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Differential Cytokine Responses following Marek’s Disease Virus Infection of Chickens Differing in Resistance to Marek’s Disease

Pete Kaiser,* Greg Underwood,† and Fred Davison

Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom

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The production of cytokine mRNAs, in addition to viral DNA, was quantified by real-time quantitative reverse transcription-PCR (RT-PCR) (cytokines) or PCR (virus) in splenocytes during the course of Marek’s disease virus (MDV) infection in four inbred chicken lines: two resistant (lines 61 and N) and two susceptible (lines 72 and P). Virus loads were only different after 10 days postinfection (dpi), increasing in susceptible lines and decreasing in resistant lines. Gamma interferon (IFN-γ) mRNA was expressed by splenocytes from all infected birds between 3 and 10 dpi, associated with increasing MDV loads. For other cytokines, differences between lines were only seen for interleukin-6 (IL-6) and IL-18, with splenocytes from susceptible birds expressing high levels of both transcripts during the cytolymphocytic phase of infection, whereas splenocytes from resistant birds expressed neither transcript. These results indicate that these two cytokines could play a crucial role in driving immune responses, which in resistant lines maintain MDV latency but in susceptible lines result in lymphomas.

Marek’s disease virus (MDV) is a highly cell-associated, lymphotropic alphaherpesvirus that causes Marek’s disease (MD) in chickens. Clinical signs include immunosuppression, polyneuritis, and T-cell lymphoma formation in the visceral and ectoderm-derived tissues. Although MD has been controlled by vaccination for over 30 years, it continues to be a serious threat to the health and welfare of poultry, and there is growing evidence that intensive use of vaccines is driving the virus to increasing virulence (62). Infection with virulent strains of serotype 1 MDV causes an early cytolymphocytic infection (3 to 7 days postinfection [dpi]), primarily in B lymphocytes with temporary, often profound, immunosuppression (14) and T-cell activation. Once activated, T lymphocytes themselves become susceptible to infection, which can be lytic, but after about 7 dpi, the virus enters latency (54).

Mechanisms underpinning establishment and maintenance of latency have not been elucidated, but are clearly influenced by a complex set of interactions involving viral genes and the host’s immune responses (15, 48). Cytokines have been implicated in the maintenance of latency, especially alpha interferon (IFN-α) (59, 60) and a soluble latency-maintaining factor (12).

All chicken genotypes are susceptible to MDV infection, but they differ greatly in their resistance or susceptibility to clinical MD. Several genetic loci are involved (14). The chicken major histocompatibility complex (MHC) has a strong influence on MD resistance (7, 38). Genes within the class I (B-F) region are most influential, with the B21 haplotype (line N) expressing the lowest levels of class I but possessing the highest order of resistance (29), whereas the B19 haplotype (line P) expresses the highest levels of class I and represents the highest level of susceptibility. Other forms of genetic resistance are associated with genes outside of the MHC, the most notable examples being lines 61 and 72, which are homozygous for the B2 haplotype (14, 17). Line 61 is highly resistant, and line 72 is highly susceptible to clinical MD (32). MD resistance in these lines has been mapped to genetic loci outside of the MHC, most importantly a cluster of genes (MDV1 locus) on chicken chromosome 1 (9). This region has synteny with the mammalian lectin-like natural killer (NK) cell antigen complex (31, 65) that contains the Cmv1 locus for resistance to murine cytomegalovirus (8, 33, 47). The outcome of MDV infection is associated with MDV load during the early stages of infection, a significant association between viral load in peripheral blood leukocytes and the development of clinical MD being evident as early as 4 dpi (10).

Until recently the role of cytokines in the pathogenicity of and immune responses to MD has been poorly understood. Xing and Schat (63, 64) investigated the effects of MDV infection on transcription of a number of cytokines, both in vitro and in vivo. IFN-γ transcription was increased from as early as 3 dpi until at least 15 dpi, the time the experiment terminated. There was also upregulation of interleukin-1β (IL-1β), IFN-α, and, after 6 dpi, inducible nitric oxide synthase (iNOS). Xing and Schat (64) proposed that IFN-γ plays a pivotal role in the early pathogenesis and immune responses to MDV infection.

