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THE ROLE OF INTERLEUKIN-8 LIKE GENE (vIL-8) IN THE PATHOGENESIS OF MAREKS'S DISEASE VIRUS

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THE ROLE OF INTERLEUKIN-8 LIKE GENE (vIL-8) IN THE PATHOGENESIS OF MAREK'S DISEASE

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ABSTRACT

THE ROLE OF INTERLEUKIN-8 LIKE GENE (vIL-8) IN THE PATHOGENESIS OF MAREK'S DISEASE

By

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Marek's disease (MD) is a lympho-proliferative disease of chickens caused by an alpha-herpesvirus called Marek's disease virus (MDV). MDV can interact differently with different types of cells, resulting in productive infection, latent infection or cell transformation. The molecular basis of pathogenesis of this virus has not been clearly defined. The oncogenic serotype 1 MDV encodes a virokine vIL-8, with general homology to cellular CXC chemokines such as IL-8 and Gro-α. To study the function of the vIL-8 gene, we deleted both copies of the vIL-8 gene residing in the TR_L and IR_L region of the viral genome and generated a vIL-8 deleted virus rMd5/ΔvIL-8. Growth kinetics studies showed that the vIL-8 gene is dispensable for virus replication in cell culture. *In vivo*, vIL-8 gene is involved in early cytolytic infections in lymphoid organs as indicated by less viral antigen expression detected by immunohistochemical staining. The rMd5/ΔvIL-8 virus is unimpaired in virus spreading. Similar viremia titers as the rMd5 virus at 6 and 8 dpi indicated that vIL-8 gene is not necessary for virus reactivation and is not involved in latency. Nevertheless, deletion of the vIL-8 gene compromised

transformation of the virus with reduced number of transformed cells at 5 weeks post inoculation and less gross tumors developed *in vivo*. The revertant virus that restored the expression of vIL-8 gene showed the same phenotype as the rMd5.

The role of vIL-8 in affecting the virulence and pathogenesis of MDV was also elucidated by rMd5/ΔvIL-8. The rMd5/ΔvIL-8 virus not only had decreased virulence but also showed the ability to protect against challenge with the very virulent plus (vv+) MDV strain 648A. Studies on cell tropism between the virus rMd5/ΔvIL-8 and the parental virus rMd5 inoculated chickens were carried out using flow cytometry assays (FACS). During the early cytolytic infection, there was significantly more B cells in the rMd5/ΔvIL8 virus inoculated chickens than the parental rMd5. Significant differences were also demonstrated in the virus induced numbers of activated T cells.

One of the interesting sequential differences between the vIL-8 gene and other cellular homologue is that vIL-8 encodes a "DKR" motif instead of "ELR". To study the role of the variation of this motif in MDV pathogenesis, we generated recombinant MDV, rMd5/vIL-8-ELR, carrying "ELR" motif by mutagenesis. Both *in vitro* and *in vivo* studies showed that rMd5/vIL-8-ELR has the same phenotype as rMd5.

DEDICATION

To my family, my advisors, friends.....

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INTRODUCTION

Marek's disease (MD), a contagious lymphoma of chickens, is considered a major disease problem in commercial poultry. Marek's disease virus (MDV) is classified as a member of the family *Herpesviridae*. Based on its genomic organization and significant sequence similarity, it is classified into subfamily *Alphaherpesvirinae* (Buckmaster et al. 1988; Roizman 1992a). The MDV as well as the disease itself has been studied for several decades. Many MDV genes have been characterized and the mechanisms of the pathogenesis of the disease have been explored step by step. There are three serotypes of MDV of which, only serotype 1 MDV strains are pathogenic and causing lymphomas. The most specific difference in the genomic information among different serotypes is clearly seen in the inverted repeat regions.

The vIL-8 is one of the unique genes located in this inverted repeat region. The studies described in this dissertation were designed to characterize the function of the virus encoded chemokine like gene, vIL-8, in the viral replication and pathogenesis of MDV. Chapter 1 provides a review of information on the involvement of MDV and chemokines in host immune responses. Different mechanisms served by large DNA virus to defend against the host inflammatory responses are also discussed. Chapter 2 describes a detailed analysis of mutagenesis and pathogenesis of three recombinant MDVs, rMd5/vIL-8-ELR which carries ELR (Glu-Leu-Arg) motif in the vIL-8 gene, vIL-8 knockout virus rMd5/ΔvIL-8 and its revertant, rMd5/ΔvIL-8-RV. It is demonstrated in this chapter,

that MDV encoded vIL-8 gene is involved in early cytolytic infection and transformation but dispensable to latency. Various *in vivo* studies in Chapter 3 showed that the rMd5/ΔvIL-8 virus had attenuated virulence, and was also highly protective against challenge with very virulent MDV, 648A (vv+). Meanwhile, the immunohistochemical staining demonstrated that the deletion of vIL-8 gene impaired the expression of pp38 gene and delayed the expression of Meq gene *in vivo*. In the final Chapter, future directions are suggested to further characterize the protein and its function in the mechanisms responsible for the MDV replication and transformation.

CHAPTER ONE

LITERATURE REVIEWS

I. History of Herpesvirus

The *Herpesviridae* family has numerous large DNA viruses. More than 80 viruses have been reported in different hosts including humans, mammals, and vertebrates, and in one case, an invertebrate (Le Deuff 1994; Minson 2000). Current molecular analysis have tracked herpesvirus evolution back to more than 200 million years, parallel to vertebrate evolution (McGeoch 1994, 1995). The genomes of herpesviruses of mammals and birds clearly indicate descent from a common ancestor, but with a great range of variation in terms of nucleotide substitution, gene content, and genomic arrangement (McGeoch 1999).

All herpesviruses have similar morphology with a large enveloped icosahedral nucleocapsid which contains double-stranded DNA. Despite the similar morphology, the genome varies widely with the genomic size range from 120 to 240 kbp and numbers of genes varies from 70 to 200. The base composition and pattern of repeated sequences also vary considerably (McGeoch 1989a; Roizman 1992a). It is common that all herpesviruses establish latency following primary infection and the latent infection may last throughout the life of the host, but the biological and the pathological aspects of different herpesviruses vary greatly. Herpesviruses have been classified into the three subfamilies, the *Alpha-*, *Beta-*, and *Gammaherpesvirinae*, based on their distinct biological

properties and genomic attributes (McGeoch 1995; Roizman 1992a). *Alphaherpesvirinae* are neurotropic viruses which have the capacity to establish latent infections primarily in ganglia. The examples of this subfamily include herpes simplex virus types 1 and 2 (HSV-1, HSV-2), varicella zoster virus (VZV), bovine herpesvirus and pseudorabies virus (PRV). *Betaherpesvirinae* have a narrow host range and establish latency in the secretory glands and lymphoreticular cells. Members of this subfamily are human cytomegalovirus (HCMV), human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7). The third group, *Gammaherpevirinae* cause mostly lymphoproliferative diseases and the host range is highly restricted. Virus latency is established with lymphoid cells. Members of this subfamily include Epstein-Barr virus (EBV), Herpesvirus saimiri (HVS), and Kaposi's sarcoma herpesvirus or human herpesvirus 8 (HHV-8) (Table 3).

Because of the lymphotropism and oncogenicity, Marek's disease virus (MDV) was originally classified as a gammaherpesvirus along with EBV. However, analysis at the molecular level suggests that MDV is more closely related to the members of *alphaherpesviruses* (Brunovskis et al. 1995). MDV was then re-classified as an *alphaherpesvirus* based on its genomic structure.

II. Marek's Disease Virus

1. History of the disease

The disease was first recognized and described by Joseph Marek, a Hungarian veterinarian, in 1907 (Marek 1907). The lesions were restricted to the nervous system; therefore, he named the condition a polyneuritis. Later, this syndrome was also described in USA (Kaup 1921) and in the Netherlands (Van der Walle 1924). Their descriptions of MD suggested that the pathological changes were restricted to the central and peripheral nervous system. The neoplastic condition of the disease was not recognized until 1926, when Pappenheimer et al. (Pappenmeimer 1926, 1929a, 1929b) described lymphomatous tumor-like masses in 10% of the cases. The significance of these findings was the recognition that the disease was a lymphoproliferative process resulting in lesions in peripheral nerves. These lesions were essentially similar to the visceral tumors that occurred in some of the cases. However, the visceral tumors found in many cases of MD appear similar to those found in lymphoid leukosis caused by a group of RNA tumor viruses. It was the contribution of Campbell (Campbell 1956, 1961) and Biggs (Biggs 1968) that identified certain histological differences of the lymphoid tumors, and distinguished the neural lymphomatosis from other neoplasm and established it as MD. Successful experimental transmission of the disease was accomplished in early 1960's by inoculating young chicks with blood or lymphoma cells from chickens with MD (Biggs 1963; Sevoian 1962a; Sevoian 1962b). This was the first hallmark in

modern MD research, and the concept of MD was widely accepted by this time. Further studies on the etiology of MD by Biggs and his colleagues clearly defined the differences between MD and lymphoid leucosis (LL) (Biggs 1963, 1967). The real breakthrough came when two laboratories simultaneously isolated a herpesvirus from MD tumors by co-cultivating in chick kidney cells CKC (Churchill 1967) and in duck embryo fibroblasts (DEF) (Nazerian 1968; Solomon 1968). It was also confirmed that this infectious agent is cell-associated (Biggs 1967; Biggs 1968; Spencer 1967). The MD virus (MDV) was propagated in cell culture and established as the etiological agent of MD. Detailed studies by Calnek and his co-workers demonstrated that the antigens of the herpesvirus were consistently present in the superficial layers of the feather follicler epithelium and that enveloped herpesvirus particles were present at this site (Calnek 1969). They also showed that the cell-free extracts of skin were infectious both for chicken and cultured cells (Calnek BW 1970a; Calnek 1970b). It was also found that poultry dust and litter were infectious for long periods (Beasley 1970; Carrozza 1973; Jurajda 1970). These observations provided a satisfactory explanation as to why and how the etiological agent of MD is highly contagious and the model of its transmission.

2. Biological aspects of MD and MDV

1) Classification and pathotyping

MDV is classified as a member of the family *Herpesviridae* (Roizman 1992b). Because of its lymphotropism, which is similar to Epstein Bar virus (EBV), MDV was originally classified as a γ–herpesvirus (Roizman 1981). However, later on, based on its genomic organization and significant sequence similarity to other α–herpesviruses, such as herpes simplex virus (HSV) and varicella-zoster virus (VZV), it was classified in the subfamily *Alphaherpesvirinae* (Buckmaster et al. 1988; Roizman 1992a).

a) Serotypes

In a series of studies using the agar gel precipitation test (AGP), indirect immunofluorescence assay (IFA) and virus neutralization, it was found that there were antigenic differences between MDV isolates (Bülow 1975a, 1975b). Based on these results, the MDV group was classified into three serotypes (Bülow 1975a, 1975b; Calnek et al. 1997): Pathogenic strains of MDV and their attenuated derivatives are the prototype viruses of the MDV group, and are designated as serotype 1 MDV (MDV-1). The non-pathogenic strain of MDV from chicken origin is serotype 2 MDV (MDV-2), and that of turkey origin (HVT) is serotype 3 MDV (MDV-3) (TABLE1). The division of the MDV group into three serotypes has been supported by the differences of the restriction endonuclease digestion patterns among the genomes of viruses of the three serotypes (Hirai 1979; Ross et al. 1983; Silva et al. 1991). Furthermore, the serotype specific monoclonal antibodies have been developed which confirmed the specificity of the three serotypes and aided in viruses classification (Ikuta et al. 1982; Lee et al. 1983a).

b) Pathotypes

Based on the ability of different isolates to cause disease in vaccinated chickens, Witter (Witter 1983; Witter et al. 1985) proposed to classify the isolates with mild (mMDV), virulent (vMDV), very virulent (vvMDV) and very virulent plus (vv+MDV) (Table 1). Calnek et al. (Calnek 1998) found a correlation between early cytolytic infection and pathotype resulting in the establishement of an alternate criteria for pathotyping based on lymphoid organ atrophy. There was also a correlation between pathotype and neurological signs in SPF chicken lines (Gimeno 2002).

2) Morphology and ultra-structure

The morphology and structure of MDV virions have been studied by many laboratories (Kato et al. 1985; Payne 1976; Schat 1985). It was shown that viruses of all three serotypes have similar characteristics typical of other herpesviruses. Electron microscope studies of thin sections of infected cell cultures reveal naked hexagonal particles measuring 85-100 nm in the nucleus, and the presence of some small ring-shaped structures about 35 nm in diameter (Epstein et al. 1968; Hamdy et al. 1974; Morgan 1959; Nazerian et al. 1968; Okada et al. 1972). In negatively stained preparations, the unenveloped virion measures about 100 nm in diameter and has 162 capsomeres (Churchill 1967; Epstein et al. 1968; Nazerian et al. 1968). Usually the centrally located nucleoid, 50-60 nm in diameter, appears as an electron-dense toroid that surrounds a less

dense cylindrical mass (Nazerian 1974; Nii et al. 1975). The cylindrical mass probably consists of protein. The electron-dense toroidal structure consists of a pair of fibrils in a cohelical configuration (Okada et al. 1980). The fibrils are presumed to be viral DNA, because of the 2-3 nm diameters.

3) Cultivation and propagation of MD Virus

Both chickens and cell cultures are used for the propagation of cell-free and cell-associated MDV to provide stocks of infectious material for experimental studies. All three serotypes of MDV can be cultured in a number of types of tissue cultures. Chick kidney cells (CKC) or duck embryo fibroblasts (DEF) are the best culture condition for the propagation of low passage serotype-1 MDV isolates, especially for primary isolation and quantification of infected cells derived from chickens (Churchill 1967, 1968a; Churchill 1968b; Solomon 1968). In both cell systems, infection results in a cytopathic effect in the form of characteristic plaques which can be used for quantitative assays. The attenuated serotype-1 MDV, serotype-2 MDV and HVT propagate well in chick embryo fibroblasts (CEF) (Biggs 1972; Schat et al. 1978a). The developed discrete focal cytopathic plaque effects (CPE) consist of clusters of rounded, refractile degenerating cells. Differences in morphology of MDV-1 plaques in chick and duck cells (Biggs 1972; Schat 1985; Spencer 1969; Witter et al. 1969) have been described.

MDV can also develop virus pocks on the chorioallantoic membrane (CAM) of chicken embryo as a result of the yolk sac inoculation with cell-associated MDV

preparations (Biggs et al. 1971; Bülow 1977). The number of the pocks on the CAM is directly proportion to the dose of the virus and can be used to quantitate virus titer. The chicken embryos are also used for the evaluation of the MD vaccines. At 18th day of hatch, vaccine viruses are inoculated in the amnionic sac for *in ovo* vaccination administration (Sharma 1987).

4) Host range

By far, chickens are the most important host system for the MDV in both natural and experimental infections. Quail is another host for MDV infection. MDV isolated from either chickens or quail have been used to reproduce MD in both species (Kenzy et al. 1969; Pradhan et al. 1985; Pradhan et al. 1987). However, quail-origin MDV appeared to be more pathogenic than chicken-origin MDV for quail (Imai 1991). Furthermore, the pathogenesis of infection was also different between chickens and quail, and turkey herpesvirus (HVT) vaccine failed to protect quail against MDV challenge (Kaul et al. 1991). Chicken-quail hybrids were found susceptible to MD (Powell 1984). Experimental transmission of MD was possible in pheasants (Dandapat et al. 1994) but not in ducks. Sparrows as well as variety of mammalian species were refractory to infection with virulent MDV (Churchill et al. 1968; Hlozanek et al. 1974; Rispens et al. 1972b; Sharma et al. 1973; Sharma et al. 1972).

3. Pathology of MD

1) Clinical signs

MD induced clinical signs are characterized by asymmetric progressive paresis and later, the complete paralysis of one or more of the extremities. The incidence of MD is quite variable in commercial flocks and depends on strain, dose of virus, age at exposure, maternal antibody, host gender, genetics and several environmental factors including stress. A particular characteristic of MD is the unilateral paresis or paralysis of the leg resulted in a typical presentation where one leg is stretched forward and the other backward (Biggs 1968). With the extensive use of vaccines, the transient paralysis syndrome is only seen in experimental trial and seldom in the field. The affected chickens display varying degrees of ataxia and partial or whole body paralysis beginning 8-15 days after virus inoculation and last for 1-2 days. During this stage a few affected birds may die without specific viscera lesions and result in nonspecific MD mortality. Most of the birds recover only to succumb from clinical MD a few weeks later. It is clear now that the transient paralysis syndrome appear to be the result of vasogenic brain edema (Kornegay 1983; Swayne et al. 1988; Swayne 1989). Infection of MDV in the iris results in blindness in chickens and is another specific clinical sign of MD. This is normally caused by neoplastic mononuclear cell infiltration (Calnek 1997).

2) Gross and histological lesions

Lesion distribution appears to be similar for naturally occurring or experimental disease (Pappenmeimer 1926; Payne 1967). Nerve enlargements are one of the most consistent gross lesions in affected birds. Various peripheral nerves, particularly the vagus, brachial, and sciatic, become enlarged and lose their striations (Goodchild 1969; Sevoian 1962b). Diffuse or nodular lymphoid tumors may be seen in various visceral organs, particularly the liver, spleen, gonads, heart, lung, kidney, muscle, and proventriculus (Payne 1985; Payne 1976). Both the genetic strain and the virus strain can influence the location of lesions. Enlarged feather follicles may be noted in broilers after defeathering during processing and are a cause for condemnation. But the skin lesions are usually associated with, but not limited to, feather follicles (Benton 1957).
Non-neoplastic lesions of MD include severe atrophy of the bursa of Fabricius and thymus as well as degenerative lesions in the bone marrow and various visceral organs (Jakowski 1970; Witter 1980b). These are the result of intense cytolytic infections that can result in death of chickens at an early age before the development of lymphomas.

Histologically, the lesion consists of a mixed population of small, medium, and large lymphoid cells plus plasma cells and large anaplastic lymphoblasts. These histopathologic changes associated with MD appear in both the peripheral nerves (Lawn 1979) and the visceral organs (Payne 1967). The mixed cell populations in MD associated lesions undoubtedly include both tumor cells and reactive

inflammatory cells. The bursa rarely develop tumor, but when it is involved, the infiltrated cells typically appear in interfollicular areas.

3) Differential diagnosis

Depending on the etiologic agent, virus induced neoplasms of poultry are divided into two main groups: 1) Herpesvirus induced Marek's disease, and 2) retroviruses induced avian leucosis/sarcoma (ALV) and reticuloendotheliosis virus (REV). Differential diagnosis has been considered difficult in the field, because MD gross lesions may resemble those of lymphoid leucosis (LL), or reticuloendotheliosis (RE). RE, although rare, can easily be confused with MD because both diseases feature enlarged nerves and T-cell lymphomas in visceral organs. However, LL can usually be differentiated from MD by its common involvement of the bursa of Fabricius and uniform blast cell morphology. Also, MD occurs at any age >3 wk (most commonly in birds <16 wk), whereas LL occurs in older birds. Histochemical assays to detect cellular and viral antigens in sections of tumor tissue are techniques for differential diagnosis of MD and other avian viral tumors. Specifically, MD can be diagnosed by the demonstration of predominant T-cell populations and viral specific antigens, such as MEQ and MATSA on tumor cells by the histochemistry. Furthermore, MD lymphomas usually lack evidence of integration or alteration of the cellular oncogene c-Myc as they are in the cases of avian retroviruses (Table.2).

4) Pathogenesis

The pattern of events which occur sequentially in antibody-free, genetically susceptible chickens which ultimately die from lymphoid tumors after infection with an oncogenic strain of MDV can generally be divided into four phases (Fig.1): (1) early cytolytic, productive-restrictive infection, (2) latent infection, (3) late cytolytic, productive-restricted infection, and (4) transformation. Although essentially sequential, these are not necessarily discrete phases. A fairly sharp line demarcates the first two stages, and latent infection in certain cell types is prerequisite to transformation, but both transforming and latent infections may exist intermixed with cytolytic infections in different cell populations as lymphomas are developing in later stages (Calnek 1986, 2001).

a) Early cytolytic, productive-restricted infection

The lymphotropic nature of MDV is illustrated by the fact that the first significant sites of infection following exposure to the virus are in the major lymphoid organs. Virus generally gains entry via the respiratory tract, where it is probably picked up by phagocytic cells, but little or no active infection can be detected in the trachea, lungs, or air sacs. The productive, cytolytic infection can be detected with the identification of large amounts of the viral antigen expression in the lymphoid organs (spleen, bursa of Fabricius, and thymus) peaking at 3-6 days post inoculation (DPI). Few or no enveloped virions are produced; rather, naked intranuclear particles are present in infected cells. Like all productive or semi-productive herpesvirus infections, the consequence is cytolysis. The

necrotizing effects of this early infection provoke an acute inflammatory reaction with infiltration of various cells including macrophages, granulocytes, both immunologically committed and uncommitted lymphocytes. Ultimately, the consequence is a transient atrophy of the lymphoid organs, especially the thymus and the bursa. Depending on the virulence of the challenge strain, birds may recover between 8 to14 DPI or the atrophy may become permanent (Calnek et al. 1998). Using a variety of techniques to separate lymphocyte populations, Shek et al. (Shek et al. 1983) found that bursa-derived lymphocytes (B-cells) are the primary target cells for the MDV infection in all lymphoid organs. Apparently, thymus-derived lymphocytes (T-cells), which are in the resting state, either in the central organ from which they originate or in peripheral locations are relatively refractory to infection during this early phase. However, using a sensitive dual fluorescence technique with monoclonal antibodies to detect surface markers on circulating T-cells, it was later identified that cytolytically infected T-cells also present during the early infection period. The massive number of apoptotic thymocytes may possibly be the consequence of viral-induced cytokine changes. Recently, the presence of the vial homologue of IL-8 (vIL-8) has been reported in serotype 1 MDV strains. Schat and Xing speculated that production of vIL-8 during the lytic infection in B-cells may be essential in attracting T-cells to the areas of virus replication, and transfer of infection from B-cells to activated T-cells (Schat et al. 2000).

