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Understanding Marek's Disease Immunity: A Continuing Challenge

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Abstract: Immune responses to infection with Marek's disease virus (MDV) have been studied intensively since the isolation of MDV in cell cultures and the development of the vaccines around 1970. More than 30 years later, the understanding of the importance of different components of the acquired and innate immune responses is still limited. Antibody responses are less important than cell-mediated immune (CMI) responses due to the cell-associated nature of MDV, although maternal antibodies can interfere with the efficacy of vaccines. The proteins that are detected by the maternal antibodies remain unknown. CMI responses can be directed against a number of glycoproteins as well as immediate early and early proteins, but the relative importance of the responses against individual proteins is unknown. Information concerning the CMI epitopes is still lacking. The importance of antiviral versus antitumor immunity has not been settled. Different types of innate immune responses to MDV (e.g., NK cells, NO, macrophages, interferon-γ) have been described. Information on the nature of the target cells recognized by NK cells is lacking. Do NK cells recognize specific MDV proteins or is the down regulation of MHC class I antigens sufficient to activate the NK cells? NO can inhibit MDV replication *in vitro* and *in vivo*, but infection with very virulent + strains results in increased levels of NO. It is certainly feasible that excessive NO production can lead to pathology. Finally, the relationship between different mechanisms of genetic resistance and immune responses is still incompletely understood.

Key words: Marek's disease, acquired immunity, innate immunity, cytotoxic T lymphocytes

Introduction

Marek's disease (MD), caused by an alpha-herpesvirus remains an economically important disease in chickens even in the face of successful vaccination programs. The reasons for the continued economical importance are a combination of relatively high vaccination costs, a continued evolution of the MD virus (MDV) strains to ever more virulent strains (Witter, 2001a), early MDV challenge prior to vaccine-induced protection, and the economical realities of broiler production. Condemnation rates for MD ("skin leukosis") of 1% are already considered "vaccine breaks" causing financial problems for the broiler industry. This is mostly caused by prolonged processing time resulting in increased labor costs. That most of the broiler flocks experience far less than 1% MD condemnations is actually a remarkable tribute to the efficacy of current vaccine strategies. This paper will briefly review the current knowledge of the immunological basis of vaccine-induced protective immunity and indicate areas that need to be addressed in the future. For more detailed information on MDV and immunity the interested reader is referred to recent review articles (Schat, 2001; Schat and Markowski-Grimsrud, 2001; Schat and Xing, 2000).

Early immunity: One of the key problems in protection against MD is that chicks are challenged with MDV, as well as other pathogens, as soon as they are placed on the farms, which is in general within 1 to 3 days after hatching. Vaccine-induced protection can be demonstrated as early as 5 days post vaccination (dpv) under controlled conditions, e.g., in laboratory challenge experiments (Witter, 2001b). Vaccination at embryo day (ED)18 (Sharma and Burmester, 1982) was developed based on the hypothesis that an increase in time between vaccination and the earliest possible challenge may allow the development of a more solid protective immunity. However, it is also plausible that the highly efficient administration of the *in ovo* vaccine reduces the percentage of chicks that are not properly vaccinated, thus reducing the number of chicks at risk. Although *in ovo* vaccination for MD is now widely and successfully used in the USA (Avakian *et al*., 2000; Ricks *et al*., 1999), the mechanisms for the early protection have not yet been elucidated. Sharma (1989) examined the production of interferon (IFN) in lung homogenates of chicks between 3 and 7 days post vaccination (dpv). *In ovo* vaccination resulted in significantly higher levels of IFN at 3 and 5 dpv than after vaccination at 1 day of age (Fig. 1). It is not clear if IFN-α or IFN-γ was detected, but IFNs can upregulate NK cell activity, increase expression of major histocompatibility complex (MHC) class I and II antigens, and several components needed for the presentation of nonapeptides by MHC class I antigens. In addition, IFN-γ can stimulate macrophages to produce inducible nitric oxide (NO) synthase (iNOS)

