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Immune responses against Marek's disease virus

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Abstract

It is more than a century since Marek's disease (MD) was first reported in chickens and since then there have been concerted efforts to better understand this disease, its causative agent and various approaches for control of this disease. Recently, there have been several outbreaks of the disease in various regions, due to the evolving nature of MD virus (MDV), which necessitates the implementation of improved prophylactic approaches. It is therefore essential to better understand the interactions between chickens and the virus. The chicken immune system is directly involved in controlling the entry and the spread of the virus. It employs two distinct but interrelated mechanisms to tackle viral invasion. Innate defense mechanisms comprise secretion of soluble factors as well as cells such as macrophages and natural killer cells as the first line of defense. These innate responses provide the adaptive arm of the immune system including antibody- and cell-mediated immune responses to be tailored more specifically against MDV. In addition to the immune system, genetic and epigenetic mechanisms contribute to the outcome of MDV infection in chickens. This review discusses our current understanding of immune responses elicited against MDV and genetic factors that contribute to the nature of the response.

Keywords: immune response, Marek's disease, vaccine, chickens, herpesvirus, genetic resistance

Introduction

The disease condition in chickens, which was first reported as polyneuritis by Joseph Marek, in 1907, is named Marek's disease (MD) (Marek, 1907; Biggs, 2001). MD caused significant damage to the poultry industry across the world in the 1960s. However, due to the introduction of effective vaccines, the impact of this disease on the poultry industry is significantly reduced. Nevertheless, it is estimated that the annual worldwide losses associated with MD are US$1–2 billion (Morrow and Fehler, 2004). These losses are due to carcass condemnation or immunosuppression and the ensuing secondary infections.

The agent that causes MD is a herpesvirus (MD virus or MDV), which belongs to the subfamily *Alphaherpesvirinae*. There are several species in this subfamily, including MDV (gallid herpesvirus type 2 (GaHV-2)), GaHV-3 (MDV-2) and turkey herpesvirus (meleagrid herpesvirus type 1 (MeHV-1)) (Osterrieder and Vautherot, 2004). Based on the 'Cornell model' (Calnek, 1986), MDV pathogenesis encompasses four phases in the host. During the first phase, also known as the early cytolytic phase, B cells undergo cytolysis between 2 and 7 days post-infection (dpi). Activated T cells become infected during this period and MDV becomes latent around 7 to 10 dpi in these cells. At about 18 dpi, depending on the pathotype of the virus and genotype of the host, infected $CD4^+CD8^-$ T cells may undergo transformation, but cytolysis might occur in this phase as well (Calnek, 2001; Baigent and Davison, 2004). Transformation leads to development of lymphomas and cytolysis results in immunosuppression (Calnek, 2001). The lymphomatous lesions result in blindness, paralysis and death (Calnek, 2001). Some infected chickens may also suffer from transient paralysis (TP) which is due to vasculitis that

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leads to brain edema and, subsequently, flaccid paralysis (Schat and Nair, 2008).

In response to MDV infection, both non-specific (innate) and specific (adaptive) host responses are elicited. Innate defense mechanisms emerge soon after infection, whereas adaptive immune responses are usually detectable around 5 to 7 dpi and include the development of MDV-specific antibodies and cytotoxic T lymphocytes (CTL) (Davison and Kaiser, 2004). In addition to the above responses, cytokines are involved in the orchestration of both arms of the immune system.

Innate defense mechanisms

Upon infection of chickens by MDV, host innate responses are elicited, including activation of macrophages and natural killer (NK) cells, secretion of type I interferons (IFNs) and pro-inflammatory cytokines. In addition, other components of the innate immune system may be triggered, such as Toll-like receptors (TLRs) and antimicrobial peptides (AMPs) (Akbari *et al.*, 2008; Abdul-Careem *et al.*, 2009), although the role of TLRs and AMPs in induction of immune responses to MDV needs further investigation.

Macrophages have phagocytic, microbicidal and tumoricidal functions. They can also control the outcome of the adaptive immune response by serving as antigen presenting cells (APCs) (Qureshi *et al.*, 2000). After being activated, macrophages exert their role in defense mechanisms by internalizing the pathogen via phagocytosis and the release of various mediators such as nitric oxide (NO) and cytokines. It is hypothesized that macrophages may transport MDV from the respiratory site of infection to primary lymphoid organs including the bursa of Fabricius (Calnek *et al.*, 1970; Schat *et al.*, 1982). Barrow *et al.* (2003) detected very virulent (vv) MDV in macrophages and suggested that the virus may actually replicate in these cells as well as being transferred from the lungs to the bursa of Fabricius. Abdul-Careem *et al.* (2008a) also reported an infiltration of macrophages in the bursa during the early stages post-infection suggesting a role for macrophages in virus distribution. Despite the role of macrophages in transfer of MDV to lymphoid tissues, these cells do not appear to play a part in transfer of the virus to the feather follicle epithelium (FFE), in which the virus can replicate and produce infective particles (Calnek *et al.*, 1970; Johnson *et al.*, 1975).