Several factors can modify the early pathogenesis. Prior vaccination or the presence of maternal antibodies reduces the cytolytic infection. The reduction in

cytolytic infection will also reduce the number of latently infected cells and reduce tumor development.

b) Latency

Herpesvirus latency is defined as the presence of the viral genome without detection of infectious virus except during episodes of reactivation (Feldman 1991; Fraser 1992; Garcia-Blanco 1991; Stevens 1989; Wager 1991). Maintenance of a reservoir of latently infected cells within an immunocompetent host is critical to viral persistence. Like other α–herpesviruses, MDV establishes a life-long latent infection, but differs from other members of the subfamily in that it goes latent not in sensory nerve ganglia but in activated T-cells (Calnek et al. 1984; Calnek et al. 1981; Calnek 1997). MDV can be reactivated by cell culture propagation of T-cells isolated from infected chickens (Calnek et al. 1984). At about 6-8 days, the infection becomes latent when cytolytic infection can be no longer demonstrated and tumors are not yet detectable. The development of latency coincides with the development of immune responses. T- cell mediated immunity (CMI) plays a central role in this switch (Calnek and Spencer, 1985; Payne, 1985). The interactions between virus and cells during the induction of latency are not completely understood.

c) Productive infection in FFE.

MDV replication in FFE is also a cytolytic infection. It is the only site that produces cell-free infectious virus due to complete virus replication (Calnek et al.

1970). MDV is most likely transferred to the FFE by infected lymphocytes. The lymphoid aggregates consist of small nuclear inclusion in FFE. These aggregates can either develop into a necrotic area with feather follicle epithelial cells or degenerated lymphocytes, or into cutaneous tumors.

d) The second phase of cytolytic infection

The second phase of cytolytic infection does not always happen. The development and extent of this phase depends on genetic resistance of the host and the virulence of the MDV strain. The second phase of cytolytic infection coincides with permanent immunosuppression. It is much more severe than the early cytolytic infection and affects not only lymphoid organs but also other visceral including kidney, ovary, pancreas, adrenal gland, proventriculus, etc. The details of the second lytic infection have not been clearly defined.

e) Transformation and lymphoma

Susceptible chicken lines will progress past the latent stage and develop a second wave of cytolytic infection. It is at this stage that lymphoproliferation and T-cell tumors would be developed (Buscaglia et al. 1988; Calnek 1997). The composition of lymphomas consists of a mixture of neoplastic, inflammatory and immunologically committed and noncommitted cells. Both T and B-cells are present, but T-cells are the predominant type (Hudson et al. 1973; Ross et al. 1973). The usual target cells for transformation are $CD4^+$, activated T-cells (Schat et al. 1991). But under certain experimental conditions, other T-cell

subsets, including $CD4^+$, $CD8^+$ and $CD4^-/CD8^-$ cells are transformable (Schat et al. 1991). But continuous cell lines of MD tumor cells provided uniform population of cells for study (Matsuda et al. 1976a; Nazerian et al. 1975; Ross et al. 1975; Schat et al. 1989). Several antigens are found associated with MD tumors cells or cell lines established from tumors, including Marek's disease tumor-associated surface antigen (MATSA) (Matsuda et al. 1976b; Powell 1984; Witter et al. 1975), now known as a marker associated with normal activated T-cells (McColl et al. 1987), chicken fetal antigens (Murthy et al. 1979; Powell et al. 1983a), and heterophil and Forssman antigens (Ikuta et al. 1981). Because of MATSA's association with activated T-cells, it is reasonable to expect that transformed cells would express this antigen. Ross et al. recently described another MD tumor associated antigen, AV37 (Ross et al. 1997). The authors speculated that AV37 is linked with activation and may actually play a role in pathogenesis.

Lymphomagenesis is the consequence of MDV infection that is most commonly associated with MD. Lymphomatous tumors can appear as early as 12-14dpi following infection of young, genetically susceptible chickens by a highly virulent strain of MDV. Tumors may not appear until several weeks or months after infection with less virulent viruses or under influencing factors such as vaccinal immunity, maternal antibodies, genetic resistance, and age of infection. Lymphomas may occur in almost any visceral organ.

5) Virus genes associated with oncogenicity

The MD system provides a number of approaches to identify viral genes that are responsible for, or contribute to, the transformation of T-cells and the maintenance of that state. First, the genomes of virulent serotype 1 viruses can be compared with their attenuated derivatives. Second, genes present in virulent serotype 1 viruses but are absent in serotype 2 viruses and HVT can be identified for further study. Third, genes that are expressed in lymphoblastoid cell lines derived from MD lymphomas and in lymphomas cells isolated from the chicken can be examined. Using these approaches, a number of genes and alterations in gene structure have been identified.

a) Phosphorylated protein 38 (pp38)

MDV phosphprylated protein pp38, is an unique serotype 1 gene within internal repeat long region. It was expressed in a lymphoblastoid cell line and in lymphoma cells in MD tumors (Naito 1986; Nakajima 1987). This finding suggests that it might have a role in transformation of lymphoid cells. However, pp38 homolog gene has been reported in an attenuated serotype serotype 1 strain (Ross et al. 1993) and non-oncogenic serotype 2 and 3 strains (Ono et al. 1994; Ono et al. 1995; Smith 1995). The fact that the pp38 gene is expressed in productive infections and is found in attenuated virus suggests that it may not play a direct role in oncogenecity. It was shown that pp38 is involved in the maintenance of latency and early cytolytic infection and is essential for transformation (Reddy et al. 2002; Xie et al. 1996).

b) Meq gene

A 339 amino acid protein is enoced with the MDV *Eco* Q fragment, therefore it is designated Meq. Meq gene is located in the repeat long regions, thus two copies of the Meq gene are presented in the MDV genome. Like other genes in the repeat long regions, Meq gene is encoded by serotype-1 MDV but not by serotype-2 (SB-1) or serotype-3 (HVT). Meq is consistently expressed in MDV latently infected or tumor cells (Jones et al. 1992). It codes for a basic-leucine zipper (bZIP) domain at the N-terminus (Qian 1996) and is closely related to the Jun/Fos oncoproteins (Liu 2000). The C-terminal proline-rich domain structurally resembling the WT-1 tumor suppressor gene (Qian 1995). Because of its structural properties and specific function, Meq gene has been characterized by different groups as a transcription factor, an oncogene, and as an immunogen. As a reflection of its multifunctional roles, Meq has an interesting subcellular localization pattern (Kung 2001). The cytoplasmic location of Meq is cell-cycle-dependent and is only being detected in S phase. Meq has two stretches of basic amino acids which signal the translocation of Meq into nucleus and nucleolus (Liu 1997). Meq is also localized in the coiled body, which is correlated with transformation and increased metabolic activity (Liu 1999). Several factors have defined Meq as an oncogene and transformation factor. Over-expression of Meq induces morphological transformation and anchorage-independent growth of rat-2 cell (Kung et al. 2001). Similarly, inhibitors of Meq in MDV-transformed T-cells results in decreased growth (Xie et

al. 1996). This and other evidence strongly support the role of Meq in transformation.

4. MDV Immunity

MDV immunity has conversed significances: Immunological responses of the host to MDV infection may be the basis for resistance but the virus can also induce immunosuppression. Vaccinal immunity is the prime means of control, but the immunologic response may contribute to the cellular mass of the lymphoma (Calnek 1997).

1) Cell-mediated immune response

Infection with pathogens normally results in the activation of nonspecific and specific immune responses. MDV infection has been controlled effectively by vaccination using nononcogenic and/or attenuated oncogenic MDV strains. Thus far, there is little knowledge on the role of cell-mediated immune (CMI) responses during MDV infection or vaccination.

MDV infections cause nonspecific responses involving macrophages, Natural Killer (NK) cells, and production of specific cytokines and interleukins (IL). Thus far, there is limited information on cytokine activation after MDV infection. Infection or vaccination with MDV results in an early activation of NK cells (Lessard et al. 1996; Schat 1996). NK cells may also play a role in tumor

regression (Calnek 1997). Several groups have reported that macrophages can inhibit virus replication *in vitro* (Haffer et al. 1979; Kodama et al. 1979; Lee 1979; Powell et al. 1983b). Depletion of macrophages from spleen cell preparations enhanced the level of virus replication significantly. The mechanism for the inhibition of virus replication has not been elucidated, but it has been suggested that Interferons (IFNs) or macrophage activating factor (MAF) could be involved (Bülow 1984). Specific immune responses are antigen-dependent and require lymphocyte activation to produce specific antibodies and antigen-specific $CD4^+$ and $CD8^+$ T-cells. MDV specific responses can be detected as early as 5–7 DPI with the appearance of antibodies and antigen-specific CTL. Omar reported that virus specific $CD8^+$ CTL but not $CD4^+$ effector cells are responsible to the MDV specific syngeneic cell-mediated immune response (Omar et al. 1996; Omar et al. 1998).

2) Humoral immune response

MDV induced precipitating and virus-neutralizing antibodies can be detected within 1-2wk and persist throughout the life of the chicken. The presence of maternal antibodies may delay or reduce virus replication (Ball et al. 1971; Chubb et al. 1969) and may interfere with vaccine-induced immunity, especially when cell-free vaccines are used (Schat 1987). The protective role of the passively acquired humoral antibody in chickens is thought to cause immune resistance to MD by neutralizing and reducing the level of infection but may not be able to

exclude virus replication. The exact mechanism of how the humoral antibody can cause immune resistance to MD is not clear.

3) Immunosuppression

Much of the impact of MD in broiler chickens is considered to be due to immunosuppression induced by MDV. These infections result in atrophy of the thymus and bursa of Fabricius leading to reduced numbers of circulating T lymphocytes and B lymphocytes. It is also noticed that MDV immunosuppression greatly increased susceptibility to *E. coli* infection but reduced infectious bursa disease (IBD) antibody titer. Calnek et al. proposed three criteria of immunosuppression in MDV: 1) persistence of early cytolytic infection (i.e., a delay or failure to enter latency) in lymphoid organs, 2) atrophy of the bursa of Fabricius and thymus as measured by organ weight proportional to body weight at 8 and 14 DPI, and 3) histopathologic evidence of necrosis and atrophy in lymphoid organs (Calnek et al. 1998). By comparing the relationship between the immunosuppressive potential and the pathotype of MDV isolates, they suggested that the degree of immunosuppression is linked to virulence and that a simple measure of atrophic changes (relative organ weights) in the bursa of Fabricius and thymus might be useful in determining the pathotype classification of new MDV isolates. The MDV serotype-3 virus HVT alone caused mild depletion of T and B lymphocytes but had no effect on immune organ weight or IBD titer.

4) Vaccinal Immunity

Although MD vaccines have been used since 1970, the mechanism for protection is still poorly understood. Vaccination results in a decrease in viral replication of pathogenic MDV strains during the early lytic infection as well as a lower level of infected lymphocytes afterwards (Lee et al. 1999; Volpini et al. 1995). It has been assumed that this is caused by the generation of antigen-specific CTL, but recent work from Schat's group suggested that nonspecific responses may also be involved (Schat et al. 2000). Vaccinal immunity is largely against viral antigens, and possibly tumor antigens. Therefore, vaccination has high efficacy in protecting against tumor induction and mortality but does not prevent viral replication or shedding and can not eradicate the disease.

5) Types of vaccines and evolution of virulence

Prior to vaccination, MD was economically the most costly poultry disease. For the past 30 years, a series of avirulent or attenuated live virus vaccines have provided protections to chickens in both commercial layer and breeder flocks (Churchill et al. 1969; Rispens et al. 1972a). The development of successful MD vaccines is a significant achievement both in avian disease and basic cancer research. It is now the most effective strategy for prevention and control of MD.

Since early 1970s, several MD vaccines have been used with success in the field and generally, they could be grouped into four generations. The first effective vaccine was attenuated serotype1 virus, HPRS-16att (Biggs et al. 1970; Churchill et al. 1969), it was then soon replaced by the second generation, the naturally non-oncogenic turkey herpesvirus (HVT) of serotype 3 MDV (Okazaki et al. 1970; Witter et al. 1970). The third generation introduced in 1983 was a bivalent vaccine (Schat et al. 1978b) composed of HVT and SB-1 (serotype-2, non-oncogenic chicken herpesvirus). About 10 years after using the bivalent vaccine, more virulent virus strains appeared, which are named very virulent plus (vv+) strains. In mid-1990s, an attenuated serotype 1 vaccine virus, strain CVI988 (also called Rispens) was introduced in US, either alone or in combination with other vaccines, against vv and vv+ MDV strains (Witter 1992). But, CVI988 has been used in Europe for much longer timer.

However, the foregoing succession of MD vaccines paralleled the continued evolution of field strains of serotype 1 MDV to greater virulence (Witter 1997) (Figure 2). Until now, MD vaccines have been able to keep pace with the challenges of continued evolution of MDVs towards greater virulence. The future of sustainable poultry production would be in jeopardy if MD vaccines are unable to keep pace with evolving MDV. As it was stated, after about 8-10 years of efficient protection from one generation of vaccine, more virulent viruses emerge that require the introduction of more efficientive vaccines. The evolution of the virulence appears to be caused by the mutations of MDV. And it is possible that the selection pressure created by intensive vaccination could be responsible for

MDV evolution. Concerns exist that the continued evolution of MDV toward greater virulence could break through the CVI988 vaccine-induced immunity and have devastating effects on poultry. In order to meet the challenges raised by the continued increases in virulence of MDV strains, the mystery of the cycle of mutation of MDV around vaccinal immunity and how to prevent future cycle of mutation is critical to eradicate the disease.

5. Molecular Biology of MDV

1) Physical MDV map and genomic structure

Like other herpesviruses, MDV contains a linear double-stranded DNA genome that has a density of 1.705 g/cm^3 in CsCl, and a base composition of 46% guanine and cytosine. The molecular weight of MDV DNA is about 108-120 x 10^6 Dalton (Da) (Lee et al. 1971), equivalent to 166-184 kilo base pairs (kbps) (Cebrian et al. 1982; Fukuchi 1984; Hirai 1979). The composition and density of HVT DNA is similar to MDV-1 DNA (Lee et al. 1971). The genomic structure and gene arrangement of MDV DNA are similar to that of α- herpesviruses, including HSV and varicela-zoster virus (VZV) (Buckmaster et al. 1988; Davison 1986; Karlin et al. 1994; Roizman 1992a; Wu et al. 1988), although biological properties of MDV are close to γ–herpesviruses, such as Epstein-Barr virus (EBV).

The genomic sequences of all three serotypes of MDV have been completely sequenced which include two serotype 1 MDVs, GA (virulent strain) (Lee et al.

2000) and Md5 (very virulent strain) (Tulman et al. 2000), one serotype 2 MDV, HPRS-24 strain (Izumiya et al. 2001), and the serotype 3 MDV, FC126 (HVT strain) (Afonso et al. 2001). The DNA sequence data indicates that the genomic size differs between serotypes. The MDV genome consists of unique long (U_L) and unique short regions (U_S), each bounded by inverted repeats, called terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S) and terminal repeat short (TR_S) (Figure 3).

Several direct repeats have also been identified in the MDV genome (Hirai 1988). These direct repeat sequences are mostly located within the internal and terminal repeat regions. A heterogeneous expansion region, containing multiple tandem 132 bps repeats, has been identified in attenuated MDV and mapped to the inverted regions flanking the U_L region of the genome, TR_L and IR_L (Fukuchi et al. 1985a; Maotani et al. 1986; Silva 1985), adjacent to the MDV origins of replication (Ori), and are designated as DR1. These DR1 can be expanded *in vitro* by passages of virulent MDV-1 strain in primary CEF cell culture (Fukuchi et al. 1985b; Maotani et al. 1986). There exist two copies of 132 bps in virulent MDV-1, and multiple copies in their attenuated derivates. Kawamura et al. suggested that the function of this 132 bp repeats may be associated with virual oncogenicity (Kawamura 1991). With the establishment of cosmid clone of MDV Md5 strain, Silva et al. constructed the recombinant virus with the deletion of two copies of DR1 repeats. The deletion virus has similar virulence and tumorgenicity as the parental virus Md5 (Witter 1980a).

Based on the physical maps of *Bam*HI restriction endonuclease (RE) fragments constructed from MDV-1 (Figure 3) (Fukuchi et al. 1985a), MDV-2 (Ono et al. 1992), and HVT (Igarashi et al. 1987), it was shown by cross hybridization data (Yoshida et al. 1994) that all three serotypes differ in their RE digestion patterns (Silva 1991), but share significant homology at the DNA level. The RE maps have become a basis for most gene identification and localization (Figure 4), and also are useful for comparative studies.

2) Cosmid and Bacterial Artificial Clone (BAC) systems for recombinant MDV.

Although the members of the Herpesvirus family are responsible for a wide variety of human and animal diseases, advances in the understanding of viral molecular mechanisms of pathogenesis have been hampered by the large size of herpesvirus genomes, rendering the viruses difficult to experimentally manipulate. In recent years, manipulation of the large herpesvirus genomes have been facilitated by using cosmid DNA library and bacterial artificial chromosome (BAC) vectors (Arvin 2001; Cohen 2001; Schumacher et al. 2000). The genomes of murine and human cytomegaloviruses (HCMV) (Ehsani et al. 2000; Messerle 1997), herpes simplex virus type 1 (Cohen et al. 1993; Suter 1999), pseudorabies virus (PRV) (Smith 1999), and Epstein-Barr virus (Delecluse 1998; Tomkinson et al. 1993) have been cloned either as overlapping cosmid clones or infectious BACs, or both. These techniques also facilitated mutagenesis of herpesvirus

genomes, allowing for the assessment of the role of specific viral gene products in replication, immunity, and pathogenesis.

Studies on MDV have been carried out for decades, but because of its strong cell-associated features and lack of efficient tools, MDV gene characterizations have been limited to *in vitro* methods (Cui et al. 1990; Jones et al. 1992). To understand the viral oncogenicity of MDV, it is necessary to have extensive knowledge about MDV genes functions, especially their roles *in vivo*. The best way to characterize the role that proteins play in oncogenesis is to introduce site-specific mutations in the viral genome.

The finding in early 1990s that purified MDV DNA was infectious in cell culture (Wilson et al. 1991) indicated that MDV recombinants could be generated by cotransfection with selectable markers. From then on, generation of mutant MDV has always been done by the marker rescue method (Bublot et al. 1999; Parcells et al. 1995). The disadvantages of using this method in MDV are obvious. Since this method requires the introduction of a selectable marker in the mutant virus as well as several rounds of plaque purification, the whole process of selection of recombinant viruses is laborious which can cause the introduction of unexpected mutations elsewhere in the genome and even attenuation of the recombinant virus. In addition, the introduction of a foreign marker gene in the viral genome could also generate the effect to the viral oncogenecity, although it has not been reported thus far (Parcells et al. 2001).

Recently, overlapping MDV cosmid clones have been generated and was successfully used to introduce mutations into a vvMDV strain, Md5 (Reddy et al. 2002). This method does not require repeated rounds of plaque purification and insertion of selective marker genes. Thus far this is the only tool available to introduce site-specific mutations into oncogenic MDV. Using this recombinant technology it is shown that pp38 is not essential for replication in cultured cells and dispensable for tumor induction, but deletion of pp38 severely impaired *in vivo* replication in lymphoid organs (Reddy et al. 2002). Several other MDV specific genes have been targeted for characterization now using this method. It is believed that easy generation of mutant viruses with this technique will lead to a better understanding of the mechanisms of MDV pathogenesis.

Bacterial artificial chromosome (BAC) technique is the other useful tool to introduce mutations to large DNA virus genomes. Thus far, all of the MDV BAC clones were derived from attenuated virus, such as 584Ap80C (Schumacher et al. 2000; Tischer et al. 2002), and vaccine strain CVI988 (Petherbridge et al. 2003). MDV BAC clones could not be used for pathogenesis studies presently. But it has been reported that MDV BAC clone DNA is capable of inducing protection as a vaccine, possibly through the reconstitution of live virus *in vivo*. This represents a new generation of DNA-based vaccines against herpesviruses (Petherbridge et al. 2003; Tischer et al. 2002).

III. Chemokine family and Virokines

1. Introduction to chemokines family

Chemokines constitute a growing superfamily of intercellular messengers which play multiple roles in the development and homeostasis of different organ systems, particularly the hematopoietic system, as well as the generation of both innate and adaptive immune responses. It is now known that chemokines and their receptors are expressed by a wide variety of nonhematopoietic cells, and that chemokine function extends far beyond leukocyte physiology. For example, the knowledge of the relation among chemokines, their receptors and human immunodeficiency virus (HIV) infection broadens the previously narrow focus on chemokines as mere chemoattractants. Chemokines are small molecules, with molecular weights in the range of 8 to 12 KDa, but there are exceptions which involve proteins comprised of multiple domains, (Bazan 1997; Ernst 1994). Chemokines have four highly conserved cysteine residues. Based on the number of amino acids which separate the first two cysteine residues, chemokine families are classified into four groups: CXC (α class), CC (β class), CX3C (γ class) and C (δ class) (Baggiolini 1997). CXC family can be further divided into two subgroups of molecules (ELR^+ and non-ELR) according to the presence or absence of an "ELR" (Glu-Leu-Arg) motif located immediately before the first cysteine residue. The presence of "ELR" seems to correlate to the neutrophil chemoattractant (Rollins 1997b). But there are also some exceptions, such as chicken Chemotaxis and Angiogenic Factors (cCAF), 9E3/CEF. The chicken

cCAF gene has an "ELR" motif, but it does not attract neutrophils, only monocytes (Martins-Green 2001)(Table 4).