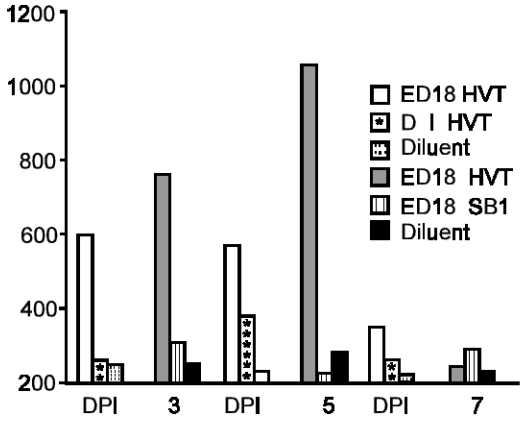

Fig. 1: Interferon in lung homogenates 3 to 7 days post vaccination with HVT or SB-1 at embryo day 18 or at one day of age (Sharma, 1989)

leading to the production of NO, which can inhibit MDV replication (Xing and Schat, 2000a; Djeraba *et al.*, 2000). It still remains to be determined if *in ovo* vaccination induces an earlier maturation of the innate immune responses and/or induction of acquired immune responses. An answer to this question will become especially important if manipulation of innate and acquired immune responses through the use of recombinant cytokines becomes a reality as suggested by Lowenthal *et al.* (2000). Inoculation of 2-week-old chicks with avian myelomonocytic growth factor (cMGF) resulted in a prolonged survival after challenge with the very virulent RB-1B strain. The combination of cMGF and HVT treatments improved the overall protection against RB-1B (Djeraba *et al.*, 2002). However, the use of recombinant cytokines may also have unexpected, negative effects on immune responses. For example, the use of recombinant chicken IFN-α administered as a protein in drinking water or expressed in a recombinant MDV vaccine strain caused an unexpected decrease in NK cell activity rather than the anticipated increase (Jarosinski *et al.*, 2001). Clearly, it will be important to gain a better understanding of the mechanism(s) inducing protective immunity after *in ovo* vaccination in order to examine the potential *in ovo* use of different cytokines to enhance immune responses.

Development of innate and acquired immune responses: Innate and acquired immune responses associated with MDV infection have been reviewed extensively and these reviews provide most of the references for this section (Schat, 2001; Schat and Markowski-Grimsrud, 2001; Schat and Xing, 2000). The key events leading to the activation of innate immune responses and the development of specific immune responses occur during the first 7 dpi. These events are closely associated with the events of the early pathogenesis of infection (Fig. 2a and b). MDV was detected in spleens as early as 36 to 48 hours post infection after intratracheal infection with feather follicle epithelium (FFE)-derived cell-free virus. Spleen cells were harvested from these chicks at 12-hour intervals and transferred into susceptible recipients to determine the presence of MDV (Schat and Xing, 2000). Most studies have used cell-associated MDV to infect chickens and virus can be detected between 3 and 4 dpi in spleen cells by virus isolation, viral protein detection by immunofluorescence, or histochemistry assays. The early lytic infection occurs in B cells, and shortly afterwards MDV can be detected in activated T cells, but not in resting T cells. The transfer of MDV from B to T cells may be facilitated by the production of vIL-8 during the lytic infection as was hypothesized originally by Schat and associates (Schat, 2001; Schat and Xing, 2000). Parcells *et al.* (2001) demonstrated that vIL-8 can attract peripheral blood mononuclear cells in support of this hypothesis. Between 5 to 7 dpi the infection becomes latent in activated T cells. During that same period and at least until 14 dpi IFN-γ transcripts were detected at increased levels, but IL-2 transcription was not influenced (Xing and Schat 2000b). Transcripts for iNOS were also detected starting at 4 dpi (Kaiser *et al.*, 2003, Jarosinski *et al.*, 2002). However, the virus strain influenced relative transcript levels for both IFN-γ and iNOS. Interestingly, there was no obvious relation between the presence or relative level of transcription of IFN-γ and iNOS (Jarosinski *et al.*, 2002). Other cytokines were also activated but the increased expression of IL-6 and IL-18 was apparently correlated with the genetic resistance of the chicken line to MD (Kaiser *et al.*, 2003). A poorly characterized cytokine, latency maintaining factor or LMF, has been described that is able to keep MDV latent in lymphocytes, when these lymphocytes are cultured *in vitro* (Buscaglia and Calnek, 1988). The importance of LMF for maintenance of latency *in vivo* has not been established.

Increased levels of NK cell-mediated lysis of LSCC-RP9 has been reported between 3 and 4 dpi when chickens were vaccinated with SB-1 or HVT before 3 to 4 weeks of age and especially when the bivalent combination was used (Heller and Schat, 1987). Vaccination at a later age did not result in increased NK cell activity, suggesting that vaccination at one day of age may lead to enhanced maturation. Differences in NK cell activation have been reported between different genetic strains after inoculation with virulent or very virulent MDV (Sharma, 1981; Garcia-Camacho *et al.*, 2003). The latter group reported increased NK cell activity in N2a line chickens from 4 to 12 dpi while P2a chickens were only positive at 8 dpi.

