of MDV-infected cells with these agonists for 2 hrs was believed to be sufficient to induce transcription and translation of pro-inflammatory cytokines and/or type I interferons. The treated SPCs were then washed and incubated with CEF, in triplicate for each treatment/virus. MDV plaque formation was conducted via incubation at 37^0C for 5 days, when the monolayers were fixed with cold 95% ethanol. The remaining procedure was followed similar to plaque analysis in CEFs, mentioned above.

2.7.2 Statistical Analysis

We counted plaque numbers in triplicate 60 mm dishes of infected CEF or infected SPC for each of the recombinant virus for each agonist treatment and the control. We then compared the differences in mean counts between each treatment and the control (medium only) of the respective viruses using Student t-test, assuming

unequal variance. For the plaque area size, a total of 150 plaque areas (in triplicate 60 mm dishes, 50 plaque areas per dish) were measured (or total plaque numbers if less than 50), and the mean differences in plaque area size between each agonist treatment and the control for each virus (medium only) were compared using Student t-test assuming unequal variance. Differences were considered significant at $p < 0.05$.

Chapter 3

RESULTS

3.1 Replication of viruses

3.1.1 *In vitro* replication in CEF

In order to characterize the replication properties and competency of the recombinant viruses, we infected CEFs with 100 PFU dose of each of the recombinant virus and performed single-step replication kinetic analysis at different time points post-infection by qPCR. In addition to replication, the recombinant viruses retained the ability to spread, based on the visual appearance and relative size of the plaques (data not shown)

Comparison of the replication of pRB-1B (parent), pRB1BΔIRL CVI meq and pRB1BΔIRL N meq viruses at 37˚C showed that both recombinant viruses (pRB1BΔIRL CVI meq and pRB1BΔIRL N meq viruses) replicated to comparable levels to parent RB-1B. The DNA copy numbers reached their peak level by 96 hr time point. The replication curves (Figure 4.) shows that all the viruses were able to replicate in CEFs at comparable levels.

3.1.2 *In vivo* replication in SPC (in chickens)

Replication characteristics of the recombinant and parental viruses were also studied in infected chickens (Figure 5). We monitored replication of the viruses in chicken spleen cells (SPC) harvested from infected, SPF chickens. Genome copy

numbers for each virus, at various time points post-infection, were plotted on the curve. Replication of recombinant viruses in SPC depicted a similar trend, that was comparable to the replication of RB-1B (Figure 5.). Following our initial sampling time point on Day7 post-infection, the genome copy numbers peaked on Day 14, representative of early cytolytic phase (7-14 days post-infection), but rapidly decreased by Day 21, indicative of a suppression of viral lytic replication as latency was established.

Once the secondary cytolytic phase ensued (after day 21), the reactivation of virus caused increased replication, and so a rapid increase in viral genome copies. Alternatively, this is a period of transformation, in which malignantly-transformed T-cells, each harboring multiple genome integration events, proliferate

Post reactivation, the pRB1B parent displayed higher viral genome copies than pRB1BΔIRL CVI meq after reactivation/transformation, whereas pRB1BΔIRL N meq copy numbers were intermediary to pRB1B parent and pRB1BΔIRL CVI meq. One contrary observation to our hypothesis was that pRB1BΔIRL N meq displayed lower genome copy numbers than pRB1B and pRB1BΔIRL CVI meq (except Day 28) at all time points observed. These data therefore suggested that in unvaccinated, maternal antibody-free chickens, changes in Meq coding sequence conferred no inherent pathogenicity increase (or decrease) for the mutations examined.

3.2 Tumor incidence and mortality

To understand the effects of Meq mutations on the pathogenicity and tumorigenicity, we infected SPF chickens with the recombinant and parent viruses and recorded the mortality and tumor incidence in these chickens. The target dose was

1000 PFU/bird for each of the viruses, but actual back-titrated doses were slightly higher (Table 1.), indicating that the birds received sufficient doses to elicit infection.

Contrary to our hypothesis, the highest mortality was recorded for pRB1BΔIRL CVI *meq* treatment group while the lowest mortality was recorded for pRB1BΔIRL N *meq* treatment group. Similarly, tumor incidence was highest for pRB1BΔIRL CVI *meq*-infected and lowest for pRB1BΔIRL N *meq*-infected chickens. From the tumor and mortality incidence data (Table 1), it is clear that our data do not support our hypothesis that Meq mutations alone are sufficient to confer increased mortality, because the *meq* gene from vaccine strain-containing virus (pRB1BΔIRL CVI *meq*) showed the highest level of mortality and tumor incidence (47.1% and 94.11%, respectively), whereas the *meq* gene from the highest virulent virus (N strain, a vv+ MDV) showed the lowest mortality and tumor incidence (5.9% and 29.41%, respectively). Survival curve analysis showed a similar trend in the data (Figure 6.).

3.3 Confirmation of virus structure and *meq* sequences

Given our unexpected results, we sought to ensure that the samples were not mixed during the study, we confirmed the identity of recombinant viruses by amplifying the *meq* loci of viruses reisolated from spleen cells at 2 weeks post infection (VR2) (Figure 7.). In each case, amplicons were cloned and their DNA sequences determined. The PCR fragments obtained were the expected corresponding size of the *meq* gene of these viruses. All reisolated viruses were found to contain their original mutations, indicating that viruses did not revert to the RB-1B original form and that the samples had not been mixed-up during inoculation.

To further confirm our observations, we also performed Southern blot analysis to evaluate the genomic structures of the original BAC clones, of the *in vitro*

reconstituted input virus stocks (infected-CEF), of the reisolated viruses *in vivo* (from spleen cells), and from primary tumor-derived cell lines (Figure 8).

As the IR_L-deleted versions of the BAC viruses are allowed to replicate in the CEF or SPC, homologous recombination events between the identical regions flanking deleted IR_L and the intact TR_L are expected to isomerize and reconstitute the entire IR_L. As expected, Southern blot results showed that both copies were restored in all the viruses upon replication in CEF *in vitro*, SPC *in vivo,* and in cell lines derived from lymphomas (Figure 8.). These observations confirmed that the recombinant viruses have not undergone significant mutations (duplications, deletions, etc.) during propagation in cell culture or *in vivo* and that changes in pathogenicity were not due to differences in gene dosage.

3.4 Establishment of MDCC-UD 39 and -UD 40 cell lines

We processed numerous tumor samples derived from pRB1BΔIRL CVI *meq*-, pRB1BΔIRL N *meq*-, and pRB1B parent-infected birds and attempted to establish cell lines. After 30+ passages, we were able to establish two cell lines, and designated them MDCC-UD39 and –UD40. Cell line UD39 was established from an pRB1BΔIRL CVI *meq*- induced kidney lymphoma, while the UD40 cell line was established from an pRB1BΔIRL N *meq*-induced intestinal tumor. Table 2 shows the results of the immunophenotypic analysis on these two MDCCs, compared to a parental RB-1B induced cell line, UD35. These results depict that the cell lines MDCC-UD39 and –UD40 have immunophenotype chacracteristic of MDV-transformed, CD4+ T cells. None of these cell lines expressed B cell or CTL (CD8) surface markers. These cell lines expressed various levels of CD3, CD4, CD28, CD30, CD44, CD45, MHC-I, MHC-II, and integrin β1. All cell lines showed TCR2 (αVβ)

with UD39 also showing some TCR3 ($\alpha V_{\beta 2}$ co-expression (Table 2). These findings are consistent with MDCC immunophenotypes, suggesting that they are T-helper cells phenotype (95). Both cell lines showed a high level expression of CD30 and integrin β1, which are found highly expressed in MDV transformed cell lines and MD lymphomas cells (95).

3.5 Lymphoma composition

Despite the unexpected apparent increase in virulence of the RB-1BΔIRL CVI-*meq* virus, compared to parental RB-1B and RB-1B ΔIRL N-*meq* viruses, we hypothesized that mutations in Meq may affect tumor composition. Consequently, we characterized the cell populations and activation markers present in primary tumors caused by these viruses.

Flow cytometric analysis was performed on tumor cell homogenates using antibodies to BU-1 (pan-B-cell marker), CD3, CD4, KUL01(macrophage), MHC-I, MHC-II, CD44 (leucocyte migration), CD45 (leucocyte common antigen, leucocytes), CD30 (tumor necrosis factor receptor family), different T-cell receptors TCRs, etc. Results from these analyses are summarized in Table 3. All the tumors derived from recombinant virus-infected chickens were found to express MHC-I and MHC-II, but were more positive for MHC-I compared to MHC-II. T-cell activation in these lymphomas was found with 26-50% cells being positive for CD28 and CD30. CD30 was found to be responsive to Meq expression (17), however, in our analysis, we did not see a strict correlation of CD30 expression with the transactivation potential of Meq (i.e., vMDV<vvMDV<vv+MDV). For the percentages of CD30+ cells in the lymphomas, there was a rough correlation with Meq isoform, in that RB-1B CVI-Meq-induced lymphomas showed a consistent low percentage of CD30+ cells (22 –

24%), the RB-1B-induced showed a somewhat higher percentage (35 -37%), and RB-1B N-Meq-induced lymphomas showed one quite high percentage (71%) but one with much less (spleen lymphoma, 7%). As this was the only spleen lymphoma examined, it may have had a lower level of transformed cells composition (~4% CD4+ to ~10% CD8 cells).

CD107 (aka, lysosomal-associated membrane protein, LAMP-1) was also highly expressed in these lymphomas. CD107 is highly expressed on highly metastatic cancers, suggesting it may have this role in MDV-transformed cells. All the lymphomas were of T-cell origin (or contained some levels of T-cells), as they were found to be positive for CD3 antigen, while totally negative for BU-1 antigen, and were found to express TCR-2 receptors. Similarly, these lymphomas were largely negative for KUL01 (macrophage marker), with the exception of the RB-1B ΔIRL N *meq*-induced lymphomas, which had 1-2% macrophages, suggesting that these lymphomas may have a greater inflammation-associated component.

3.6 Meq mutations and Innate signaling manipulation

We performed plaque analysis to see if the respective Meq mutations (on a common backbone of RB-1B BAC) have the ability to overcome the agonist innate signaling elicited by specific agonists, by monitoring MDV replication and spread via plaque number and plaque area measurements post-treatment. Based on the relative specificity of the agonists, we could also assess the signaling most important driving the selection for Meq mutation.