2. Chemokine and virus

The successful propagation of viruses within the host requires skillful evasion or manipulation of the host immune arsenal (Alcami 2000; Lalani 1999a; McFadden 2000; Murphy 2001). Large DNA viruses, particularly the poxviruses and herpesviruses, provide some of the most extensive inventories of gene products that serve to defend these viruses against the aggressive assault executed by the host inflammatory response. The chemokine network represents a significant target for disruption or exploitation by these viruses (Lalani et al. 2000; Murphy 2001; Rosenkilde et al. 2001). From a functional standpoint, two major groups of chemokines can be distinguished: housekeeping (HK) chemokines (Baggiolini et al. 1994; Butcher 1996), which are generally expressed constitutively under physiological conditions and play essential roles in development and homeostasis; and proinflammatory (PI) chemokines (Cook 1995), which are typically inducible and participate in the generation of both innate and adaptive immune responses. The relation between virus and the chemokine system is characterized by a complex blend of enmity and attraction. Chemokines are key regulators of innate and adaptive immune responses against invading microorganisms, including viruses (Baggiolini 1998; Baggiolini et al. 1995). As immune system "traffic officers", chemokines control leukocyte migration under either physiological or pathological conditions, meanwhile,

chemokines could modulate the induction, amplification, and cytokine-secretion pattern of antiviral responses. Indeed, the physiological effects of chemokine-elicited intracellular signaling are not only limited to the activation of chemotaxis. Chemokines also serve as co-stimulators that amplify both proliferative and cytokine-secretive T-cell responses which allow antiviral immune responses to proceed beyond a critical threshold under conditions of low antigenic load or during infection by non-cytopathic viruses (Taub 1996).

3. Virokines

Viruses have succeeded in turning the chemokine system into an ally. During the course of a long parallel evolution, viruses have captured from their hosts the genetic information for encoding chemokines and chemokine receptors and have reprogrammed it for evading the control of the immune system. The large DNA viruses, particularly the poxviruses (Lalani 1999b) and herpesviruses, have developed several mechanisms to corrupt the normal functioning of the chemokine network by either mimicking chemokines or chemokine receptors (Seet 2002). These include U83 of HHV-6 (Zou 1999), vMIP-I, –II and -III of HHV-8 (Chen 1998; Dairaghi 1999) and M131 of murine CMV (Table 5). All these chemokines are CC chemokines. Liu et al. first reported the identification of vIL-8 in MDV (Liu et al. 1999) and about the same time, Penfold et al. reported the discovery of UL146 and 147, two IL-8-like chemokines in human CMV (Penfold et al. 1999). These are the only viral CXC chemokines identified thus far. Among all these virokines thus far identified, many of them have been

characterized. Knowledge of how viruses function to oppose chemokines is providing important insights into how the immune system combats viral infection and contributes to a better understanding of how to better control unwanted chemokine-mediated inflammation. Because of the elucidation of the complex relationship between HIV and the chemokine system, a cautious optimism on the possibility of developing effective strategies for the control of HIV infection has been raised (Baba 1999; Bashoff 1997; Faure 2000).

4. Chemokine and angiogenesis

Several members of the CXC family are among the first chemokines identified as regulators of angiogenesis, acting either as angiogenic or angiostatic factors (Strieter et al. 1995a). Angiogenesis, defined as the growth of new capillaries from preexisting vessels, is a pervasive biological phenomenon that is at the core of many physiologic and pathologic processes. An opposing balance of angiogenic and angiostatic factors regulates angiogenesis. Examples of physiologic processes that depend on angiogenesis include embryogenesis, wound repair, and the ovarian/menstrual cycle (Strieter et al. 2002). A common characteristic of all solid tumor growth is the presence of neovascularization. This is a basic requirement for solid tumor progression, since the absence of new vessels does not allow tumors to grow beyond a few millimeters. In addition, blood-borne metastasis cannot be initiated without neoplastic cell access to blood vessels. Tumor cells may induce angiogenesis via release of growth factors, such as prostaglandins and fibroblast growth factor 2 (FGF-2), and by the

attraction of inflammatory cells which in turn release multiple angiogenic stimuli (Bernardini et al. 2003).

Interestingly, the presence or absence of an "ELR" motif in the amino acid sequence seems to correlate with an angiogenic or angiostatic activity, respectively (Strieter et al. 1995b). The requirement of the "ELR" motif has been demonstrated by site-directed mutagenesis substitution of the "ELR" motif of an angiogenic chemokine (i.e. IL-8/CXCL8) with a non-ELR motif of the angiostatic molecule (Mig/CXCL9) and vice versa. The shift in angiogenic properties of the mutated chemokines in both *in vitro* and *in vivo* assays, strongly supports the importance of the "ELR" motif as a structural domain for angiogenic activity (Strieter et al. 1995c).

The angiogenic members of the CXC chemokine family include interleukin (IL)-8 (IL-8/CXC ligand [CXCL8), epithelial neutrophil activating protein (ENA)-78 (ENA-78/CXCL5), growth-related genes (GROs) [GRO-α, GRO-β, and GRO-γ; CXCL1, CXCL2, and CXCL3, respectively], granulocyte chemotactic protein (GCP)-2 (GCP-2/CXCL6), and NH_2-terminal truncated forms of platelet basic protein, which include connective tissue activating protein-III, ß-thromboglobulin, and neutrophil activating protein (NAP)-2 (NAP-2/CXCL7) (Strieter et al. 1995d) (Table 4).

The angiostatic members of the CXC chemokine family include platelet factor-4/CXCL4, monokine induced by IFN-γ (MIG) [MIG/CXCL9], and IFN-γ–inducible protein (IP)-10 [IP-10/CXCL10] (Zlotnik 2000). All three

IFN-inducible ELR- CXC chemokines specifically bind to the CXC chemokine receptor, CXCR3 (Zlotnik 2000).

Angiogenesis is regulated by an opposing balance of angiogenic and angiostatic factors. CXC chemokines comprise a unique cytokine family which contain members that exhibit on a structural/functional basis either angiogenic or angiostatic biological activity. As a family, the CXC chemokines appear to be important in the regulation of angiogenesis associated with the pathogenesis of chronic inflammatory/fibroproliferative disorders. These findings support the notion that therapy directed at either inhibition of angiogenic or augmentation of angiostatic CXC chemokines may be a novel approach in the treatment of chronic fibroproliferative disorders.

5. MDV encoded vIL-8 gene

The MDV encoded vIL-8 gene is located within the repeated sequences flanking the unique long (U_L) region of the MDV genome. The vIL-8 gene is produced by multiple splicing with a conserved exon-intron organization. The protein is 134-amino-acid long. It is designated as IL-8 like gene based on the high sequence and structure similarity compared with the cellular IL-8 genes, including both mammalian and avian IL-8 (Figure 5) (Hughes et al. 2000; Kaiser et al. 1999; Martins-Green 2001; Sick et al. 2000). Similar to other cellular homologs, vIL-8 has a signal peptide at the N-terminal end which signals the secretion of the vIL-8 molecule and vIL-8 does have the four conserved cysteine residues that function in forming two disulfide bridges in the protein tertiary

structure that might have effects on the stability of the structure and also be essential in maintaining biological function of the protein (Rajarathnam et al. 1999). However, there are interesting structural differences between vIL-8 and cellular IL-8. Most notably, vIL-8 carries extra sequences in the C-terminal domain. Secondly, the NH2 terminus of the majority of the CXC chemokines contains three amino acid residues (Glu-Leu-Arg: the "ELR" motif), which precedes the first cysteine amino acid residue of the primary structure of these cytokines, while MDV encodes vIL-8 protein it does not have an "ELR" motif, instead it is Asp-Lys-Arg (DKR). Based on this sequence feature, vIL-8 belongs to ELR$^-$CXC chemokine family. Because of its chemokine-like properties, it is hypothesized that the function of vIL-8 is to attract cells for MDV as target cells for virus infection and replication. Since MDV is a cell associated virus, target cells have to come in contact with the infected cells to become infected. It is possible that vIL-8 plays a critical role in attracting naive cells for infection. The role of vIL-8 in virus replication *in vitro* and *in vivo*, and the role of vIL-8 in lytic infection, latency, or transformation, is evalulated in the studies reported herein.

	Serotype	Pathotype	prototype virus	Oncogenicity
Serotype 1 (MDV-1)	1	mild (mMDV)	CU2	Chicken
		virulent (vMDV)	GA	Chicken
		very virulent (vvMDV)	Md5	Chicken
		very virulent plus (vv+MDV)	648A	Chicken
Serotype 2 (MDV-2)	2		SB1	no
Serotype 3 (MDV-3)	3		HVT	no

Table 1. **Classification of MDV strains by serotypes and pathotypes.**

	MD	LL	RE/Bursal	RE/Nonbursal
Virus type	herpesvirus	retrovirus	retrovirus	retrovirus
Chicken age at onset	3wk	>14wk	>14wk	
Gross lesion:				
nerve	+	-	-	-
bursa	atrophy	enlarge	enlarge	
Histology	Pleomorphic	Homogeneity	Homogenity	pleomorphic
B cell marker	-	+	+	-
T cell marker	+	-	-	+
pp38	+	-	-	-
Meq	+	-	-	-
MATSA	+	-	-	-
c-Myc	-	+	+	+

Table 2. **Differential diagnosis of MD and other chicken lymphoma diseases.**

MD: Lymphoma by Marek's disease virus; LL: Lymphoid leucosis by ALV; RE/Bursal: Bursa lymphoma by Reticuloendotheliosis virus (REV); RE/Nonbursal: Non bursa lymphoma by REV; +: Present; -: Absent.

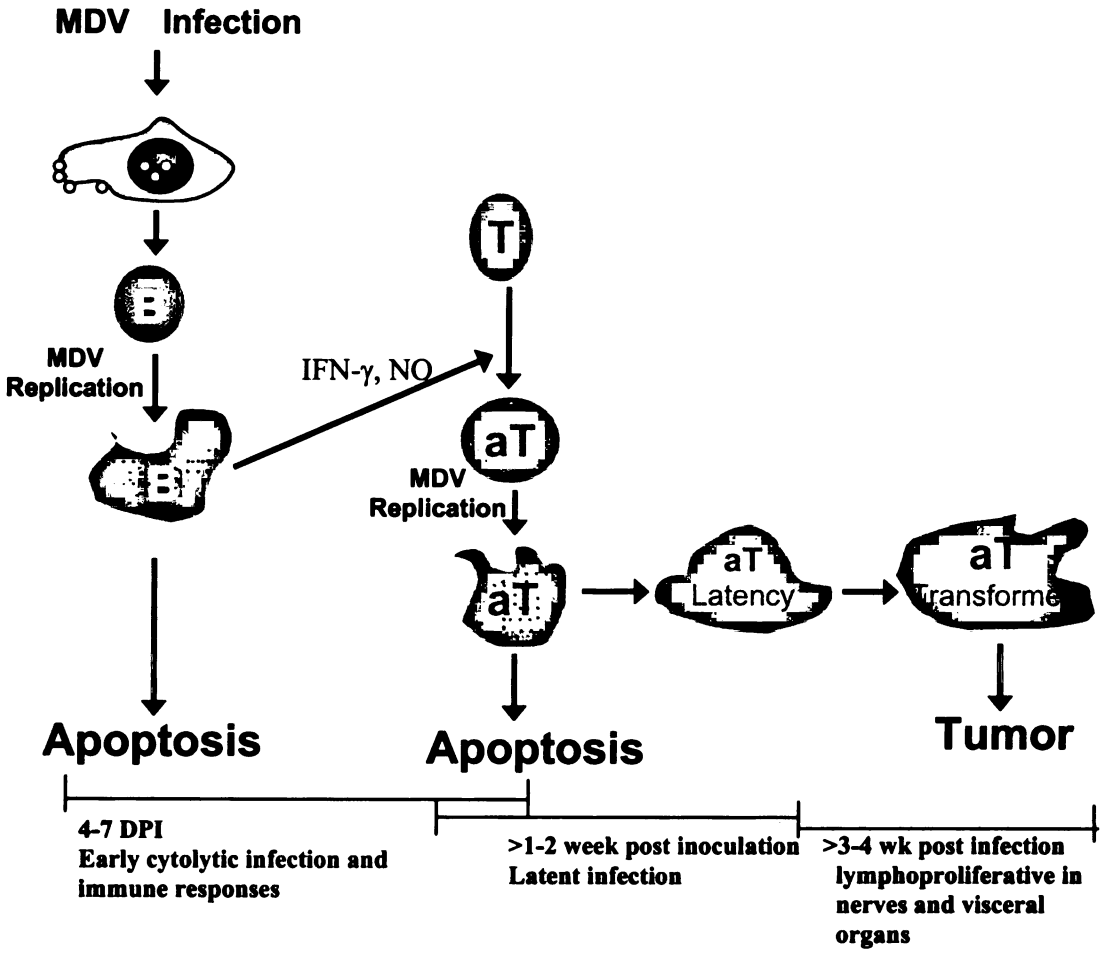

Figure 1. **Possible sequential events in the pathogenesis of Marek's disease.** MDV infected cells or dander are phagocytized by macrophages followed by infection of B-cells. The infected B-cells initiate the early cytolytic infection. Some of these B-cells undergo apoptosis. Infected B-cells may induce an early immune response, releasing other cytokines and chemicals such as IFN-γ or NO, which will assist in activating T-cells. Only the activated T-cells (aT) but not the resting T-cells are the targets for MDV infection. Some of the aT-cells undergo apoptosis, others enter into the latency (aT-Latency) phase or become transformed (aT-Transformed), finally leading to the development of tumors.

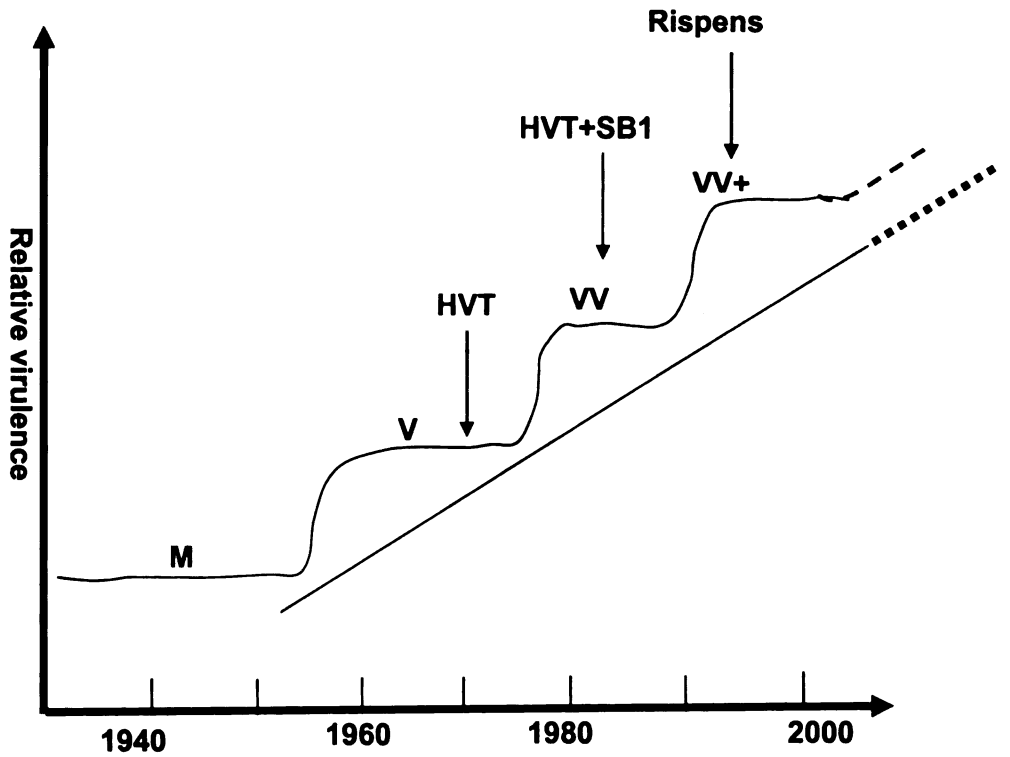

Figure 2. **The continued evolution of MDV towards greater virulence parelled with the foregoing succession of MDV vaccines.** MDV virulence has increased from low virulent mild (mMDV) strain, to the virulent (vMDV) strain, to high virulence of the very virulent (vvMDV) strain and, finally to the very virulent plus (vv+MDV) strain. MDV vaccines have also been kept pace with the continued evolution of MDV virulence. HVT was used in 1970s, and was only efficient against vMDV. Bivalent vaccine (HVT+SB1) was introduced in mid 1980's, and was used to protect chickens against vvMDV strains. With the emergence of vv+MDV, Rispens strain has become the most efficient vaccine against vvMDV and vv+MDV strains since 1990s.

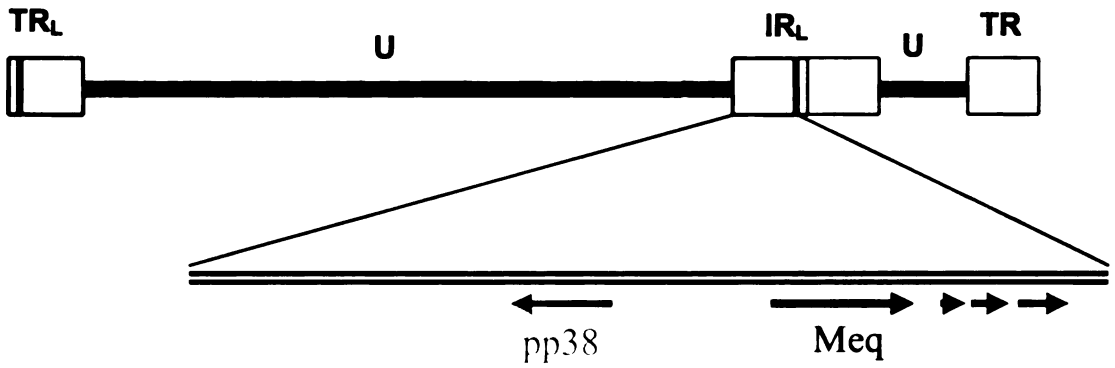

Figure 3. **Schematic map of MDV genome and the locations of genes Meq, pp38 and vIL-8.** Double stranded DNA structure of MDV consists of unique long (U_L) and unique short (U_S) regions each bounded by inverted repeats, named terminal repeat long (TR_L), internal repeat long (IR_L), internal repeat short (IR_S) and terminal repeat short (TR_S). MDV-1 unique genes, such as pp38, Meq and vIL-8, are located in the repeat long region as indicated in the figure.

This figure is in color.

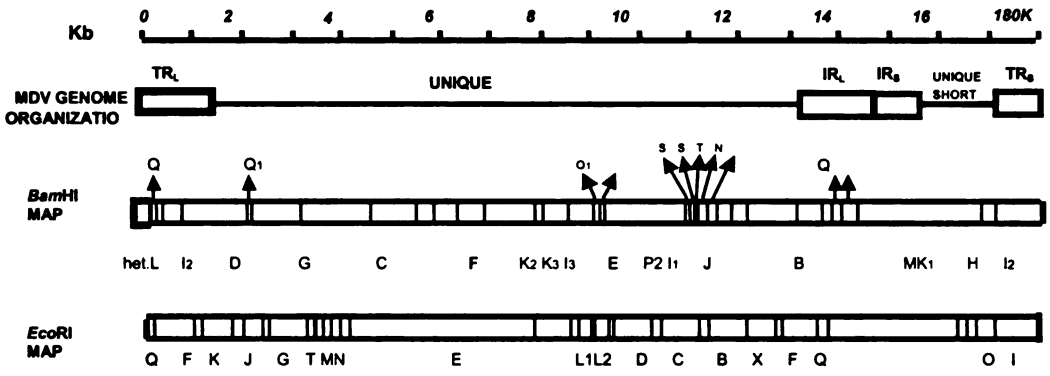

Figure 4. **Schematic structure and restriction map of the MDV genome.** The size of MDV genome and its organization are shown in the top panel. The location of *Bam*HI and *Eco*RI restriction endonucleus sites are shown in the bottom (adapted from Fukuchi et al., 1984; Silva and Witter, 1985)

Virus	subfamily	Natural Host	Associated disease	Oncogenic	Other species
Marek's disease virus	alpha	chicken	T-cell Lymphoma	yes	Turkey, quail
Human simplex virus	alpha	human		-	
varicella zoster virus	alpha	human	Vericella, zoster	?	
pseudorabies virus	alpha	pig	Aujeszky's disease	?	
Human herpesvirus 6	beta	human	T-cell lymphomas	?	
Human herpesvirus 7	beta	human	T-cell lymphomas	?	
human Cytomegalovirus	beta	human			
Epstein-Barr virus	gamma	human	Burkitt's lymphoma Nasopharyngeal carcinoma	yes	?
Kaposi sarcoma virus	gamma	human	Kaposi sarcoma	yes	?
Herpesvirus saimiri	gamma	squirrel monkey	lymphoma	no	other monkeys
Herpesvirus ateles	gamma	spider monkey	lymphoma	no	other monkeys
Herpesvirus papio	gamma	Baboon	lymphoma	yes	Marmosets
Herpesvirus sylvilagus	gamma	Cotton rabbit	lymphoma	yes	?
Lucke herpesvirus	?	Frog	Renal carcinoma	yes	?
Chenoid herpesvirus	?	sea turtle	Skin tumors?	yes	?

