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Host responses are induced in feathers of chickens infected with Marek's disease virus

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Abstract

Control measures are ineffective in curtailing Marek's disease virus (MDV) infection and replication in the feather follicle epithelium (FFE). Therefore, vaccinated birds which subsequently become infected with MDV, shed the virulent virus although they remain protected against disease. The present study investigated host responses generated against MDV infection in the feather. We observed that in parallel with an increase in viral genome load and viral replication in the feather, there was a gradual but progressive increase in infiltration of CD4+ and CD8+ T cells into the feather pulp of MDV-infected chickens, starting on day 4 and peaking by day 10 post-infection. Concomitant with infiltration of T cells, the expression of interleukin (IL)-18, IL-6, interferon (IFN)-γ and major histocompatibility complex class I genes was significantly enhanced in the feather pulp of MDV-infected chickens. The finding that host responses are generated in the feather may be exploited for developing strategies to control MDV infection in the FFE, thus preventing horizontal virus transmission.

Keywords: Chicken; Feather follicle; Marek's disease virus; Immunity; Cytokine; Major histocompatibility complex

Introduction

Infection of susceptible chickens with Marek's disease virus (MDV), an alphaherpesvirus, results in neurological manifestations and lymphomas in various tissues. Chickens are infected with MDV naturally by inhalation and the source of infection is usually poultry house dust containing feather dander (Beasley et al., 1970; Calnek et al., 1970). The initial viral replication, 1–3 days post-infection (d.p.i.), occurs in the lung tissue and then spreads to lymphoid tissues mainly via infected macrophages (Barrow et al., 2003). This is followed by the early cytolytic infection in B and activated T lymphocytes that lasts for up to 6 d.p.i. (Calnek, 2001). MDV infection becomes latent in T lymphocytes around 7 d.p.i. (Calnek, 2001). The infected lymphocytes, mostly activated T cells, circulate in the blood spreading the infection to various tissues including the feather follicle epithelium (FFE). MDV infection in the FFE is productive and is the sole source of enveloped fully infectious virus for infecting susceptible chickens (Calnek et al., 1970; Johnson et al., 1975). Once chickens are infected, they become virus carriers for their entire life (Witter et al., 1971). In addition to the importance of the FFE for MDV infection and viral transmission, lesions that vary from an initial inflammatory to late proliferative types, also develop in the feather pulp that may be observed after 7 d.p.i. (Moriguchi et al., 1982, 1984, 1986; Cho et al., 1996, 1998). The inflammatory lesions of the feather pulp are composed mainly of lymphocytes (Moriguchi et al., 1982; Cho et al., 1998). As various cell types such as CD4+ and CD8+ T cells are involved in the response to MDV infection in other tissues (Gimeno et al., 2001; Burgess et al., 2001), it is important to further characterize host response and define the infiltrating cell populations in the feather pulp in response to MDV infection.

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Although host genetic resistance and vaccination may prevent lymphoma formation (Cole, 1968; Okazaki et al., 1970) accompanied by a significant reduction in viral infectious particles (Lee et al., 1999) as well as viral genome load in lymphoid organs (Kaiser et al., 2003), host responses appear unable to control viral replication in the FFE (Baigent and Davison, 2004; Islam et al., 2006; Abdul-Careem et al., 2007). For instance, viral genome load in the feather follicle is several fold higher than in the spleen (Baigent et al., 2005; Abdul-Careem et al., 2007). The apparent differential host response in lymphoid tissues and the feather follicles may be due to the lack of development of protective immune response against MDV in the feather (Baigent et al., 2005). As suggested by Davison and Nair (2005), viral replication in feather tips in the face of vaccination and genetically mediated resistance has implications for horizontal transmission of MDV and evolution of viral virulence.

Cytokine responses associated with cell-mediated immune response to MDV infection have been characterized, particularly in the spleen (Kaiser et al., 2003) and peripheral blood lymphocytes (Quere et al., 2005). MDV infection is associated with a significant increase in the expression of interferon (IFN)-α (Quere et al., 2005), IFN-γ (Xing and Schat, 2000; Djeraba et al., 2002; Jarosinski et al., 2005; Quere et al., 2005; Abdul-Careem et al., 2007), interleukin (IL)-1β (Xing and Schat, 2000; Jarosinski et al., 2005), IL-6 (Kaiser et al., 2003; Jarosinski et al., 2005; Abdul-Careem et al., 2007), IL-8 (Xing and Schat, 2000; Jarosinski et al., 2005), IL-10 (Abdul-Careem et al., 2007) and IL-18 (Kaiser et al., 2003; Abdul-Careem et al., 2007). However, little is known about the process of induction of immune response to MDV in the feather where fully infectious virus particle formation takes place. The objective of the present study was to determine whether MDV infection in feather follicles stimulates host responses marked by expression of cytokines and infiltration of immune system cells. In this study, the generation of immune response was assessed by measuring the expression of genes that are involved in antigen processing and presentation pathways, including major histocompatibility complex (MHC) class I, MHC-II and transporter associated with antigen presentation (TAP) as well as cytokine genes, such as IL-6, IL-18 and IFN-γ.

Results

Generation of standard curves

Standard curves for relative quantification of TAP-2, MHC-I, MHC-II and MDV glycoprotein B (gB) were generated. The values recorded for slope of the curve were −3.323, −3.592, −3.660 and −3.492 and that yielded PCR efficiency (E) of 2.0, 1.90, 1.875 and 1.93 for TAP-2, MHC-I, MHC-II and MDV gB, respectively. The regression coefficients recorded for TAP-2, MHC-II and MDV gB were 1.00 and that for MHC-I was 0.99.

MDV genome load in feather tips of MDV-infected chickens

MDV genome load was quantified in feather tips, which harbor the productive cytolytic phase of the virus life cycle. Initial screening of feather tip DNA by conventional PCR determined that uninfected controls had remained MDV free, whereas meq could be amplified from all tested DNA samples derived from MDV-infected chickens. Feather tip DNA originated from MDV-infected chickens was further analyzed by real-time PCR and the data are illustrated in Fig. 1(A). MDV-infected chickens that were sampled at 10 d.p.i. had significantly higher copy numbers of the MDV genome (32,249±32,498) in feather tips compared to MDV-infected chickens that were sampled 4 (14±10) and 7 (73±94) d.p.i. ($P \leq 0.05$). Furthermore, there was a significant difference ($P \leq 0.001$) between MDV genome copy numbers in feather tips of MDV-infected chickens on 14 d.p.i. (860,550±527,948) compared to feather tips of chickens at all other sampling time points.