3.6.1 Effects of innate agonist treatment on MDV replication in CEF

The change in plaque counts in CEF (Figure 9.) showed that all the treatments (LPS, Poly I:C and cGAMP) caused a significant decrease ($p < 0.05$) in the number of plaques when compared to the medium-only control for RB-1B CVI *meq*. For the RB-1B parent virus, there was a decrease in the plaque number with LPS and Poly I:C agonist treatments, however, cGAMP did not affect the plaque number compared to control (medium only). The RB-1B N *meq* strain was similarly not affected by cGAMP treatment, however, was affected by the treatment with Poly I:C and to a lesser extent, LPS. For all three viruses, Poly I:C treatment led to a decrease in plaque number compared to their respective controls.

Similarly, plaque area determination in CEF (Figure 10.), revealed that LPS and Poly I:C treatments caused statistically significant ($p < 0.05$) decreases in plaque area for all three viruses compared to the medium-only treatment. However, cGAMP treatment caused a significant decrease in plaque area for the RB-1B CVI *meq* virus, but did not statistically affect RB-1B or RB-1B N *meq* viruses. The plaque area size in CEF monolayers without any treatment was largest for RB-1B CVI *meq* ($118404 \pm 5760\ \mu m^2$), followed by RB-1B parent ($62115 \pm 2497\ \mu m^2$) and lowest for RB-1B N *meq* strain ($46753 \pm 1505 \mu m^2$). Consequently, changes in plaque area may be more readily detected with the RB-1B CVI *meq* virus. Given the common backbone and passage history of the viruses, the relative decreased size of plaque number and area for the different viruses suggests that the lower virulence form of *meq* (CVI988) may generally increase viral replication in CEF.

3.6.2 Effects of innate agonists on MDV replication coming out of spleen cells

Since *in vivo*, the target of MDV replication is lymphocyte (B- and T-cells), and feather follicle epithelium, and to a lesser degree, macrophages, we sought to test the effects of innate immune agonists on MDV replication in this context. To evaluate effects on viral load, MDV-infected spleen cells were washed *ex vivo* and treated for 2 hrs with medium, LPS, Poly I:C, or cGAMP and then washed again and plated at a constant number of cells/dish.

The graph of plaque counts on CEF co-cultured with the treated SPC (Figure 11.), showed that there was no statistically significant difference in the plaque number upon treatment with all the agonists, except for LPS, when compared to medium treatment for all three viruses. LPS treatment, however, caused a significant decrease in the plaque number in RB-1B CVI *meq* and the RB1B parent virus, but not for RB-1B N *meq* strain. The RB-1B N *meq* virus was not affected by any of the agonist treatment, in terms of plaque numbers.

Similar to the evaluation for plaque number, the plaque area size of RB-1B N *meq* virus was not statistically affected by any of the agonist treatments compared to the medium control, as shown in the graph for plaque area in SPC (Figure 12). RB-1B CVI *meq* and RB-1B parent viruses were affected by all the agonists, showing significant decreased plaque area compared to their respective controls. Plaque area size was again largest for RB-1B CVI *meq* (84905 ± 4960 μm^2) followed by RB1B parent (70646 ± 2447 μm^2) and smallest for RB-1B N *meq* (67587 ± 4938 μm^2).

When the effects of innate immune agonists were examined for percent decrease compared to the medium-only controls (Figure 13), the RB-1B CVI *meq* virus showed a decrease for each agonist of ~30%, the RB-1B parent virus showed a decrease for each agonist of ~10%, while the RB1B N *meq* showed a decrease of ~4%

for each agonist. Although not a perfect system for assessing the effects of innate immune stimulation as a selection for Meq mutations, our data suggest that given the common background strain of these viruses, with only the Meq coding sequences being changed, these mutations appear to mediate greater resistance to innate repression of virus replication, particularly in lymphocytes.

Chapter 4

DISCUSSION

Previous work from our lab (128), showed a correlation between the amino acid mutations of the oncoprotein Meq, and the virulence level of the MDV-1 strains. This observation has been subsequently made by others examining strains of difference virulence from around the world (25, 40, 85, 86, 108, 131, 134-137, 140, 142, 152-154). This had led us to hypothesize that these mutations may be mediating the virulence evolution of these MDV-1 strains. To address this hypothesis, our laboratory has examined the biochemical properties of these Meq isoforms in terms of transcriptional activation potential, ability to induce proliferation in cell line models (DF-1, HTC), and differences in binding proteins via proteomics (Arumugaswami dissertation, Kumar dissertation, and data not shown). Moreover, we were able to select for Meq-expressing MDVs via transfection of the *meq* loci from vMDV (JM16), vvMDV (RB-1B) and vv+MDV (N strain) with rMd5ΔMeq and injection of these transfected cells into chickens (64). As rMd5ΔMeq is highly inflammatory and a potent vaccine (78), the selection of RB-1B Meq and N strain Meq-containing viruses, but not JM16 Meq-containing viruses, suggested a positive selection for these higher virulence forms of Meq in the context of *in vivo* infection.

To more definitively address the roles of these mutations, on MDV virulence, our collaborator constructed a number of recombinant MDVs in the background strain RB-1B. Previously, the RB-1B BAC has already been characterized as a vvMDV-1, and its ability to replicate in *in vitro* and *in vivo* conditions is well established (101).

For the construction of the viruses for these studies, an RB-1B derivative was used (RB-1BΔIRL), which allows the mutation of genes in the RLs via a targeted mutation of the non-deleted copy, which undergoes isomerization during replication in CEF (as we have observed, as well in Figure 8.).

Upon investigating the replication and virulence of these recombinants in SPF chickens, the mortality percentage and tumor incidence data showed that the mutations in Meq are not directly responsible for mediating increased virulence of these strains. In fact, our data contradicts our hypothesis in that the vv+ N strain was least pathogenic in terms of mortality and tumor incidence. Also, the overt signs of vv+ strains like transient paralysis, early mortality and distinct neurological signs were not observed.

The kinetics of replication of these strains *in vivo* also corresponded to the Cornell model of MDV pathogenesis (21), suggesting that these BAC-derived viruses display similar kinetics with the reference RB-1B strain to induce MD in chickens. Given the provision that these viruses have replicated similarly to cause pathogenesis like the field strains, it can be concluded that the mutations in the Meq oncoprotein are not entirely responsible for the evolution of MDV-1. Supporting results from PCR on the *meq* loci, sequencing on the *meq* loci, and Southern blotting have confirmed that no aberrant mutations have occurred and that both the copies of *meq* (in TR_L and IR_L) have been retained in these viruses. These data further suggest that the viruses constructed do not contain mutations that have affected the virulence of the recombinant strains.

We also sought to see if these mutations in Meq could affect the tumor composition. Flow cytometric analysis on the lymphomas harvested from birds

infected with different recombinant viruses showed that these were predominantly of CD4+ T-cell in origin, and highly expressed MHC-I and MHC-II and Hodgkin's lymphoma-associated Reed Sternberg ($CD30^{hi}$) antigen. This is consistent to previous findings (95). These lymphomas also exhibited high expression of CD107, also known as lysosomal associated membrane protein 1(LAMP1), which is expressed on the surface of tumor cells in the case of highly metastatic cancers like human colon cancer and melanoma (4, 115). Similarly, the immunophenotype of the cell lines was also as expected, being primarily T-cell in origin and highly expressing the CD30 antigen, a characteristic of MDV transformed cell lines.

This study was conducted using maternal antibody negative (MAb-), specific pathogen free (SPF) chickens, whereas in a real world field situation, the evolution of MDV-1 strains has been associated with overcoming the protective effects of vaccines. To actually emulate the field conditions, challenge studies with the viruses in our study have to be performed in monovalently (HVT), or bivalently (HVT/SB1) *in ovo* vaccinated commercial chickens.

The MDV-1 strains have evolved in virulence due to their increased ability to overcome vaccinal immunity. Although the vaccines are able to limit tumor formation, they do not prevent MDV-1 lytic replication and transmission of virus. As a result, the viruses are presumed to increase their virulence due to the selective pressure imposed on these viruses, survive, replicate and spread in the face of vaccinal innate immune responses. Hence, the other hypothesis of our study was that the mutations in Meq are selected in such a manner that the virus can overcome vaccinal immune responses via interruption or deregulation of the innate signaling during early post-vaccination and natural exposure.

To address this hypothesis, we first allowed the recombinant viruses to grow in the presence of innate agonists specific to various innate signaling pathways, and then observed the replication of recombinant viruses in terms of change in plaque counts and plaque sizes in CEFs. The findings from the plaque counts and plaque size in CEFs showed that Poly I:C remarkably decreased the number and size of plaques in these viruses. This suggests involvement of NF-kB via TLR3 and/or MDA5 signaling which ultimately activates the transcription factors IRF3 and IRF7 (although IRF3 is absent in the chicken) and that these activated transcription factors drive the expression of type I interferon genes.

Previously, *in vitro* infection of CEFs with RB1B and HVT has been reported to upregulate IRF1 and IRF3/7 in micro-array analysis (55, 83). We speculate that treatment with Poly I:C would be able to enhance the downstream activation of IRF3/IRF7 in particular to limit the replication and spread, and would explain the remarkable decrease in plaque sizes in case of all three viruses, irrespective of the *meq* gene encoded. Rapid induction of IFN- α and IFN- β via TLR3 signaling has been observed in chickens, upon treatment with Poly I:C (56) and IFN- α has been shown to reduce viral replication in both *in vitro* and *in vivo* studies (48, 71, 146). Moreover, Poly I:C treatment was found to induce sufficient amounts of type I IFN and IL-6 in freshly prepared chicken spleen cells (124). In addition, TLR3 via TRAF6 also activates NF-kB and MAP kinases p38 and JNK, consequently Poly I:C treatment combines much of the signaling of LPS (TLR4) and cGAMP (STING) and likely has an additive effect of interferon and pro-inflammatory signaling.

LPS treatment caused decreased in plaque numbers in RB-1B CVI *meq* and RB-1B parent viruses, but did not affect the plaque number for RB-1B N *meq* virus,

but caused a decrease in plaque area of all three viruses. This means that TLR4 innate signaling seems to be another pathway involved in innate anti-viral defense. LPS activation of chicken TLR4 and co-receptors has been found to induce production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-18 and soluble mediator NO via iNOS activation (29, 33, 43). NO is specially of mention here because iNOS is produced from activated macrophages that leads to production of NO and the inhibitory effects of NO on MDV replication have been confirmed both in *in vitro* and *in vivo* (156).

Interestingly, cGAMP treatment caused decrease in plaque number and plaque area size for RB-1B CVI *meq*, but did not affect RB-1B parent or RB-1B N *meq* viruses. cGAMP which operates as a downstream molecule of cGAS activation and upstream of the STING/TBK-1 pathway, ultimately leads to activation of IRF3/IRF7. From our data, it can be inferred that RB-1B parent (vv) and RB-1B N *meq* (vv+) viruses are able to deregulate, disrupt, or overcome cGAMP innate signaling, while not so in the case of RB-1B CVI *meq* (mild) virus, at least *in vitr*o. However, this is only a preliminary speculation, and further experiments need to be carried out to support this hypothesis.