Table 3. **Classification of oncogenic herpesviruses** (Adapted from (McGeoch 1999; McGeoch et al. 2000).

Name	Target Cells	Angiogenic	Angiostatic
ELR			
IL-8	Neutrophils, T-cells, basophils ?endothelial cells	yes	
GRO-α (MGSA)	Neutrophils, melanoma cells	yes	
	?endothelial cells		
GRO-β (MIP-2)	Neutrophils, ?endothelial cells	yes	
GRO-γ (MIP-2)	Neutrophils, ?endothelial cells	yes	
ENA-78	Neutrophils	yes	
GCP-2	Neutrophils	yes	
9E3/CEF	Mononuclear cells ?endothelial cells	yes	
K60	Mononuclear cells ?endothelial cells	yes	
Platelet basic protein	-	-	
CATP-III	Fibroblasts	?	
Thromboglobulin	Fibroblasts	?	
NAP-2	Neutrophils, basophils	?	
Non-ELR			
Platelet factor 4	Fibroblasts, endothelial cells activated T-cells,		yes
IP-10	?endothelial cells, ?NK cells		yes
MIG	activated T-cells,		yes
SDF-1	T-cells,?B-cells		yes

Table 4. **CXC chemokines, their target cells and angiogenesis activity**

(Adapted and edited from (Rollins 1997a)).

Virus	Coding gene	Cellular homologues	Proposed functions
MDV	vIL-8	IL-8	?
MCMV	m131 (MCK-1)	CC Chemokines	chemokine agonist(target cell recruitment?)
	m33	CCR1	Functional receptor (disseminate infected cells)
HCMV	UL146 (VCXC-1)	IL-8	chemokine agonist (target cell recruitment?)
	UL147 (VCXC-2)	IL-8	?
	US27	?	?
	US28	CCR1	Functional CC chemokine receptor HIV-1coreceptor
HHV-6	UL33	CCR1	?
	UL83	CC Chemokines	chemokine agonist (target cell recruitment?)
	UL12	CC Chemokines receptors	Functional CC chemokine receptor
	UL51	CC Chemokines receptors	chemokine sequestraion?
HHV-7	UL12	CC Chemokines receptors	?
HHV-8	K6(VMIP-I)	MIP-1	chemokine agonist on CCR8, Angiogenesis
	K4(VMIP-II)	MIP-1	chemokine agonist on CCR3, Angiogenesis
	K4.1(VMIP-II)	MIP-1	chemokine agonist on CCR4, Angiogenesis
HSV	ORF74	CXCR2	constitutive signaling, cellular transformation
	ORF74/ECRF3	CXCR2	functional CXC chemokine receptor

Table 5. **Herpesviruses encoded chemokine or chemokine receptor homologues** (adapted from (Lusso 2000)).

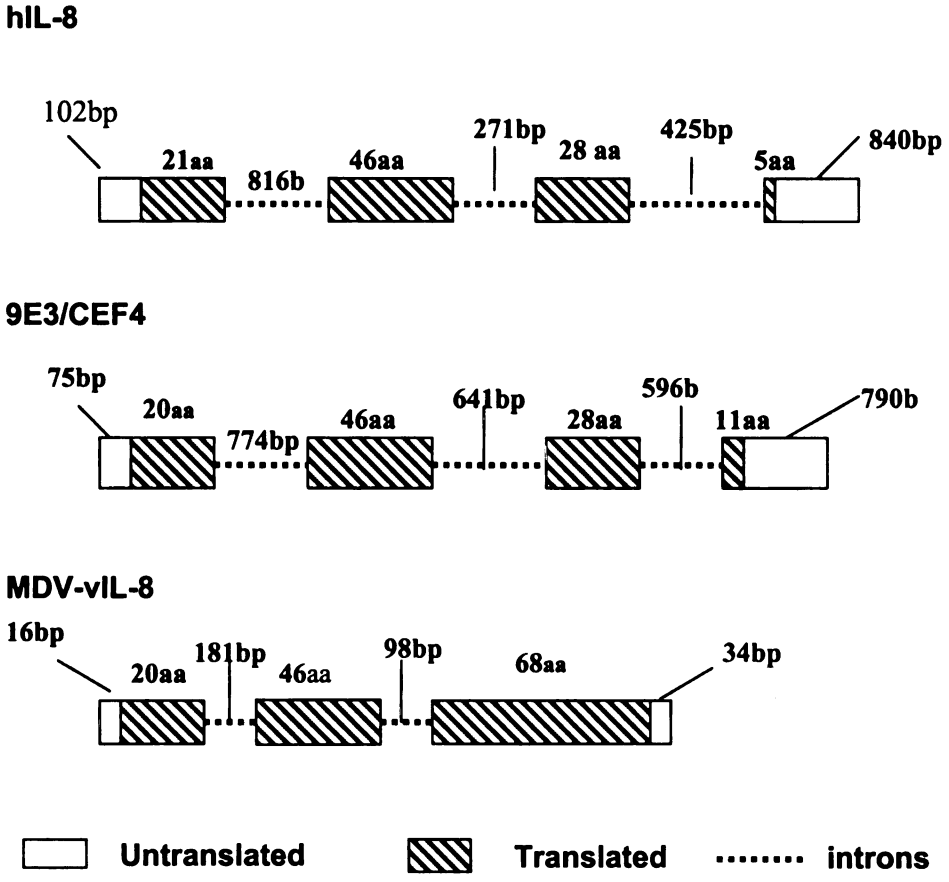

Figure 5. **Structure comparison of IL-8.** Cellular IL-8s inculding hIL-8 and chicken IL-8 9E3/CEF consist of four exons and three introns. The MDV encoded vIL-8 gene only has three exons and two introns, and it codes for a much longer exon 3. The number of nucleotides for each intron or untranslated region and the number of amino acids for each translated region are marked in the figure.

CHAPTER TWO

MDV ENCODED vIL-8 GENE IS INVOLVED IN EARLY CYTOLYTIC INFECTION AND TRANSFORMATION BUT DISPENSABLE TO LATENCY

ABSTRACT

Marek's disease, a lympho-proliferative disease of chickens, is caused by an alpha-herpesvirus MDV (Marek's disease virus). This virus encodes a virokine vIL-8, with general homology to cellular CXC chemokines such as IL-8 and Gro-α. To study the function of vIL-8 gene, both copies of vIL-8 residing in the TR_L and IR_L regions of the viral genome were deleted. The vIL-8 deleted virus rMd5/ΔvIL-8 was generated. Growth kinetics studies showed that vIL-8 gene is dispensable for virus replication in cell culture. *In vivo*, vIL-8 gene is involved in early cytolytic infections in lymphoid organs as indicated by less viral antigen expression detected by immunohistochemical staining. But the rMd5/ΔvIL-8 virus is unimpaired in virus spreading. Similar viremia titers as the rMd5 virus at 6 and 8 dpi indicated that vIL-8 gene is not necessary for virus reactivation and is not involved in latency. Nevertheless, deletion of vIL-8 gene compromised transformation of the virus with reduced number of transformed cells at 5 week post inoculation and less gross tumors development *in vivo*. The revertant virus that restored the expression of vIL-8 gene showed the same phenotype as the rMd5.

One of the interesting sequence differences between the vIL-8 gene and other cellular homologue is that vIL-8 encodes a "DKR" motif instead of "ELR". To study the role of the variation of this motif in MDV pathogenesis, we generated recombinant MDV, rMd5/vIL-8-ELR, carrying ELR motif by mutagenesis. Both *in vitro* and *in vivo* studies showed that rMd5/vIL-8-ELR has the same phenotype as rMd5.

INTRODUCTION

Marek's disease (MD) is a contagious, lymphoproliferative disease of domestic chickens in which mononuclear infiltration, demyelination of peripheral nerves, and T-cell lymphomas are common features (Biggs 1975). The etiological agent of MD is a lymphotropic, oncogenic herpesvirus, MD virus (MDV). The MDV genome is about 180 kbps in length and is most related genetically and structurally to the genomes of alpha-herpesviruses, such as herpes simplex virus and varicella-zoster virus (Buckmaster et al. 1988; Lupiani et al. 2001). Recently, the complete nucleotide sequences have been determined for all serotypes of MDV(Afonso et al. 2001; Lee et al. 2000). The data showed that MDV and other alpha herpesviruses are colinear in the unique long and short regions but differ substantially in the adjacent repeats (Lee et al. 2000; Tulman et al. 2000). MDV is grouped into three serotypes: serotype 1 consists of all pathogenic virus strains, serotype 2 comprises the naturally occurring, non-oncogenic strains in chickens, and serotype 3 includes the non-pathogenic herpesvirus of turkeys (HVTs) (Becker et al. 1992; Bülow 1975c; Kaaden 1977; Lee et al. 1983b). MD incidence has largely been controlled by vaccination with all three serotypes of MDV, often in bi- and multivalent combinations since the 1970s (Witter 1991, 2001). However, there is a continuation of an apparent evolutionary trend of MDV towards greater virulence, which results in recent increased losses from Marek's disease (MD) in vaccinated flocks (Calnek 1997). A thorough understanding of the genes involved in the replication, immune modulation and oncogenesis holds keys to the development of improved live-vaccines, based on

targeted mutations of the MDV genome. We have focused on genes specific to the oncogenic serotypes of MDV and developed a cosmid-based recombinant virus approach to study their functions *in vivo* (Reddy et al. 2002). In this study, the findings on vIL-8, a virokine encoded by an oncogenic serotype of MDV is reported.

vIL-8 is located in the repeat region of the MDV genome and like other virokines of herpesviruses, may be involved in viral replication and/or host immune-modulation (McGeoch 1989b). MDV vIL-8 shares significant homology to cellular CXC chemokines such as IL-8 and GRO-α, and is the only one found in alpha-herpesvirus. Cytomeglavirus, a beta-herpevirus, encodes two CXC virokines (i.e., UL146 and 147) (Penfold et al. 1999). Most other virokines belong to the CC family of chemokines. Mutagenesis studies of ELR+ chemokine (IL-8) and ELR- chemokine (Mig) revealed that the presence of the ELR motif correlated well with the chemokines' ability to attract neutrophils during inflammation (Baggiolini et al. 1997) and induce angiogenesis in tumorigenesis (Strieter et al. 1995b).

Liu et al. reported the identification of MDV vIL-8 and the initial characterizations of this virokine (Liu et al. 1999). It was found that vIL-8 has a DKR motif in place of ELR, and in a chemotaxis assay, the major cell types targeted by vIL-8 are mononuclear cells rather than heterophils (chicken equivalent of neutrophils). A vIL-8 deletion mutant in the genetic background of RB1B strain of MDV was constructed by inserting a soluble-modified green

fluorescent protein (smGFP) expression cassette at the site of deletion. This vIL-8 virus was found to replicate well in cell culture, but much less so *in vivo* and had a weak oncogenic phenotype (Parcells et al. 2001). In this early study, revertant virus was not developed and inadvertent mutations responsible for some of the observed phenotypes cannot be completely ruled out. Nevertheless, the results have provided an important framework for the understanding of the general properties of MDV vIL-8.

In this dissertation, an extended study was carried out by using the newly established MDV cosmid DNA library (Reddy et al. 2002) to construct recombinant MDVs. This approach permits the efficient construction of revertant virus, unattainable by the previous approach. The *in vivo* infection course of the mutant virus was also characterized in more detail. The results demonstrated that vIL-8 plays an important role in the establishment of early infections, presumably functioning to recruit target cells for MDV infection. To test whether the DKR is critical in target cell selection, a mutant virus where DKR is replaced by ELR was also developed and the properties of the mutants is discussed.

MATERIALS AND METHODS

Antibodies. Monoclonal antibody (Mab) H19 specific to viral protein pp38 (Cui et al. 1990) was used to detect pp38 by IFA and IHC. Rabbit antiserum specific to viral protein vIL-8 was used to detect vIL-8 in IFA and western blot.

Chickens. Chickens used in the study were specific pathogen free MD susceptible F1 progeny (15x7) of Avian Disease and Oncology Laboratory line $15I_5$ males and line 7_1 females. All the chickens were wing-banded at hatch, and randomly sorted into different experimental groups (17 chickens per group) and held in modified Horsfall-Bauer isolators.

Cells and viruses. Primary duck embryonic fibroblasts (DEF) were used for virus propagation and DNA transfections. Recombinant viruses were generated from cosmids derived from a very virulent MDV strain, Md5 (Witter 1980a).

Plasmids. A 3.1kb fragment (nt. 1,451 to 4,543) containing the entire sequence of the vIL-8 gene was obtained by digesting the SN5 cosmid with *Bam*HI and was cloned into the same site of pUC19 generating the transfer vector pUC19/SN5*Bam*HI. Subsequently, the pUC19/SN5*Bam*HI was digested with *Cla*I (nt. 2,807) and *Nco*I (nt. 3,605), blunt ended and religated to generate the vIL-8 deletion transfer vector, pUC19/SN5*Bam*HI/ΔvIL-8.

Substitution of the DKR (Asp-Lys-Arg) motif of vIL-8 with the ELR (Glu-Leu-Arg) motif in the plasmid pUC19/SN5*Bam*HI was carried out using the QuickChange^{TM}XL site-directed mutagenesis kit (Stratagene, CA), according to

the manufactures' instructions. The primers used were: primer 1: 5'G AGT CTC GCT GTC GA**G CT**G AGG TGC AAG TGC G 3' and primer 2: 5'C GCA CTT GCA CCT C**AG C**TC GAC AGC GAG ACT C 3' where the introduced mutations are indicated by bold and underline. The presence of the corresponding mutations in the plasmid pUC19/SN5*Bam*HI/ELR was confirmed by sequencing.

Cosmids. MDV cosmid clones SN5, P89, SN16, A6 and B40, from the very virulent strain, Md5 (Witter 1997), encompassing the entire MDV genome were used to generate recombinant Md5 viruses (Reddy et al. 2002) (Figure 6). Cosmid clones A6 and SN5, containing a copy of the complete coding sequence of the MDV unique gene vIL-8 in opposite orientation, were used to introduce vIL-8 specific mutations.

The RecA-assisted restriction endonuclease (RARE) cleavage method (Ferrin et al. 1991) was used to delete the vIL-8 gene from the SN5 and A6 cosmid DNA. Briefly, the SN5 and A6 cosmids were incubated with RecA protein, ADP/ATP γs and two oligonucleotides, vIL-8blkF (5'-GCC CGC ATC TCG CAG CCC CCG GAT CCG ATC CCG CAG ACC C-3') and vIL-8blkR (5'-TCC CCT GCT AGC CCT GCC CTA GGT AAT GCA TTT TAA ATC T-3'), overlapping the two *Bam*HI sites flanking the vIL-8 sequence (nt. 1,451 to 4,543) to protect these sites from methylation. The protected cosmid DNAs were methylated with *Bam*HI methylase, denatured and digested with *Bam*HI to generate SN5/Δ*Bam*HI and A6/Δ*Bam*HI. These cosmid DNAs were treated with CIP (calf intestinal alkaline phosphatase) and were purified by phenol-chloroform extraction followed by ethanol precipitation.

To introduce the vIL-8 deletion into the SN5 and A6 cosmids, the pUC19/SN5*Bam*HI/ΔvIL-8 transfer vector was digested with *Bam*HI and the SN5*Bam*HI/ΔvIL-8 fragment ligated to SN5/Δ*Bam*HI and A6/Δ*Bam*HI, generating SN5/ΔvIL-8 and A6/ΔvIL-8, respectively.

Similarly, to introduce the ELR motif into the vIL-8 coding sequence, the pUC19/SN5*Bam*HI/ELR transfer vector was digested with *Bam*HI and the SN5*Bam*HI/ELR fragment ligated to SN5/Δ*Bam*HI and A6/Δ*Bam*HI to generate SN5/vIL-8-ELR and A6/vIL-8-ELR, respectively.

Transfections. Parental, P89, SN16 and B40 and mutant, SN5/ΔvIL-8, A6/ΔvIL-8, SN5/vIL-8-ELR and A6/vIL-8-ELR, cosmid DNA were digested with NotI and purified by phenol chloroform extraction and ethanol precipitation. To generate a mutant virus with vIL-8 gene deletion, rMd5/ΔvIL-8, 500 μg of each digested cosmid DNA (P89, SN16, B40, SN5/ΔvIL-8, and A6/ΔvIL-8) along with 2 μg of sheared salmon sperm DNA were used to transfect $5x10^5$ DEF in 35mm dishes by the calcium phosphate method (Morgan et al. 1990). Four days after transfection, cells were trypsinized, seeded onto a 100 mm dish and monitored daily for cytopathic effect (CPE). Viral stocks were subsequently made in DEF for further analysis. A MDV mutant virus carrying the "ELR" motif, rMd5/vIL-8-ELR, was generated in a similar method using P89, SN16, B40, SN5/vIL-8-ELR and A6/vIL-8-ELR cosmid DNA.

Revertant Virus. To generate a revertant virus from rMd5/ΔvIL-8 containing the vIL-8 gene, transfer vector pUC19/SN5*Bam*HI was digested with *Bam*HI and

co-transfected onto DEF cells with the purified rMd5/ΔvIL-8 viral DNA. After the CPE was evident, transfected cells were overlayed with 1.25% of Bacto-Agar and more than 400 viral plaques picked by trypsinization. Cells from each plaque were divided into two aliquots, one was used to re-infect a fresh 60mm dish of DEF and the other was used for PCR analysis. Integration of vIL-8 gene into the rMd5/ΔvIL-8 genome was detected by PCR using primers *Cla*IF: 5'-GGC GCA GCA CTG AAT AAG CC-3', and *Bam*HoriR: 5'-GGA GTA ATC TGC GTT-3' which would generate a 2200bp and 1400bp fragments in the revertant and deletion mutant virus, respectively (Figure 7).

Indirect immunofluorescence assay (IFA) and Immunohistochemistry (IHC). IFA of cosmid transfected DEF cells was carried out as previously described (Cui et al. 1988). For IHC, lymphoid organs (thymus, spleen, bursa of Fabricius) and feather follicles of infected and uninfected chickens were embedded in O.C.T. (optimal cutting temperature) compound (Sakura Finetek U.S.A., Inc. CA), immediately frozen in liquid nitrogen and stored at -80°C until use. Four to eight micron thick cryostat sections of tissue blocks were prepared, fixed with cold ethanol for 5 minutes and air-dried. Immunostaining was carried out using the Vectastain ABC Kit (Vector Laboratories, Inc. CA) according to the manufacture's instructions. In IFA, Mab H19 and rabbit polyclonal anti-vIL-8 were used at 1:300 and 1:50 dilutions, respectively. In IHC, Mab H19 was diluted at 1:3000.

Growth kinetics. Growth kinetics of rMd5, rMd5/ΔvIL-8 (clone 1 and 2) and rMd5/vIL-8-ELR (clone 1 and 2) viruses were studied as described (Cohen et al. 1993). Briefly, 100 plaque forming units (pfu) of the different viruses were inoculated onto DEF cells seeded on 60-mm plates. On days 1, 2, 3, 4 and 5 post inoculation the infected cells were typsinized, serial dilutions were inoculated on fresh DEF cells seeded on 35-mm plates, and plaques of different dilutions counted 7 days post infection.

Southern blot. DNAs from rMd5, rMd5/ΔvIL-8, rMd5/vIL-8-ELR and rMd5/ΔvIL-8-RV infected or uninfected DEF were isolated as previously described (Reddy et al. 2002). Five micrograms of each viral DNA were digested with *Eco*RI, or *Bam*HI, or double digested with *Bam*HI and *Sal*I. The DNA fragments were then separated on a 1% agarose Tris-borate/EDTA (TBE) gel and transferred to nylon membranes. Two individual [32P]dCTP-labeled DNA probes, one from the total genomic viral DNA (SN5, P89, SN16, A6 and B40 cosmid DNA fragments) and one from the 3.1kb *Bam*HI fragment containing the vIL-8 gene were generated by random priming and hybridization was carried out using standard protocols.

Western blot. Supernatants from rMd5, rMd5/ΔvIL-8, rMd5/vIL-8-ELR and rMd5/ΔvIL-8-RV infected DEF as well as the supernatant from the non-infected DEF were collected 3 days post infection. Sixteen microliters of each supernatant were separated on 15% SDS-PAGE gel using standard procedures. Proteins were then transferred to nitrocellulose membranes, blocked with 5% dry

milk for 1hr at room temperature, and probed with rabbit anti-vIL-8 polyclonal antibody (1:200 diluted in TBS buffer(20mM Tris-HCl (pH 7.5), 0.8%NaCl) at 37°C for 1hr. After 3 washes with TBS buffer, horseradish peroxidase conjugated goat anti-rabbit antibody (1:3000) (Kirkegaard & Perry Laboratories, Inc.) was added to the blots and incubated at 37°C for 1 hour. Following 3 washes with TBS, antibody bound specific antigens were detected by incubating with ECL™ western blotting detection reagent (Amersham Bioscience, UK) and exposed to X-ray film.