Acquired immune responses consisting of cytotoxic T lymphocytes (CTL) and virus-neutralizing (VN) antibodies can be detected starting at 6 to 7 dpi (Schat

Table 1: MDV proteins recognized by cytotoxic spleen cells obtained from resistant N2a (MHC: $B^{21}B^{21}$) and susceptible P2a ($B^{19}B^{19}$) chickens 7 days post inoculation with serotype 1 or 2 MDV strains. Proteins associated with protective immunity (see text) are printed in bold face

IE genes		Early genes		Glycoproteins	
N2a	P2a	N2a	P2a	N2a	P2a
ICP27	ICP27	**pp38**	**pp38**	**gB**	**gB**
ICP4		Meq	Meq	**gI**	**gI**
				gC	gE
				gK	
				gH*	
				gL*	
				gM*	

* CTL were not demonstrated in all assays.

Fig. 2 a

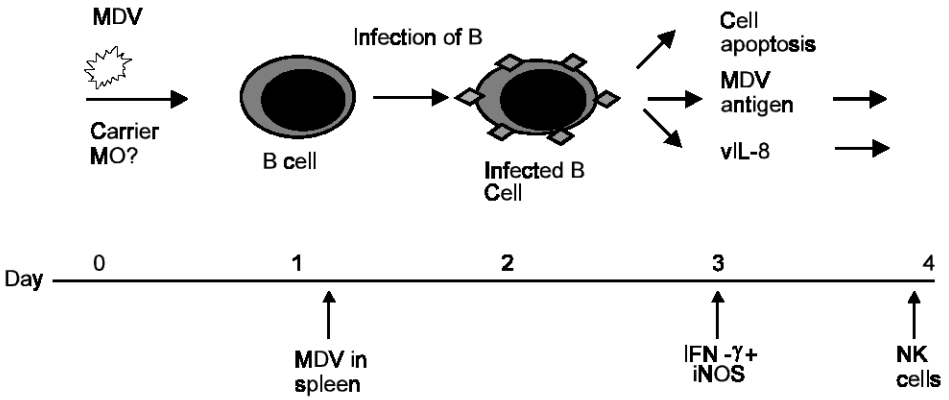

Fig. 2 b

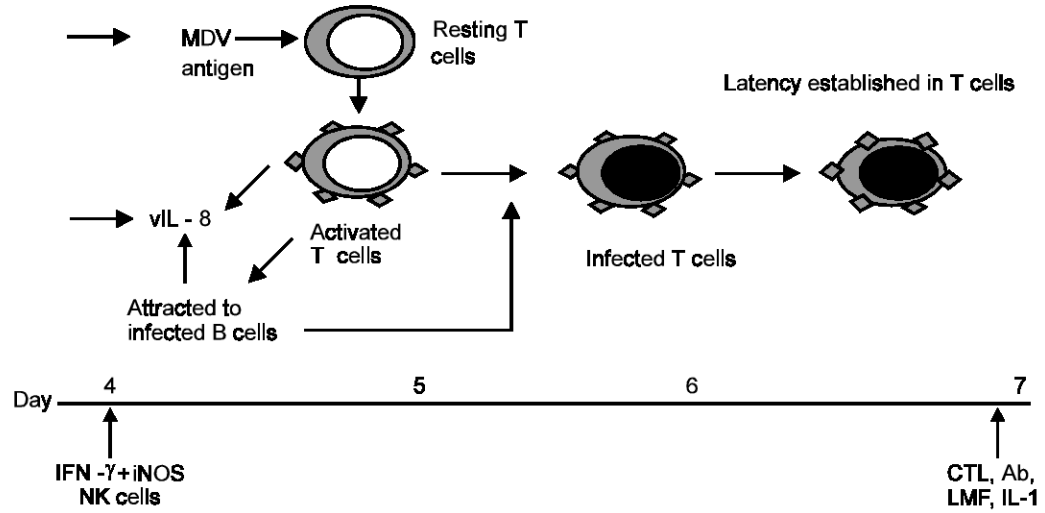

Fig. 2: Pathogenesis of infection with cell-free Marek's disease virus in relation to the development of immunity. Panel 2A: events from day 0 until day 4; panel 2B: events from day 4 to day 7