Since the MDV-1 replicates in lymphocytes, with a predilection for B-cells, we further extended the innate agonist experiment using the spleen cells (SPC at 21 dpi) harvested from birds infected with the recombinant viruses. This would better emulate the actual host environment and elucidate our hypothesis. Plaque analysis from infected and treated SPC showed that except for the LPS treatment, the other treatments did not have statistically relevant effects on the plaque numbers for all three viruses. However, all the innate agonist treatments caused a significant decrease in

plaque area for RB-1B CVI *meq*, and RB-1B parent viruses, but not in RB-1B N *meq*. It is noteworthy to mention that plaque number and area for the RB-1B N *meq* recombinant were not affected by any of the agonist treatments in both CEF and SPC. Thus, the N strain-*meq*, seems to have overcome innate signaling immunity triggered via either of TLR3, TLR4 or cGAS-STING pathways, possibly by interfering with signaling integrators (TBK1, IKKε, IKKα/β/γ, NFκB, IRF7) that are common to all three pathways. It seems that plaque area size is a better gauge than plaque number to determine the effect on MDV-1 replication, because the data on plaque area size in both CEF and SPC seemed to better correlate with the virulence level of strains.

The results from plaque analysis do not precisely mimic the actual condition in the chicken, as we have not used an actual lymphocyte model (B-cell and T-cell) for our study. We have used SPCs as source of lymphocytes from the chickens infected with different recombinant viruses to further infect the CEFs. In chickens, the virus infects more lymphocytes (B-cells) and not CEFs. Further study using a total lymphocyte model is hence necessary before concluding that Meq mutations actually manipulate innate signaling to drive MDV-1 to higher virulence.

Chapter 5

CONCLUSIONS

In this study, we tested our hypothesis that Meq mutations are directly responsible for the virulence and pathogenicity of the MDVs. However, based on our data on mortality and tumor incidence, followed by the confirmatory results on the involvement of both copies of *meq* gene and the absence of any aberrant mutations on the *meq* loci, we report that these mutations are not directly responsible for the observed difference in MDV virulence levels, at least in SPF chickens. In addition, we did not observe any difference in the composition of primary lymphomas and the established cell lines, compared to the previously reported findings.

Furthermore, upon examining a follow up hypothesis that Meq mutations may still be relevant and can act indirectly via dysregulation of innate immune signaling, we report that Meq mutations may be selected based on the ability of the MDVs to overcome innate signaling early post-vaccination in chickens. We hereby recommend future studies to employ an *in vitro* lymphocyte infection model to replicate our findings and then test the pathogenicity of the recombinant viruses of our study in maternal antibody positive commercial chickens upon challenge in the context of vaccination.

TABLES

Table 1. Tumor incidence and mortality

Viruses	Target Dosage (PFU/bird)	Actual Dosage (PFU/bird ± SD)	% Mortality	% Tumor Incidence
pRB-1BΔIRL parent	1000	1813 ± 142	12.5 (2/16)	50 (8/16)
pRB-1BΔIRL CVI *meq*	1000	1533 ± 170	47.1 (8/17)	94 (16/17)
pRB-1B ΔIRL-N *meq*	1000	1167 ± 155	5.9 (1/17)	29 (5/17)

Table 2. Immunophenotype of cell lines

Antigen	UD35	UD39	UD40
CD3	++	++	++
CD4	+++	++	++
CD8	-	-	-
CD8b	-	-	-
BU-1	-	-	-
KUL01	NA	-	-
CD25	NA	-	-
CD28	-	++	+
CD30	++	++	++
CD44	++	++	++
CD45	+/-	+	-
CD107	NA	++	++
MHC-I	+++	++	++
MHC-II	++	++	++
TCR-1	-	-	
TCR-2	++	++	+++
TCR-3	-	+	-
Integrin β1	+++	++	+

-Indicates no expression detected; +/- indicates not all cells showed expression; +, ++, +++ indicate levels of fluorescence expression above control, NA indicates not available (data for UD35 cell lines were obtained from (64))

Table 3. Parental and Recombinant RB-1B-induced Lymphoma Composition

Antigen	RB-1B FU5195 Testis (%)	RB-1B FU5204 Ovary (%)	CVI-Meq GR3816 Ovary (%)	CVI-Meq GR3822 Kidney (%)	N-Meq RD53 Spleen (%)	N-Meq RD57 Kidney (%)
BU-1	-	-	-	-	-	-
KUL01	-	-	-	-	1.00	2.10
CD3	13.30	55.20	67.80	55.00	46.90	25.30
CD4	42.10	82.80	83.00	79.00	4.50	26.6
CD8	5.30	-	82.40	-	10.9	1.60
CD8b	-	1.60	53.10	-	1.00	3.40
CD25	-	-	36.60	2.20	-	1.30
CD28	44.60	77.20	56.50	50.00	7.00	10.70
CD30	34.90	36.60	21.80	23.60	7.10	70.40
CD44	44.10	82.00	72.80	62.90	15.50	76.40
CD45	7.60	2.40	7.30	-	1.70	5.40
CD107	79.90	82.30	71.80	86.60	24.90	63.20
MHC-I	96.60 (324)[1]	98.30 (349)	93.20(168)	89.60(181)	80.00(166)	74.00(177)
MHC-II	89.00(88)	92.00(70)	84.30(69)	26.60(125)	30.10(79)	42.50(65)
TCR-1	-	-	-	-	-	3.40
TCR-2	43.00	50.30	79.10	18.00	10.00	10.30
TCR-3	1.40	-	1.20	1.30	2.70	6.10

FU5195, FU5204, GR3816, GR3822, RD53 and RD57 denote the bird numbers from which the lymphomas were harvested.

[1] – Numbers in parentheses denote peak channels of fluorescence for MHC-I and -II

FIGURES

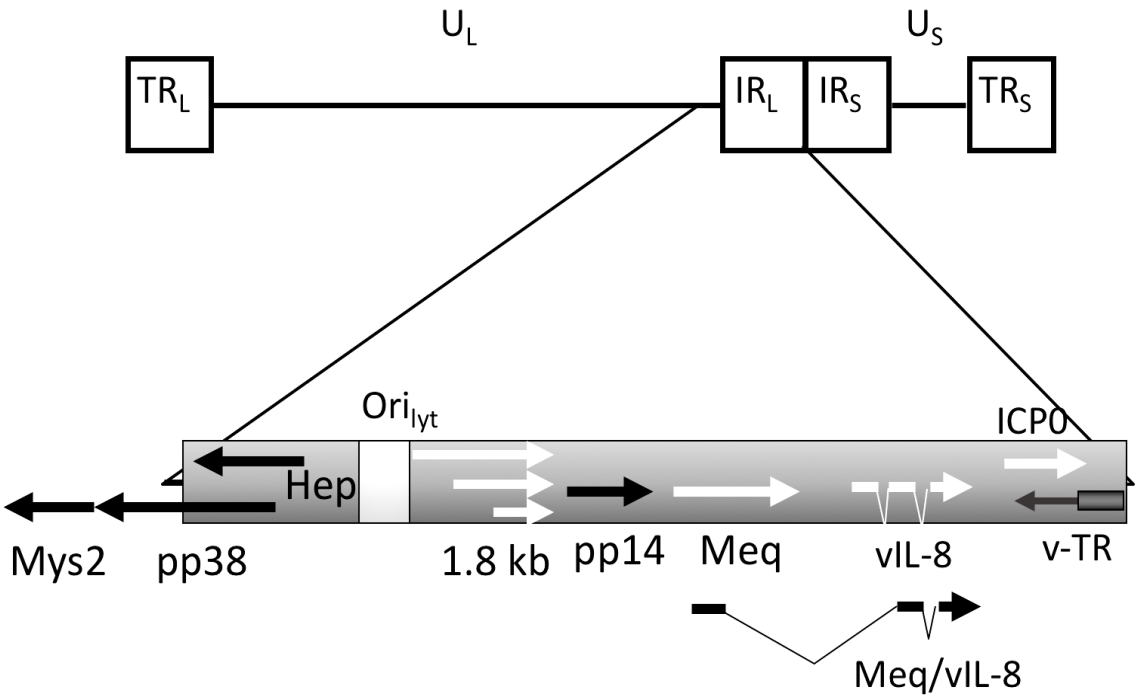


Figure 1. MDV-1 genome. Unique long ($\mathbf{U_L}$) and Unique short regions ($\mathbf{U_S}$) are flanked by repeat regions. Some of the major proteins implicated in oncogencity and pathogenicity are **Meq** -**M**DV **E**coR1 **Q** encoded protein, **vTR**-**V**iral **T**elomerase **R**NA, **1.8kb**-Family of transcripts with 132 bp repeats. Origin of lytic replication (**Orilyt**) acts as bidirectional promoter encoding for **pp14**-phosphoprotein 14 and **pp38**-phosphoprotein 38 in opposite directions. Meq also forms spliced products with **vIL8**-**V**iral **I**nterleukin 8. The other proteins shown are **ICP0**-**I**nfectious **C**ell **P**rotein 0, **Hep**-BamHI-H-encoded protein and **Mys2**-**M**ysery protein (aka, LORF12).

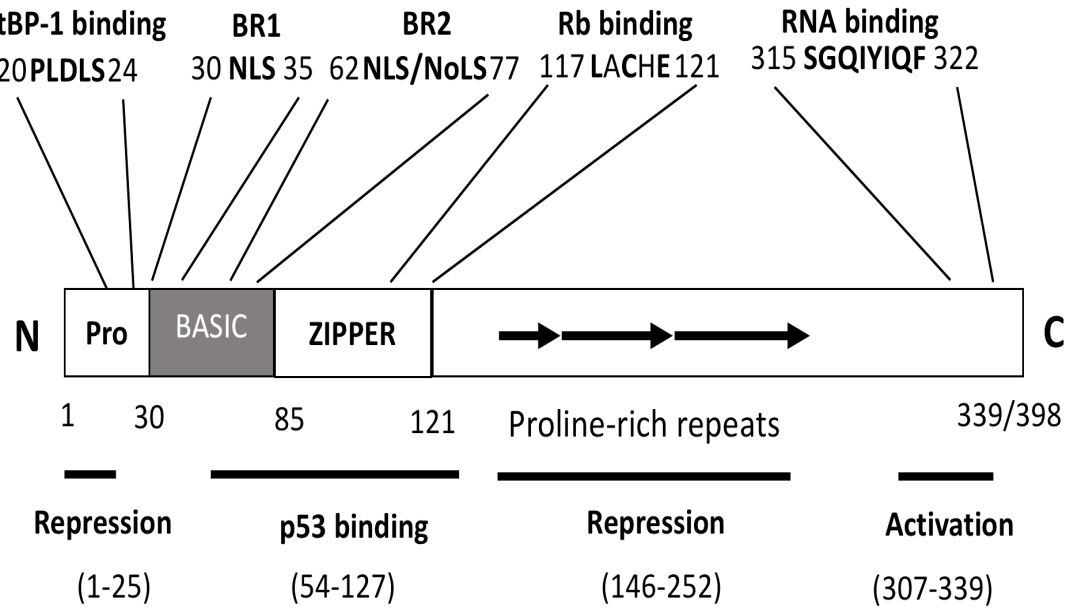


Figure 2. Structure of Meq (MDV EcoRI Q encoded) protein. Meq protein consists of N terminal and C terminal domains. N terminal domain includes Proline rich domain (**Pro**), Basic region (**BR**) and a leucine zipper (**ZIP**). C terminal domain includes proline rich repeats and transactivation domain. Basic region 1 acts as Nuclear Localization Signal (**NLS**) and Basic region 2 acts as both Nuclear as well as Nucleolar Localization Signal (**NoLS**). Meq binds to C-terminal binding protein 1 (**CtBP-1**) via **PLDLS** motif and binds to Retinoblastoma protein (**Rb**) via **LXCXE** motif. It also contains RNA binding motif at amino acid position from 315 to 322.