Chicken Chorioallantoic Membrane assay. Recombinant rMd5 and rMd5/vIL-8-ELR viruses were inoculated onto fresh DEF at the same multiplicity of infection (MOI) and supernatants collected 72 hours post infection. Gelatin sponges (1 mm3; Upjohn Company Kalamazoo, USA) were absorbed with 100ul supernatant from each virus and placed on the chorioallantoic membrane (CAM) of 8 day old embryonating eggs. Phorbol 12-myristate 13-acetate (TPA) (0.1 ug) was also absorbed to gelatin sponges and used as positive control. Sponges alone and sponges absorbed with supernatant from non-infected DEF cells were used as negative controls. On day 12 of embryonation, CAMs were photographed in ovo.

Pathogenesis studies. Specific pathogen free MD-susceptible F1 progeny (15x7) of the Avian Disease and Oncology Laboratory line $15I_5$ males and line 7_1 females were used in all the studies. These progeny were free of maternal antibodies against MDV. Chickens were wing-banded at hatch, and randomly sorted into different experimental groups (17 chickens per group) and held in

modified Horsfall-Bauer isolators. Day old chickens were inoculated with 2000 pfu of rMd5, rMd5/ΔvIL-8, rMd5/vIL-8-ELR or rMd5/ΔvIL-8-RV, subcutaneously. All the chickens that died during the trial or were killed at the end of the experiment (8 weeks post inoculation) were necropsied and evaluated for tumor incidence.

Viremia assay. To examine *in vivo* virus replication and reactivation, 5 birds from each group were randomly selected and bled at 6, 8, 14 and 35 days post inoculation. Buffy-coats were obtained by centrifuging at 700 rpm for 5 minutes. Lymphocytes were then counted and diluted to 10^6cells/ml. For each chicken sample, duplicated 35-mm plates of freshly seeded DEF monolayers were inoculated with both 10^5 and 10^6 lymphocytes and plaques were counted 7 days post inoculation.

RESULTS

MDV vIL-8 is dispensable for *in vitro* replication, latency and *in vivo* transmission but important for early *in vivo* cytolytic infection and transformation. In order to determine the role of MDV vIL-8 in viral replication and pathogenesis, rMd5/ΔvIL-8 virus was constructed in which the entire coding sequence of the vIL-8 gene was deleted (Figure 6C). Cosmids SN5/ΔvIL-8 and A6/ΔvIL-8, lacking the entire coding sequence of vIL-8, were transfected, along with parental SN16, P89, and B40, into DEF and transfected cells observed for CPE. To confirm the deletion of vIL-8 gene, transfected cells showing CPE were examined by IFA with Mab H19 (anti-pp38) and rabbit anti-vIL-8 polyclonal sera. As expected, rMd5 virus expressed both pp38 and vIL-8 (Figure 8A and B) while rMd5/ΔvIL-8 expressed only pp38 (Figure 8C and D). Similarly, Western blot analysis of rMd5 and rMd5/ΔvIL-8 infected DEF supernatants indicated that a 18 kDa band, corresponding to vIL-8, was present in supernatants from rMd5 but absent in supernatants from rMd5/ΔvIL-8 infected cells (Figure 9). To verify that rMd5/ΔvIL-8 had the expected genome structure, Southern blot of rMd5 and rMd5/ΔvIL-8 genomic DNA digested with EcoRI was performed. As shown in figure 10A, both viruses showed no detectable difference in pattern of DNA fragments, suggesting that there were no gross rearrangements in the rMd5/ΔvIL-8 genome (Figure 10A lanes 5-6). In addition, Southern blot analysis of viral DNA digested with BamHI and probed with the 3.1 Kb fragment purified from pUC19/SN5BamHI resulted in a 3.1 Kb band in rMd5 viral DNA (Figure 10B

lane2) and a 2.3 Kb band in the rMd5/ΔvIL-8 DNA, reflecting the deletion of 798 bp spanning the vIL-8 gene (Figure 10B lanes 5-6).

To determine if vIL-8 plays a role in the in vitro replication of MDV in DEF, the growth kinetics of rMd5, rMd5/ΔvIL-8-1 and rMd5/ΔvIL-8-2 were compared with each other. As seen in Figure 11, viral titers at all time points tested were very similar for all three viruses indicating that expression of vIL-8 is dispensable for viral replication in cell culture.

To examine if vIL-8 plays a role in the in vivo viral replication, day old 15x7 chickens were inoculated with rMd5 and rMd5/ΔvIL-8 viruses. Six days post-inoculation, lymphoid organs (bursa, thymus and spleen) from 3 chickens from each group were collected and examined for virus replication by immunohistochemistry. As seen in figure 12, there was a high level of expression of pp38 in the lymphoid organs of rMd5 inoculated chickens (Figure 12D, E and F). However, there was significantly lower level of pp38 expression in rMd5/ΔvIL-8 infected chickens (Figure 12G, H and I). These results indicated that vIL-8 gene is important for early cytolytic infection in lymphocytes.

MDV virus transmission takes place by virus replication in the feather follicle and release of infectious virus in the dander. To examine if vIL-8 is necessary for virus transmission, viral replication in the feather follicle of chickens infected with rMd5, and rMd5/ΔvIL-8 were examined 2 weeks post inoculation. As shown in Figure 13, both rMd5, and rMd5/ΔvIL-8 viruses had similar level of pp38 expression in the feather follicle suggesting that rMd5/ΔvIL-8 can be transmitted

horizontally like parental MDV. In addition, sentinel chickens housed in the same isolator as rMd5/ΔvIL-8 inoculated chickens developed high titer anti-MDV antibodies (data not shown), indicating that deletion of the vIL-8 did not affect shedding of viral particles into the environment.

To examine if vIL-8 affects MDV latency, viral titers in peripheral blood lymphocytes of infected chickens were tested at 6, 8, 14 and 35 days post inoculation. As shown in the Figure 14, both rMd5 and rMd5/ΔvIL-8 viruses reach the highest viral titer 8 days post-inoculation indicating that vIL-8 is neither involved in latency nor in reactivation from latently infected cells. However, at both 14 days and 35 days post-inoculation, there were significantly less reactivated latently infected PBL in rMd5/ΔvIL-8 infected chickens (Table 6, Figure 14). This may be due to reduced number of transformed PBL in rMd5/ΔvIL-8 infected chickens compared to parental rMd5 at later stages of infection.

In order to determine if the deletion of vIL-8 affects the pathogenic properties of MDV, chickens inoculated with rMd5 or rMd5/ΔvIL-8 were examined for gross tumors and mortality for a period of 8 weeks. As indicated in Table 7, the incidence of mortality was significantly lower in the group inoculated with rMd5/ΔvIL-8 (4.3%) than in the group inoculated rMd5 (88.2 %). In addition, in agreement with the low level of replication of rMd5/ΔvIL-8 in lymphoid tissues, no atrophy of bursa and thymus was observed in this group of chickens compared with massive atrophy of these organs in the rMd5 inoculated group (data not

shown). Similarly, the tumor incidence in the group inoculated with rMd5/ΔvIL-8 was much lower (17.6%) than that observed in the group inoculated with rMd5 (76.7%). These data, all together indicate that the deletion of the gene vIL-8 significantly decreases the virulence of the recombinant virus, rMd5/ΔvIL-8.

To verify that the phenotypic changes observed in the in vivo replication and pathogenesis of rMd5/ΔvIL-8 were only due to the deletion of vIL-8, a revertant virus, rMd5/ΔvIL-8-RV, was generated by cotransfection of rMd5/ΔvIL-8 viral DNA with a transfer vector, pUC19/SN5BamHI, containing the vIL-8 gene. Revertant viruses were selected by plaque purification and screened for the presence of vIL-8 gene by PCR (Figure 7). In addition, expression of vIL-8 in supernatant of DEF infected cells was confirmed by Western blot (Figure 9 lane 1). As seen in table 7, the pathogenic properties of the revertant rMd5/ΔvIL-8-RV virus were very similar to those of parental virus with regards to mortality (100%) and tumor incidence (76.7%). These results confirm that vIL-8 plays and important role in MDV pathogenesis.

Generation and *in vitro* characterization of an ELR mutant virus, rMd5/vIL-8-ELR. Most CXC chemokines present an ELR motif that has been associated with attraction of neutrophils. However, MDV vIL-8 presents a DKR motif in place of the ELR motif and attracts mononuclear cells instead of heterophils (chicken neutrophils). In order to determine the role of the DKR motif in the biological properties of vIL-8, a recombinant MDV virus, rMd5/vIL-8-ELR, was generated, in which the DKR motif was substituted by ELR. Using site

directed mutagenesis and RARE cleavage, two cosmid DNA, SN5/vIL-8-ELR and A6/vIL-8-ELR, were modified, in which the nucleotides *CAA* were changed to *GCT* resulting in two amino acids substitutions in the vIL-8 gene from DK (Asp-Lys) to EL (Glu-Leu). Interestingly, these mutations resulted in the loss of a *Sal* I site in the vIL-8 gene and this feature was used for the selection of recombinant clones prior to sequencing (Figure 15A). Transfection of DEF with cosmids, P89, SN16, B40, SN5/vIL-8-ELR and A6/vIL-8-ELR resulted in a recombinant virus with *in vitro* growth properties similar to those of wild type rMd5 (Figure 11). In addition, Southern blot analysis of *Eco*RI (Figure 10A, lane3-4) or *Bam*HI digested viral DNA (Figure 10B, lane3-4) showed the same DNA pattern as the parental virus while the *Bam*HI /*Sal*I double digested DNA (Figure 10B, lane3'-4') showed the lost of *Sal*I restriction enzyme site indicating the presence of the introduced mutations and no other major rearrangements.

Immunofluorescence analysis of rMd5/vIL-8-ELR infected DEF (Figure 8E and F) and Western blot analysis of supernatants of these cells (Figure 9, lane5-6) indicated that vIL-8-ELR was expressed and secreted like parental virus.

Mutant vIL-8 protein carrying the ELR motif induces strong angiogenic activity in CAM assay. The presence of an ELR motif of CXC chemokines has been shown to correlate well with their ability to induce angiogenesis in tumorigenesis (Strieter et al. 1995b). CAM assay has been used to study angiogenic and anti-angiogenic properties of compounds. In order to examine if a change from DKR to ELR induces angiogenic activity in vIL-8, gelatin sponges

absorbed with 100ul supernatant from rMd5 and rMd5/vIL-8-ELR infected DEF were placed on the CAM of 8 day old embryonating eggs. On day 12 of embryonation, macroscopic examination showed that sponges treated with 0.1ug of TPA (positive control) were surrounded by numerous allantoic vessels that developed radially towards the implant in a 'spoked-wheel' pattern (Figure 16A). A similar picture was produced by sponges loaded with supernatant of virus rMd5/vIL-8-ELR infected DEF (Figure 16B). However, when sponges were treated with supernatant from parental virus rMd5 infected DEF (Figure 16C); no vascular response was detectable around the sponges, as was the case with control specimens containing supernatant from non-infected DEF (Figure 16D).

ELR mutations have no effect on the pathogenic properties of rMd5/vIL-8-ELR. To examine if the DKR to ELR mutation had any effect on the pathogenicity of rMd5, rMd5/vIL-8-ELR was inoculated into day old 15x7 chickens and its effects in viremia, early cytolitic infection, mortality and tumor induction were examined. As seen on table 2 and figure 14, chickens infected with rMd5/vIL-8-ELR presented similar viremia titers as rMd5 at all four time points tested. Similarly, viral replication in lymphoid organs (bursa, thymus and spleen) determined by pp38 expression at 6 dpi, showed levels similar to those of rMd5 (Figure 12) indicating that the mutations did not have any effect on the early cytolytic infection of rMd5/vIL-8-ELR. In addition, mortality (94.1%) and tumor incidence (88.2%) induced by rMd5/vIL-8-ELR were very similar to those observed with rMd5 indicating that the mutations had no effect on the pathogenic properties of the virus.

DISCUSSION

In this report, a detailed analysis of pathogenesis of three recombinant MD viruses, rMd5/vIL-8-ELR which carries ELR motif in the vIL-8 gene, vIL-8 knockout virus rMd5/ΔvIL-8 and its revertant, rMd5/vIL-8-RV is described. The use of comid-based strategy significantly facilitated the construction of these viruses(Reddy et al. 2002). The results demonstrate that vIL-8 is not required for *in vitro* replication but plays an important role in *in vivo* propagation and pathogenesis. The gross MD tumor incidence caused by the vIL-8 deletion mutant is reduced to 17.6 % of infected birds (compared to 76.7% for chickens infected with the parental virus). Importantly, the revertant completely restores pathogenecity, conclusively demonstrating the crucial role of vIL-8 plays in this process. The vIL-8-ELR mutant has a pathogenic pattern similar to that of the wild type, indicating ELR does not significantly shift the tropism or affect the angiogenic properties of vIL-8.

While all the mutant viruses replicate equally well on fibroblasts *in vitro*, the infection patterns *in vivo* are quite different. A time-course analysis of infection in different organs was conducted. MDV induces an early phase of cytolytic infection in bursa during the first week, which is followed by latency entry and reactivation, resulting in the second phase of cytolyic infections occurring around two weeks. It was found that rMd5/ΔvIL-8 virus is significantly impaired in the early phase of cytolytic infections in lymphoid organs (6 days PI). It is well documented that reduction or absence of early cytolytic infection correlates with

absence or reduced incidence of lymphomas (Calnek 1972; Calnek et al. 1979; Calnek et al. 1983; Payne et al. 1973). This may account for the low virulence and lymphoma incidence of the vIL-8 deletion mutant. The role of vIL-8 in the second lytic infection of MDV was studied at 14 days post infection which correlates with the beginning of MDV latency. It was showed that rMd5/ΔvIL-8 virus replicated well in pheripheral blood lymphocytes and feather follicle epithelium (FFEs). FFE is the only site for productive infection resulting in cell-free infectious viral particles, which are transmitted to contact birds. It thus seems that vIL-8 plays a minor, if any, role in the second lytic infection phase as well as virus shedding and spreading.

The development of latency in MDV is not fully understood, but it is known that MDV can be reactivated by DEF cells co-cultivated with lymphocytes isolated from infected chickens (Calnek et al. 1981), a standard way of measuring viremia titers. Therefore, viremia titers in chickens can reflect both the degree of virus reactivation from latency and the number of latently infected cells. It has been shown that a successful cytolytic infection of B and T-cells is a prelude to latent-infection and transformation of T-cells. At 8 dpi, both vIL-8 deletion virus and the parental virus reached a similar peak of viremia titer, indicating that the vIL-8 gene is not essential for virus reactivation from latency. Nevertheless, at 14 and 35 days post inoculation, the viremia titers were significantly reduced in the deletion virus inoculated chickens compared to that of the pathogenic viruses. This data indicated that deletion of the vIL-8 gene compromised virus

transformation and reduced the number of transformed cells and was also confirmed by the lower tumor incidence.

The vIL-8 deletion mutant not only was less pathogenic in chickens, but also can induce protective immunity, when challenged with the very virulent plus strain, 648A virus (data not shown). Witter showed that a partially attenuated serotype 1 virus can induce protection when challenged with highly virulent viruses (Witter 2001). In this regard, rMd5/ΔvIL-8 might be considered a good candidate for the development of an improved vaccine for MDV.

The ELR mutant, rMd5/vIL-8-ELR, has both *in vitro* and *in vivo* properties similar to pathogenic parental virus, rMd5, a finding initially surprising to us. In mammals, ELR+ CXC chemokines engages CXCR2, a G-protein coupled receptor expressed in endothelial cells. It is speculated that the presence of ELR in vIL-8 would induce angiogenic activity, thus facilitating tumor growth. The preliminary study using the chicken chorioallantonic membrane assay (CAM) also showed that mutation to ELR motif is essential in inducing angiogenic activity by the mutated vIL-8 protein. It was thus speculated that vIL-8-ELR mutant virus would induce more aggressive tumors. The fact that it did not suggests that either the interaction of chicken chemokine or its respective receptor is significantly different, the tissue distribution of chicken CXCR2 is different from that of mammals, or infection of MDV induces significant level of cellular angiogenic factors such as cellular IL-8 or VEGF, such that the contribution by vIL-8 is inconsequential. At present little knowledge was known about the chicken CXCRs, nor their

expression patterns. It is also not known whether vIL-8 is able to trigger signals necessary for angiogenesis. A possible scenario is that vIL-8 is able to bind certain receptors and attract target cells, but unlike its cellular counterpart lacks the ability to trigger intracellular signals required for the proliferation of endothelial cells. Further investigation is required to sort out these questions. It is, however, known that MDV infection results in the release of cellular IL-8 homologs, 9E3/CEF and K60 (Carol Cardona personal communication), both of which contain the "ELR" motif and at least for 9E3/CEF, its angiogenic effect has been demonstrated (Martins-Green 2001; Martins-Green et al. 1998). At the same time, ELR containing chemokines are known to be chemoattractants for neutrophils, which might reduce virus load and impede tumorigenesis. The results would argue that the replacement of DKR by ELR does not change significantly its tropism toward target cells. It is noted that vIL-8 has a significantly long c-terminal domain, which is also considered important in chemotactic functions. The presence of this domain may diminish its ability to attract or activate neutrophils, even in the presence of ELR.

In summary, findings in this study are consistent with a model indicating that MDV encoded vIL-8 gene is involved in the early phase of cytolytic infections, presumably through the recruitment of B or T lymphocytes. Deletion of this gene has little impact on either virus reactivation from latency or virus spreading/shedding. Impaired early cytolytic infection due to the deletion of vIL-8 leads to weak inflammatory / immune responses, and reduced numbers of transformed cells, significantly decreased pathogenecity and tumor incidence.

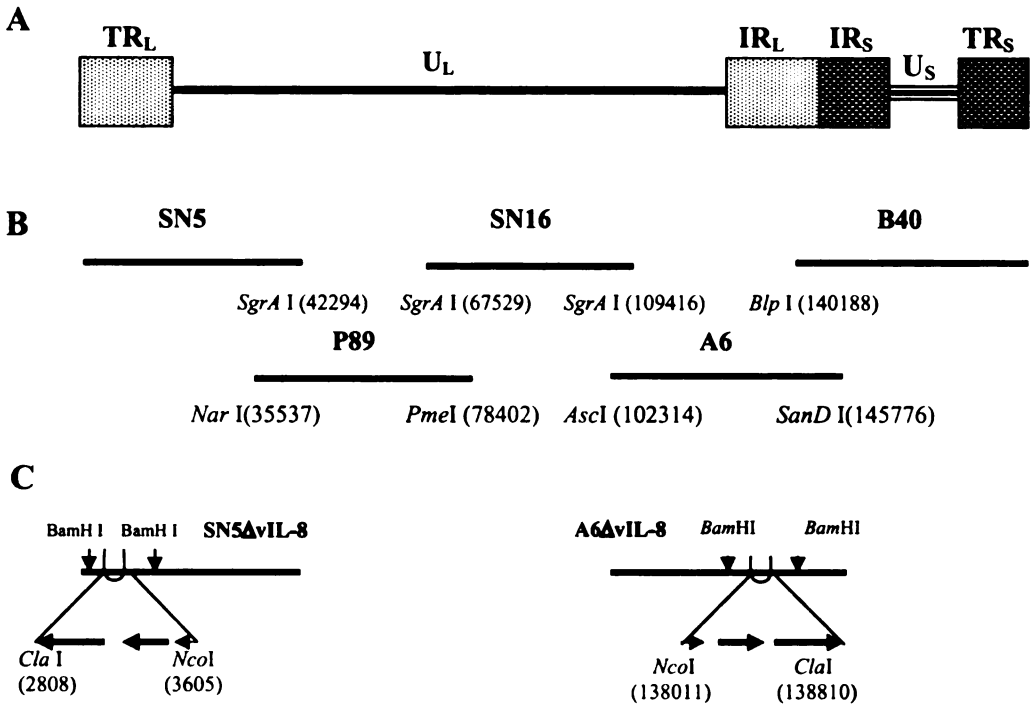

Figure 6. **Construction of recombinant virus with deletion of vIL-8 gene.** **A.** The MDV genome consists of terminal repeat long (TR_L) and short (TR_S), internal repeat long (IR_L) and short (IR_S), unique long (U_L) and unique short (U_S) DNA segments. **B.** Schematic representation of overlapping clones generated to reconstitute an infectious virus from a very virulent strain of MDV (Md5). The restriction enzymes used to generate each cosmid clones and their positions are indicated. **C.** Cosmids SN5/ΔvIL-8 and A6/ΔvIL-8 have the vIL-8 coding sequences deleted by *Cla*I and *Nco*I digestion. The locations of the restriction enzymes used to introduce the deletions are indicated.

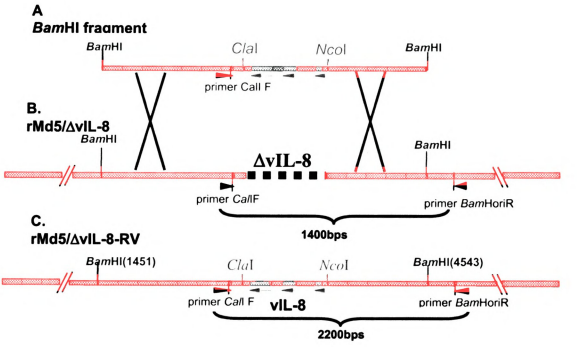

Figure 7. **Generation of rMd5/ΔvIL-8 revertant virus by homologous recombination.** (A) 3.1 kb *Bam*HI fragment (1451-4543) of rMd5 which contains the complete vIL-8 coding sequence. (B) *Bam*HI fragment of rMd5/ΔvIL-8 lacking the vIL-8 gene, but the remaining sequences in the *Bam*HI region are homologous to the *Bam*HI fragment in panel A. Homologous recombination between the DNA in A and B would generate a revertant virus rMd5/ΔvIL-8-RV (C) which includes a 3.1 kb *Bam*HI fragment (1451-4543) contains the complete vIL-8 coding sequence. The location of *Cal*IF and *Bam*HoriR primers and the size of the PCR product in each one of the virus is indicated.