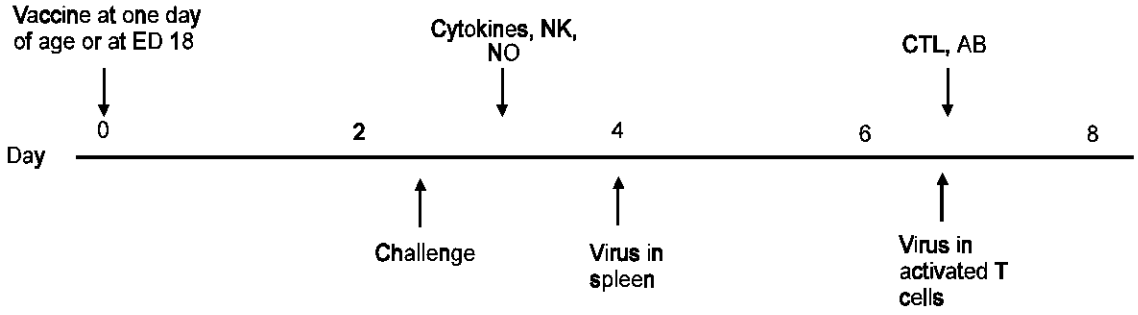

Fig. 3: Pathogenesis of infection in chickens that are vaccinated at embryo day 18 or at one day of age and challenged between 2 and 3 days of age as is the usual situation in commercial poultry production

and Xing, 2000; Schat and Markowski-Grimsrud, 2001). The relevance of the VN antibodies for protective immunity is not clear. MDV is highly cell-associated *in vivo* and the actual moment of infection is the only time that cell-free virus may be neutralized. Unfortunately, the events from the moment of exposure with cell-free virus until virus enters and replicates in B lymphocytes are poorly understood. Maternal antibodies may reduce the early lytic infection and vaccine efficacy to a limited degree probably through complement lysis of infected cells. The mechanism for antibody-mediated reduction of the lytic infection after infection with cell-free virus is poorly understood, but macrophages may be able to more efficiently eliminate antibody-virus complexes (Schat and Calnek, 1978). CTL are developing at the time that virus enters into latency and are probably more important to control virus replication during reactivation from latency than during the first 7 dpi. These CTL have been characterized as $CD4^-CD8^+$ T cell receptor (TCR)$\alpha\beta^+$ cells (Omar and Schat, 1997).

The basic events of the pathogenesis and the development of innate and acquired immunity as outlined in Fig. 2a and 2b can be modified by several factors. For example, the virulence of the MDV strain or infection at 1 day of age may prolong the lytic infection and delay or reduce the development of immune responses. The major modifying effect affecting the majority of commercial chickens is the use of vaccination. Assuming that chicks are vaccinated at the day of hatch or ED18 and placed in an MDV-contaminated environment at 3 days of age, vaccine virus will already be replicating at the time of challenge (Fig. 3). The developing immune responses will modify the pathogenesis resulting in reduced replication of the challenge virus (Schat *et al.*, 1982), because by the time challenge virus is replicating, activated cytokines, NO producing macrophages, vaccine-activated NK cells, and MDV-specific CTL are available and able to curtail virus replication. Unfortunately, studies have not been conducted to determine how challenge virus interacts with the developing vaccine-induced immunity during its initial replication. A better understanding of these interactions will be essential for the development of more effective vaccines that will be needed when MDV viruses become more virulent at some point in the future.

What are the relevant antigens?: There is a paucity of information on the relevance of specific MDV proteins that may be able to trigger innate responses. MDV-induced responses have been reported for IFN-γ and iNOS as early as 3 to 4 dpi, but cell-associated viruses were used in these studies. It is possible that MDV-infected chicken embryo fibroblasts (CEF) or chicken kidney cells (CKC) contain cells expressing cytokines, which may influence cytokine responses in the vaccinated chickens through feed-back loops. Xing and Schat (2000b) reported that the addition of rChIFN-γ and/or lipopolysaccharide (LPS) upregulated the expression of mRNA for iNOS, IL-1β, and Il-6, while the addition of LPS alone upregulated IFN-γ. It can not be excluded that inoculated MDV-infected cells complicate the analysis of the early events immediately after inoculation. Future experiments studying the immediate responses need to use cell-free virus derived from FFE. However, if research is directed toward an understanding how vaccination activates early innate immune responses, cell-associated virus will be appropriate because the large majority of vaccines are cell-associated.