Production of NO by activated macrophages is an important innate response, critical for bactericidal activity of macrophages and inhibition of viral replication (Xing and Schat, 2000a, b; Bogdan, 2001). MDV infection induces the expression of inducible NO synthase (iNOS) resulting in increased production of NO, which may inhibit MDV replication (Xing and Schat, 2000a, b; Djeraba *et al.*, 2002; Jarosinski *et al.*, 2002). Up-regulation of iNOS by viruses has been linked to the production of pro-inflammatory cytokines such as IFN gamma (IFN-γ). Associations between NO and IFN-γ expression in various tissues have been well documented after MDV infection (Xing and Schat, 2000a, b; Kaiser *et al.*, 2003; Abdul-Careem *et al.*, 2007, 2008a). However, Jarosinski *et al.* (2005) found that a strong pro-inflammatory response with high levels of NO production could lead to central nervous system signs in genetically resistant lines infected with vv+ strains of MDV. Recently, Buscaglia *et al.* (2009) reported that genetic selection for increased levels of NO production increased MD incidence in a pure broiler breeder line.

NK cells act as a first line of defense by inducing rapid cell death in their targets, such as virus-infected or tumor cells, by a serine protease and a pore-forming protein, granzyme and perforin, respectively. Although NK cells have not yet been fully characterized in chickens and cell markers have not been completely established, studies in the early 1980s have shown an increase in activity of NK-like cells after infection in both resistant and infected chicken and after vaccination (Sharma and Okazaki, 1981; Heller and Schat, 1987). Expression levels of mRNA of perforin, granzyme A and NK-lysin, an AMP from CTL and NK-like cells, were shown to be up-regulated at 4 and 7 dpi in infected birds compared to uninfected birds (Sarson *et al.*, 2008a).

In chickens, there are three groups of type I IFNs that have been identified, IFN-α, -β and -λ (Kaiser *et al.*, 2005). IFN-α and -β have antiviral activity and when secreted, they act as potent regulators of the innate immune system particularly through the enhancement of NK cell cytotoxicity (Biron, 1998). Treatment of chicken embryo cell cultures with recombinant chicken IFN-α (rChIFN-α) inhibited replication of the vv MDV RB-1B strain *in vitro*, and oral treatment of chickens with rChIFN-α reduced MDV R2/23 replication *in vivo* (Jarosinski *et al.*, 2001). Transcripts of IFN-α and IFN-β genes are present in virus-infected chicken cells suggesting their roles in host response to viral infection. Signal transducers and activators of transcription (STAT)1 and 2 heterodimers, a family of signal transduction molecules, together with IFN response factor (IRF)-9 are involved in type I IFN signalling. The involvement of the signalling pathway in immunity to MD can be elucidated from the findings of Sarson *et al.* (2008b), where a higher expression of STAT2 in resistant birds was seen at 4 dpi as compared to genetically susceptible birds in which the expression was only up-regulated at later stages of viral pathogenesis, for example at 14 and 21 dpi, when the second phase of cytolysis or transformation begins.

TLRs play an important role in recognition of pathogens, including viruses, by activating intracellular signaling pathways that initiate production of various cytokines including IFNs. TLRs recognize pathogen associated molecular patterns (PAMPs), such as double-stranded (ds) or single-stranded (ss) RNA and unmethylated CpG DNA, as well as viral proteins. TLRs (1, 2, 3, 4, 5, 7, 9, 15 and 21)

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have been characterized in chickens (Fukui *et al.*, 2001; Kogut *et al.*, 2005; Boyd *et al.*, 2007; Jenkins *et al.*, 2009), but information on the interaction of MDV-encoded molecular patterns and TLRs that recognize these PAMPs are not available. In mammals, antiviral responses primarily involve TLR3, 7, 8 and 9. Using polyI:C, lipopolysaccharide (LPS), ssRNA, and oligodeoxynucleotides (ODN) which are specifically recognized by TLR3, TLR4, TLR7/8 and TLR9, Schwarz *et al.* (2007) showed that these ligands induce substantial amounts of type I IFN and interleukin-6 (IL-6) in freshly prepared chicken splenocytes. When expressed in human 293 cells, chicken TLR3 strongly responded to polyI:C demonstrating that it recognizes the same ligand as the mammalian TLR3. The involvement of TLR3 in antiviral immune responses was also shown by the up-regulation of chicken TLR3 and IFN-β expression during infection with H5N1 virus. In addition, IFN-α and -β readily induce expression of TLR3 (Karpala *et al.*, 2008). It remains to be seen what, if any, interactions exist between MDV and these host molecules. However, we have recently observed a strong positive correlation between MDV replication in respiratory mucosa and expression of TLR3 in this tissue (M. F. Abdul-Careem, unpublished observations). This observation raises the possibility that MDV-derived PAMPs may be recognized by chicken TLRs.

AMPs possess antiviral activities against various viruses including herpesviruses (Carriel-Gomes *et al.*, 2007) and play a significant role in host innate immunity. AMPs are divided into two main groups: defensins and cathelecidins. In addition to their direct antimicrobial activities, defensins have a wide spectrum of other immunological functions such as a chemoattraction, induction of dendritic cell maturation and polarization of effector T cells (Selsted and Ouellette, 2005). The role of defensins in immunity against viruses, especially against enveloped viruses is known (Ganz, 2003). However, the importance of these molecules against viral infections in chickens, including MDV, has not been explored.

Adaptive immune responses

Adaptive immune responses, which by nature are antigen-specific, encompass secretion of antibodies against various MDV proteins by plasma cells, in addition to the responses mounted by $CD4^+$ T helper (Th) or $CD8^+$ CTL against virus-infected or tumor cells (Kindt *et al.*, 2007).

Antibodies are produced against a wide range of MDV proteins such as glycoprotein (g)B, gE and gI among which anti-gB neutralizing antibodies have a known protective role via blocking virus entry into host cells (Churchill *et al.*, 1969a; Chen and Velicer, 1992; Schat and Markowski-Grimsrud, 2001). Because MDV is a highly cell-associated virus, antibody-mediated immune responses are regarded as not being as important as T cell-mediated responses. However, antibodies can play a role in the establishment of immunity against MD (Davison and Kaiser, 2004). For instance, presence of maternal antibodies reduced clinical signs of MD, tumor formation, morbidity and mortality, although it also interfered with vaccination against MD, especially in the case of cell-free vaccines (Calnek, 1982).

Non-neutralizing antibodies were proposed to coat infected cells and abrogate cell-to-cell spread of the virus. These antibodies might induce antibody-dependent cell-mediated cytotoxicity (ADCC) that further aids the lysis of MDV-infected cells (Schat and Markowski-Grimsrud, 2001). It was also demonstrated that MD lymphomas express Hodgkin's disease antigen CD30 and that anti-CD30 antibodies develop after challenge with MDV in genetically resistant chickens (Burgess *et al.*, 2004). The latter observation raises the possibility that some antibodies against self-antigens may be involved in protection (Burgess *et al.*, 2004).

Following the induction of innate defense mechanisms and alongside the antibody-mediated immune responses, $CD8^+$ CTL responses against various envelope glycoproteins of herpesviruses in mammals and in avian species play an essential role in the control of herpes virus infection (Mester *et al.*, 1990; Mester and Rouse, 1991; Omar and Schat, 1996; Schat and Xing, 2000; Markowski-Grimsrud and Schat, 2002). Lymphocytes derived from MDV-infected chickens inhibited plaque formation in chicken kidney cells (CKC) infected with MDV (Ross, 1977) and the absence of $CD8^+$ T cells compromised immunity conferred by MD vaccines (Morimura *et al.*, 1997). The phenotype of CTL in avian species was determined to be $CD3^+CD4^-CD8^+$ TCRαβ1 T cells, in a series of *in vitro* assays in which reticuloendotheliosis virus (REV)-infected target cells were lysed in an major histocompatibility complex (MHC) class I-restricted manner by effector cells obtained from REV-infected chickens (Maccubbin and Schierman, 1986; Lillehoj *et al.*, 1988; Weinstock *et al.*, 1989; Merkle *et al.*, 1992). The same phenotype of cytotoxic T cells was reported in chickens challenged with a non-oncogenic vaccine strain of MDV (Omar and Schat, 1997).

To further elucidate the role of CTL in eliciting protective immune response against MDV, REV-transformed chicken cell lines (RECC) with defined MHC haplotypes were developed and transfected with genes encoding various MDV proteins (Pratt *et al.*, 1992; Schat *et al.*, 1992; Uni *et al.*, 1994; Omar and Schat, 1996; Schat and Xing, 2000; Markowski-Grimsrud and Schat, 2002). Then, syngeneic lysis of RECC transfected with coding sequences for MDV gB, pp38, meq and ICP4 by effector cells obtained from spleens of chickens infected with JM16 (a virulent strain of GaHV-2 or MDV-1), SB-1 (GaHV-3 or MDV-2) as well as herpesvirus of turkey (HVT) (MeHV-1 or MDV-3) was assessed using chromium release assays. gB induced the strongest lysis in all three infection groups while meq elicited the weakest lysis compared to other

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proteins in the first two groups (Uni *et al.*, 1994; Omar and Schat, 1996). Splenocytes isolated from SB-1 and HVT-immunized chickens induced a CTL response against pp38-transfected cells due to the fact that both viruses encode a homologue of pp38 which was previously believed to be a GaHV-2-specific protein (Cui *et al.*, 1991; Chen *et al.*, 1992; Ono *et al.*, 1995; Smith *et al.*, 1995). The epitope of gB recognized by CTL was mapped to the carboxyl-terminal 100 amino acids (Schat and Xing, 2000). The protective role of CTL response against MDV was further confirmed by immunizing chickens with a recombinant fowlpoxvirus expressing gB (rFPV-gB). Vaccination with rFPV-gB elicited neutralizing antibodies as well as a $CD8^+$ $TCR\alpha\beta1^+$ CTL response that protected chickens against challenge with virulent strains of MDV, including RB1B and GA (Nazerian *et al.*, 1992; Omar *et al.*, 1998). Additional evidence to support the involvement of CTLs was provided by Sarson *et al.* (2008a) who demonstrated that the expression of perforin and granzyme A was up-regulated at 4 and 7 dpi in spleens of MDV-infected chickens.

CTL responses play a pivotal role in genetic resistance to MD as well. RECC lines with $B^{19}B^{19}$ and $B^{21}B^{21}$ haplotypes were transfected with MDV ICP4 and viral glycoproteins C, D, E, I, K, L and M. CTL responses by MDV-stimulated syngeneic splenocytes from the resistant line against ICP4, gC, gK, gH, gL and gM were detected which were not present in splenocytes from the susceptible line (Omar and Schat, 1996; Markowski-Grimsrud and Schat, 2002). $CD4^+$ helper cells, as mentioned previously, are target cells for transformation (Calnek, 2001). Further research is needed to map MHC-II-restricted antigenic epitopes of various MDV proteins and elucidate their roles in the initiation of immune response via $CD4^+$ T cells as well as the differences among epitopes that might play a role in the genetic resistance versus susceptibility to MD.

Cytokine and chemokine production in response to MDV

Cytokines are important mediators that are involved in induction and regulation of immune responses to infection and are secreted by numerous cell types, including NK cells, DCs, T cells, B cells, cells of the monocyte/macrophage lineage and cells that are not typically considered immune system cells, such as endothelial and epithelial cells. A complex milieu of cytokines coordinates innate defense mechanisms as well as adaptive immune responses against MD. Engagement of some of the innate receptors, such as TLRs, with PAMPs results in triggering of downstream pathways, including the IFN pathway. Interestingly, the expression of IRF-1, IRF-3 and IFN-inducible protein genes is altered following MDV or HVT infection of chicken embryo fibroblasts (CEF) (Morgan *et al.*, 2001; Karaca *et al.*, 2004). Activation of the IRF pathway leads to an antiviral response, mediated by type I IFNs, which are the main antiviral cytokines produced by the innate immune system (Mossman and Ashkar, 2005). In relation to MDV infection, the expression of IFN-α has been observed in MD susceptible chickens (Quéré *et al.*, 2005). In addition to the direct action of IFN-α against MDV replication, this cytokine enhances the activity of NK cells against MD tumor cells (Ding and Lam, 1986).

Several chemokines are relevant to innate host defenses in response to MDV infection in chickens. Buza and Burgess (2007) identified two chemokines, CXCL14 and RANTES, which are expressed in MD tumor cells. These two chemokines are involved in attracting monocyte/macrophages in mammalian species. IL-8 is another chemokine that acts as a chemoattractant for neutrophils in mammals (Baggiolini and Clark-Lewis, 1992). IL-8 is up-regulated in brains, spleens (Jarosinski *et al.*, 2005) and lungs (M. F. Abdul-Careem, unpublished observations) after MDV infection. MDV also encodes a homolog of chicken IL-8 (vIL-8) that may function as a chemoattractant for T cells facilitating MDV replication cycle (Liu *et al.*, 1999; Parcells *et al.*, 2001; Cui *et al.*, 2004).

Cytokines may be classified based on the cell type that secretes them and type of immune response that they drive. In general, cytokines are classified as pro-inflammatory, such as IL-1β, IL-6 and the IL-17 family, Th1 or type I, including IL-2, IFN-γ, IL-12, IL-15, IL-16 and IL-18, Th2 or type II, including IL-3, IL-4, IL-13 and regulatory, such as transforming growth factor beta (TGFβ) and IL-10 (Kaiser *et al.*, 2004; Giansanti *et al.*, 2006). In response to MDV infection, the expression of both type I and type II cytokines can be altered in the early cytolytic, latent and late cytolytic phases as well as the transformation phase and the expression of these cytokines may be detected in spleen, brain and blood.

Xing and Schat (2000b) studied the expression of cytokine genes in splenocytes following MDV infection *in vivo* and reported an up-regulation of IFN-γ, IL-1β, IL-2 and IL-8 genes. Similarly, an increase in the expression of IFN-γ gene was observed following MDV infection in splenocytes, predominantly at 7 dpi (Djeraba *et al.*, 2002). The expression of cytokine genes in relation to MDV genome load in splenocytes isolated from MD-resistant and -susceptible chickens has also been studied (Kaiser *et al.*, 2003). MDV genome accumulation in splenocytes was associated with the increased expression of cytokine genes, such as IFN-γ, IL-6 and IL-18. Of these cytokines, IL-6 and IL-18 were found to be associated with susceptibility rather than resistance to MD (Kaiser *et al.*, 2003). The work of Quéré and colleagues (2005) indicated that the expression of IFN-γ gene is influenced by the genetic background of chickens, although Kaiser *et al.* (2003) did not observe a differential IFN-γ response in susceptible and resistant chickens. The expression of cytokine genes in chicken spleen and brain could be influenced by the virulence of MDV (Jarosinski *et al.*, 2005). Although the expression of IFN-γ, IL-1β, IL-6 and

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IL-8 is up-regulated in response to MDV infection, only the expression of IFN-γ, IL-1β and IL-8 is differentially regulated by the genetic background of chickens (Jarosinski *et al.*, 2005). The latter study provides evidence that the virulence of MDV as well as the genetic background of the chicken influences the expression of cytokine genes in splenocytes. In an attempt to further underline the expression of cytokines from different cell populations, we have shown that there is a significant up-regulation in the expression of IFN-γ, IL-18 and IL-6 at 4 and 21 dpi in $CD4^+$ and $CD8^+$ T cell subsets (Parvizi *et al.*, 2009b). The outcome of the cytokine milieu was inclined toward the induction of type I immune response at 4 and 21 dpi (Parvizi *et al.*, 2009b).

The expression of Th2 (type II) cytokine genes, such as IL-4 and IL-13, is up-regulated in chickens in response to helminth infections (Degen *et al.*, 2005). Type II responses may also be elicited following MDV infection. Morgan *et al.* (2001) studied the expression of genes in CEF cells following infection with MDV and showed that the IL-13 receptor α chain gene is up-regulated early following infection. In a microarray experiment, GATA-3 was found to be up-regulated in spleens of MDV-infected chickens (Sarson *et al.*, 2006). GATA-3 is a transcription factor that regulates the expression of type II cytokines, including IL-4, IL-5 and IL-13 (Maneechotesuwan *et al.*, 2007). Along with these observations, the expression of IL-13 and IL-4 genes in response to MDV infection is increased during the cytolytic and latent phases of MDV infection (Heidari *et al.*, 2008). The expression of regulatory cytokines, specifically IL-10, is also enhanced in chickens with MD (Abdul-Careem *et al.*, 2007). These latter studies indicate that MDV can induce type II and regulatory cytokine profiles in the spleen. In support of these findings, the proteomic study conducted by Buza and Burgess (2007), using MDV-transformed cell lines, showed that cytokines, their receptors and transcription factors belonging to both type I (IL-12, IL-18, IRF-3 and IRF-4), type II (IL-4) and regulatory (IL-10 and IL-10Rα chain) cytokines are expressed by MDV transformed cells. Therefore, MDV may skew cytokine expression to type I, type II or regulatory depending on various phases of its replication cycle. In addition, the cytokine milieu may vary in a tissue- and MDV strain-dependent manner.

Cytokines expressed in response to MDV in the central nervous system have been studied in relation to viral replication and genome accumulation (Jarosinski *et al.*, 2005; Abdul-Careem *et al.*, 2006). Jarosinski *et al.* (2005) found a correlation between the expression of cytokine genes IFN-γ, IL-1β, IL-6 and IL-8 and virulence of MDV in brains of infected chickens. For example, vv+MDV strains such as RK-1 induced significantly higher cytokine expression in brain tissues than JM-16, a vMDV. Abdul-Careem *et al.* (2006) showed that chickens infected with vvMDV with clinical signs of TP had higher levels of IL-6, IL-12 and IFN-γ mRNA in their brain tissues than asymptomatic MDV-infected chickens. Overall, the above findings underscore the importance of cytokines not only in immunity against MDV but also in the pathogenesis of infection.

Genetic factors involved in the induction of the immune response to MDV infection in chickens

The observation that chicken lines may be selected for various degrees of MD resistance and susceptibility has been known for a long time (Biely *et al.*, 1933; Cole, 1968). MD might be one of the most distinct examples of the association of genetics and resistance to an infectious disease in livestock animals (Gavora and Spencer, 1979). Numerous studies have demonstrated a high degree of heritability of resistance phenotype against MD in chickens (Schat and Davies, 2000; Bacon *et al.*, 2001; Bumstead and Kaufman, 2004).

Although the mechanisms of genetic resistance to MD are still under active investigation, the most significant association has been observed between chicken MHC and disease resistance (Bacon *et al.*, 2001). Given its significant association as well as its pivotal role in the induction of immune response, numerous studies have been performed to dissect out the underlying mechanisms of MHC-mediated MD resistance.

MHC class I and II molecules play a key role in the orchestration of immune responses via presentation of antigens to $CD8^+$ T cells and $CD4^+$ T cells, respectively. The chicken MHC or the B-complex encodes B-F and B-L proteins with functional and structural similarity to mammalian MHC class I and II molecules, respectively. Given the importance of MHC in mediation of both the innate and adaptive components of the immune response, it is not surprising that different chicken MHC haplotypes have a high degree of association with susceptibility of chickens to various infectious diseases, including MD (Kaufman and Salomonsen, 1997; Juul-Madsen *et al.*, 2000; Bumstead and Kaufman, 2004).

Several studies have demonstrated that B haplotypes confer various degrees of resistance in relation to susceptibility to MD. Briles *et al.* (1977) reported that chickens with the B^{21} MHC haplotype were highly resistant to tumors caused by MDV. In addition, Abplanalp *et al.* (1984) reported that chicken with B^2, B^Q and B^{21} MHC haplotypes demonstrated more resistance to disease caused by three strains of MDV including JM-10, GA-5 and RB1B than chickens with other haplotypes. In general, MHC haplotypes including B^1, B^4, B^5, B^{12}, B^{13}, B^{15} and B^{19} have been associated with susceptibility and B^2, B^6 and B^{14} have been associated with moderate resistance, whereas B^{21} is associated with resistance to MD (Hepkema *et al.*, 1993; Bacon *et al.*, 2001; Bumstead and Kaufman, 2004). A classic example is the selection of N and P lines for MD resistance and susceptibility, respectively, at Cornell University using a virulent strain of MDV where all chickens in the former line possessed the B^{21} MHC

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haplotype, while 97% of the chickens in the latter line possessed the B^{19} MHC haplotype (Bacon *et al.*, 2001).

Despite several studies that have underlined the influence of MHC in MD resistance, the genes within the *MHC* locus that are involved in conferring resistance or susceptibility to MD are not well explored (Kaufman *et al.*, 1999; Dalgaard *et al.*, 2003). Hepkema *et al.* (1993) narrowed down the search to the B-F/B-L region that encodes MHC class I and II molecules, respectively. There are several hypotheses that attempt to explain the associations between MHC and MD. For instance, it has been speculated that the association between MHC and resistance against MD may be related to the level of surface expression of MHC molecules on cells of resistant versus susceptible birds (Kaufman *et al.*, 1995). Furthermore, it has been suggested that there may be a difference in the repertoire of peptides presented by MHC molecules of haplotypes associated with resistance compared to those that are associated with susceptibility. As such, some of the peptides associated with B^{19} and B^{21} haplotypes *in vitro* have been identified and also peptide-binding motif for B^{19} haplotype has been established (Haeri *et al.*, 2005; Cumberbatch *et al.*, 2006; Koch *et al.*, 2007). In addition, crystallography of MHC class I of the B^{21} haplotype and sequencing of the peptides presented by these molecules have revealed that B^{21} MHC-I molecules are able to bind a wide range of peptides (Koch *et al.*, 2007). This may, at least partly, explain the fact that this haplotype is highly associated with resistance to MD. Collectively, these studies enable the examination of MDV epitopes that are differentially presented by these haplotypes and further elucidate the role of MHC in resistance versus susceptibility to MD. Discovery of epitopes will be a major advancement in the area of genetic resistance to disease and will open several new avenues for further research, for example in the area of dynamics of T cell response to MDV in genetically defined chickens. To this end, we have developed chicken MHC class I and II tetramers for B^{19} and B^{21} haplotypes (Niemiec *et al.*, 2006; and unpublished results), which can be loaded with MDV epitopes and employed for studying elicitation and regulation of T cell responses in infected chickens.

Non-MHC genes and quantitative trait loci (QTLs) associated with MD resistance

Non-MHC genes play a role in resistance or susceptibility to MD (Bacon *et al.*, 2001). Three non-MHC loci, *TH1*, *LY4* and *BU1* are associated with resistance or susceptibility to MD. These loci contain genes which encode various antigens on thymocytes and bursal lymphocytes, respectively (Bacon *et al.*, 2001). Moreover, genes that encode mitochondrial phosphopyruvate carboxykinase (PEPCK-M) (Li *et al.*, 1998) and vitamin D receptor (Praslickova *et al.*, 2008) may also be involved in differential resistance to MD (Bumstead, 1998). Furthermore, it has been determined by a protein interaction assay that other genes such as lymphocyte antigen, *LY6* locus E (Liu *et al.*, 2003), growth hormone (*GH*) (Liu *et al.*, 2001a, b) and lymphotactin gene (*SCYC1*) are among the candidate genes responsible for MD resistance. However, the role of such associations in the context of MDV pathogenesis has yet to be elucidated.

Several QTLs have been associated with susceptibility or resistance to MD (Vallejo *et al.*, 1998; Xu *et al.*, 1998; Lipkin *et al.*, 2002; McElroy *et al.*, 2005). A study using a large number of microsatellite markers identified 15 QTLs with some overlapping identities with previous studies and demonstrated a strong association with the MHC haplotype (Heifetz *et al.*, 2007). Furthermore Cheng *et al.* (2007) using susceptible 7_2 and resistant 6_3 lines have demonstrated the occurrence of significant epistatic interactions between various QTLs (Cheng *et al.*, 2007). More recently, a total of 21 QTL regions (QTLR) were identified that affected survival time in challenged birds (Heifetz *et al.*, 2009).

In addition to various QTLs that are involved in resistance versus susceptibility to MD, epigenetic mechanisms, such as DNA methylation, have been implicated as well. For example, the role of DNA methylation profiles of DNA methyl transferase genes (DNMT3a, DNMT3b and DNMT1) and their association with tumorogenesis in chickens have been studied (Yu *et al.*, 2008). The methlylation pattern of DNMT3b in four tissues was not significantly different between resistant versus susceptible lines (resistant line 6_3 and susceptible line 7_2). However, the methylation pattern of DNMT1 was different between the two chicken lines. In addition, tissue-specific methylation profile of DNMT1 was described. Finally, the association of DNA methylation profiles of DNMT1 and DNMT3a with oncogenesis of MDV in chickens was underscored in MDV-infected chickens (Yu *et al.*, 2008). The evidence presented in the preceding section points to the complexity of genetics of host–MDV interactions.

Several studies have investigated the changes in gene expression in response to MDV infection irrespective of genetic background of the infected chickens (Morgan *et al.*, 2001; Sarson *et al.*, 2006). Sarson *et al.* (2006) reported differential gene expression in the spleen of RB1B infected SPF chickens at 4, 7, 14 and 21 dpi. Based on their investigation, genes that are involved in expression of cell surface molecules, transcription factors, metabolic mediators as well as cytokine and cytokine receptors were expressed differently in infected versus control groups (Sarson *et al.*, 2006). Interestingly, granzyme-A, which is involved in cytotoxicity mediated by NK cells and CTLs, was up-regulated in infected groups at different time-points (Sarson *et al.*, 2006). In addition to the above studies, a limited number of investigations have focused on the differential gene expression between MD-resistant and susceptible chicken lines (Liu *et al.*, 2001a; Sarson *et al.*, 2008b). Liu *et al.* (2001a) analyzed gene expression changes in peripheral

blood lymphocytes of East Lansing lines 6_3 and 7_2 after infection with a virulent strain of MDV using a microarray that contained 1200 gene elements. Among several genes that were differentially expressed between lines, notably GH was identified as a putative candidate gene associated with MD resistance (Liu *et al.*, 2001b). Furthermore, a recent study from our laboratory compared gene expression in the spleen of B^{19} and B^{21} chickens (i.e. susceptible and resistant, respectively, to MD) in response to intra-abdominal infection with the virulent JM-16 strain of MDV at 4, 7, 14 and 21 dpi (Sarson *et al.*, 2008a, b). In this study, several genes such as chemokine *AH221*, B cell marker *Bu1*, IgM, IgG, IgA, MHC class II β chain, granzyme A and STAT2 were differentially expressed at various time points and treatments. Among other genes that were differentially regulated between the two lines at different time points, immunoglobulin genes, IgG and IgM, were expressed more than two-fold in susceptible birds at 7 dpi and repressed during the subsequent sampling time point (i.e. 14 dpi).

Differential expression of cytokines in tissues and cellular subsets of resistant/susceptible lines of chickens has also been studied. Using a laser capture micro-dissection approach, it was shown that the tissue microenvironment in L6 (resistant) and L7 (susceptible), which have the same MHC haplotype, inclines toward Th1 and Th2 microenvironments, respectively (Kumar *et al.*, 2009). We have also profiled the expression of cytokines in CD4+ and CD8+ cell subsets of B^{19} and B^{21} chickens and while we have noted significant changes in expression of cytokine over time in both lines, there was no significant association between these patterns and resistance or susceptibility to MD (Parvizi *et al.*, 2009a).

Vaccination against MD

Churchill *et al.* (1969b) were the first to report the use of live attenuated virus, HPRS-16/Att, to immunize chickens against MDV. A year later, HVT characterized by Witter *et al.* (1970) was used to immunize chickens (Okazaki *et al.*, 1970). Since then, HVT has been used worldwide to protect commercial flocks against MD alongside various other vaccines (Bublot and Sharma, 2004). In addition to HVT, several other types of vaccines have been described, including CVI988 attenuated serotype I MDV (Rispens *et al.*, 1972a, b) and non-oncogenic serotype 2 (Schat and Calnek, 1978), which are all currently in use with the exception of HPRS-16/Att. Currently, combinations of CVI988, HVT and SB-1 are commonly used as bivalent or trivalent vaccines (Bublot and Sharma, 2004). MD vaccines have been administered mostly via the subcutaneous route (Witter, 2001). However, *in ovo* vaccination has replaced subcutaneous application in broilers in most of the world (Gimeno, 2008). The *in ovo* route does not reduce hatchability and protects against MDV (Sharma and Burmester, 1982).

Despite the widespread use of vaccines, MD outbreaks occur in various countries (Baigent *et al.*, 2006). The outbreaks take place due to a variety of factors such as improper storage or administration, presence of maternal antibodies, suppression of the immune system by other pathogens or stress, and emergence of vv or vv+ MDV in the field (Baigent *et al.*, 2006). On the other hand, administration of vaccines exerts pressure on MDV to evolve into more virulent pathotypes which, in turn, may override immunity conferred by vaccination (Schat and Baranowski, 2007).

MD vaccines protect chickens against virus replication and tumor formation, but MDV can still spread from vaccinated to unvaccinated birds (Baigent *et al.*, 2006). Therefore, virulent virus may be shed along with feather dander from infected chickens that have been vaccinated (Abdul-Careem *et al.*, 2007). Furthermore, it has been reported that after vaccination, infection with a virulent MDV can result in an increase in shedding of vaccine viruses, such as HVT and MDV-2 in feather dander (Islam and Walkden-Brown, 2007). To gain more insight into the process of immune response to MDV in feathers, we have examined the expression of host immune response genes and have determined that in addition to MHC-I, IL-18, IL-6 and IFN-γ genes are up-regulated in feathers of infected chickens compared to uninfected control birds (Abdul-Careem *et al.*, 2008b). This observation points to the presence of an active immune response against MDV in feathers, which is clearly ineffective in curtailing virus replication and shedding. We have also obtained evidence that both HVT and Rispens strains of vaccine virus enter the feathers and can elicit immune responses in this tissue (Abdul-Careem *et al.*, 2009). Despite the aforementioned observations, the mechanisms of protection induced by MDV vaccines are not well understood. It has been shown that NK cell activity is enhanced due to vaccination (Heller and Schat, 1987). In addition, T cell-mediated immune responses especially $CD8^+$ T cells play a key role in elicitation of immunity against MDV (Omar and Schat, 1997; Garcia-Camacho *et al.*, 2003; Gimeno *et al.*, 2004). We have also previously reported that the expression of cytokines such as IL-6, IL-10 and IL-18 is decreased in spleens of vaccinated chickens compared to unvaccinated and challenged ones (Abdul-Careem *et al.*, 2007). IL-10 and IL-18 can skew the immune response to a type II immune response (Leite-De-Moraes *et al.*, 2001; Rothwell *et al.*, 2004), raising the possibility that a type I response may be correlated with protection and a type II response associated with lack of protection. Kano and co-workers (2009) have also reported that vaccinated chickens produce higher amounts of IFN-γ in the latent phase infection compared to unvaccinated birds. Therefore, it was concluded that IFN-γ plays a key role in vaccine-mediated protection.

Immune response to MD vaccines may be genetically regulated. Bacon *et al.* have also shown that B haplotypes affect the efficacy of the vaccine in both congenic and

commercial chickens (Bacon and Witter, 1994b, 1995). Serotype 2 vaccines, for instance, provided more protection in chickens with B5 haplotype (Bacon and Witter, 1994a). Therefore, it might be essential to choose the vaccine based on the B haplotype of the flock (Bacon and Witter, 1993).

Several strategies have been employed to enhance efficacy of MD vaccines, such as including cytokines in vaccine formulations. For example, Djeraba *et al.* (2002) have shown that chicken myelomonocytic growth factor can improve protection conferred by MD vaccines. Tarpey *et al.* have also used a recombinant HVT that expressed chicken IL-2. The recombinant IL-2/HVT was used via the *in ovo* route that resulted in an increase in neutralizing antibodies against HVT. However, IL-2 expression did not enhance the protective efficacy of the vaccine (Tarpey *et al.*, 2007). Virulent and vv strains of MDV have also been modified by cell-culture passage, back passage in chickens and insertional mutagenesis to enhance their efficacy. In terms of efficacy, although the modified strains are protective, their efficacy does not significantly exceed that of the currently available vaccines (Witter and Kreager, 2004).

Due to the fact that evolution of MDV may lead to enhancement of virulence and possible disease outbreaks in infected flocks, there is an urgent need to increase the efficiency of the current vaccines by using strategies such as the use of cytokines and TLR ligands as adjuvants, use of different vaccines, and breeding for resistant flocks (Gimeno, 2008).

Conclusions

There is a complex and intricate interplay between MDV and its chicken host. Our understanding of the interactions between MDV and the chicken immune system has been broadened in the last few decades. Several observations have underscored the role of innate defense mechanisms and adaptive immune responses against MDV. However, the role of various immune system molecules as well as different cell populations in the elicitation of protective immunity against MDV needs to be further elucidated. With the advent of modern immunological techniques, it is feasible to further dissect the role of various soluble factors, such as AMPs and cytokines in the induction of protective immunity against MD. In addition, the results of these investigations can be further incorporated into designing more efficacious prophylactic methods against MDV.

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