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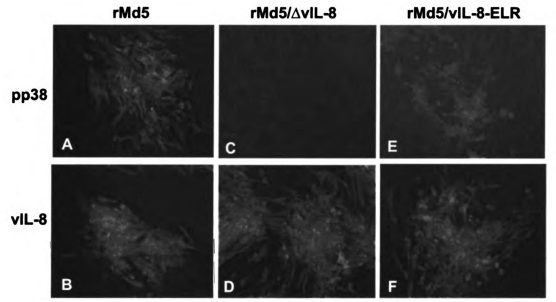

Figure 8. **Immunofluorescence staining of parental and recombinant MD viruses infected DEF using anti-pp38 and anti-vIL-8 antibodies.** PP38 could be detected in all three viruses infected DEF, rMd5 (A), rMd5/ΔvIL-8 (C), and rMd5/vIL-8-ELR (E). The vIL-8 can only be detected in rMd5 (B) and rMd5/vIL-8-ELR (F), but not in the vIL-8 deleted virus rMd5/ΔvIL-8 (D).

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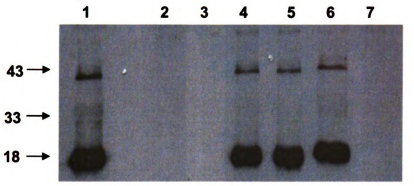


Figure 9. **Western blot analysis of supernatant** from rMd5/ΔvIL-8-RV (lane 1), rMd5/ΔvIL-8 (lanes 2 and 3), rMd5 (lane 4), rMd5/vIL-8-ELR (lanes 5 and 6) and mock infected (lane 7) DEF using rabbit sera against vIL-8. The size of vIL-8 protein is about 18kDa.

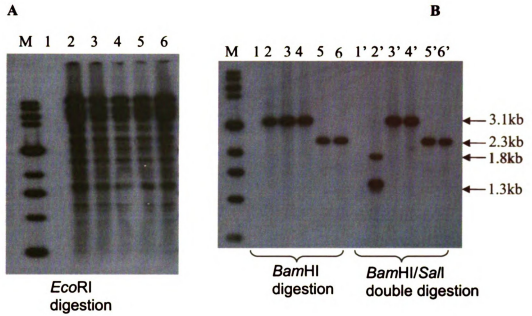
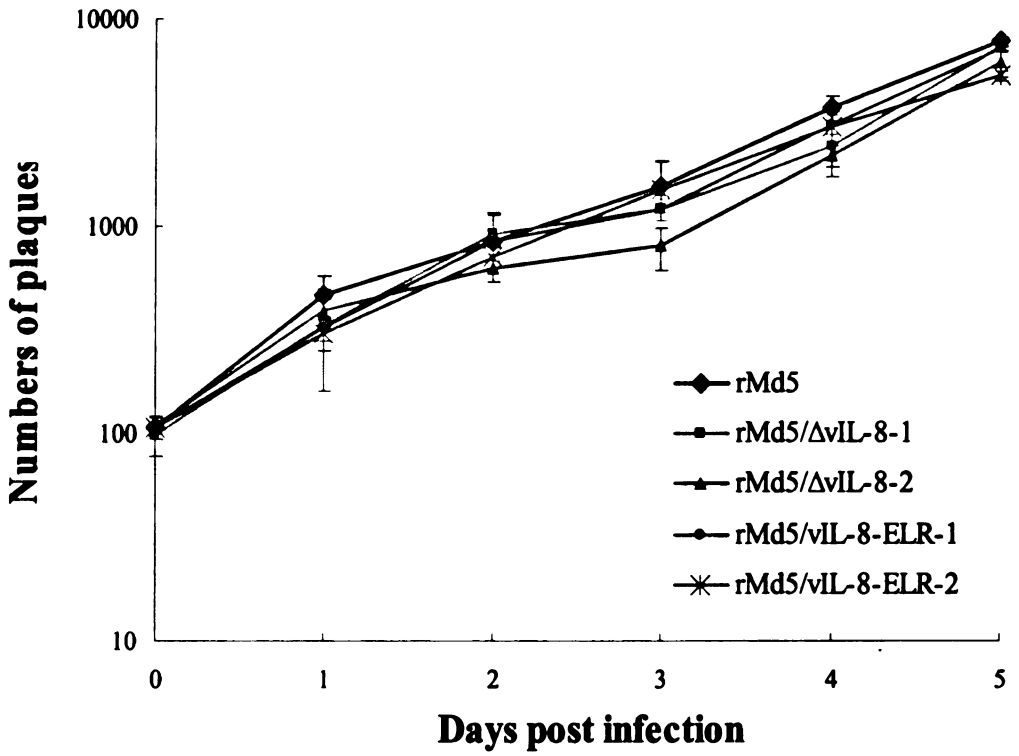


Figure 10. **Southern blot analysis of DNA isolated from nucleocapsids of wild type and recombinant MDV viruses.** (A) Viral DNA was digested with *Eco*RI and probed with all five radiolabeled cosmids. (B) Viral DNA was digested with *Bam*HI (lanes 1-6) or double digested with *Bam*HI/*Sal*I (lanes 1'-6') and hybridized with the 3.1 kbps MDV *Bam*HI fragment containing the vIL-8 gene. Lanes: 1 and 1' uninfected CEF; 2 and 2' rMd5; 3-4 and 3'-4' rMd5/vIL-8-ELR; 5-6 and 5'-6' rMd5/ΔvIL-8. *Bam*HI single digestion produces a 3.1 kb band in both rMd5/vIL-8-ELR and rMd5 viruses and a 2.8 kb fragment in rMd5/ΔvIL-8 virus. *Bam*HI/ *Sal*I double digestion results in two bands (1.8 kb and 1.3 kb) in rMd5 and a single band (3.1 kb) in rMd5/vIL-8-ELR.



Error bars in the figures show the standard deviation of the mean (S.D.).

Figure 11. ***In vitro*** **growth kinetics of rMd5, rMd5/vIL-8-ELR-1, rMd5/vIL-8-ELR-2, rMd5/ΔvIL-8-1, and rMd5/ΔvIL-8-2 viruses.** DEF were infected with aproximately 100 PFU of the indicated viruses and at 1, 2, 3, 4, and 5 days post-infection, the cells were harvested and their titer determined in fresh DEF after infection and titered on fresh DEF. The experiment was performed in duplicate, and the titer (logarithm of the mean number of plaque-forming units per dish) is indicated. Error bars in the figures show the standard deviation of the mean (S.D.).

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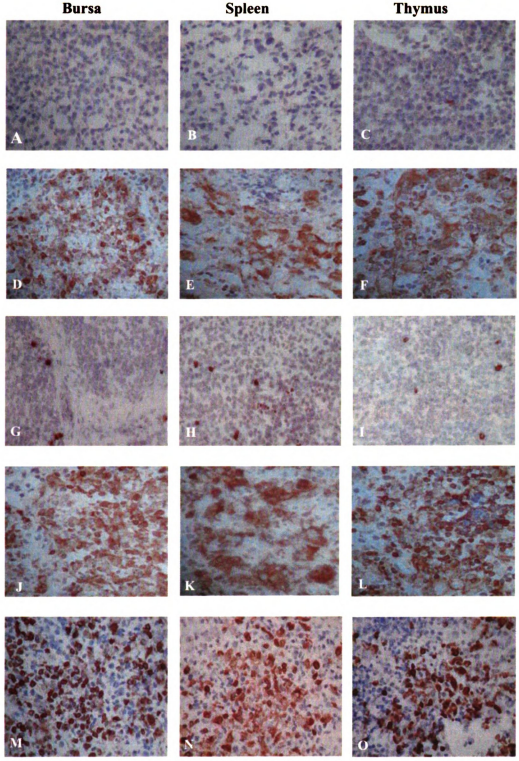
Bursa
Spleen
Thymus
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 12. **Immunohistochemistry of lymphoid organs (bursa, spleen and thymus)** of 15x7 MDV maternal Ab-negative chickens 6 days after inoculation with: no virus (A, B, C), rMd5 (D, E, F), rMd5/ΔvIL-8 (G, H. I) rMd5/vIL-8-ELR (J, K, L), or rMd5/ΔvIL-8-RV (M, N, O) Monoclonal antibody against pp38 (H19) was used for the staining. Antigen expression in lymphoid organs is severely impaired only in rMd5/ΔvIL-8, showing that vIL-8 is involved in early cytolytic infection in lymphocytes.

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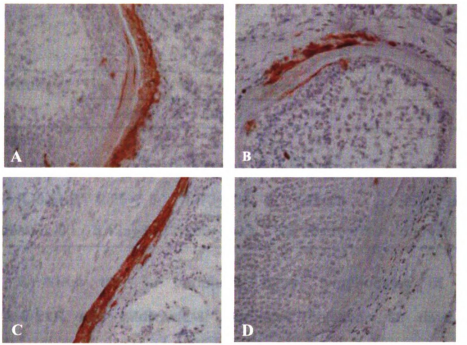

Figure 13. **Immunohistochemical analysis of Feather Follicle Epithelium (FFE) cells from inoculated chickens.** FFEs were sampled at 2 weeks post inoculation. All of the recombinant viruses rMd5 (A), rMd5/vIL-8-ELR (B), and rMd5/ΔvIL-8 (C) expressed viral antigen in FFEs, indicating that the second lytic infection is not impaired in either the vIL-8 gene mutations or the vIL-8 gene deletion. No viral antigen was detected in the control chickens (D).

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Virus	Viremia[§]			
	6dpi	8dpi	14dpi	35dpi
Mock	0	0	0	0
rMd5	7.25±4.87	803.5±337.2	223.25±38.7	1835±271.5
rMd5/vIL-8-ELR	2.25±0.5	1273±336	139±64	2529±992
rMd5/ΔvIL-8	5.125±3.5	1280±349.4	79.75±10.9**	355±51.96***

TABLE 6. **Virus titer in peripheral blood lymphocytes of rMd5, rMd5/vIL-8-ELR and rMd5/ΔvIL-8 inoculated chickens 35 days post inoculation.**

§: The values represent the average of the 5 birds from each inoculated group. The results shown are the mean value ± S.D.

*: The value in rMd5/ΔvIL-8 inoculated birds is smaller than the other two viruses inoculated birds. Differences in viremia were compared among the viruses using the Student's *t* test. A significant difference in viremia was observed at $p \leq 0.05$ (2 asterisks) and $p \leq 0.001$ (3 asterisks).

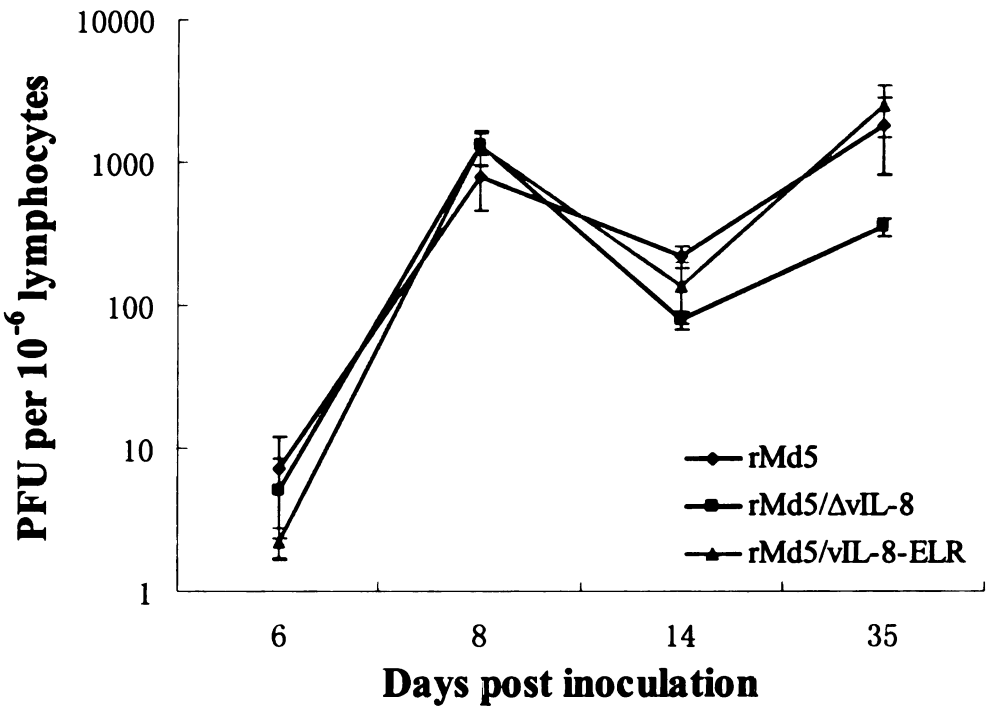

Figure 14. **Viral titers, at 6, 8, 14, and 35 days post-inoculations, in peripheral blood lymphocytes of 15x7 chickens inoculated with rMd5, rMd5/vIL-8-ELR, and rMd5/ΔvIL-8.** Three birds from each experimental group were tested and titrations performed in duplicate. The titer is indicated as the logarithm of the mean number.

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Virus[¶]	Mortality	Tumor incidence (%)
Mock	0/17 (0)	0/17 (0)[∞]
rMd5	15/17 (88.2)	13/17 (76.7)
rMd5/ΔvIL-8	1/17 (4.3)[*]	3/17 (17.6)[*]
rMd5/vIL-8-ELR	16/17 (94.1)	15/17 (88.2)
rMd5/ΔvIL-8-RV	17/17 (100)	13/17 (76.7)

TABLE 7. **Comparison of the pathogenecity of rMd5, rMd5/ΔvIL-8, rMd5/vIL-8-ELR and rMd5/ΔvIL-8-RV in 15x7 antibody negative chickens.**[§]

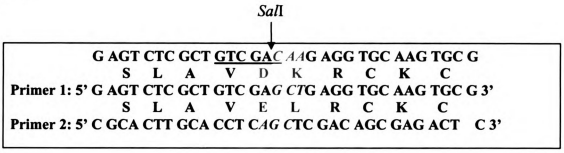
§: This experiment was repeated two times separately.

∞: Data in parentheses indicate percentage of affected chickens.

¶: All chickens were inoculated with 2000 PFU of the indicated viruses.

*: Student's *t* test ($p \leq 0.001$) analysis indicated that these values were significantly different from the other groups.

A.



B.

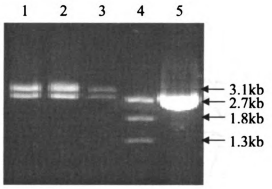


Figure 15. **Mutation of DKR to ELR motif in the MDV vIL-8 gene.**

A. Wild type DNA sequence of the DKR motif. Primers 1 and 2 were used to introduce 3 nucleotides changes in the vIL-8 coding sequence, which results in the loss of the *Sal*I restriction endonuclease site and a change from DKR to ELR.

B. *Bam*HI and *Sal*I double digestion of ELR mutant (pUC19/SN5-ELR) (lanes 1-3) and wild type (pUC19/SN5) (lane 4) transfer vectors confirms the absence of *Sal*I site in the ELR mutant vector. Mutagenesis results in the loss of *Sal*I RE site, and in turn changes the digestion pattern of the plasmid DNA. Plamid vector (lane 5) alone is used as control. The size of the restriction fragments obtained is indicated on the right in kb.

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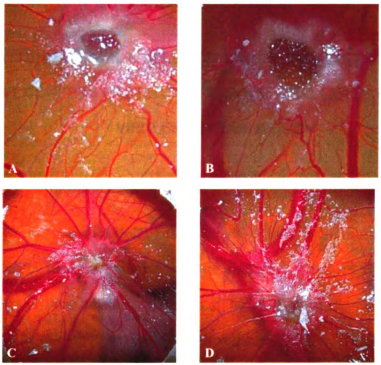

Figure 16. **Angiogenic effect of supernatants from uninfected (A) and rMd5 (B) and rMd5/vIL-8-ELR (C) infected DEF.** Chorioallantoic membranes (CAMs) of 10-day-old chicken embryos were incubated for 5 days with sponges absorbed with the indicated supernatants. Supernatant from rMd5/vIL-8-ELR infected cells showed a "spoked-wheel" patterned neovascularization reaction similar to the TPA positive control (D). Supernatants from rMd5 infected and uninfected DEF produced no neovascularization reaction.

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CHAPTER THREE

AN ATTENUATED vIL-8 DELETION MAREK'S DISEASE VIRUS (MDV) MUTANT CONFERS PROTECTION AGAINST CHALLENGE WITH A VERY VIRULENT STRAIN OF MDV

ABSTRACT

Marek's disease virus (MDV) encoded vIL-8 gene, a chemokine-like gene, was studied by using a recombinant virus, rMd5/ΔvIL-8, in which both copies of the vIL-8 gene were deleted. Deletion of the vIL-8 gene attenuated the virulence of the virus as demonstrated by the pathological studies in chickens. Both the MD maternal Ab^+ and Ab^- chickens inoculated with rMd5/ΔvIL-8 developed a much lesser number of gross lesions compared to the parental virus rMd5. Similarly, the mortality and tumor incidence in groups of chickens inoculated with rMd5/ΔvIL-8 were also significantly lower than rMd5 inoculated chicken groups. The revertant virus, rMd5/ΔvIL-8-RV, which restored the vIL-8 gene back in the rMd5/ΔvIL-8 viral genome, also restored the pathological phenotype as the parental virus. With the successful generation of revertant virus, one can make a more convincing explaination of the *in vitro* and *in vivo* phenotype characteristics of vIL-8 deleted virus.

Interestingly, the rMd5/ΔvIL-8 virus also had the ability to protect chickens against challenge with a MDV very virulent plus (vv+MDV) strain, 648A. This finding provides new optimism regarding the generation of recombinant virus

vaccines for control of MDV. Using the flow cytometry assay, the T and B lymphocytes populations were studied along with the splenocytes of virus inoculated chickens. Compared to the non-infected chickens or chickens with the inoculum of rMd5/ΔvIL-8, the numbers of B lymphocytes in the rMd5 inoculated chickens were dramatically reduced, while the numbers of activated T-cells were increased at 7 DPI. The loss of B-cells may due to cytosis or apoptosis resulting from the effective cytolytic infection of the rMd5. The early cytolytic infection resulted in stimulation of the immune response and activation of T-cells to become the next major target cells. With the deletion of vIL-8, the early cytolytic infection was impaired, therefore, the number of B-cells remained similar to the non-infected chickens, and activation of T-cells was also reduced. Since vIL-8 is a chemokine-like gene, it is also reasonable to speculate that the presence of vIL-8 may be functioning as a chemoattractant for B-cells and activated T-cells in assisting MDV infection and replication. A hypothesized model for the possible role of vIL-8 in the pathogenesis of MDV is proposed.

INTRODUCTION

MDV is classified as a member of the family *Herpesviridae* (Roizman 1992b). Because of its lymphotropism, which is similar to Epstein Bar virus (EBV), MDV was originally classified as a γ–herpesvirus (Roizman 1981). However, later on, based on its genomic organization and significant sequence similarity to other α–herpesviruses, such as herpes simplex virus (HSV) and varicella-zoster virus (VZV), it was classified in the subfamily *Alphaherpesvirinae* (Buckmaster et al. 1988). The MDV group was divided into three serotypes (Bülow 1975a): Pathogenic strains of MDV and their attenuated derivatives are the prototype viruses of the MDV group, and are designated as serotype 1 MDV (MDV-1). The nonpathogenic strains of MDV are designated serotype 2 MDV (MDV-2) and HVT, a nonpathogenic herpesvirus of turkeys is grouped into serotype 3 MDV (MDV-3).

The pathogenesis of MDV is divided into four distinct phases, the early cytolytic infection, a second lytic infection, latent infection, and transformation. The molecular mechanisms of all these phases have not yet been fully understood. MD incidence has largely been controlled by vaccination with all three serotypes of MDV, often in bi- and multivalent combinations (Witter 1991, 2001). However, there is continuation of an apparent evolutionary trend of MDV towards greater virulence which has resulting in increased losses from MD in vaccinated flocks (Witter 1997). The attenuated MDV-1 strain virus, CVI988, is currently the most protective vaccine in use. If the MDV evolutionary trend continues, vaccine may fail even with CVI988. The live virus vaccines for MDV

are efficient in controlling disease by protecting chickens against tumor induction and mortality, but do not prevent viral replicating or shedding. To effectively control this disease, understanding the mechanisms of the virus replication and virus interaction with the host cells is the key.

Recently, the successful establishment of MDV cosmid clones has provided a molecular tool to introduce targeted mutations into the genome of a pathogenic strain of MDV. This recombinant technology will be extremely helpful in the exploration of the mechanisms of MDV pathogenesis and identify the roles of individual genes in the four distinct pathogenesis phases. Based on this technique, a recombinant MDV, rMd5/ΔvIL-8 was generated. And using homologous recombination, the revertant virus that restored the vIL-8 gene in the rMd5/ΔvIL-8 viral genome was also successfully generated and provided comparative information to the rMd5/ΔvIL-8 and parental virus. It is known that vIL-8 is involved in the early cytolytic infection and transformation, but not in latency or virus reactivation from latency. The absence of vIL-8 does not impair virus spreading and shedding either. In this study, the pathologic studies of rMd5/ΔvIL-8 and rMd5/ΔvIL-8-RV were carried out. The results demonstrated that the virulence of rMd5/ΔvIL-8 was attenuated and for the first time in marek's disease, it is reported that an attenuated recombinant virus has the ability to protect chickens against challenge from vv+MDV strains.