There is more information concerning the knowledge of relevant antigens for the acquired immune responses. This information is derived, in part, from vaccination experiments using purified glycoproteins (Ikuta *et al.*, 1984) recombinant herpesvirus of turkeys (HVT) (Ross *et al.*, 1996) or recombinant fowlpox virus (rFPV-gB) (Nazerian *et al.*, 1996, 1992; Yanagida *et al.*, 1992) vaccines expressing different MDV proteins. The general conclusion is that VN against gB are generated, but is it not clear if this is indeed the mechanism for protection. Omar *et al.* (1998) reported that chickens vaccinated with rFPV-gB generated gB-specific CTL.

Antigen-specific CTL have been demonstrated in MDV-infected or vaccinated chickens. These CTL were

directed against several MDV proteins. Some of these proteins have been associated with protective immunity in rFPV vaccines. For example, gB protects against challenge as mentioned before. In addition, rFPV-gI induces a limited level of protection (Lee *et al.*, 2003) and rFPV-pp38 reduces viremia levels but does not protect against the disease (Nazerian *et al.*, 1992). Interestingly, there are differences in responses between resistant and susceptible lines (Table 1) (Schat and Xing, 2000; Omar and Schat, 1996; Markowski-Grimsrud and Schat, 2002). For example, ICP4, gC, and gK are recognized by CTL from genetically resistant N2a chickens but not by CTL from susceptible P2a chickens. On the other hand, P2a CTL from infected birds can lyse target cells expressing gE. These data suggest that the genetic difference in resistance to MDV between P2a and N2a lines may depend in part on the CTL responses that are generated after infection or vaccination. However, other differences such as earlier and more persistent NK cell activity in N2a versus P2a chickens, differences in NO and cytokine responses can all contribute toward genetic differences in MD resistance. In addition to lines that differ in MHC there are also chicken lines that have the same MHC background but differ greatly in their genetic resistance, e.g., line 6 versus line 7 (Bacon *et al.*, 2001). Obviously, genetic resistance to MD is multifactorial and additional studies are needed to gain a better understanding of these processes and to use this knowledge for the development of more resistant chickens and/or develop better vaccine that are targeted for use in specific chicken lines.

One of the ways to develop more specific vaccines is to identify which epitopes are recognized by CTL from genetically different chickens and to determine if one or more of these epitopes are recognized by CTL from different MHC backgrounds. Preliminary studies have delineated a 100 amino acid fragment of gB that is recognized by CTL from P2a chickens (Schat and Xing, 2000). However, target cell lines pulsed with nonapeptides synthesized on this information failed to yield positive results. It is not clear if this was simply caused by technical problems such as lack of replacement of peptides normally present by the synthesized peptides or that the modeling system was incorrect (C.J. Markowski-Grimsrud and K.A. Schat, unpublished data). One of the ways to address this question is to generate TAP-deficient reticuloendotheliosis virus (REV) cell lines expressing MHC class I with empty pockets. This approach is currently in progress in our laboratory.

One of the unresolved issues is the existence of anti-tumor immunity. Burgess *et al.* (2001a) suggested that anti-tumor responses were important for the regression of lesions. This group suggested that the immune response could be directed towards an over expressed host cell antigen that is detected by monoclonal antibody AV37 (Burgess and Davison, 2002), which detects the chicken homologue of CD30 (Burgess *et al.*, 2001b), but it is not clear if this protein is indeed a target antigen for the immune response. Further studies to determine if anti-tumor immunity against non-virally coded proteins occurs will be needed to resolve this question.

Modifying factors: It was mentioned before that the virulence of the virus strain, the genetic background of the chicken, and vaccine status can influence the outcome of infection. However, there are some important additional factors that can interfere with the effectiveness of immune responses. Chicken infectious anemia virus (CIAV), a member of the Circoviridae (Schat, 2003) can effectively interfere with the development of CTL when CIAV is replicating at the time that CTL against MDV, REV, or other pathogens are generated (Markowski-Grimsrud and Schat, 2003). Fehler and Winter (2001) reported late MDV breaks and linked these breaks to the presence of CIAV infection. It is certainly plausible that if CIAV replication occurred while MDV was reactivated or infecting chickens that CTL responses were impaired. It will be important to further study the influence of CIAV on cell-mediated immune responses to MDV as well as its impact on other pathogens.

Conclusions: Considerable progress has been made during the last decade in the understanding of the molecular biology of MD, but this has not resulted in the same level of progress of the understanding of the immune responses to MDV. It is expected that with the recent advances in the identification of many of the avian cytokines in the chicken, significant progress can be made in our understanding of the MD immunity. For example, the availability of micro-arrays for chicken lymphocytes and macrophages is expected to provide useful information on the activation of genes involved in the activation and regulation of MD immunity.

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