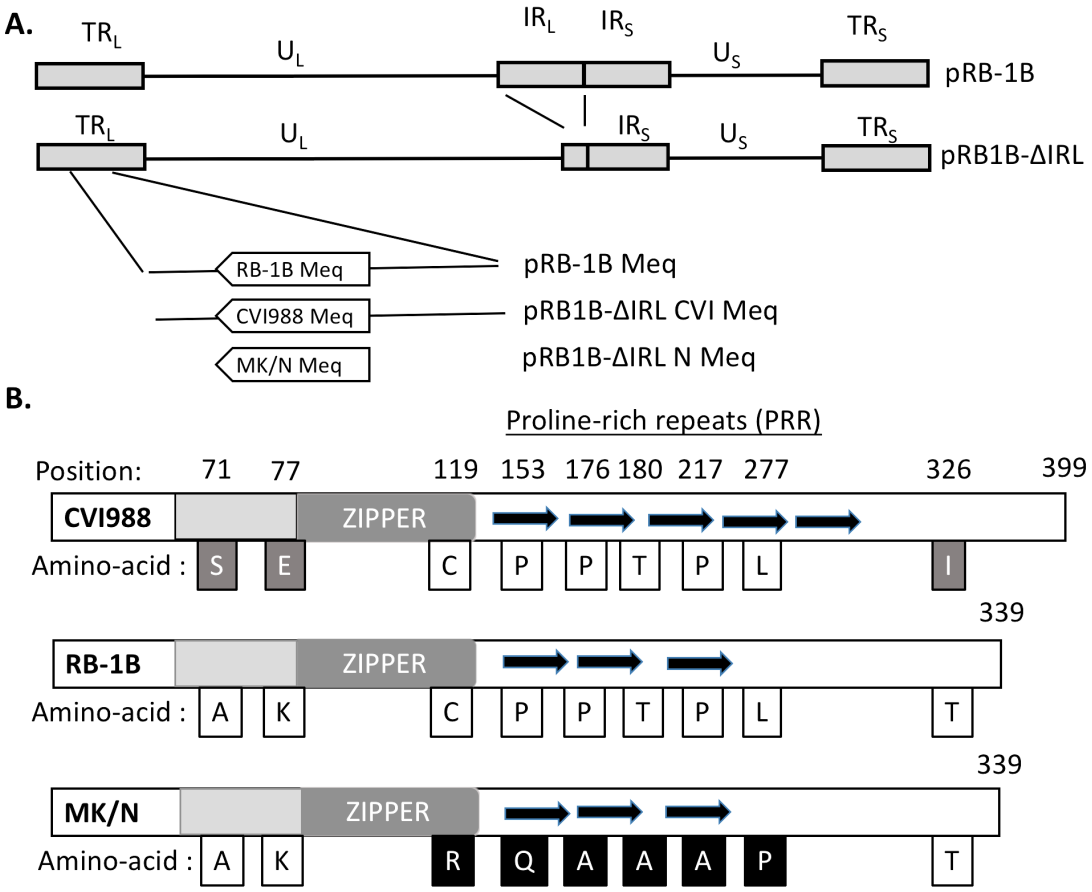


Figure 3. A. Overview of the MDV genome and Meq mutant viruses. Shown here is the schematic representation of the MDV pRB-1B genome consisting of two unique regions, long (UL) and short (US), flanked by terminal and internal repeats long (TRL and IRL) and shorts (TRS and IRS), respectively. Recombinant pRB-1B with a deletion of most of the IRL(pΔIRL) in which the *meq* gene is deleted (pRB1BΔIRLΔMeq). Into pRB1BΔIRL, various representative *meq* isoforms were inserted.

B. Meq oncoprotein of mutant viruses. The amino acid positions along with the mutations in amino acid for different mutant viruses are shown.

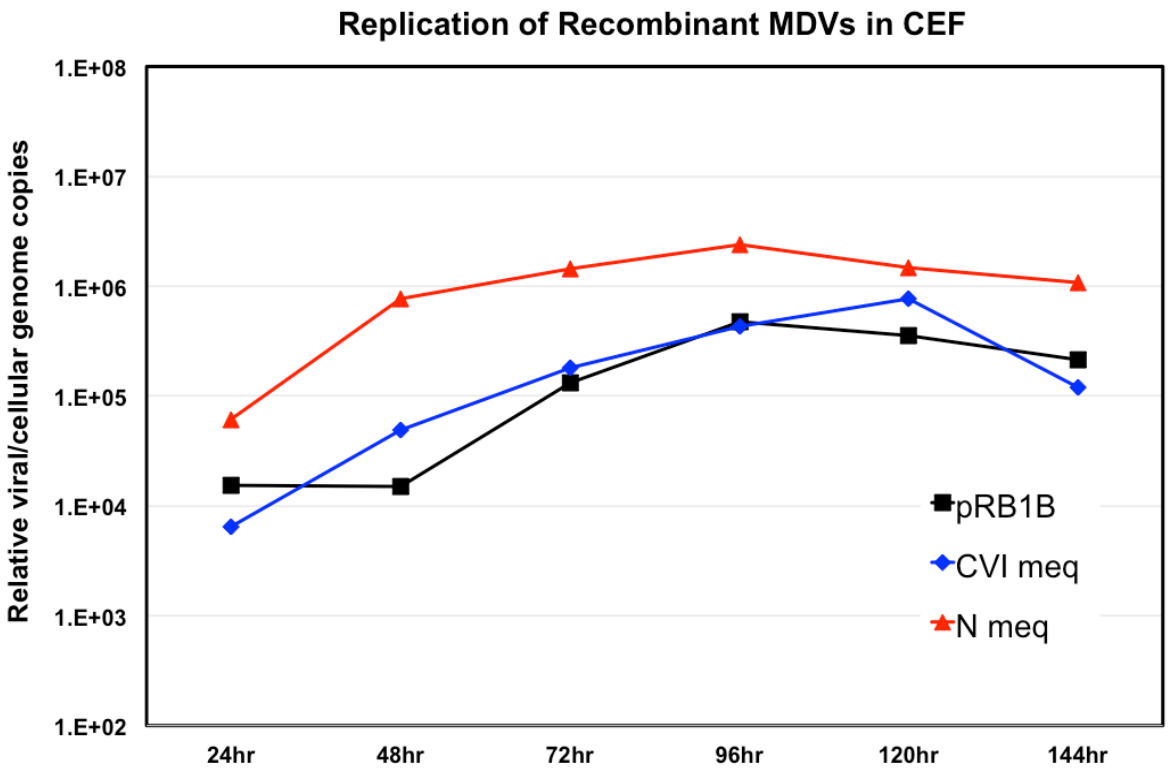


Figure 4. Comparison of Cell Culture Replication of Recombinant MDVs. The graph shows the comparison of replication in CEF among pRB1B parent virus control, pRB1B del IRL CVI988 *meq* (designated as CVI Meq) and pRB1B del IRL N *meq* (designated as N Meq) over the course of six days. Each point represents the mean genome copy number compared to the cellular ovotransferrin gene of triplicate dishes of infected CEF. Virus replication in cell culture was measured with a glycoprotein B-specific real-time qPCR on DNA samples from spleen cells collected from three birds from each group. Each point represents the mean genome copy number compared to the cellular ovotransferrin gene. Genome copies per 10,000 cells were obtained by multiplying the viral/cellular genome copy ratio by 10,000.