MDV-gB gene expression in feather tips of MDV-infected chickens

Feather tip cDNA preparations from RB1B MDV-infected chickens were evaluated for the expression of gB gene by real-time RT-PCR (Fig. 1B). MDV-infected chickens that were sampled at 14 d.p.i. had significantly higher transcripts of the MDV gB gene in their feather tips compared to MDV-infected chickens that were sampled 4 and 7 d.p.i. ($P \leq 0.05$). Compared to 4 d.p.i., MDV gB transcripts in feather tips were increased by

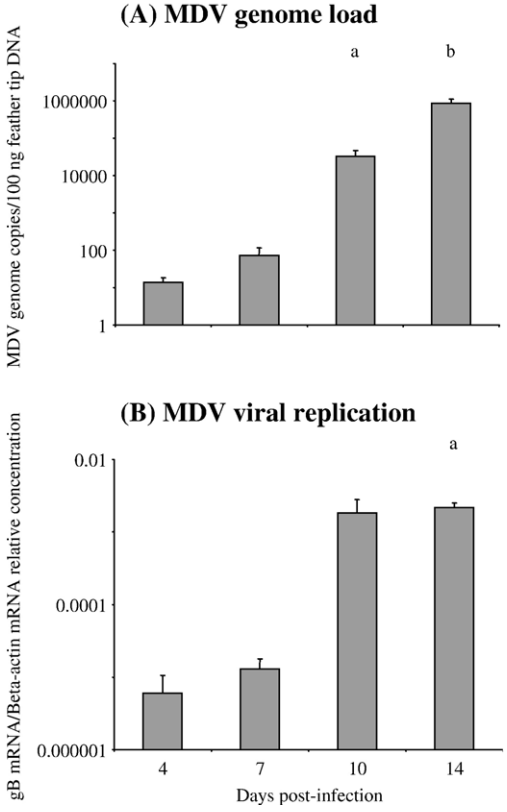

Fig. 1. MDV genome load and gB transcripts in feather tips of chickens infected with MDV. Chickens were infected with MDV and sampled on 4, 7, 10 and 14 d.p.i. Mean MDV genome load (A) and gB mRNA expression relative to β-actin mRNA expression (B) are presented and the error bars represent standard error of the mean. a=significant when compared to MDV-infected chickens sampled on 4 and 7 d.p.i. ($P \leq 0.05$), b=significant when compared to MDV-infected chickens sampled on 4, 7 and 10 d.p.i. ($P \leq 0.001$).

359-fold by 14 d.p.i. There was also an increase (303-fold) in MDV gB transcripts in feather tips on 10 d.p.i. compared to that observed on 4 d.p.i., but the difference was not statistically significant ($P=0.071$).

Histological observation

In response to MDV infection, the distribution of infiltrating cells in the tissue varied depending on the time of observation (Figs. 2 and 3). In general, CD4+ and CD8+ T cells and macrophages were quantifiable in the feather pulp area of the feather. Appreciable differences in infiltration of cells, particularly CD4+ and CD8+ T cells were observed between 4, 7, 10 and 14 d.p.i. and also between the feather pulp of MDV-infected and that of control chickens. CD4+ and CD8+ T cells were distributed in the feather pulp cavity around the perivascular areas and areas close to the basement membrane of the FFE. However, there was no difference in the number of macrophages present in the feather pulp of MDV-infected birds compared to those present in uninfected control chickens (data not shown). There were no B cells detected in the feather pulp of MDV-infected or control chickens (data not shown).

At 4 d.p.i., there was a mild infiltration of CD4+ T cells in the feather pulp of MDV-infected chickens. The number of