MATERIALS AND METHODS

Antibodies. Phosphoprotein 38 (pp38) specific Mab, H19 (Cui et al. 1990), and rabbit antiserum against MEQ (Lucy Lee, Avian Disease and Oncology Laboratory, East Lansing, MI,) were used in this study. Monoclonal antibodies (Mab) CT4 and CTLA3 reacts specifically to chicken CD4 and CD8 respectively (Southern Biotechnology Associate, Inc., Birmingham, AL) were used to detect the transformed cell type of the tumors. Mab anti-chicken MHC class I-R-PE (R-phycoerythrin), Mab anti-chicken class II-R-PE, Mab anti-chicken CD3-FITC and Mab anti-chicken Bu1a/1b-FITC (Southern biotechnology associate, Inc, Birmingham, AL) were used in flow cytometry experiments.

Cells and viruses. Primary duck embryonic fibroblasts (DEF) were used for virus propagation and DNA transfections. Both the MDV vaccine strain CVI988 and challenge virus 648A were kindly provided by Richard Witter, Avian Disease and Oncology Laboratory (East Lansing, MI). Recombinant viruses were generated from cosmids derived from a very virulent MDV strain, Md5 (Reddy et al. 2002).

Chickens. Chickens used in the study were specific pathogen free (SPF) MD susceptible F1 progeny (15x7) of Avian Disease and Oncology Laboratory line $15I_5$ males and line 7_1 females. All the chickens were wing-banded at hatch, and randomly sorted into different experimental groups (17 chickens per group) and held in modified Horsfall-Bauer isolators.

Pathogenesis studies. Chickens from either unvaccinated breeder hens free of antibodies to all three MDV serotypes (Ab-negative) or from breeder hens vaccinated with all three serotypes of MDV (Ab-positive) were used in these studies. Day old chickens were inoculated with 2000 plaque forming units (PFU) of rMd5, rMd5/ΔvIL-8, or rMd5/vIL-8-RV, subcutaneously. Chickens that did not receive any inoculum were used as negative controls. Chickens were raised in isolators for 8 weeks, each group comprised of 17 chickens. The chickens in Ab^- groups were observed daily for signs of transient paralysis. All the chickens that died during the trial or were killed at the end of the experiment (8 weeks after inoculation) were necropsied and evaluated for gross and histological lesions and mortality. Lymphoid organs and feather follicles were collected in a time course (4, 6, 14, 35 DPI) for the expression kinetics of MDV viral proteins (pp38 and Meq) by immunohistochemical staining. Tumor tissues were also collected freshly at termination by embedding in O.C.T. (optimal cutting temperature) compound (Sakura Finetek USA, Inc., CA) and immediately frozen in liquid nitrogen. Immunohistochemical staining of frozen sections were carried out to identify the phenotype of the transformed cells.

Immunohistochemical staining. Lymphoid organs (thymus, spleen, bursa of Fabricius) and feather follicles of infected and uninfected chickens were collected and embedded in O.C.T. compound, immediately frozen in liquid nitrogen and stored at -80°C. Four to eight micron thick cryostat sections of tissue blocks were mounted on poly-L-lysine (Sigma Diagnostics, St. Louis, MO)-coated sides and fixed for 5 minutes in ethanol, air dried at -20°C for at least

30 minutes, and stored at -80°C until staining. An avidin-biotin-peroxidase complex (ABC) method (Vectastain® ABC kit, Vector Laboratories, Inc., Burlingame, CA) was used for immunohistochmistry. Samples were equilibrated for 15 minutes in isotonic phosphate-buffered saline (PBS), pH 7.4. Between each remaining step, sections were washed three times for 10 minutes each in PBS. Sections were preincubated for 20 minutes with normal blocking serum (provided with the kit) to decrease nonspecific background staining. Samples were then incubated at room temperature with primary antibodies in an appropriate concentration for 30 minutes. Mab H19 (Cui et al. 1990) specific to antigen pp38 was used at a working dilution of 1:3200, rabbit antiserum against Meq was used at a working dilution of 1:2500. Mab CT4, which is specific to CD4 and reacts with T-cell subsets expressing CD4, and Mab CTLA3, which reacts with the T-cells subsets expressing CD8 were diluted at the concentration recommended by the manufacturers (Southern Biotechnology Associate, Inc., Birmingham, AL) to examine the MDV tumor cells. A biotinylated secondary antibody (provided with the kit) was incubated with the slides for another 30 minutes at room temperature after the primary antibody incubation. The working concentration of the secondary antibody was also used according to manufacturers' Instruction. Following a PBS wash, the sections were incubated for 30 minutes with the ABC. The immunohistochemical reaction was visualized following incubation with a solution of hydrogen peroxide and 3, 3'-diaminobenzidine (DBA) (Vector® SK-4100, Vector Laboratories) for 7 minutes. All sections were then lightly counterstained with Gill's hematoxylin NO2 (Fisher

Scentific, Fair Lawn, NJ), dehydrated, and mounted in DPX mountant (BDH Laboratory Supplier).

Flow cytometry assay. Antibody negative chickens (line 15x7) were inoculated with 2000 PFU of parental virus rMd5 or mutant virus rMd5/ΔvIL-8. Uninoculated chickens were used as negative controls. On 4 and 7 days post inoculation, splenocytes from 5 chickens from each group were examined for the presence of activated T-cells (MHC class $II^+/CD3^+$) and B-cells (MHC class $I^+/Bu1a^+/1b^+$). To determine the number of the activated T-cells, 10^7 splenocytes were double stained with mouse anti-chicken MHC class II-R-PE (red) and mouse anti-chicken CD3-FITC (green). The numbers of B-cells were determined by double staining splenocytes with mouse anti-chicken Bu1a/1b-FITC (green) and mouse anti-chicken MHC class-I-R-PE (red). After 1 hour incubation at 40°C, the cells were washed three times with PBS and analysed by Fluorescent Activated Cell Sorter, BD FACSCalibur system (BD Bioscience, San Jose, CA). The significance of cell population between each group was analyzed by student-*t*-test. Difference were considered to be significant when $p< 0.05$.

Protection experiments. The protection efficacy of rMd5/ΔvIL-8 was compared to that of vaccine virus CVI988. A vv+MDV strain, 648A, was used as a challenge virus. MDV maternal Ab^+ chickens were used for protection experiments.

Groups of 17 chickens were inoculated subcutaneously at 1 day of age with 2000 PFU per chicken of vaccine virus. Two groups of chickens were inoculated

with the test vaccine strain, rMd5/ΔvIL-8. One group of chickens was inoculated with vaccine strain CVI988. The vaccinated groups and one unvaccinated virus control group were challenged with very virulent plus MDV strain, 648A, 500 PFU per chicken subcutaneously at 5 days post-vaccination. One more group of chickens was set as control with no vaccination and no challenge.

Chickens that died during the experiment or were killed at the termination at 8 weeks post-challenge were examined for gross MD lesions, which include enlarged peripheral nerves, visceral lymphomas, and bursal/thymic atrophy.

MD responses were expressed as a percentage (% MD) based on the chickens with MD lesions divided by the number of chickens at risk and multiplied by 100. The percentage protection (% Protection) was calculated as the % MD in unvaccinated challenged controls minus the % MD in vaccinated challenged group divided by the % MD unvaccinated challenged controls multiplied by 100. Data pooled from the two replicates were also summarized for % MD and % Protection.

RESULTS

The recombinant mutant virus, rMd5/ΔvIL-8, has an attenuated virulence. The pathological lesions induced from the mutant viruses (rMd5/ΔvIL-8 and the revertant virus rMd5/ΔvIL-8-RV) was compared with the parental virus rMd5 in both MDV maternal $Ab^+$and Ab^- chickens (Table 8, 9) by evaluation for transient paralysis (TP), gross lesions, mortality and microscopic lesions. During the 8 weeks post inoculation with virus rMd5/ΔvIL-8, no mortality was observed in the antibody positive chickens and only one bird died in the antibody negative group, while in the groups inoculated with either the parental virus rMd5 or the revertant virus rMd5/ΔvIL-8-RV, high mortality was observed in both Ab^- chicken groups (88.2-100%) and Ab^+ chicken groups (64.7%). The incidence of nerve lesions and visceral tumors in rMd5/ΔvIL-8 inoculated groups was also much lower in both Ab^- chickens (17.6%) and Ab^+ chickens (5.9%) compared to the groups inoculated with parental virus rMd5 or rMd5/ΔvIL-8-RV (76.7-88.2%). It was also observed that none of the rMd5/ΔvIL-8 inoculated chickens developed TP in Ab^- chickens. There was severe atrophy in the bursa of Fabricius and thymus in rMd5 and rMd5/ΔvIL-8-RV inoculated chickens. However, no atrophy was observed in chickens inoculated with rMd5/ΔvIL-8. Based on the results of gross lesions, the deletion of the vIL-8 gene significantly attenuated the virulence of the recombinant virus, rMd5/ΔvIL-8. Nevertheless, it was observed that rMd5/ΔvIL-8 still induced microscopic lesions in nerves and visceral organs (Figure 17). The lesions consisted of a mixed population of small, medium, and large lymphoid cells including plasma cells and large anaplastic lymphoblasts.

Phenotype of the target cells for transformation is not affected by vIL-8. During the pathological studies, only one bird developed gross tumor in the group of chickens inoculated with rMd5/ΔvIL-8. The populations of transformed cells in the gross tumor were characterized by immunohistochemical staining with specific T-cell markers. Tumors developed from rMd5 inoculum were used as controls. The immunohistochemical results revealed that both rMd5 and rMd5/ΔvIL-8 virus transformed $CD4^+/CD8^-$ subset of T-cells (Figure 18). The results suggested that the phenotype of the tumor cells was not influenced by the absence of vIL-8.

Deletion of vIL-8 gene delayed MEQ expression and impaired pp38 expression in lymphoid organs. A time course study on the expression of MDV viral proteins Meq and pp38 in the rMd5/ΔvIL-8 inoculated chickens was compared to those in the parental virus rMd5 inoculated chickens (Table 10). In the rMd5 inoculated chickens, pp38 expression was detected from 4 to 35 DPI in lymphoid organs, with peak detection at 6 DPI. In the FFEs of rMd5 inoculated chickens, pp38 expression was positive from 6 DPI and reached the peak at 14 DPI. Compared to rMd5, rMd5/ΔvIL-8 inoculated chickens had almost no detectable pp38 expression in the lymphoid organs while a normal level of pp38 expression was detected in the FFEs at 14 DPI. This data shows that deletion of vIL-8 gene impairs the expression of pp38 in lymphoid organs, but not in the FFE.

The expression of Meq in the rMd5 inoculated chickens was detected from 6 to 35 DPI in both lymphoid organs and FFEs. No Meq expression was detected in

rMd5/ΔvIL-8 inoculated chickens at 4 and 6 DPI, however, Meq expression was detected later at 14 and 35 DPI in both lymphoid organs and FFEs of rMd5/ΔvIL-8 inoculated chickens. The numbers of Meq positive cells in the tissue of rMd5/ΔvIL-8 inoculated chickens were comparatively lower than that in the rMd5 inoculated chicken tissues.

The nucleus morphology of Meq positive cells was changed from single lobed at 6 DPI, to multi-lobular like pattern at 14 DPI and later (Figure 19). The same multi-lobular like staining pattern was observed in rMd5/ΔvIL-8 inoculated chickens at 14 DPI and later. This indicated that at 14 DPI or later, MDV infected cells were undergoing transformation (Liass 1985; Russo et al. 1988). The rMd5/ΔvIL-8 induced a lower level of transformation.

Change in lymphocyte population following early cytolytic infection with rMd5 and rMd5/ΔvIL-8. The proportions of activated T-cells and B-cells in the splenocytes of virus inoculated chickens were analyzed using the FACS. The data showed significant differences between chickens that received rMd5 inoculum and chickens inoculated with rMd5/ΔvIL-8 at 7 DPI but not at 4 DPI. The number of B-cells dramatically decreased at 7DPI in rMd5 inoculated chickens because of a robust early cytolytic infection. However, the number of B-cells in the rMd5/ΔvIL-8 inoculated chickens remained significantly higher than rMd5 inoculated chickens and no significant difference to the non-infected group indicating that vIL-8 is essential for a robust early cytolytic infection (Figure 20). The number of activated T-cells was increased at 7 DPI in rMd5 inoculated

chickens, but not in the rMd5/ΔvIL-8 virus inoculated chickens or the non-infected groups. Activated T-cell was the other target cells for MDV infection. Deletion of vIL-8 reduced the number of the activated T-cells indicating that vIL-8 plays an important role in the activation of T-cells, which is critical for establishment of MDV infection.

Recombinant virus rMd5/ΔvIL-8 protected chickens against very virulent MDV. The protective efficacy was compared between rMd5/ΔvIL-8 and strain CVI988. Two replicates were conducted in the experiment. The MD responses and percentage of protection for each group were evaluated in each replicate and summarized (Table 11).

The results showed that chickens vaccinated with rMd5/ΔvIL-8 and challenged with 648A had a lower percentage incidence of MD than that of CVI988 vaccinated chickens. As shown in Table 11, with vaccination with rMd5/ΔvIL-8, there was only 23.5% of MD incidence in replicate 1 and 17.6% MD incidence in replicate 2, while vaccination with CVI988 the MD incidence was 47.1% and 41.2% in replicate 1 and 2, respectively. Without vaccination, 648A induced a 100% incidence of MD in both replicates. So, based on MD incidence, the percentage of protection of rMd5/ΔvIL-8 was 76.5% in replicate 1 and 82.4% in replicate 2, and the percentage of protection with CVI988 was 52.9% in replicate 1 and 58.8% in replicate 2. Data pooled from two replicates were summarized in Table 11 and demonstrated that rMd5/ΔvIL-8 induced about a 79.4% protection

index against 648A, and CVI988 induced about a 55.9% of protection index against 648A.

DISCUSSION

This study reports the pathological characterization of the attenuated recombinant vIL-8 deleted MDV, rMd5/ΔvIL-8, and its ability to protect chickens against challenge with a vv+MDV, 648A.

Since the first report of reconstitution of MDV with cosmid clones, only the pp38 gene deleted virus was studied and reported (Reddy et al. 2002). This vIL-8 deleted virus, rMd5/ΔvIL-8, is the first cosmid clone reconstituted mutant virus characterized by detailed *in vivo* studies. Results presented here from experiments in both Ab^+ and Ab^- chickens indicate that the virulence of rMd5/ΔvIL-8 was attenuated and caused a much lower incidence of gross lesions and mortality. Moreover, a revertant virus that restored the vIL-8 gene back to the rMd5/ΔvIL-8 viral genome was also successfully generated. The successfully restored pathological phenotype of the revertant virus strongly assisted in demonstrating that the impairments in rMd5/ΔvIL-8 pathogenicity specifically resulted from the deletion of the vIL-8 gene.

In order to investigate the interaction between vIL-8 and other viral proteins *in vivo*, lymphoid tissues and FFEs from rMd5/ΔvIL-8 inoculated chickens were examined for pp38 and Meq expression level in a time course study with IHC and compared to that of rMd5 inoculated chickens. The results demonstrated that deletion of vIL-8 impaired the expression of pp38 in lymphoid organs, but not in FFEs. Furthermore, deletion of vIL-8 delayed Meq expression *in vivo*, and the number of Meq positive cells was also reduced in comparison to that of rMd5

inoculated chickens. Meq is expressed in the nucleus, from 6 DPI to 14 DPI, and later. The multi-lobular-like nuclear staining pattern of Meq-positive cells at later stages of MDV infection suggested that the cells were undergoing transformation. Although MEQ positive stained cells were detected in rMd5/ΔvIL-8 inoculated chickens at 14 DPI with the same multi-lobular like nucleus, the percentage of positive cells in each sample was lower than the rMd5 inoculated chickens. The pathogenic studies also showed that rMd5/ΔvIL-8 inoculated chickens developed a lesser number of gross tumors. Collectively, the results may indicate that vIL-8 gene is involved in pathogenecity and transformation of the disease. Moreover, neither pp38 nor Meq expression in lymphoid organs at 6 DPI confirmed again the rMd5/ΔvIL-8 induced impairment of early cytolytic infection in lymphoid organs.

B-cells and activated T-cells are the major target cells for MDV infection and replication. As the primary target cells, infected B-cells at the early cytolytic infection phase will either undergo apoptosis or cytosis, which will induce early immune responses and release cytokines and other immune factors such as IFN-γ, Nitric Oxide (NO) etc, to activate resting T-cells (Schat et al. 2000). The activated T-cells then become the major target cells. It was also observed that deletion of vIL-8 resulted in a reduction in the number of B-cells infected and also a reduced number of activated T-cells. It was demonstrated earlier that rMd5/ΔvIL-8 had impaired early cytolytic infection, which could result in reduced infection of B -cells, followed by less apoptosis or cytosis, and therefore induce a weak immune response to activate T-cells. Furthermore, as a chemoattractant, it is also reasonable to speculate that vIL-8 may function to attract target cells

(B-cells and activated T-cells) and facilitate virus infection and dissemination. Although it has been reported that *in vitro* over-expressed vIL-8 protein attracted mononuclear cells, further studies are necessary to characterize the specific mononuclear cell type.

Calnek has established a model for the sequential events during the pathogenesis of MDV infection (Calnek 1985). Based on our studies of the vIL-8 gene, a modified model of MD pathogenesis is proposed with reference to the function of vIL-8 (Figure 21). Specifically, during the early phase of MDV replication (4-7 DPI), vIL-8 is critical in attracting B-cells for MDV infection and replication, and consequently, vIL-8 gene functions in activating the resting T-cells to become MDV susceptible activated T-cells and attract activated T-cells to the infection sites. Similarly, the MDV infected activated T-cells secrete large amounts of vIL-8 which further aid in activating and attracting more T-cells to establish a robust early cytolytic infection.

The other important finding in this study is that this recombinant virus has the ability to protect chickens against challenge with vv+MDV strain, 648A. The protection index was even higher than what was observed with the commercial vaccine strain, CVI988. The mechanism of how the MDV vaccine protects chickens against challenge with a highly virulent virus strain is not quite clear, but Calnek et al. have speculated that vaccination could alter the pathogenesis of MDV infection by severely curtailing or eliminating the early cytolytic phase, thus decreasing the number of target cells for the establishment of latency or

transformation (Calnek et al. 1997). As it was proved in this study and the earlier work reported in this dissertation, deletion of vIL-8 gene resulted in depletion of viral antigen expression in the lymphoid organs at 6 DPI, and no bursa/thymus atrophy in Ab^- chickens during the experimental trial. So it is reasonable to conclude that rMd5/ΔvIL-8 could act like the vaccine virus strains in impairing the early cytolytic infection, and therefore block the latent infection and transformation of T-cells and protect chickens. This is the first report of a recombinant serotype 1 MDV vaccine.

Attenuation of serotype 1 MDV vaccines was achieved by virus passages in cell culture. In order to adapt to the *in vitro* growth environment, the virus would introduce mutations in the genome resulting in attenuation of virulence. The mutations happened randomly in certain viral copies but not all. After several *in vitro* passages, attenuated virus in fact consists of a mixture of mutant and intact viral genomes. Witter recently reported that partially attenuated serotype 1 MDV vaccines induce stronger protection (Witter 2002). The disadvantage of using partially attenuated serotype 1 MDV vaccine is also apparent. The copies of intact viral genome in the mixture of virus have the advantage to replicate *in vivo*, so after backpassage of the attenuated virus in the chickens for several generations, the virus will gain back the virulence and cause disease. Because the attenuated recombinant virus such as the rMd5/ΔvIL-8 was produced from a pure clone, backpassage in chickens would not change the virulence of the virus. So, it is likely that recombinant virus vaccines will possibly become the next generation of MD vaccines in the near future.

Virus Inoculum	Transient Paralysis	Gross Lesions[§]		Microscopic Lesions	Total Mortality
		Nerves	Visceral		
None	0/17(0)[*]	0/17	0/17		0
rMd5	12/17(70.6)	13/17(76.7)	10/17(58.8)	NT[¶]	15/17(88.2)
rMd5/ΔvIL-8	0/17(0)	1/17(4.3)	2/17(13.3)	9/17(52.9)	1/17(4.3)
rMd5/ΔvIL-8 -RV	11/17(64.7)	13/17(76.7)	11/17(64.7)	NT	17/17(100)

Table 8. **Pathological lesions in MDV maternal antibody negative chickens inoculated with rMd5, rMd5/ΔvIL-8, and rMd5/ΔvIL-8-RV viruses.**

[*]Data in parentheses indicate percentage of chickens.

[¶]Not all chickens were examined by histopathology. NT: Not Tested.

[§]Gross lesions include nerve enlargement and visceral tumors.

Virus inoculum	Gross Lesion[§]		Microscopic Lesions	Total Mortality
	Nerve	Visceral		
None	0	0	0	0
rMd5	13/17(76.5)*	6/17(35.3)	6/6(100)	11/17(64.7)
rMd5/ΔvIL-8	0/17(0)	1/17(5.9)	9/17(52.9)	0/17(0)
rMd5/ΔvIL-8-RV	15/17(88.2)	7/17(41.2)	4/4(100)	13/17(76.5)

Tabel 9. **Pathological lesions in MDV maternal antibody positive chickens inoculated with recombinant rMd5, rMd5/ΔvIL-8, and rMd5/ΔvIL-8-RV viruses.**

*Data in parentheses indicate percentage of chickens.