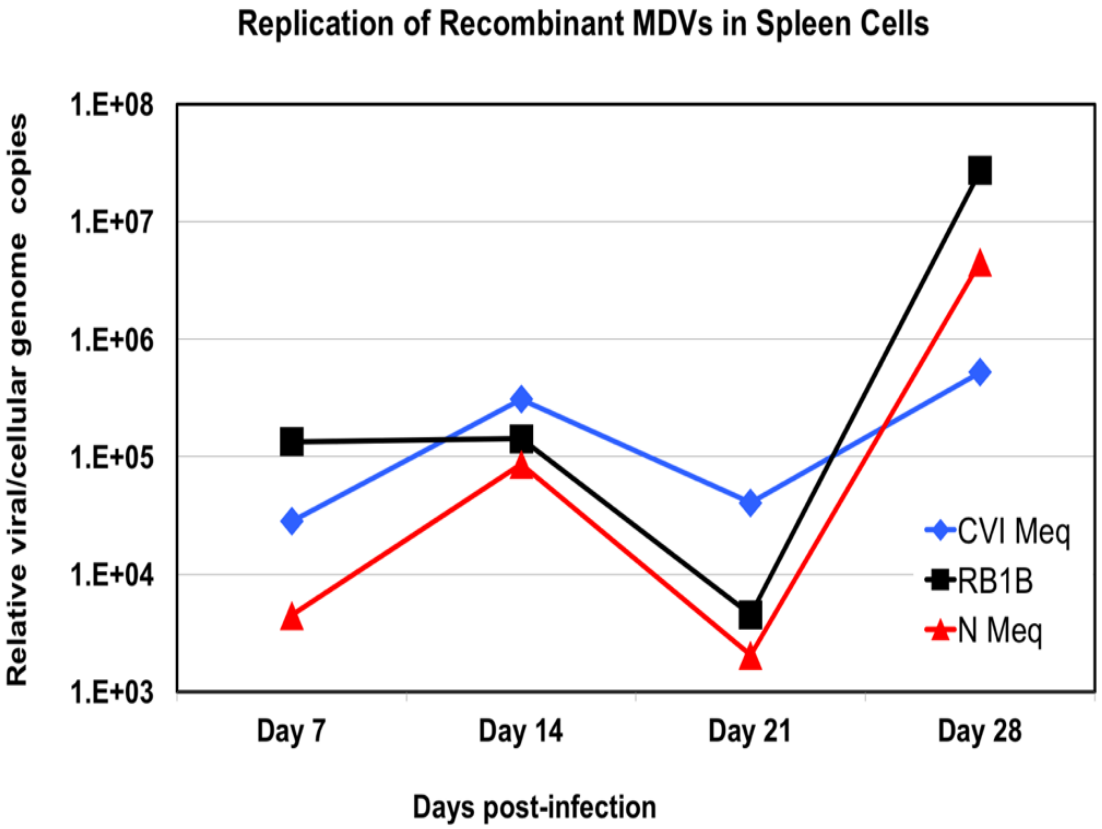


Figure 5. Comparison of *in vivo* Replication of Recombinant MDVs. The graph shows a comparison of replication of recombinant viruses in chicken spleen cells on 7, 14, 21 and 28 dpi. The viruses used were; the pRB1B parent virus control, pRB1B del IRL CVI988 Meq and pRB1B del IRL N Meq. Virus replication *in vivo* was measured with a glycoprotein B-specific real-time qPCR on DNA samples from spleen cells collected from three birds from each group. Each point represents the mean genome copy number compared to the cellular ovotransferrin gene. Genome copies per 10,000 cells were obtained by multiplying the viral/cellular genome copy ratio by 10,000.

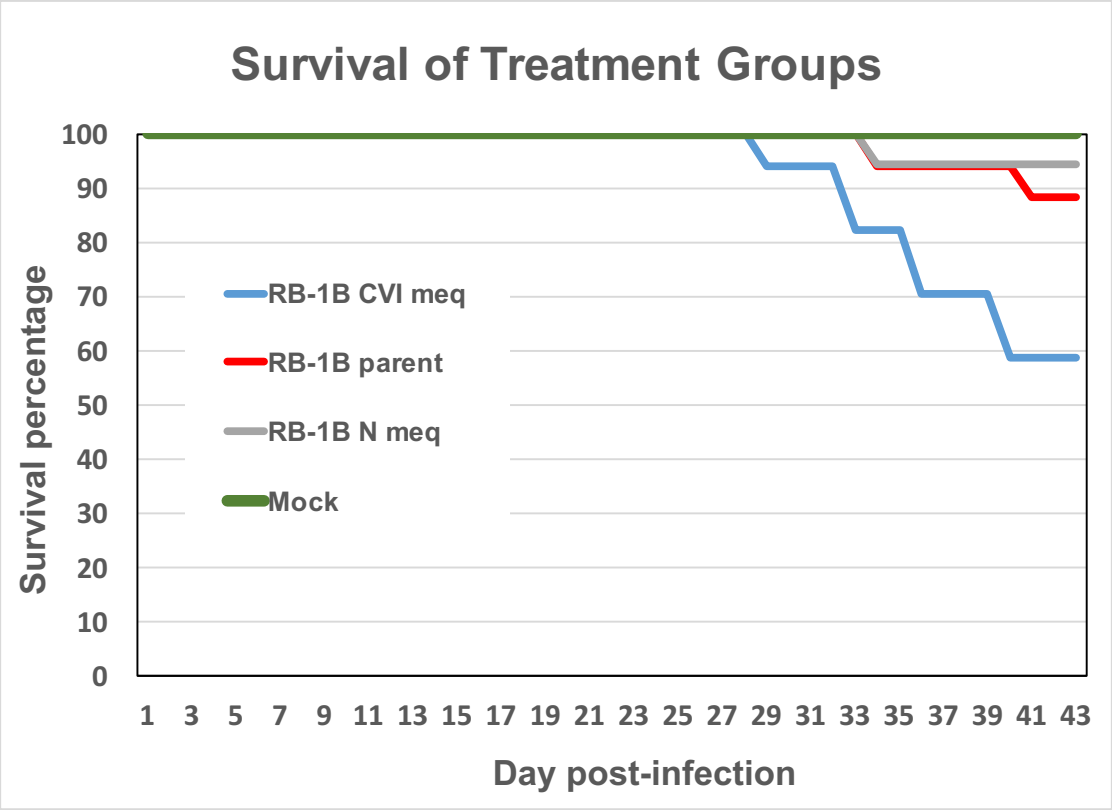


Figure 6. Comparison of Survival Percentage of the Recombinant MDVs. Survival data was estimated over the period of 42 days.

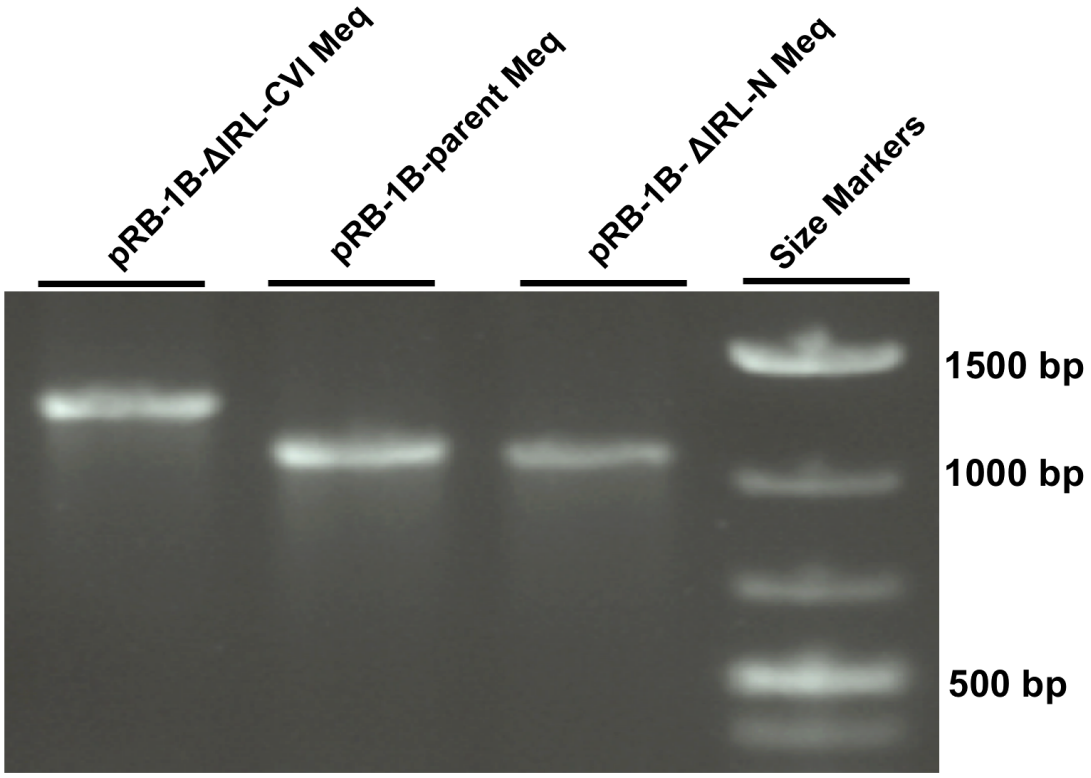
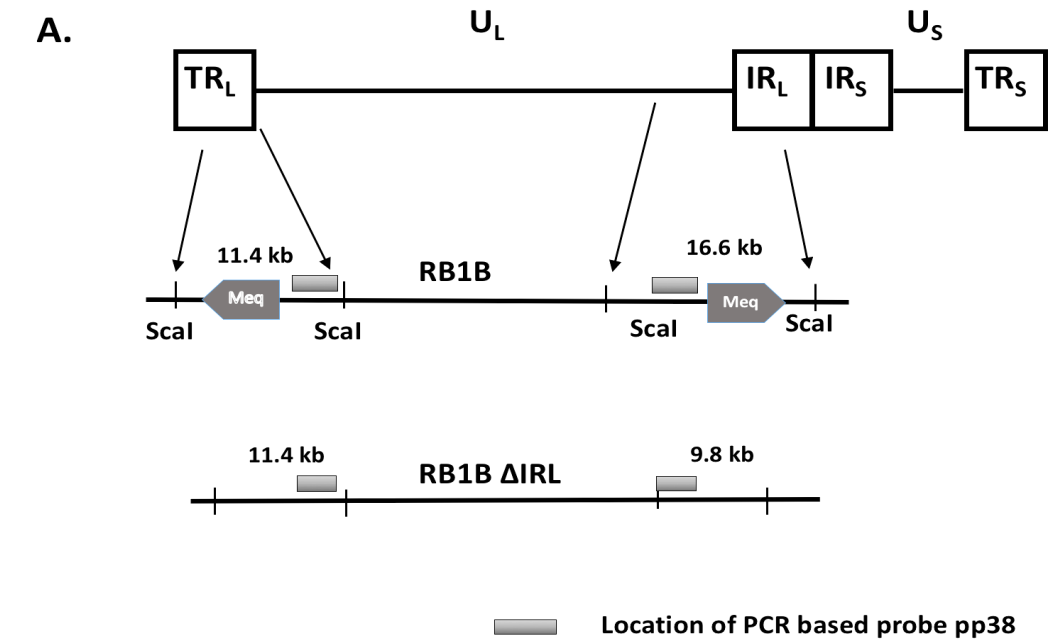
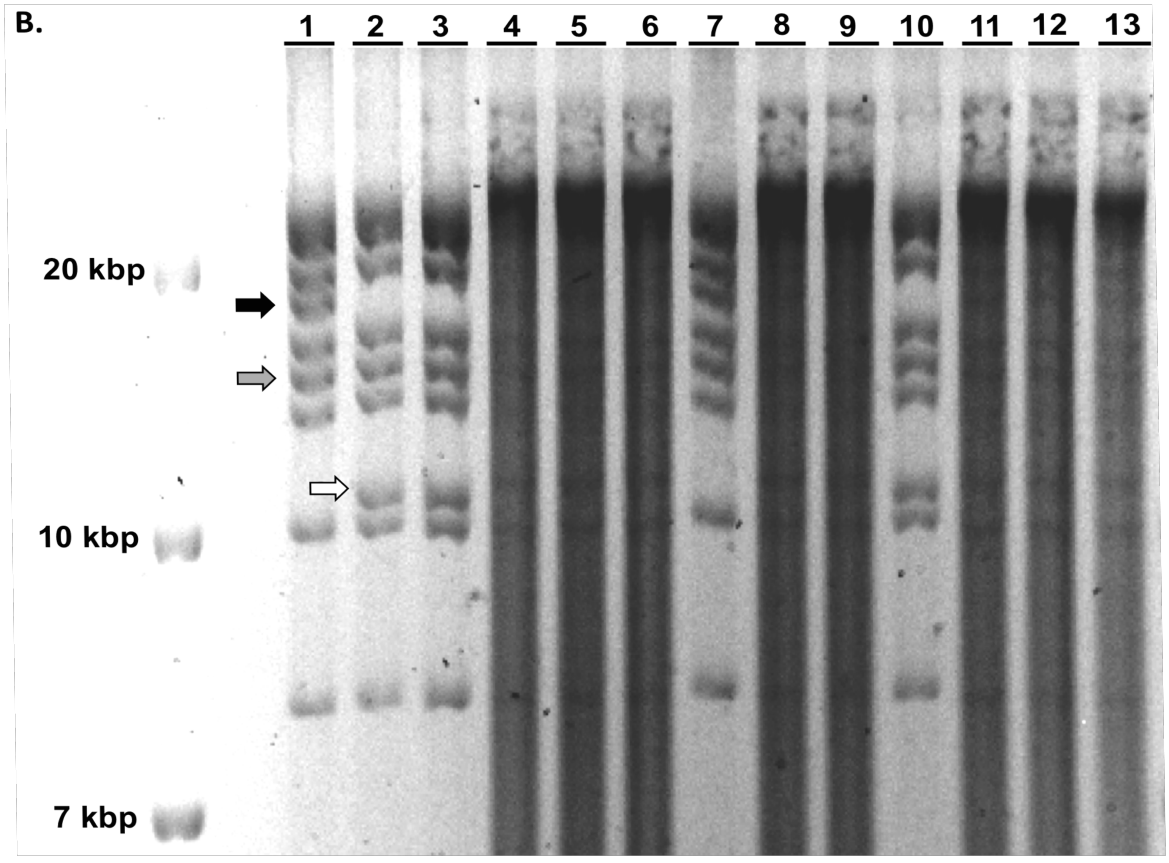