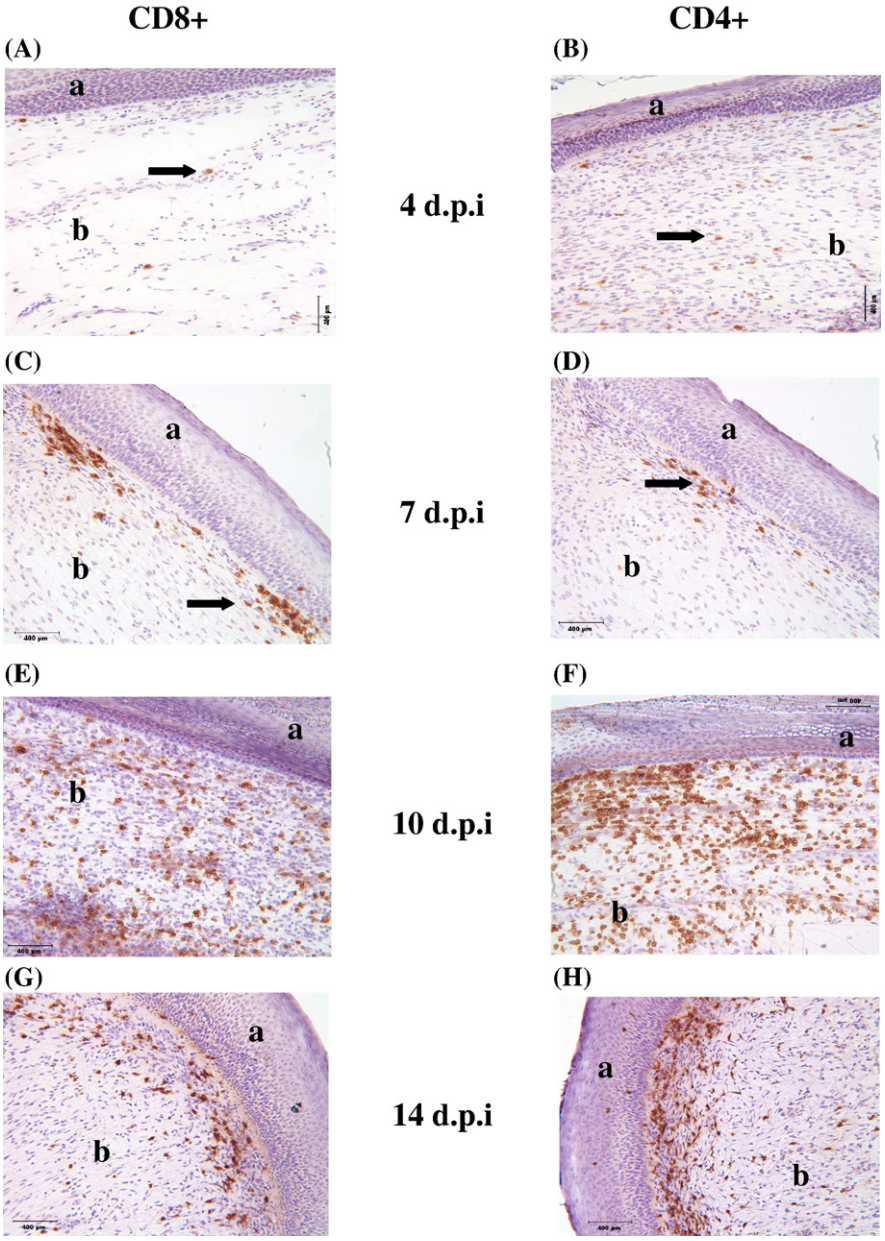

Fig. 2. Immunohistochemistry analysis of feather tips of chickens infected with MDV. A=CD8+ T cell infiltration on 4 d.p.i., B=CD4+ T cell infiltration on 4 d.p.i., C=CD8+ T cell infiltration on 7 d.p.i., D=CD4+ T cell infiltration on 7 d.p.i., E=CD8+ T cell infiltration on 10 d.p.i., F=CD4+ T cell infiltration on 10 d.p.i., G=CD8+ T cell infiltration on 14 d.p.i., H=CD4+ T cell infiltration on 4 d.p.i., a=FFE lining the feather pulp cavity, b=feather pulp cavity. Scale bar=400 μm. Arrows show positively stained cells.

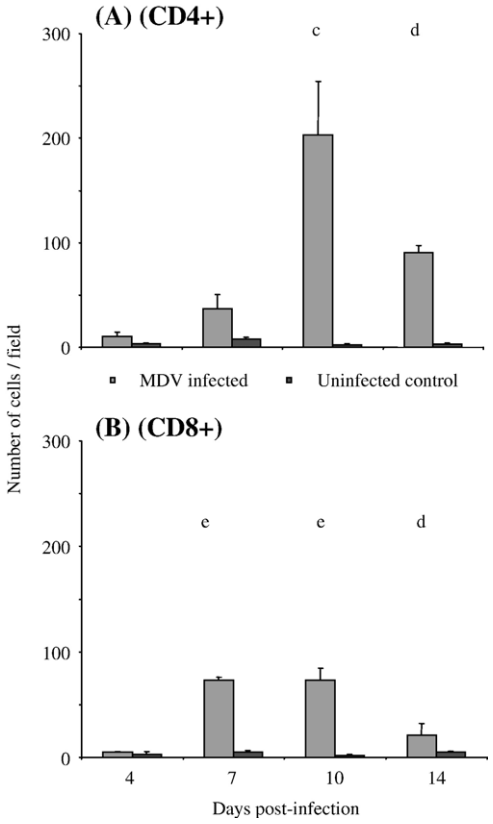

Fig. 3. Distribution of T cell subsets in the feather pulp of chickens infected with MDV. The groups were as follows: MDV-infected=chickens that were infected with MDV and sampled on 4, 7, 10 and 14 d.p.i.; and Uninfected controls=age-matched chickens that were not infected. Group mean number of CD4+ (A) or CD8+ T cells (B) per ×40 microscopic field is presented and the error bars represent standard error of the mean. c=significant when compared to age-matched controls and MDV-infected chickens sampled on 4, 7 and 14 d.p.i., d=significantly higher when compared to age-matched controls and MDV-infected chickens sampled on 4 d.p.i., e=significant when compared to age-matched controls and MDV-infected chickens sampled on 4 and 14 d.p.i.

CD4+ T cells in the tissue gradually increased and peaked by 10 d.p.i., and then declined by 14 d.p.i. There was a significant difference between the degree of CD4+ T cell infiltration at 10 d.p.i. and all other time points ($P \leq 0.001$). Also, there was a significantly more CD4+ T cell infiltration at 14 d.p.i. compared to 4 d.p.i. ($P \leq 0.001$).

The number of CD8+ T cells in the feather pulp of MDV-infected chickens at 4 d.p.i. was mild, peaked by 7 d.p.i. and then declined at the remaining two time points. There was a significant difference in the degree of CD8+ T cell infiltration at 7 and 10 d.p.i. compared to 4 and 14 d.p.i. ($P \leq 0.001$). The increase in CD8+ T cells observed on 14 d.p.i. was, however, significantly higher when compared to the age-matched controls or to MDV-infected chickens at 4 d.p.i. ($P \leq 0.001$).

The relative number of CD4+ and CD8+ T cells per X40 microscopic field was compared. At 4 and 7 d.p.i., the two subsets had equally infiltrated the tissue. However, at 10 d.p.i., the number of infiltrating CD4+ T cells was marginally higher than the number of CD8+ T cells ($P=0.068$). By day 14 post-infection, there were more infiltrating CD4+ than CD8+ T cells ($P \leq 0.001$).

TAP-2, MHC-I and MHC-II gene expression in feather tips of MDV-infected and uninfected chickens

The expression of TAP-2 and MHC-I in feather tips of MDV-infected and control chickens is illustrated in Figs. 4A and B. The expression of these two genes was constitutively quantifiable in feather tips of both MDV-infected and control uninfected chickens at all time points. Although an increase in the expression of TAP-2 was observed in MDV-infected compared to uninfected chickens, the difference was not statistically significant ($P>0.05$). At 14 d.p.i., the expression of TAP-2 was lower in both infected and uninfected groups than that observed at other time points, but the difference was not statistically significant ($P>0.05$). The expression of MHC-I gene also increased over time in MDV-infected chickens. The expression of MHC-I gene in MDV-infected compared to uninfected chickens was significantly higher on 10 and 14 d.p.i. ($P \leq 0.05$). MHC-II gene expression was only detected in one sample (data not shown).

Cytokine gene expression in feather tips of MDV-infected and uninfected chickens

Although the expression of IL-6 gene (Fig. 4C) did show an increase over time in feather tips of MDV-infected chickens, only on 10 d.p.i. was the expression significantly higher in feather tips of MDV-infected chickens when compared to age-matched uninfected controls ($P \leq 0.05$).