Recent progress in the cloning of additional chicken cytokines has resulted in the development of a more comprehensive panel of reagents for investigating the innate and acquired immune mechanisms controlling responses to avian diseases, at both cellular and molecular levels. This provides an opportunity to investigate the involvement of key cytokines in MD pathogenesis in the context of resistant and susceptible chicken genotypes. Chicken orthologues of the Th1 cytokines IFN-γ,

* Corresponding author. Mailing address: Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom. Phone: (44) 1635 777277. Fax: (44) 1635 777263. E-mail: pete.kaiser@bbsrc.ac.uk.
† Present address: Bioproperties (Australia) Pty. Ltd., Ringwood North, Victoria 3134, Australia.
IL-2, and IL-18 (18, 50, 56) and the proinflammatory cytokines IL-1β (61) and IL-6 (51) have also been cloned and sequenced. Genomic sequences and gene structures for IFN-γ (28), IL-2 (26), IL-18 (Kaiser, unpublished data), IL-1β (Kaiser, unpublished data), IL-15 (Kaiser, unpublished data), IL-8 (25), and IL-6 (Kaiser, unpublished data) have been fully determined. This information now makes it possible to design probes and primers to specifically quantify cytokine mRNA by using real-time quantitative reverse transcription-PCR (RT-PCR), and so monitor changes in cytokine transcription during the course of MD.

**Experimental design and sample preparation.** We determined MDV load and levels of the abovementioned cytokines in spleen cells at various times after infection with MDV and compared them with those in uninfected age-matched controls. Differences in the levels of cytokine mRNA between resistant and susceptible lines of birds during the course of MDV infection should help delineate mechanisms underlying the resistance/susceptibility profiles.

Chicks were obtained from four inbred lines of White Leghorns that were unvaccinated and maintained under disease-free conditions at the Institute for Animal Health, Compton, Berkshire, United Kingdom. At 2 weeks of age, chicks were randomly selected and placed in “infected” and “control” groups. Due to the need for contemporaneous sampling of both control and infected chickens and comparisons between the four inbred lines, the experiment was carried out in two parts. The first involved only the MD-susceptible lines 72 and 72, and the second involved the MD-resistant lines 61 and N. In each case, infected birds of both lines were housed in separate cages in the same filtered-air, positive-pressure isolation room and injected intra-abdominally with 1,000 PFU of the virulent serotype 1 MDV strain HPRS-16 (46) in a chick kidney cell (CKC) suspension (0.2 ml). Similarly, controls were kept together in a separate isolation room and injected intra-abdominally with the same number of uninfected CKC (0.2 ml). Spleens from three infected chickens and two controls of each group were randomly selected and placed in positive-pressure isolation room. DNA was extracted by standard procedures, and MDV DNA was quantified with TaqMan PCR. DNA was extracted by standard procedures, and samples were stored at $-20^\circ\text{C}$. The MDV-specific probe (Table 1) was designed to be specific for the meq gene (24) and

<table>
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<tr>
<th>Target</th>
<th>Probe or primer$^a$</th>
<th>Sequence</th>
<th>Exon boundary</th>
<th>Accession no.$^b$</th>
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$^a$F, forward; R, reverse.

$^b$Genomic DNA sequence.