Figure 7. Gel picture of amplified *meq* genes from viruses. PCR of the DNA samples from VR2 SPC using *meq* primers gave the expected bands for the respective viral *meq* genes in a 0.8% agarose gel.


A.
U_L
U_S
TR_L
IR_L
IR_S
TR_S
11.4 kb
RB1B
16.6 kb
Meq
Meq
ScaI
ScaI
ScaI
ScaI
11.4 kb
RB1B ΔIRL
9.8 kb
Location of PCR based probe pp38


B.
1
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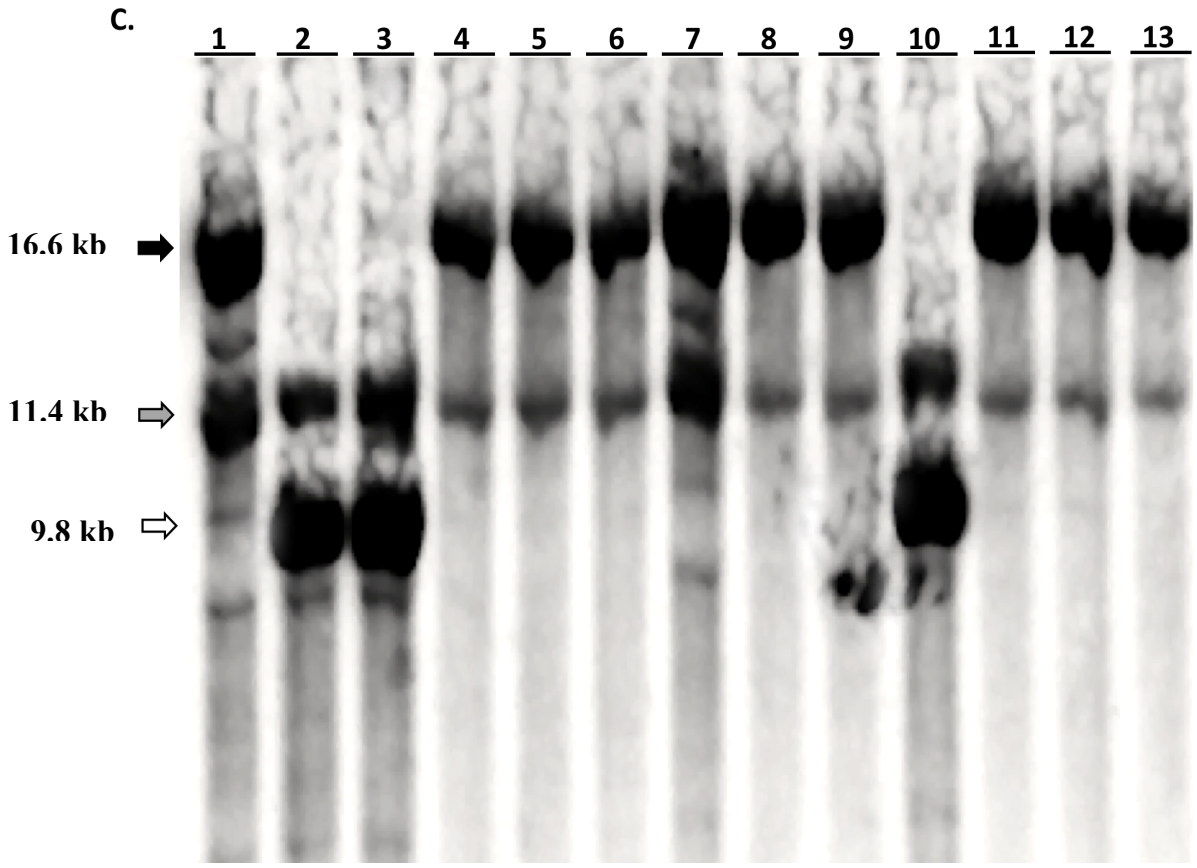

Figure 8. Structural confirmation of viruses via Southern blotting. A. MDV genome showing *meq* loci of original RB1B parent and the deleted IRL form of RB1B. Relative differences in the size of fragments as expected upon digestion with ScaI are shown.

B. Gel picture for the Southern blot. Arrow heads indicate fragments differing between the original and deleted IRL forms. Lane number in black represent different DNA samples loaded in the order: 1. RB1B parent BAC, 2. RB1B del IRL parent BAC, 3. RB1B del IRL CVI988 Meq BAC, 4. CVI988 Meq-infected CEF DNA, 5. CVI988 Meq-infected SPC DNA, 6. UD39 (CVI988 Meq) cell line DNA, 7. RB1B

parent BAC, 8. RB1B del IRL infected CEF DNA, 9. RB1B del IRL infected SPC DNA, 10. RB1B del IRL N Meq BAC, 11. RB1B del IRL N Meq infected DNA, 12. RB1B del IRL N Meq infected SPC DNA, 13. UD40 (N Meq) cell line DNA. Size markers (1 kb plus DNA ladder, Thermo Scientific) are in far left and right lanes.

C. Southern blot obtained after digestion with ScaI enzyme and hybridization with pp38 probe. Lanes numbered in black are in the same order as in B. Presence of both *meq* genes in different BAC viruses when compared to the original and del IRL forms is evident from Southern blot based upon pp38 probe.

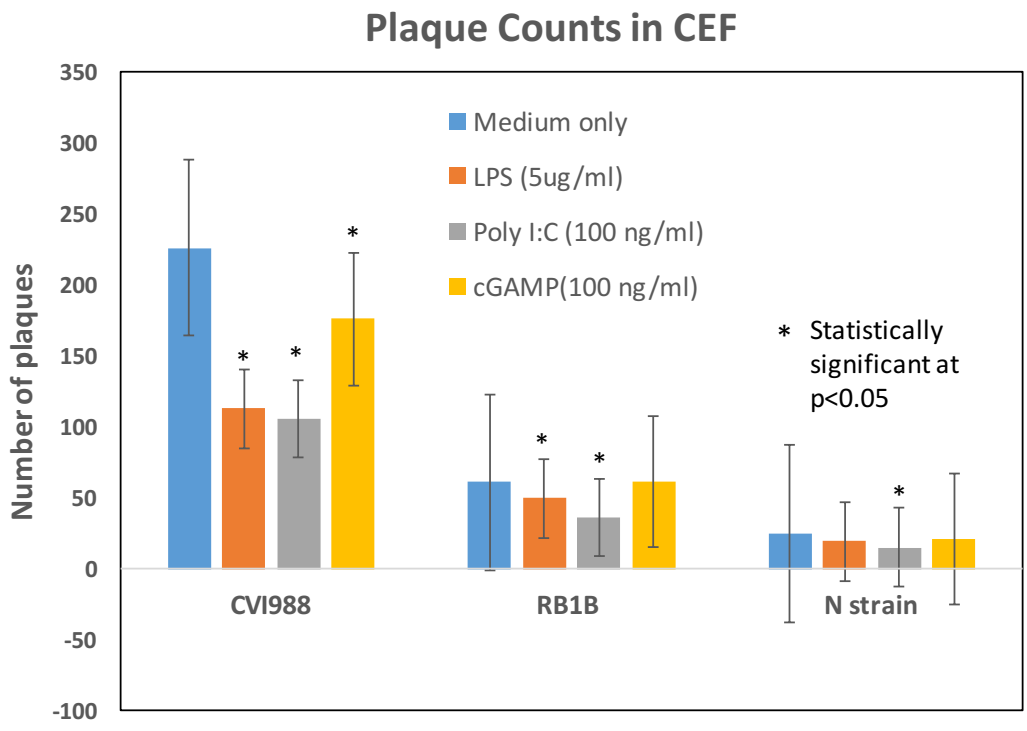

Figure 9. Plaque counts in CEF upon treatment with different agonists. The bar graph shows the effect on MDV plaque counts in CEF when treated with different agonists of innate signaling. Plaques were counted after 5 days of treatment when the viruses were allowed to replicate. Treatments include medium only as control, LPS (5 µg/ml) as TLR4 agonist, Poly I:C (100ng/ml) as TLR3 and MDA5 agonist and cGAMP as STING agonist. Astriks (∗) denote the significant decrease in the plaque number compared to the control for each virus at $p<0.05$.