[§]Gross lesions include nerve enlargement and viceral tumors.

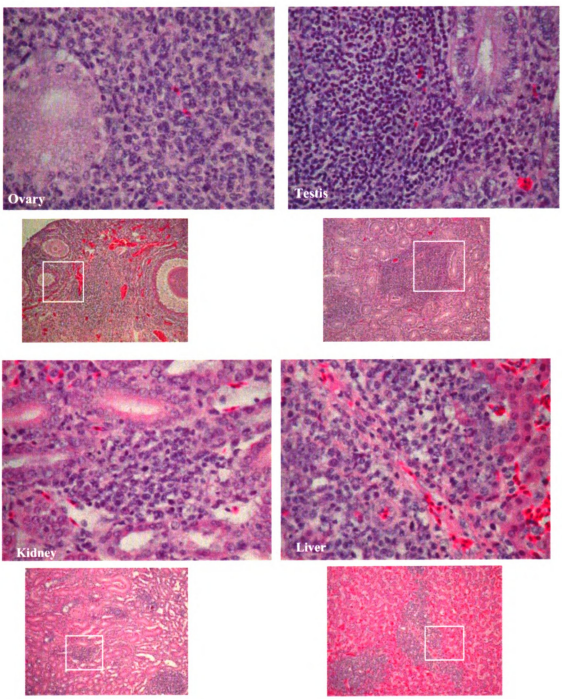
Ovary
Testis
Kidney
Liver

Figure 17. **Histopathological lesions derived from infection with the recombinant viruses.** The deletion virus rMd5/ΔvIL-8 induced a much lower frequency of gross lesions, but histological lesions were present in various organs (Ovary, testis, kidney, liver). A mixed population of infiltrating small, medium, and large lymphoid cells infiltration replaced the normal structure of the tissue.

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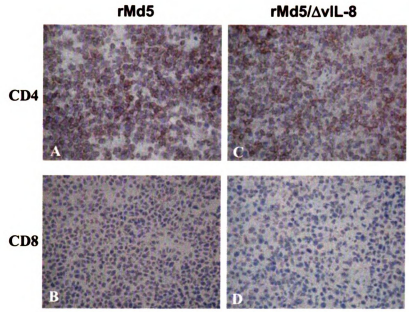

Figure 18. **Immunohistochemical staining of tumor tissues derived from all three recombinant viruses using a Mab against chicken CD4 and CD8.** Cells in the tumor tissues derived from rMd5 (A, B), and rMd5/ΔvIL-8 (C, D) infected chickens express CD4 (A, C) but not CD8 (B, D). The transformed cell types in both viruses are the $CD4^+/CD8^-$ subset of T cells.

This figure is in color.

	pp38		MEQ	
	LO	**FFE**	**LO**	**FFE**
rMd5				
4 DPI	+	-	-	-
6 DPI	++++	+	+++	+
14 DPI	+++	++++	+++	+++
35 DPI	+++	+++	++++	+++
rMd5/ΔvIL-8				
4 DPI	-	-	-	-
6 DPI	-	-	-	-
14 DPI	-	++++	++	++
35 DPI	-	+/-	++	++

Table 10. ***In vivo* expression kinetics of the viral protein pp38 and MEQ.** With parental virus rMd5, the expression of viral protein (pp38) in lymphoid organs (LO) is present at all time points examined (4, 6, 14, and 35 DPI), whereas there was no pp38 expression in rMd5/ΔvIL-8 inoculated chickens. pp38 expression in FFE (feather follicle epithelium) was similar to that observed in rMd5 inoculated chickens. MEQ expression in rMd5/ΔvIL-8 inoculated chickens was delayed in both lymphoid organs and FFE until 14DPI compared to rMd5, and the level of Meq expression was lower in rMd5/ΔvIL-8 inoculated chickens.

Note: the level of viral protein expression was scored as negative (-), low (+), medium (++), high (+++), very high (++++), minimal to none (+/-).

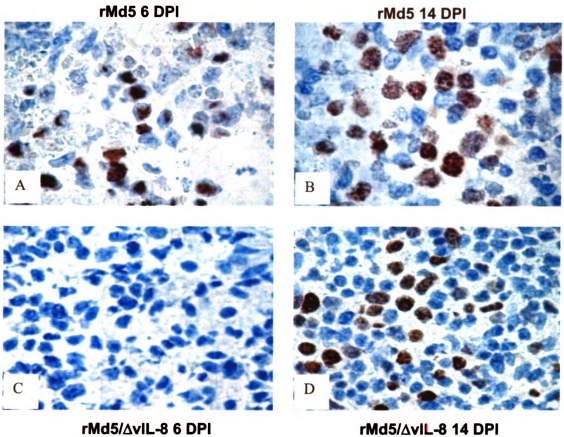

Figure 19. **Immunohistochemical staining of spleens using antibody against MEQ.** In the MDV infected cells, Meq has a nuclear localization. At 6 DPI with parental virus rMd5, during the early cytolytic infection phase, MEQ positive cells appear to have a single lobe staining pattern in the nucleus (A), while at 14 DPI, the Meq positive cells have multi-lobular-like staining pattern in the nucleus (B). No viral expression was detected in the rMd5/ΔvIL-8 virus inoculated chickens at 6 DPI (C), but MEQ was expressed at 14 DPI in rMd5/ΔvIL-8 inoculated chickens (D).

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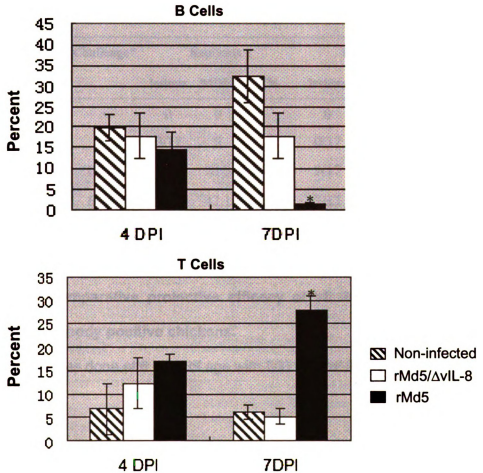

Figure 20. **Flow cytometry analysis of spleen cells from virus infected chickens.** The parental virus, rMd5 (black) destroyed large numbers of B-cells in chickens at both 4 and 7 DPI, and induced a higher level of activated T-cells. The levels of B-cells and activated T-cells in chickens infected with the deletion virus rMd5/ΔvIL-8 (white) have no significant difference to the non-infected control (strip) chickens.

* = $p<0.05$ between indicated group and the others.

Vaccine	Challenge*	Replicate 1			Replicate 2			Summary	
		lesions	MD%	PI†%	lesions	MD%	PI%	MD%	PI%
None	-	0	0		0	0		0	
rMd5/ΔvIL-8	-	0/17	0		0/17	0		0	
rMd5/ΔvIL-8	648A	4/17	23.5	76.5	3/17	17.6	82.4	20.6	79.4
CVI988	648A	8/17	47.1	52.9	7/17	41.2	58.8	44.1	55.9
-	648A	17/17	100		17/17	100			

Table 11. **Comparative protective efficacy of vIL-8 deletion virus in MD maternal antibody positive chickens.**

* Challenge was done at 6 days of age with 500 PFU of 648A.

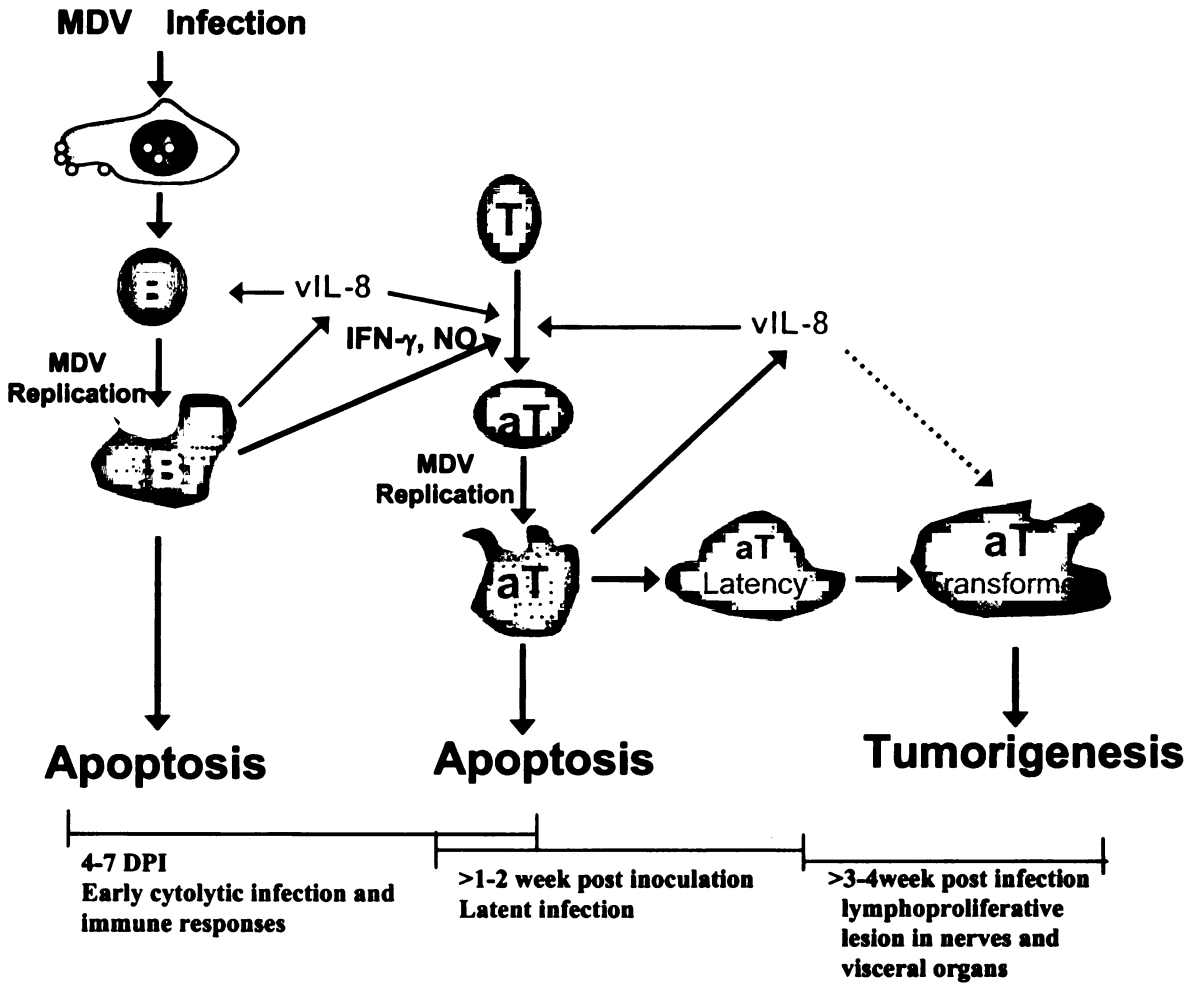
MDV Infection
B
vIL-8
IFN-γ, NO
MDV Replication
B
T
vIL-8
aT
MDV Replication
aT
aT Latency
aT
Apoptosis
Apoptosis
Tumorigenesis
4-7 DPI
Early cytolytic infection and immune responses
>1-2 week post inoculation
Latent infection
>3-4week post infection
lymphoproliferative lesion in nerves and visceral organs

Figure 21. **The possible roles of vIL-8 in the sequential pathogenesis of MDV infection.** MDV infected cells are phagocytized by macrophages followed by infection of B-cells. MDV replicates in the infected B-cells and initiate an early cytolytic infection. Some of these B-cells undergo apoptosis. Infected B-cells may induce an early immune response, release other cytokines and chemicals such as IFN-γ or NO, which activate the T-cells.

Only the activated T-cells (aT) but not the resting T-cells are the target cells for MDV infection. Infected aT-cells express viral antigens, such as vIL-8, which results in attracting more B and aT-cells for virus infection. Some of the aT-cells undergo apoptosis, some aT enter into the latency (aT-Latency) phase or become transformed (aT-Transformed), finally leading to the development of tumors.

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CHAPTER FOUR

DISCUSSION AND FUTURE RESEARCH

DISCUSSION

As a member of the alpha herpesvirus family, MDV has a genomic organization and protein composition homologus to VZV and HSV. The major difference between MDV and other alpha herpesvirus is viral tropism. VZV and HSV are neurotropic, in contrast, MDV is lymphotropic, causing latent infection and transformation of lymphocytes.

Several MDV proteins have been identified based on sequence homology to HSV proteins, although very few have been functionally characterized. The most intriguing MDV proteins are those encoded in the invert repeat regions, unique to MDV serotype 1 strains. The vIL-8 gene described in this dissertation is one of the MDV-1 unique genes. Virus encoded chemokines or chemokine receptors have been reported in different viruses, especially in herpesviruses (Seet et al. 2002) and poxviruses (Haig 2001). Some of them have characterized functions. It is likely that during the long course of evolution, viruses have been successful in taking advantage of their hosts' genetic information and reprogramming these chemokines or chemokine receptors as viral allies to aid in viral immune evasion.

To study the function of vIL-8 gene, both copies of vIL-8 residing in the TR_L and IR_L region of the viral genome was deleted and generated the vIL-8 deleted virus, rMd5/ΔvIL-8. Growth kinetics study showed that the vIL-8 gene is

dispensable for virus replication in cell culture. *In vivo*, rMd5/ΔvIL-8 inoculated chickens have much less viral antigen expression in lymphoid organs at 6 DPI, but normal level of viral antigen expression in the feather follicle epithelium at 14 DPI, indicating that vIL-8 gene is involved in early cytolytic infection in lymphoid organs but not in lytic infection in the feather follicle epithelium. The rMd5/ΔvIL-8 and the wild type virus also showed similar viremia titers at 6 and 8 DPI, a period where the virus titer comes primarily from reactivated latent genomes, demonstrating that vIL-8 gene does not appear to be important for virus reactivation or latency entry. Nevertheless, because of the impaired cytolytic infections, the overall transformation efficiency of the vIL-8 deleted virus is much lower, as reflected by the reduced number of transformed cells at 5 weeks post inoculation and fewer gross tumors. The virulence of rMd5/ΔvIL-8 was also dramatically reduced in both MDV maternal antibody negative and positive chickens. It has been documented that a reduction or absence of an early cytolytic infection with MDV correlates with the absence or reduction in the incidence of lymohomas. Impairment of the early cytolytic infection caused by deletion of the vIL-8 gene may also have an essential role in reducing the virulence of rMd5/ΔvIL-8. Another interesting finding was that the deletion virus protected chickens against challenge with a very virulent plus MDV strain. Although it is well known that MD can be efficiently prevented and controlled by vaccination, little is known about the mechanisms of how vaccines protect chickens. Importantly, the revertant virus that restored the expression of vIL-8

gene also restored the wild type phenotype, indicating the deficient phenotypes are the result of vIL-8 deletion.

Since vIL-8 is highly homologous to the members of the chemokine family, it is speculated that the function of vIL-8 is that of a chemoattractant. It was first hypothesized that vIL-8 may function in MDV to attract target cells for virus infection and replication. Preliminary *in vitro* migration assays carried out in Dr.Kung's laboratory identified that vIL-8 attracts cells different than their cellular homologues. They found that unlike the cellular IL-8, vIL-8 does not attract neutrophils, but attracts mononuclear cells instead. The lymphocyte population change following early cytolytic infection with rMd5 and rMd5/ΔvIL-8 was also studied using the flow cytometry assay. Because rMd5 virus is able to cause a robust early cytolytic infection, the numbers of B lymphocytes in the rMd5 inoculated chickens were dramatically reduced, while the numbers of activated T-cells were increased at 7 DPI. The loss of B-cells may due to cytosis or apoptosis resulting from the effective cytolytic infection of the rMd5. The early cytolytic infection stimulates the immune response and activates T-cells to become the next major target cells. With the deletion of vIL-8, the early cytolytic infection was impaired. Therefore, the number of B-cells in infected chickens was not significantly different from the non-infected chickens. It is speculated that the vIL-8 gene may play a necessary role in attracting B-cells, which are the primary target cells for virus infection. This could also account for the weak inflammatory/immune responses, resulting in less activation of T-cells and significant decreased number of transformed cells, and low virulence.

The vIL-8 gene was transcribed in all tested serotype 1 MDV genomes by Northern blot probing. The signal peptide on the N-terminal of the gene was also functional. Sequence and structural analysis indicates that, there are two notable differences among this vIL-8 gene and other cellular IL-8 gene. First, vIL-8 gene carries a "DKR" motif but not an "ELR" motif which is strictly reserved in most of the cellular IL-8 genes. As was mentioned earlier, vIL-8 and other cellular IL-8 attract different types of cells. It is speculated that this motif variation could be one of the factors to explain the difference in attraction of different cell types. The "ELR" motif is also interesting because it is related to angiogenesis which is extremely important to tumor formation, while a chemokine protein without "ELR" motif normally will have the opposite, angiostatic reaction, which does not favor tumor growth. The other difference is that vIL-8 has a much longer C-terminal sequence. The siginificance of this is yet to be identified, but may be involved in cell signaling unique to vIL-8.

To study the significance of this variation, recombinant MDV, rMd5/vIL-8-ELR, was generated carrying "ELR" motif. Both *in vitro* and *in vivo* studies revealed that the "DKR" motif is as competent as "ELR" in sustaining the vIL-8 functions. Preliminary studies using the CAM assay demonstrated that the mutant vIL-8-ELR protein induced clear neovascularization in the chicken chorioallantonic membrane. But, the mutant virus rMd5/vIL-8-ELR did not show any difference in *in vitro* replication and *in vivo* pathogenesis compared to the parental virus, which suggests that, an "ELR" motif in the virus does not facilitate more aggressive tumor growth. With these surprising results, sereval hypotheses

have been made here. Although vIL-8 is highly homologous to the cellular IL-8, the role of of extra C-terminal domain in vIL-8 is not known. This unique feature could be responsible for signaling events essential for establishment of a robust infection during the early cytolytic infection. The other hypothesis is that vIL-8 does not have a role in inducing angiogenesis, but only functions in attracting target cells. With stimulation by infection with MDV, the release of cellular chemokines or cytokines (Xing et al. 2000) may serve as the factors that contribute to the angiogenic reaction. Since few studies have been done on the chicken cellular chemokines and its receptors, the signaling pathway in this area is unclear. Further investigations are necessary.

FUTURE RESEARCH

1. Construct and characterize mutant MDV-2 and MDV-3 viruses containing the vIL-8 gene.

Based on the data reported in this dissertation, deletion of the vIL-8 gene results in impaired cytolytic infection and virus attenuation. This result indicates that vIL-8 is necessary for the pathogenecity of the virus. It is suggested that vIL-8 plays a role in attracting target cells for virus infection and replication. It is also known that the vIL-8 gene is only encoded in MDV-1 viruses, which replicate at a much higher level *in vivo* than serotype 2 and 3 viruses. Future research that could be carried out is to insert the vIL-8 gene into MDV-2 and MDV-3. Two goals could be tested after insertion of the vIL-8 gene into MDV-2 and MDV-3: 1) Evaluation of the *in vivo* and *in vitro* replication level of the mutant MDV-2 and MDV-3; and 2) Determine the virulence of the mutant MDV-2 and MDV-3 carrying vIL-8 gene. This would help determine whether vIL-8 is a necessary gene for MDV replication *in vivo* and whether or not it is essential to the virulence of the virus. If the presence of the vIL-8 gene increases virus replication in chickens, MDV-2 and MDV-3 recombinants may offer better protection. Comparisons among the mutant MDV-2 and MDV-3 viruses coding the vIL-8 gene with the parental viruses and mutant MDV-1 virus with no vIL-8 may also assist to further understand the role of vIL-8 in MDV pathogenesis.

2. Characterize the relation of vIL-8 and cIL-8 during infection *in vivo*.

With the work described herein, it is possible to elucidate the agonist or antagonist role of vIL-8 gene to the cellular IL-8 (cIL-8) gene. First, by using the vIL-8 deletion virus, the transcription levels of both vIL-8 and cIL-8 could be studied by RT-PCR from the infected chicken tissues. Since replication starts in lymphoid organs, an analysis to monitor the transcript levels at different times after inoculation could be performed. If vIL-8 plays an agonist role, a high level of both vIL-8 and cIL-8 transcripts in parental virus would be expected. The cIL-8 should have a similar level in both parental and the vIL-8 deletion viruses. If cIL-8 transcription level is higher in the vIL-8 deletion virus than in the parental virus, this may mean that vIL-8 suppresses the function of cIL-8 in parental virus. To study the important function cIL-8 may play in MDV infection, another mutant virus with the insertion of the cIL-8 in place of vIL-8 could be generated. A comparison of the sequence and structural differences between the cIL-8 and vIL-8 could be done to explain the difference in attracting different target cells, or other biological properties. This information would also help in answering several questions regarding the MDV encoded vIL-8 gene. Why does MDV-1 encode this chemokine-like gene? Is a chemokine gene necessary for the virulence and pathogenecity of MDV? Can other homologous chemokine genes replace its function in MDV infection and replication? Will the replacement of cIL-8 change the cell tropism in MDV infection? Will this change be related to MDV induced transformation or tumorigenesis? With these questions answered, a greater elucidation of the pathogenic mechanisms of MDV will be obtained.

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