ATIONAL SUMMARY: Quantitative RT-PCR was used to examine for the first time the expression of cytokines during Marek’s disease (MD) in two susceptible (72 and 72) and two resistant lines of birds. The experiment was carried out in two parts. The first involved only the MD-susceptible lines 72 and 72, and the second involved the MD-resistant lines 61 and N. In each case, infected birds of both lines were housed in separate cages in the same filtered-air, positive-pressure isolation room and injected intra-abdominally with 1,000 PFU of the virulent serotype 1 MDV strain HPRS-16 (46) in a chick kidney cell (CKC) suspension (0.2 ml). Similarly, controls were kept together in a separate isolation room and injected intra-abdominally with the same number of uninfected CKC (0.2 ml). Spleens from three infected chickens and two controls of each group were randomly selected and placed in positive-pressure isolation room. DNA was extracted by standard procedures, and MDV DNA was quantified with TaqMan PCR. DNA was extracted by standard procedures, and samples were stored at $-20^\circ\text{C}$. The MDV-specific probe (Table 1) was designed to be specific for the meq gene (24) and
TABLE 2. Standard curve data from real-time quantitative RT-PCR of total RNA extracted from splenocytes stimulated with mitogen or real-time quantitative PCR of plasmid containing the meq gene

<table>
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<tr>
<th>RNA or plasmid</th>
<th>ΔRoR</th>
<th>Log dilutions</th>
<th>C_v values*</th>
<th>R²</th>
<th>Slope</th>
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<td>meq†</td>
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<td>15-36</td>
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<td>8-22</td>
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<td>IFN-γ</td>
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</table>

* ΔRoR, change in the reporter dye.
* C_v, threshold cycle value (the cycle at which the change in the reporter dye levels detected passes the ΔRoR).
* R², coefficient of regression.
* meq, plasmid of known concentration containing the viral target sequence.

labeled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the quencher N, N, N, N'-tetramethyl-6-carboxy rhodamine (TAMRA) at the 3' end. The PCR assay used the TaqMan PCR core reagents kit (PE Applied Biosystems). Amplification and detection of specific products were carried out with the ABI PRISM 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 20 s and 60°C for 1 min.

A standard curve was prepared over a range of 10-fold dilutions with comparable reaction efficiencies to estimate virus copy number from sample threshold cycle (C_v) values. The method described by Pevenstein et al. (44) for absolute quantitation of varicella-zoster virus and herpes simplex virus was followed, using a cloned 455-bp fragment of the MDV Eco Q (meq) gene (24). Seven 10-fold serial dilutions of plasmid were made, ranging from 57 × 10⁶ to 5.7 × 10⁷ copies of plasmid per reaction. Each dilution was tested in duplicate. The plasmid concentration in the undiluted miniprep sample was shown to be 6.01 ± 0.25 μg ml⁻¹ by spectrophotometry (optical density at 260 nm [OD₂₆₀]). With a molecular mass of 4.38 × 10⁻¹² g per plasmid, the undiluted plasmid preparation was calculated to contain 1.37 ± 0.06 × 10¹² plasmids ml⁻¹. There are two copies of the meq gene in the MDV genome, and therefore virus copy number is assumed to be half the plasmid copy number. Relevant standard curve data are shown in Table 2.

Statistical analysis of mean values between multiple groups was done with one-way analysis of variance. When a level of significance was found, a Tukey's test was conducted on the data to determine the relative difference between the means within each data set. When there were only two groups of data, means were compared by using a paired Student's t test.

MDV was not detected by quantitative PCR assay in splenocytes isolated from uninfected chicks (data not shown). There were no significant differences in viral loads between the four inbred lines until 10 dpi—the onset of latency (Fig. 1). Viral loads in the two resistant lines were lower than those in the susceptible lines from 10 dpi onwards, although because of bird-to-bird variations, differences between the susceptible and resistant lines were not significant (P < 0.05) until after 10 dpi for line N and 14 dpi for line 6. Of the two resistant lines, the viral load in line 6 was greater (P < 0.05) than that in line N at 10 and 14 dpi and then decreased to the same level as that in line N by 21 dpi.