The expression of IL-18 gene (Fig. 4D) was quantifiable in feather tips of both MDV-infected and uninfected control chickens. Although the expression of IL-18 gene did show an increasing trend over time in feather tips of MDV-infected chickens, only on 7 d.p.i. was the expression of this cytokine significantly higher compared to the age-matched uninfected controls ($P \leq 0.05$). The expression of IFN-γ gene (Fig. 4E) in feather tips of MDV-infected chickens sampled 7 and 10 d.p.i. was significantly higher when compared to that of the age-matched control chickens and MDV-infected chickens sampled 4 d.p.i. ($P \leq 0.001$). The expression of IFN-γ gene in feather tips of MDV-infected chickens sampled 14 d.p.i. was significantly higher when compared to that of the age-matched control chickens ($P \leq 0.05$).

Discussion

The FFE is the only known tissue in MDV-infected chickens, which permits the formation of fully infectious MDV (Calnek et al., 1970; Johnson et al., 1975). This led us to hypothesize that host responses may not be adequately generated in the feather, which might provide a conducive milieu for virus maturation. The current study provides evidence that MDV infection indeed elicits host responses in the feather.

We discovered that the virus replicated very rapidly between 4 to 14 d.p.i. and that the increase in virus genome load from 4–10 d.p.i. was 62,090-fold. The increase in virus genome load coincided with the increase in viral replication, infiltration of CD4+ and CD8+ T cells into the feather pulp and also the

induction of immune response genes. Although MDV infection appears to initiate an active immune response in the feather, as shown in our study by the expression of cytokines and infiltration of CD4+ and CD8+ T cells into the feather pulp, prior vaccination or genetic background of chickens does not lead to induction of a protective immune response locally to preclude replication of MDV in the FFE (Baigent and Davison, 2004; Islam et al., 2006; Abdul-Careem et al., 2007). The absence of a protective response in the feather follicle may be due to the inability of the responses generated in the feather pulp

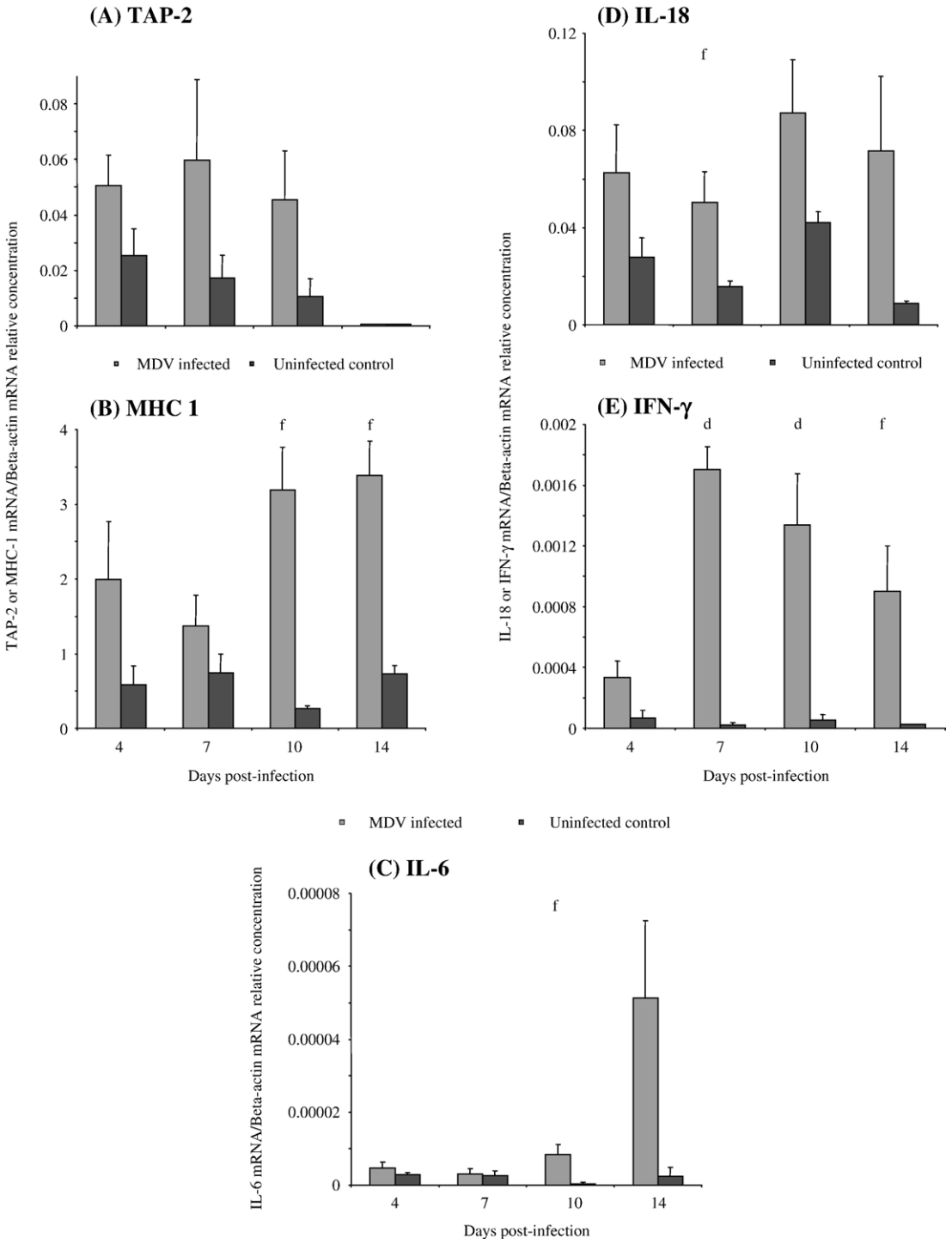

Fig. 4. Expression of immune system genes in feather tips of chickens infected with MDV. Panels A–E show TAP-2, MHC-I, IL-6, IL-18 and IFN-γ mRNA expression, respectively. The groups were as follows: MDV-infected=chickens that were infected with MDV and sampled on 4, 7, 10 and 14 d.p.i.; and Uninfected controls=age-matched chickens that were not infected. Target gene expression is presented relative to β-actin expression and normalized to a calibrator. Error bars represent standard error of the mean. f=significant when compared to age-matched controls, d=significant when compared to age-matched controls and MDV-infected chickens sampled on 4 d.p.i.

to reach the replicating virus in the FFE. As suggested by Baigent and Davison (2004), the FFE might be a privileged site that allows MDV envelop formation and egress due to the protective sheltering of the virus in cytoplasmic inclusion bodies and resistance to lysosomal activity in keratinocytes. More studies are needed to address this more directly. But in a different context, it has been shown that human papillomavirus-infected keratinocytes are resistant to effector mechanisms mediated by IFN-γ and cytotoxic T cells (Frazer et al., 1999; Leggatt et al., 2002). It is also possible that because of the difference in the life cycle of MDV in feather follicles compared to other tissues, only minimal inflammation is elicited by the virus. This is in contrast to the lytic phase of infection in lymphoid tissues which triggers inflammatory and immune responses (Payne, 2004). However, this latter explanation is unlikely, since inflammatory lesions have been documented in response to MDV infection in feather follicles (Moriguchi et al., 1982, 1984, 1986; Cho et al., 1996, 1998). Moreover, in the present study, we observed induction of proinflammatory cytokines as well as molecules involved in the presentation of viral peptides to CTL in the FFE of infected chickens.

Histologically, the feather pulp cavity is lined with the extension of the FFE of the follicle wall (Pass, 1995) and the lesions following MDV infection have been described in both the FFE (Moriguchi et al., 1982, 1986) and the feather pulp (Moriguchi et al., 1982, 1986; Cho et al., 1998). Early feather pulp lesions consist mainly of perivascular cuffing of lymphocytes (Moriguchi et al., 1982, 1986). In the present study, histological examination of the feather tip sections obtained from MDV-infected birds revealed infiltration of CD4+ and CD8+ T cells in the feather pulp, which peaked by 10 d.p.i. and declined thereafter, although at 14 d.p.i., there were still more cells in the tissue compared to 4 d.p.i. The fact that the pattern of infiltration of these T cell subsets correlated with the pattern of the gradual increase in viral genome load and viral gB transcripts in the feather at the first three time points, raises the possibility that the infiltration of CD4+ and CD8+ T cells was in response to increased viral replication in the tissue. It is also possible that at least some of the infiltrating cells were latently infected T cells carrying the virus into the feather pulp (Baigent and Davison, 2004). Once the virus is transported into the feather pulp, it can initiate the productive infection phase in the FFE (Calnek et al., 1970; Johnson et al., 1975). Since in the feather, infection is restricted to the FFE and the virus is somehow protected from immune recognition (Frazer et al., 1999; Leggatt et al., 2002; Baigent and Davison, 2004), the FFE infection may be precluding the stimulation of host response. By day 14 post-infection, the degree of T cell infiltration had diminished. It should be noted that MDV-transformed CD4+ cells may start accumulating in the feather pulp by 12–14 d.p.i. (Moriguchi et al., 1982; Cho et al., 1998; Calnek, 2001), and this may explain our observation that by 14 d.p.i., there were more CD4+ T cells than CD8+ T cells in the feather pulp.

In the present study, we did not observe a significant change in macrophage infiltration in the feather pulp between MDV-infected and control chickens. Although there was no increase in the number of macrophages in infected chickens, it is possible that resident macrophages become activated after MDV infection leading to enhanced expression of cytokines by these cells.

The importance of cell-mediated immune response to MDV infection (Sharma et al., 1975) and the potential role of cytokine gene expression in immunity against MD have been described (Xing and Schat, 2000; Djeraba et al., 2002; Kaiser et al., 2003; Jarosinski et al., 2005; Quere et al., 2005; Abdul-Careem et al., 2007). IL-6 and IL-18 are known proinflammatory cytokines (Netea et al., 2000) and the expression of IL-18 and IL-6 has been shown to increase in response to MDV infection (Kaiser et al., 2003; Abdul-Careem et al., 2007). In the present study, the expression of both of these cytokines was significantly increased in feather tips, at least at one time point. MDV infection stimulates the expression of IFN-γ in lymphoid tissues (Xing and Schat, 2000; Jarosinski et al., 2005; Abdul-Careem et al., 2007). Similarly, in feather tips, the expression of IFN-γ was significantly up-regulated on 7, 10 and 14 d.p.i. IFN-γ is an antiviral cytokine that is also known to inhibit MDV replication via macrophage activation (Lee, 1979). In addition to its direct antiviral effects, IFN-γ may activate CD8+ cytotoxic T cells to kill virus-infected cells (Whitmire et al., 2005). Indeed, CD8+ T cell numbers were significantly increased in our study on days 7, 10 and 14 post-infection in the feather pulp of MDV-infected chickens compared to infected chickens at 4 d.p.i. The role of CD8+ T cells in killing MDV-infected cells and, therefore, immunity to MDV infection has previously been shown (Markowski-Grimsrud and Schat, 2002). The increased expression of genes involved in of MHC-I antigen presentation pathway, recruitment of CD4+ and CD8+ T cells, and elevated expression of cytokines are consistent with enhanced antiviral adaptive responses in the tissues. However, it remains to be addressed why these responses are not effective in disruption of the productive phase of MDV infection.

In our study, the expression of genes encoding the endogenous antigen presentation pathway molecules, including MHC-I α-chain and TAP-2, was increased in the feather in response to MDV infection. Generally, herpesviruses through various mechanisms, such as prevention of MHC assembly and peptide loading, decrease antigen presentation by MHC-I molecules (Basta and Bennink, 2003). MDV appears to reduce cell surface expression of MHC molecules; however, its effects on MHC-I gene expression have been somewhat variable. For instance, it has been shown that cell surface expression of MHC-I protein is down-regulated in a chicken embryo fibroblast (CEF) cell line infected with MDV Md11 strain without altering the transcription and translation of MHC-I in MDV-infected cells (Hunt et al., 2001). However, Levy and coworkers (2003) have reported that MHC α-chain and β2 microglobulin transcripts are reduced in CEF cells infected with the RB1B strain of MDV. Moreover, Morgan and coworkers (2001) observed that MHC-I and β2 microglobulin transcripts are up-regulated in CEF cells 2 and 4 d.p.i. Aside from the *in vivo* nature of our study versus the previously published *in vitro* studies, it is likely that MDV infection of the FFE engages cellular pathways distinct from those activated by MDV in other cell types. The biological significance of enhanced MHC-I and

TAP-2 expression is not known, but this may be a part of anti-MDV host response, which usually appears in lymphoid tissues by day 7 (Schat and Xing, 2000).

In conclusion, MDV replication stimulates the expression of genes of the antigen presentation pathway, such as MHC-I as well as cytokine genes such as IL-6, IL-18 and IFN-γ in the feather tips. This pattern of gene expression was associated with increased infiltration of CD4+ and CD8+ T cell populations in the feather pulp. Further studies are needed to elucidate the function of the cells and cytokines identified in this study in mounting immunity against the virus in the feather. Furthermore, an important question that needs to be addressed is whether Marek's disease vaccines could induce a similar response in the feather.

Materials and methods

Infection virus strain

MDV strain RB1B (passage 9) was provided by Dr. K.A. Schat (Cornell University, NY, USA) and was used for infecting chickens (Schat et al., 1982).

Experimental animals

Specific pathogen free (SPF) chickens were obtained from the Animal Disease Research Institute, Canadian Food Inspection Agency (Ottawa, Ontario, Canada). Chicks were housed in an isolation facility (Ontario Veterinary College).

Experimental design

Thirty-six, day-old chicks were randomly divided into two groups. Twenty chickens were infected intraperitoneally on day 5 of age with 250 plaque-forming units (PFU) of the RB1B strain of very virulent MDV. The rest ($n=16$) were kept as uninfected controls. On 4, 7, 10 and 14 d.p.i., five MDV-infected chickens were euthanized by CO_2 inhalation and necropsied along with four uninfected controls. At the necropsy, 3–4 feather tips, comprised of feather pulp cavity lined by stratified FFE, were collected from each bird and preserved in RNA later (Qiagen Inc., Mississauga, Ontario, Canada) at −20 °C. Two feather tips from each chicken were also preserved in embedding medium for frozen tissue specimens (Tissue-Tek, Sakura Fine tek USA, Inc. Torrance, CA, USA) at −80 °C.

DNA and RNA extraction

DNA and RNA extraction from feather tips collected on 4, 7, 10 and 14 d.p.i. was carried out using Trizol (Invitrogen Canada Inc., Burlington, Ontario, Canada) as has been described previously (Abdul-Careem et al., 2006b).

Reverse transcription

Reverse transcription of total RNA (2 μg) was carried out using Oligo(dT)$_{12-18}$ primers (SuperScript™ First-Strand Synthesis System, Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Primers

The absolute MDV genome loads in feather tips were quantified using primers specific for the meq gene of MDV (Abdul-Careem et al., 2006a). The previously published primers were used for the relative quantification of expression of target genes (IL-6, IL-18 and IFN-γ) and β-actin that acted as the reference gene (Abdul-Careem et al., 2006b). The primers specific for chicken TAP-2, MHC-I and MHC-II genes were designed to span across exon and intron boundaries after alignment of the relevant nucleotide sequences in GenBank database (accession no. AL023516, L28959 and X07447, respectively) using the Vector NTI™ software (Version 5.5, InforMax, Inc., Frederick, MD, USA). The primers specific for MDV gB were designed after alignment of the relevant nucleotide sequence in GenBank database (accession no. AY129966). The sequences of the primers were as follows: TAP-2 (F-5′-TCGCCTTCTTCCAGAAGAC-CAC-3′, R-5′-CAAGCAGTGCCAGCATTGTCAG-3′); MHC-I (F-5′-ACAAGTACCAGTGCCGCGTG-3′, R-5′-CGCGAT-GTTGTAGC CCTTCC-3′); MHC-II (F-'CGGAGATCGAGGT-GAAGTGG-3′, R-5′-GCTTGCTCCTGCTCACATCC-3′); MDV gB (F-5′GTCTGTTCAATTCGCCATGCTCC-3′, R-5′-CCTTCCTAATGTTGCACTCGCTG-3′). The primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada).

Conventional PCR

Conventional PCR for the detection of MDV meq gene in feather tip DNA preparations was done for initial screening as has been described previously (Abdul-Careem et al., 2006a).

Preparation of constructs as standards

Real-time PCR and RT-PCR quantification of MDV genome load and gene expression, respectively, was done using standard curves. The standard curves for MDV meq gene, IL-6, IL-18, IFN-γ and β-actin have been described previously (Abdul-Careem et al., 2006a,b). Standard curves for TAP-2 and MHC-I were generated using the same protocol used for other genes as described previously (Abdul-Careem et al., 2006a). The TAP-2 forward primer binds exon 2 (starting at position 707) and the reverse primer binds exon 3 (ending at position 889) to give a 183 bp amplicon, the MHC-I forward primer binds exon 3 (starting at position 819) and the reverse primer binds exon 5 (ending at position 1015) to produce an amplicon of 197 bp in length, and the MHC-II forward primer binds exon 3 (starting at position 358) and reverse primer binds exon 4 (ending at position 579) to produce a 222 bp amplicon. The gB amplicon was 175 bp in length (between nucleotides 1368 and 1542). All PCR-amplified products were cloned into pDrive (QIAGEN® PCR Cloning Kit, QIAGEN Inc., Ontario). For construction of standard curves of target and reference genes, 10-fold serial

dilutions (10^{-1} to 10^{-9}) of the relevant plasmid DNA preparations were made and assayed in duplicate.

Real-time PCR and RT-PCR

Each real-time PCR and RT-PCR assay was run along with a dilution series of the standard that served as the calibrator. A no template control was also included with each run. All the real-time PCR and RT-PCR runs were conducted in glass capillaries (Roche Diagnostics GmbH, Mannheim, State of Baden-Württemberg, Germany) in a final volume of 20 μl of LightCycler FastStart DNA Master SYBR Green 1 (Roche Diagnostics GmbH, Mannheim, State of Baden-Württemberg, Germany) containing fast start Taq DNA polymerase for 'hot start' and DNA intercalated dye SYBR Green 1 dye for detection in a LightCycler instrument (Roche Diagnostics GmbH, Mannheim, State of Baden-Württemberg, Germany). In addition, the reaction consisted of 0.25 μM of each gene-specific primer, 3 mM $MgCl_2$, 2 μl of 1:10 dilution of cDNA or 100 ng of DNA as template and PCR grade water.

The optimum thermal cycling parameters varied according to the gene and included pre-incubation at 95 °C for 10 min; 40 cycles (50 cycles in case of IFN-γ gene) of amplification at 95 °C for 10 s, 64 °C for 5 s (4 s for meq) and 72 °C for 10 s (72 C/7 s for the amplification of meq and gB genes); melting curve analysis at 95 °C for 0 s (segment 1), 65 °C/15 s (segment 2) and 95 °C/0 s except MHC-I that needed 97 °C/0 s (segment 3) and cooling at 40 °C/30 s. Fluorescence acquisition was done at 88 °C/3 s for TAP-2, 91 °C/3 s for MHC-I, 84 °C/3 s for MHC-II and 72 °C/7 s for gB depending on the melting temperature of the PCR product of the target or the reference gene. Fluorescence acquisition conditions for other genes have been described previously (Abdul-Careem et al., 2006b).

Histological observation

The feather tip samples preserved in embedding medium for frozen tissue specimens were sectioned (thickness 5 μm) using a cryotome (LEICA CM 3050 S, Vashaw Scientific Inc., Norcross, Atlanta, GA, USA), adhered to microslides (Superfrost plus, VWR Labshop, Betavia, IL, USA) and preserved in −20 °C until used. Immunohistochemistry technique was used to assess the distribution of B cells, monocyte/macrophage and CD4+ and CD8+ T cell infiltration in feather tip sections. Monoclonal antibodies specific for chicken IgM (clone M-1), CD4 (clone CT-4) and CD8a (clone CT-8) that have been raised in mice (Southern Biotech, Birmingham, AL, USA) were used in 1:200 dilution in blocking buffer. Monoclonal antibody KUL01 specific for mononuclear phagocyte system in chicken that has been raised in mice was used (Southern Biotech, Birmingham, AL, USA) in 1:400 dilution in blocking buffer. Since it is known that KUL01 monoclonal antibody stains epidermal dendritic cells as well (Mast et al., 1998), the criterion used to identify monocyte/macrophage was the signal plus the morphology of the cells stained with immunoperoxidase. Avidin–biotin–peroxidase complex (ABC) system (Vectastain® ABC kit, Vector Laboratories, Burlingame, CA, USA) was used for immunoperoxidase staining of tissue sections according to the manufacture's instructions. Quenching of the endogenous peroxidase activity was done by treating the sections for 10–20 min with 3% hydrogen peroxide with 0.3% goat serum made in phosphate buffered saline (PBS). For the purpose of blocking, 5% goat serum in PBS was used. Biotinylated goat anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. After adding anti-chicken IgM, CD4, CD8a and macrophage monoclonal antibodies, samples were incubated for 30 min at room temperature followed by rinsing and incubation with the secondary antibody for 30 min in a humidified chamber. The antigen localization was visualized by incubation of the sections with 3,3-diaminobenzidine-H_2O_2 solution (DAB substrate kit for peroxidase, Vector Laboratories, Burlingame, CA, USA). The slides were counter stained with hematoxyline (Protocol, Fisher Scientific Company, Kalamazoo, MI, USA) and mounted in Cytoseal-60 (Richard-Allan-Scientific, Kalamazoo, MI, USA).

One feather from each of the two or three chickens in each group (MDV-infected and uninfected control) at each time point was examined in order to identify the pattern of infiltration of macrophage, B, CD4+ and CD8+ T cells. The degree of infiltration of each type of cells in the feather pulp over the time period was assessed quantitatively. Briefly, three highly infiltrated fields of ×40 were chosen from each section and number of immunoperoxidase-stained cells was counted.

Data analysis

Cells counted in three fields of X40 magnification for each chicken were averaged and subjected to statistical analysis. Quantification of MDV genome load and expression of cytokine, TAP-2, MHC-I genes by real-time PCR and RT-PCR was done as has been described previously (Abdul-Careem et al., 2006a,b). Briefly, the absolute number of MDV genomes per 100 ng of DNA of feather tips was calculated based on an external standard curve. The expression of cytokine, TAP-2 and MHC-I genes was calculated relative to the expression of β-actin gene and expressed as ratios. All data were subjected to one-way analysis of variance using the statistical package, MINITAB® release 14 (Minitab Inc., State College, PA, USA). The results from the analyses were then used in the Tukey's pairwise comparison to identify treatment differences. Comparisons were considered significant at $P \leq 0.05$.

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References

Abdul-Careem, M.F., Hunter, B.D., Nagy, E., Read, L.R., Sanei, B., Spencer, J.L., Sharif, S., 2006a. Development of a real-time PCR assay using SYBR green chemistry for monitoring Marek's disease virus genome load in feather tips. J. Virol. Methods 133, 34–40.

Abdul-Careem, M.F., Hunter, B.D., Sarson, A.J., Mayameei, A., Zhou, H., Sharif, S., 2006b. Marek's disease virus-induced transient paralysis is associated with cytokine gene expression in the nervous system. Viral Immunol. 19, 167–176.

Abdul-Careem, M.F., Hunter, B.D., Parvizi, P., Haghighi, H.R., Thanthrige-Don, N., Sharif, S., 2007. Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. Vaccine 25, 424–432.

Baigent, S.J., Davison, F., 2004. Marek's disease virus: biology and life cycle. In: Davison, F., Nair, V. (Eds.), Marek's Disease, An Evolving Problem. Elsevier Academic Press, Great Britain, pp. 62–77.

Baigent, S.J., Smith, L.P., Currie, R.J., Nair, V.K., 2005. Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol. 86, 2989–2998.

Barrow, A.D., Burgess, S.C., Baigent, S.J., Howes, K., Nair, V.K., 2003. Infection of macrophages by a lymphotropic herpesvirus: a new tropism for Marek's disease virus. J. Gen. Virol. 84, 2635–2645.

Basta, S., Bennink, J.R., 2003. A survival game of hide and seek: cytomegaloviruses and MHC class I antigen presentation pathways. Viral Immunol. 16, 231–242.

Beasley, J.N., Patterson, L.T., McWade, D.H., 1970. Transmission of Marek's disease by poultry house dust and chicken dander. Am. J. Vet. Res. 31, 339–344.

Burgess, S.C., Basaran, B.H., Davison, T.F., 2001. Resistance to Marek's disease herpesvirus-induced lymphoma is multiphasic and dependent on host genotype. Vet. Pathol. 38, 129–142.

Calnek, B.W., 2001. Pathogenesis of Marek's disease virus infection. Curr. Top. Microbiol. Immunol. 255, 25–55.

Calnek, B.W., Adldinger, H.K., Kahn, D.E., 1970. Feather follicle epithelium: a source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. 14, 219–233.

Cho, K., Mubarak, M., Kimura, T., Ochiai, K., Itakura, C., 1996. Sequential skin lesions in chickens experimentally infected with Marek's disease virus. Avian Pathol. 25, 325–344.

Cho, K.O., Park, N.Y., Endoh, D., Ohashi, K., Sugimoto, C., Itakura, C., Onuma, M., 1998. Cytology of feather pulp lesions from Marek's disease (MD) virus-infected chickens and its application for diagnosis and prediction of MD. J. Vet. Med. Sci. 60, 843–847.

Cole, R.K., 1968. Studies on genetic resistance to Marek's disease. Avian Dis. 12, 9–28.

Davison, F., Nair, V., 2005. Use of Marek's disease vaccines: could they be driving the virus to increasing virulence? Expert Rev. Vaccines 4, 77–88.

Djeraba, A., Musset, E., Bernardet, N., Le Vern, Y., Quere, P., 2002. Similar pattern of iNOS expression, NO production and cytokine response in genetic and vaccination-acquired resistance to Marek's disease. Vet. Immunol. Immunopathol. 85, 63–75.

Frazer, I.H., Thomas, R., Zhou, J., Leggatt, G.R., Dunn, L., McMillan, N., Tindle, R.W., Filgueira, L., Manders, P., Barnard, P., Sharkey, M., 1999. Potential strategies utilised by papillomavirus to evade host immunity. Immunol. Rev. 168, 131–142.

Gimeno, I.M., Witter, R.L., Hunt, H.D., Lee, L.F., Reddy, S.M., Neumann, U., 2001. Marek's disease virus infection in the brain: virus replication, cellular infiltration, and major histocompatibility complex antigen expression. Vet. Pathol. 38, 491–503.

Hunt, H.D., Lupiani, B., Miller, M.M., Gimeno, I., Lee, L.F., Parcells, M.S., 2001. Marek's disease virus down-regulates surface expression of MHC (B complex) class I (BF) glycoproteins during active but not latent infection of chicken cells. Virology 282, 198–205.

Islam, A., Cheetham, B.F., Mahony, T.J., Young, P.L., Walkden-Brown, S.W., 2006. Absolute quantitation of Marek's disease virus and herpesvirus of turkeys in chicken lymphocyte, feather tip and dust samples using real-time PCR. J. Virol. Methods 132, 127–134.

Jarosinski, K.W., Njaa, B.L., O'connell, P.H., Schat, K.A., 2005. Pro-inflammatory responses in chicken spleen and brain tissues after infection with very virulent plus Marek's disease virus. Viral Immunol. 18, 148–161.

Johnson, E.A., Burke, C.N., Fredrickson, T.N., DiCapua, R.A., 1975. Morphogenesis of Marek's disease virus in feather follicle epithelium. J. Natl. Cancer Inst. 55, 89–99.

Kaiser, P., Underwood, G., Davison, F., 2003. Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease. J. Virol. 77, 762–768.

Lee, L.F., 1979. Macrophage restriction of Marek's disease virus replication and lymphoma cell proliferation. J. Immunol. 123, 1088–1091.

Lee, S.I., Ohashi, K., Morimura, T., Sugimoto, C., Onuma, M., 1999. Re-isolation of Marek's disease virus from T cell subsets of vaccinated and non-vaccinated chickens. Arch. Virol. 144, 45–54.

Leggatt, G.R., Dunn, L.A., De Kluyver, R.L., Stewart, T., Frazer, I.H., 2002. Interferon-gamma enhances cytotoxic T lymphocyte recognition of endogenous peptide in keratinocytes without lowering the requirement for surface peptide. Immunol. Cell Biol. 80, 415–424.

Levy, A.M., Davidson, I., Burgess, S.C., Heller, E.D., 2003. Major histocompatibility complex class I is downregulated in Marek's disease virus infected chicken embryo fibroblasts and corrected by chicken interferon. Comp. Immunol. Microbiol. Infect. Dis. 26, 189–198.

Markowski-Grimsrud, C.J., Schat, K.A., 2002. Cytotoxic T lymphocyte responses to Marek's disease herpesvirus-encoded glycoproteins. Vet. Immunol. Immunopathol. 90, 133–144.

Mast, J., Goddeeris, B.M., Peeters, K., Vandesande, F., Berghman, L.R., 1998. Characterisation of chicken monocytes, macrophages and interdigitating cells by the monoclonal antibody KUL01. Vet. Immunol. Immunopathol. 61, 343–357.

Morgan, R.W., Sofer, L., Anderson, A.S., Bernberg, E.L., Cui, J., Burnside, J., 2001. Induction of host gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus. J. Virol. 75, 533–539.

Moriguchi, R., Fujimoto, Y., Izawa, H., 1982. Chronological observations of feather pulp lesions in chickens inoculated with Marek's disease virus. Avian Dis. 26, 375–388.

Moriguchi, R., Fujimoto, Y., Izawa, H., 1984. Marek's Disease in field chickens: correlation between incidence of Marek's disease and nuclear-inclusion formation in the feather-follicle epithelium. Avian Dis. 28, 331–342.

Moriguchi, R., Fujimoto, Y., Mori, F., Izawa, H., 1986. Chronological observations of Marek's disease-associated feather-pulp lesions in field chickens. Avian Dis. 30, 284–292.

Netea, M.G., Kullberg, B.J., Verschueren, I., Van Der Meer, J.W., 2000. Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1beta. Eur. J. Immunol. 30, 3057–3060.

Okazaki, W., Purchase, H.G., Burmester, B.R., 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14, 413–429.

Pass, D.A., 1995. Normal anatomy of the avian skin and feathers. Semin. Avian Exot. Pet Med. 4, 152–160.

Payne, L.N., 2004. Pathological responses to infection. In: Davison, F., Nair, V. (Eds.), Mareks Disease, An Evolving Problem. Elsevier Academic Press, Great Britain, pp. 78–97.

Quere, P., Rivas, C., Ester, K., Novak, R., Ragland, W.L., 2005. Abundance of IFN-alpha and IFN-gamma mRNA in blood of resistant and susceptible chickens infected with Marek's disease virus (MDV) or vaccinated with turkey herpesvirus; and MDV inhibition of subsequent induction of IFN gene transcription. Arch. Virol. 150, 507–519.

Schat, K.A., Xing, Z., 2000. Specific and nonspecific immune responses to Marek's disease virus. Dev. Comp. Immunol. 24, 201–221.

Schat, K.A., Calnek, B.W., Fabricant, J., 1982. Characterization of two highly oncogenic strains of Marek's disease virus. Avian Pathol. 11, 593–605.

Sharma, J.M., Witter, R.L., Purchase, H.G., 1975. Absence of age-resistance in neonatally thymectomised chickens as evidence for cell-mediated immune surveillance in Marek's disease. Nature 253, 477–479.

Whitmire, J.K., Tan, J.T., Whitton, J.L., 2005. Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. J. Exp. Med. 201, 1053–1059.

Witter, R.L., Solomon, J.J., Champion, L.R., Nazerian, K., 1971. Long-term studies of Marek's disease infection in individual chickens. Avian Dis. 15, 346–365.

Xing, Z., Schat, K.A., 2000. Expression of cytokine genes in Marek's disease virus-infected chickens and chicken embryo fibroblast cultures. Immunology 100, 70–76.