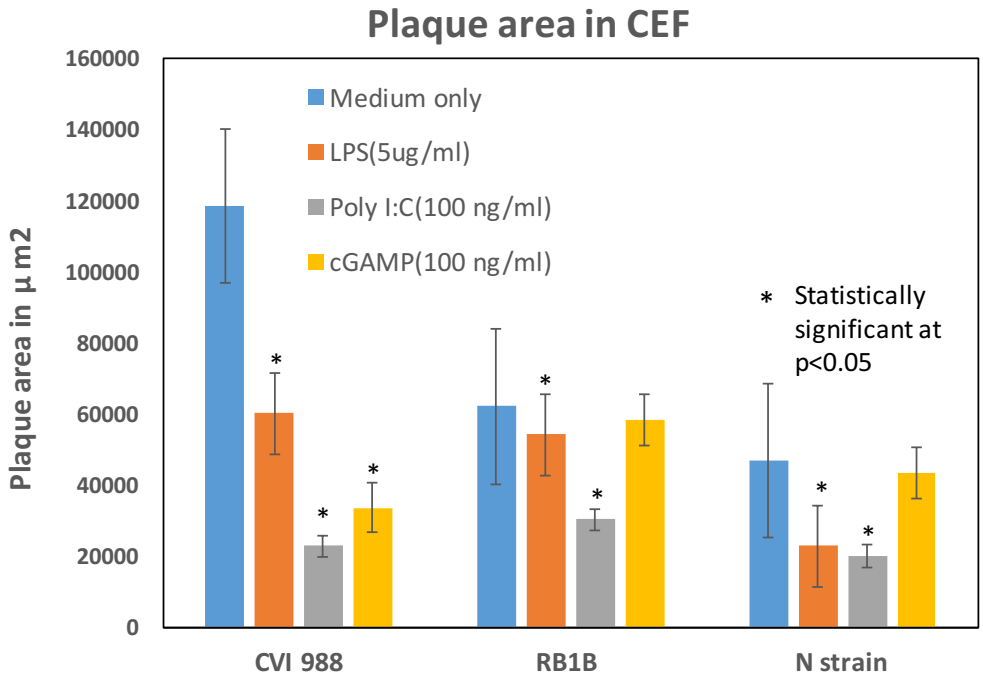

Figure 10. Plaque areas in CEF upon treatment with different agonists. The bar graph shows the effect on MDV plaque areas in CEF when treated with different agonists of innate signaling. Plaques areas were determined after 5 days of treatment when the viruses were allowed to replicate. Treatments include medium only as control, LPS (5 µg/ml) as TLR4 agonist, Poly I:C (100ng/ml) as TLR3 and MDA5 agonist and cGAMP as STING agonist. Astriks (∗) denote the significant decrease in the plaque area compared to the control for each virus at $p<0.05$.

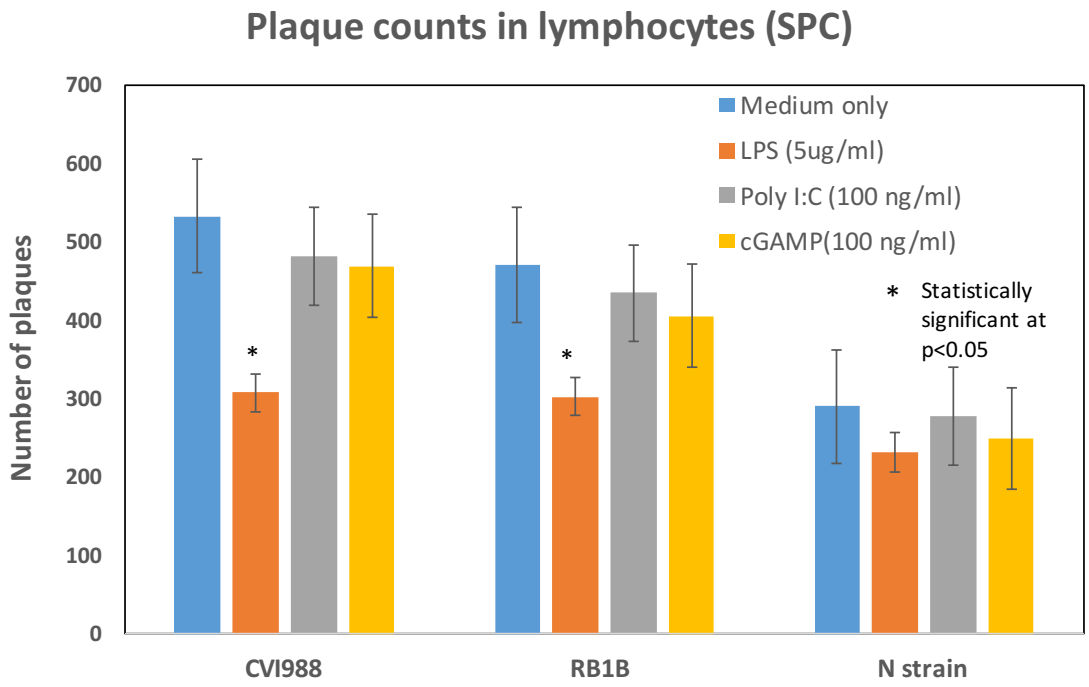

Figure 11. Plaque counts in spleen cells (SPC) upon treatment with different agonists. The bar graph shows the effect on MDV plaque counts in SPC when treated with different agonists of innate signaling. Plaques were counted after 5 days of treatment when the viruses were allowed to replicate. Treatments include medium only as control, LPS (5 µg/ml) as TLR4 agonist, Poly I:C (100ng/ml) as TLR3 and MDA5 agonist and cGAMP as STING agonist. Astriks (∗) denote the significant decrease in the plaque number compared to the control for each virus at $p<0.05$

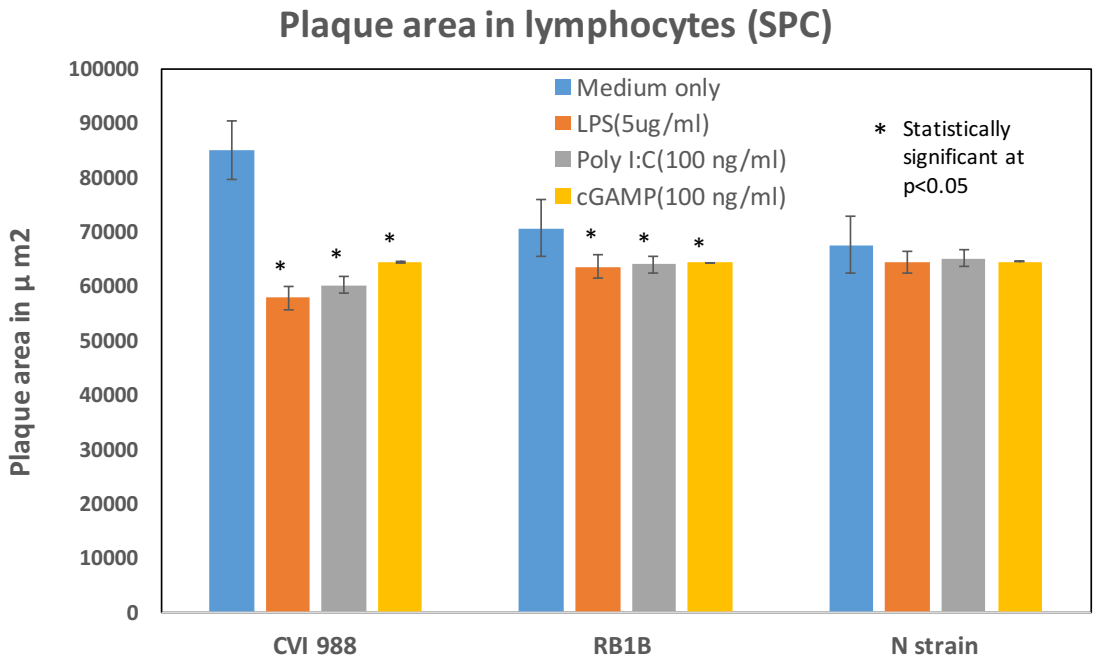

Figure 12. Plaque areas in spleen cells (SPC) upon treatment with different agonists. The bar graph shows the effect on MDV plaque areas in SPC when treated with different agonists of innate signaling. Plaques areas were determined after 5 days of treatment when the viruses were allowed to replicate. Treatments include medium only as control, LPS (5 µg/ml) as TLR4 agonist, Poly I:C (100ng/ml) as TLR3 and MDA5 agonist and cGAMP as STING agonist. Astriks (∗) denote the significant decrease in the plaque area compared to the control for each virus at $p<0.05$.

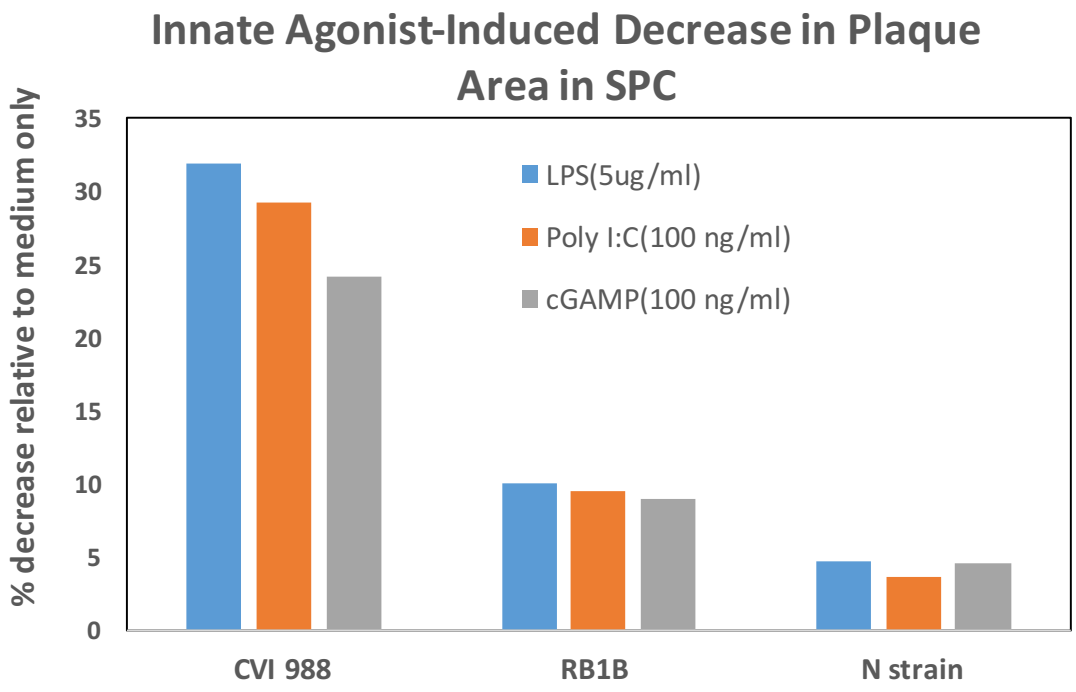

Figure 13. Innate agonist-induced decrease in plaque area in lymphocytes. Agonist treatments include LPS (5 µg/ml) as TLR4 agonist, Poly I:C (100ng/ml) as TLR3 and MDA5 agonist and cGAMP as STING agonist. Comparisons were made with the medium only control.