To our knowledge, this is the first detailed comparison of early changes in MDV copy number in spleen cells of chicken genotypes that differ markedly in their resistance and mechanisms of resistance to MD (2, 14, 49). In earlier studies, these lines have been used in pairs: line N versus line P (1, 13, 20, 39) and line 6 versus line 7 (3, 4, 32, 45). MD resistance in line N, dependent on the B21 MHC haplotype, is highly protective against clinical disease. Levels of MDV infection in the lymphoid organs during the lytic phase (first week postinfection) are essentially indistinguishable between lines N and P (1, 13, 20, 39), although markedly reduced levels of latent infection are evident in line N. Calnek (14) proposed that this MHC-related genetic resistance, mainly expressed in the latent phase of MD, has an immunological basis related to a lower level of T-cell infection. By comparison, resistance in line 6, (B2 MHC haplotype) is strongly associated with the MDV-1 locus (see earlier). It has been postulated that resistance resides at the level of target cell numbers and their susceptibility to MDV infection and is evident during the lytic phase of infection (16, 32).

Bumstead et al. (10) reported that the correlation between this PCR assay and plaque assays is high and suggested that MDV copy number per cell is rather uniform. It therefore seems reasonable to compare observations made in this study

FIG. 1. Splenic MDV load at various times after infection with MDV in four different inbred lines of chickens. MDV load was measured as MDV copy number per 10⁶ leukocytes by quantitative PCR. Each value is the mean of three samples, and vertical bars represent the standard error of the mean. R, resistant; S, sensitive.
with the PCR-based assay with earlier findings that used plaque assays.

Both susceptible genotypes had higher viral loads than the resistant lines from the onset of the latent phase (Fig. 1). These markedly different patterns of viral load in splenocytes confirm earlier findings that different resistance mechanisms must operate in susceptible and resistant genotypes (reviewed by Bacon et al. [2] and Calnek [14]). Differences in splenocyte viral loads in resistant lines from 10 dpi onwards are consistent with the hypothesis that different mechanisms operate in MHC-related and non-MHC-related MD resistance (2, 14). Splenocyte MDV load in line N receded fairly rapidly in the early latent phase, when MDV is found in latently infected T cells (54), consistent with the suggestion (14) that specific cell-mediated immune responses are an important component of MHC-related resistance when cytotoxic T lymphocytes become activated (41, 42). In contrast, the more gradual decrease in splenocyte viral load in line 61 indicates different immunological mechanisms are likely to be operating in non-MHC-related resistance.

Quantification of cytokine mRNA expression. Cytokine mRNA was isolated and the levels in infected and control samples were quantified essentially as described by Kaiser et al. (27). Total RNA was prepared from samples of purified splenocytes from three infected birds and two uninfected birds at each time point for each line by using the RNeasy mini kit (Qiagen) and following the manufacturer’s instructions. Purified RNA was eluted in 140 μl of RNase-free water and stored at −70°C. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron-exon boundaries (see Table 1 for details) and labeled with FAM at the 5′ end and TAMRA at the 3′ end. The RT-PCR assay used the TaqMan EZ RT-PCR kit (PE Applied Biosystems). Amplification and detection of specific products were also done with the ABI PRISM 7700 Sequence Detection System, but with the following cycle profile: 50°C for 2 min, 96°C for 5 min, 60°C for 30 min and 95°C for 5 min, followed by 40 cycles of 94°C for 20 s and 59°C for 1 min.

Standard curves for the cytokine and 28S rRNA-specific reactions were generated as previously described (27). Relevant standard curve data are shown in Table 2. To account for variation in sampling and RNA preparation, the C_{v} values for cytokine-specific products for each sample were standardized as previously described (27).

There were no significant differences in expression of mRNA for IL-1β, IL-8, or IL-15, compared with the values for the appropriate uninfected controls (data not shown). Increased levels of IL-2 in infected birds occurred only in line 72 at 21 dpi (data not shown). Increased levels of IFN-γ mRNA, ranging from 5- to 25-fold, were evident in infected birds of all lines between 3 and 10 dpi, except for line N, where differences were evident at 3 to 7 dpi and at 21 dpi (Fig. 2). The greatest increase was in line P, where IFN-γ mRNA levels increased steadily from 5- to 25-fold between 3 and 7 dpi. Xing and Schat (63) observed increased IFN-γ expression following MDV infection from 3 to 15 dpi. Djeraiba et al. (19) also reported strong induction of IFN-γ mRNA expression in B21 haplotype chickens at 3 and 7 dpi with a very virulent strain of MDV, RB-1B. In this study, the extents of IFN-γ mRNA upregulation were similar in all lines, with line P showing the greatest up-regulation. The present work contrasts with an earlier study by Hong and Sevoian (23) that reported IFN levels were higher in resistant (K strain) than susceptible (S strain) chickens. However, Hong and Sevoian relied on a bioassay for IFNs that did not differentiate IFN-γ from IFN-α, IFN-β, or IFN-α/β. Taken together, the present results suggest that IFN-γ production is unlikely to contribute to the differences in viral loads between the resistant and susceptible inbred lines.

Xing and Schat (64) showed that IFN-γ has a negative effect on lytic replication of MDV in cell culture. IFN-γ mRNA levels peaked earlier in splenocytes from infected resistant birds (4 dpi) than in those from infected susceptible birds (5 to 7 dpi). These early high levels of IFN-γ expression in resistant lines could drive the virus into latency earlier during lytic infection than in the susceptible lines. This would presumably limit the level of latent infection and ultimately lead to a reduction in clinical disease.

Marked differences between susceptible and resistant genotypes were evident only for IL-6 (Fig. 3) and IL-18 (Fig. 4) expression. Splenocytes from infected susceptible birds expressed high levels of IL-6 message (compared to uninfected controls) early during MDV infection (3 to 5 dpi for line 72 and 4 and 5 dpi for line P). Splenocytes from infected birds of lines 61 and 72 both expressed high levels of IL-6 message at 10 and 21 dpi. For IL-18, splenocytes from infected susceptible birds expressed high levels of message early during MDV infection (4 and 5 dpi for both lines). Splenocytes from infected birds of line 72 also expressed high levels of IL-18 message at 10 dpi. Splenocytes from infected resistant lines never expressed more IL-18 message than noninfected age-matched controls.

IL-6 is a proinflammatory cytokine, and its elevated production early in cytolytic infection (Fig. 3) may simply represent a response to increased pathology in susceptible lines, such as line 72 (3). Xing and Schat (63) failed to detect any IL-6 expression following MDV infection. Unfortunately, these
workers quantified myelomonocytic growth factor (36) as a measure of IL-6-like activity. Myelomonocytic growth factor is an avian-specific cytokine that has homology with both mammalian IL-6 and granulocyte colony-stimulating factor (G-CSF), but unlike IL-6, it does not have proinflammatory activity. The results therefore are not comparable with those for chicken IL-6 (51) reported here.

IL-18 is a proinflammatory cytokine expressed by a wide variety of cells, including monocytes/macrophages and dendritic cells. In mammals, IL-18 has three main roles: to induce IFN-γ production (40), to enhance NK cell activity (58), and to activate neutrophils (35). IL-18 is produced as a propeptide that is cleaved into an active form by the action of caspase-1 (21, 22). It must therefore be borne in mind that expression of IL-18 mRNA does not necessarily correlate with the expression of functional IL-18 protein. Assuming that IL-18 mRNA is related, at least to some extent, to the production of bioactive IL-18 and the kinetics of IFN-γ production following MDV infection are broadly similar in resistant and susceptible lines, the observed increase in IL-18 mRNA production early in the lytic infection in susceptible lines is unlikely to have a direct effect on IFN-γ expression.

Activation of NK cells after MDV has been implicated as an important component of the cell-mediated immune response in the genetically resistant line 61 (53). However, increased NK activity was only detected in the late stages (28 to 35 dpi) of MD indicating it is more likely to be involved in antitumor responses than antiviral responses. Moreover, in line 72, NK activity was actually reduced after MDV infection (52). The increased levels of IL-18 in both susceptible lines in the present study are therefore not consistent with it influencing NK activity.

Xing and Schat (63) reported that IL-8 was expressed in splenocytes from MDV-infected birds after 3 dpi. IL-8 can act as a chemoattractant for T cells, among others. Moreover, MDV has been shown to encode a CXC chemokine described as a homologue of IL-8 (vIL-8) (43). This led Xing and Schat (63) to speculate that the production of this CXC chemokine during the lytic infection of B cells might attract T cells to the areas of virus replication and, furthermore, that the observed early expression of IFN-γ following MDV infection should stimulate the expression of IL-8 receptors on T cells (cf. reference 57). There are several problems with this proposition. First, we observed no evidence of MDV-induced changes in chicken IL-8 expression in either resistant or susceptible lines. More importantly, although vIL-8 has high amino acid identity with human and chicken IL-8s, there are several important differences between this so-called vIL-8 and known IL-8s. CXC chemokines can be subdivided into two groups. Most possess an ELR motif immediately preceding the first cysteine residue, including IL-8. Other CXC chemokines, such as PF-4 and a recently discovered B-lymphocyte chemoattractant, lack this ELR motif (34). So-called “vIL-8” also lacks an ELR motif and in its place has a D KR motif (43). The presence of the ELR motif controls the tropism of the CXC chemokine, because different CXC receptors bind CXC chemokines, depending on the presence or absence of the ELR motif. In humans, the genomic structure of most CXC chemokines consists of four exons and three introns (including IL-8), whereas the genes for PF-4 and NAP-2 comprise only three exons and two introns. The gene encoding vIL-8 also has three exons and two introns. Therefore the gene product should more properly be described as a viral CXC chemokine (vCXC) rather than vIL-8.

Xing and Schat (63) also suggested that IFN-γ stimulates the production of IL-1β, which in turn upregulates IL-2 for the generation of cytotoxic lymphocytes, and iNOS, which induces macrophage nitric oxide (NO) production to inhibit MDV replication. In contrast, we did not observe an increase in IL-1β mRNA expression following MDV infection. However, IL-1β, like IL-18, is expressed as a propeptide and subsequently cleaved by caspase-1 (6, 30), so IL-1β mRNA does not necessarily correlate with protein expression.

FIG. 3. Quantification of IL-6 in splenocytes after MDV infection, expressed as fold change in cytokine mRNA levels in infected birds compared to those in age-matched, uninfected controls. Each value is the mean of three samples, and vertical bars represent the standard error. R, resistant; S, sensitive.

FIG. 4. Quantification of IL-18 in splenocytes after MDV infection, expressed as fold change in cytokine mRNA levels in infected birds compared to those in age-matched, uninfected controls. Each value is the mean of three samples, and vertical bars represent the standard error. R, resistant; S, sensitive.
This work has shown differential IL-18 expression in spleen cells of susceptible and resistant genotypes of chickens following MDV infection. However, knowledge of the basic biology of chicken IL-18 is very limited, and the functional relevance of elevated IL-18 mRNA expression during the cytolytic phase of infection in susceptible lines is difficult to account for at present. As more reagents to study this cytokine become available, mechanisms underlying and controlled by elevated IL-18 mRNA expression should be elucidated. Recently, a number of expressed sequence tags (ESTs) for chicken chemokines and their receptors have become available. Considering the different leukocyte classes involved in MD infection and the roles of chemokines as chemoattractants for leukocytes, future studies should provide further hypotheses to explain differences in resistance to MD.

The results presented here show that, although MDV loads in the two MDV-susceptible genotypes were similar, they differed markedly from those in resistant genotypes. More importantly, the changes in MDV load differed between the two resistant genotypes, consistent with the view that different immune mechanisms operate in MHC-linked and non-MHC-linked resistance (2, 14). These differences are almost certainly driven by differences in cytokine expression in the different inbred lines. There was increased expression of IFN-γ mRNA after infection with MDV in all lines, confirming the view (48) that this cytokine plays an important role in the immune response to MDV. However, there were no differences in IFN-γ mRNA levels between susceptible and resistant genotypes, but there were marked differences in IL-6 and IL-18 mRNA levels, indicating that these two cytokines could play a crucial role by driving immune responses that in susceptible lines result in tumor formation and in resistant lines result in lesion regression (11).

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