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Appendix A

AACUC APPROVAL FORM

UNIVERSITY OF DELAWARE
COLLEGE OF AGRICULTURAL & NATURAL RESOURCES
AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Continuing Approval of Protocol
for Use of Agricultural Animals in Research and Teaching

AACUC Protocol Approval Number: _ (22) 09-30-14R (3 year), UBC Approval #: 13-021 (3 year)

Please check appropriate box: Teaching/Outreach ☐ Research ☒

Proposal Title: Pathogenicity of Recombinant Marek's Disease Viruses (rMDVs)

Instructor/Principal Investigator: Mark S. Parcells, Ph.D.

Co-investigator(s) if applicable: none

Start Date: May 21, 2015 End Date: July 2, 2015

Description of Animals: Common Name: chickens (SPF)

Estimated Number: 335 (max) Breed: SCWL

Source: Sunrise Farms, Catskill, NY

Are all proposed animal care management procedures and experimental procedures (surgical, manipulative methods) defined as "pre-approved" by the Animal Care and Use Committee?

yes X no _____ To be Determined by the AACUC _____

If not, or you are not sure, or if you are making major modifications to your animal protocols or facilities, you will have to fill out a new Application for Use of Agricultural Animals at:
http://ag.udel.edu/research/aacuc/index.html

If responsibilities beyond standard animal management practices are required of animal care personnel as part of the proposed project, please attach a description of those special duties.

Mark S. Parcells 5/7/15
Signature, Instructor Principal Investigator Date

- -

Proposal Approved X Proposal Disapproved _____

Reason for Disapproval _____

Robert A. Ogden 5-19-15
Signature, Animal Care and Use Committee Date

Appendix B

UBC APPROVAL FORM

Registration #: 13-021

University of Delaware
Department of Environmental Health & Safety
Recombinant DNA Registration

Directions: Please complete this form to register recombinant DNA research with the University Biosafety Committee (UBC) as required by the most current "Guidelines for Research Involving Recombinant DNA Molecules" (NIH *Guidelines*) and University Policy 7-19.

Submit a separate form for each project. **A copy of the current *Guidelines* is available at the EHS web site: http://www.udel.edu/ehs/. For questions, please contact the Biosafety Officer at 831-8475.**

Section I- to be completed for all projects

Principal Investigator: Mark S. Parcells, Ph.D.
Department: Animal and Food Sciences (Primary), Biological Sciences (Joint)
Address: 052 Townsend Hall
Phone Number: (302) 831-0114 Fax: (302) 831-2822
Email: Parcells@UDel.edu
Labs to be used: 310 and 313 Worrilow Hall, Allen Laboratory
For exempt work: General Work Description: General cloning and molecular biological procedures
For non-exempt (covered) work: Project Title: Construction and Characterization of Recombinant Marek's Disease Viruses (rMDVs)
Proposed start date for research: 10/1/2013
Your signature below indicates that you acknowledge all requirements and restrictions of the most current NIH *Guidelines* for the biosafety level you have indicated, unless modified by the UBC, that you accept responsibility for the safe conduct of the experiments conducted at this biosafety level and that you have informed all associated personnel of the conditions required for this work. It is the Principal Investigator's responsibility to follow the NIH *Guidelines* and notify the Biosafety Officer and the UBC of any adverse events, including research-related accidents and illnesses. The Principal Investigator certifies that the work description is accurate. Any work performed which is not approved under this permit may be subject to the loss of grant funds. This registration must be updated annually. Signature of Investigator: Mark S. Parcells Date: 9/5/13

Section II- to be completed for all projects

Check the appropriate registration category for experiments covered by the NIH Guidelines:
All categories are defined in the NIH Guidelines

A. Experiments which are exempt and do not require registration.
☐ Examples include rDNA that is: not in organisms and viruses; entirely DNA segments from a single nonchromosomal or viral DNA source; entirely from a prokaryotic host including its indigenous plasmids or viruses when propagated only in that host or when transferred to another host by well established physiological means; entirely from a eukaryotic host when propagated only in that host or a closely related strain of the same species; entirely segments from different species that exchange DNA by known physiological processes; or not a significant risk to health or the environment.
If work is exempt, attach a description of the recombinant DNA procedures to be performed.

B. Experiments that Require IBC Approval, Recombinant DNA Advisory Committee Review, and NIH Director Approval Before Initiation.
☐ Deliberate transfer of a drug resistance trait to a microorganism that is not known to acquire the trait naturally, if such acquisition could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture

C. Experiments that Require NIH/ORDA and IBC Approval Before Initiation.
☐ Cloning of toxin molecules with LD_{50} of less than 100 nanograms per kilogram body weight

D. Experiments that Require IBC Approval, Human Subjects Approval, and NIH/ORDA Registration Before Initiation. Submit completed Appendix M, I-V from the NIH *Guidelines* along with this document.
☐ Deliberate transfer of recombinant DNA or DNA or RNA derived from recombinant DNA into one or more human subjects (human gene transfer)

E. Experiments that Require IBC Approval Before Initiation
☐ 1. Experiments using Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents as Host-Vector Systems
☐ 2. Experiments in which DNA from Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents is cloned into nonpathogenic prokaryotic or lower eukaryotic Host-Vector Systems
X 3. Use of infectious DNA or RNA viruses or defective DNA or RNA viruses in the presence of helper virus in tissue culture systems
X 4. Experiments involving recombinant DNA in animals or transgenic whole animals
☐ 5. Experiments involving whole plants, to include exotic infectious agents that may impact ecosystems, transmissible exotic infectious agents in the presence of their specific arthropod vectors, sequences encoding vertebrate toxins introduced into plants or associated organisms, or microbial pathogens of insects/animals associated with plants if microorganism may impact ecosystem
☐ 6. Experiments involving more than 10 liters of culture

F. Experiments that Require IBC Notice Simultaneous with Initiation
☐ 1. Formation of recombinant DNA molecules containing no more than two-thirds of the genome of any eukaryotic virus in tissue culture with no helper virus
☐ 2. Recombinant DNA modified plants that are noxious weeds or can interbreed with noxious weeds. Plants associated with recombinant DNA modified non-exotic microorganisms which have the potential for serious impact on ecosystems. Recombinant DNA modified arthropods or small animals associated with plants if these materials have no serious impact on ecosystems
☐ 3. Experiments involving recombinant DNA modified whole plants or organisms (if not included in Category E5 above)
☐ 4. Generation of transgenic rodents where genome is altered by stable introduction of rDNA into germ line, if it requires only BSL1 containment

Section III- to be completed for covered (non-exempt) projects only

1. Names of individuals participating in project, with job title: Dr. Mark S. Parcells (Professor, PI), Phaedra Tavlarides-Hontz (Research Associate I), Upendra K. Katneni (PhD Student), Sabari Nath Neerukonda (PhD Student), Wachen Peters (MS Student), Juliana Rojas-Amortegui (MS Student), Nicholas Siano (MS Student)

2. Source(s) of DNA/RNA sequences (include genus, species, gene name and abbreviation): Marek's Disease Virus, MDV (gallid herpesvirus 2, *Mardivirus* 1)

3. Is a vector required? **Yes** ☐ **No X**

If yes, identify specific phage, plasmid, or virus:

Virus vector: Adenovirus ☐ Retrovirus ☐ Other ☐

Defective: **Yes** ☐ **No** ☐

Replication competent: **Yes** ☐ **No** ☐

If viral vector, what percent of the viral genome remains?

4. If the recombinant contains viral DNA, does the insert represent more than 2/3 of the viral genome? **Yes X No** ☐

5. Is a helper virus required? **Yes** ☐ **No X** If yes, specify:

6. What is the biological activity of the gene product or sequence inserted? These strains will be bacterial-artificial chromosome (BAC)-based infectious clones of MDV (strains GA-22, RB-1B, CVI988, 686 and TKing). These will be used for (1.) basic pathogenesis studies (GA-22, RB-1B, 686, and TKing), (2.) vaccine studies (CVI988 and derivatives). Genes to be introduced are mutated versions of MDV genes (*meq*, glycoprotein L), or immune stimulatory genes (IFN-α, IFN-β, IL-1β, IL-18) under the control of keratinocyte-specific promoters.

7. Will a deliberate attempt be made to obtain expression of the foreign gene encoded in the recombinant DNA? **Yes X No** ☐

8. Host strain for propagation of the recombinant (give genus, species, and parent strain): *E. coli,* DH10β and derivatives (GS1783)

9. Target recipient of recombinant DNA (indicate species or cell lines used):

Animals: **Gallus Gallus (chickens)** Tissue Culture: **chicken embryo fibroblasts (CEF)**

Plant cells: Plants:

Gene therapy:

Specify target host(s) - human, animal species:

10. Proposed biosafety level for project (check one): 1 ☐ 2 **X** 3 ☐

11. Have all personnel involved in this project been trained to the appropriate biosafety level? **Yes X No** ☐

12. Dual Use Research- Check any categories below that apply to your project:

☐ Renders a useful vaccine ineffective
☐ Adds antibiotic resistance affecting response to a clinically useful drug
X Enhances pathogen virulence ***(non-mammalian pathogen – see description)***
X Increases pathogen transmissibility ***(non-mammalian pathogen – see description)***
☐ Widens a pathogen's host range

☐ Enables a pathogen to evade diagnostic or detection modalities ☐ Weaponization (e.g. environmental stabilization of pathogens) ☐ None of the above
13. **Be sure to attach a description of the recombinant DNA procedures to this form.** Include the following items: nature and purpose of the project; outline the procedures and techniques; risk to personnel; practices/equipment/facilities to protect the personnel; methods to inactivate and dispose of the agents. Sufficient detail must be provided to understand the project and review the rDNA procedures.

Section IV- For UBC Use Only

☐ Project/work exempt from recombinant DNA NIH *Guidelines*. (**Make sure Work Description is attached**).
☒ Project/work requires registration according to NIH *Guidelines*. The PI and staff can safely perform this work with the training, work practices, and lab facilities listed.
The following signatures indicate provisional approval by the University Biosafety Committee for this project involving recombinant DNA technology. The work is to be performed according to NIH requirements. Final approval for projects that are NOT exempt from the NIH *Guidelines* will not be granted until after review by the entire UBC at the next meeting. Non-exempt work covered under this approval cannot begin until final approval is received. **UBC Member Conducting Review** Print Name: Erin Brannick Signature: Erin Brick Date: 9/9/13 **Biosafety Officer** Print Name: Krista Murray Signature: Krista Murray Date: 9/11/13 Expiration date: 9/11/16 Final UBC approval date: UBC Representative Signature:
UBC Comments: