NOTE TO USERS

Innate resistance to cytomegalovirus infection in wild-derived mice: role of natural killer cell receptors

by

Sonia Girard Adam

Department of Human Genetics

McGill University, Montreal

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TABLE OF CONTENTS Page

ABSTRACT

Human cytomegalovirus (HCMV) infection can cause life-threatening disease in immunodeficient hosts. Experimental infection in mice has revealed that natural resistance to murine cytomegalovirus (MCMV) is genetically determined and mediated by binding of the natural killer (NK) cell activating receptor Ly49H to the pathogen-encoded glycoprotein m157. The study of genetically diverse wild-derived strains revealed natural resistance in the PWK/Pas mouse. However, intracellular staining of NK cells indicated that PWK/Pas mice do not express Ly49H. Moreover, PWK/Pas were also found to be resistant to mutant virus lacking m157. Analysis of backcrosses to a susceptible strain demonstrated that MCMV-resistance is controlled by a major dominant gene effect linked to the *Ly49* gene cluster and a modest influence of the major histocompatibility complex (*H2*). *Ly49* cDNAs encoding four novel activating receptors were identified as candidate genes for the resistance phenotype. Our results provide genetic evidence for the existence of alternative NK cell receptors that mediate resistance to viral infection in mice by mechanisms other than recognition of m157. Functionally homologous innate resistance genes may be found in human populations.

RÉSUMÉ

Le cytomégalovirus humain est responsable de maladies sévères et mortelles chez les patients immunodéficitaires. L'étude des déterminants innés de la susceptibilité ou la résistance au CMV a été facilitée par l'analyse de modèles expérimentaux d'infection dans les lignées de souris pures. Chez la lignée C57BL/6, la charge virale durant la phase précoce de l'infection est contrôlée, par l'activation des cellules NK suite à l'interaction entre le récepteur Ly49H et la protéine virale, m157. La lignée de souris PWK/Pas est de descendance sauvage et est aussi résistante au cytomégalovirus. Néanmoins, les cellules NK de PWK/Pas n'expriment pas Ly49H. De plus, la souris PWK/Pas est résistante à l'infection avec un virus mutant dépourvu du gène *m157*. Des études de liaison dans une population des souris issue du croisement entre PWK/Pas et une souche de souris sensible à l'infection ont démontré que le phénotype est contrôlé par un locus majeur et dominant, lié à la famille des gènes *Ly49*. De plus, une influence mineure du complexe majeur d'histocompatibilité (CMH) a été observée. Par clonage d'ADN complémentaire, 4 nouveaux membres activateurs de la famille *Ly49* ont été identifiés comme candidats possibles du gène de résistance. Nos résultats procurent des évidences génétiques suggérant la présence de récepteurs activateurs responsables du contrôle de l'infection chez les souris PWK/Pas, par un mécanisme différent de la reconnaissance de m157. Des gènes de résistances comportant une homologie fonctionnelle, pourraient être retrouvés chez les humains.

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LIST OF FIGURES **Page**

LIST OF TABLES **Page**

LIST OF ABBREVIATIONS

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
bp	Base Pairs
cDNA	Complementary Deoxiribonucleic Acid
CEN	Centromere
CHO	Chinese Hamster Ovary
cM	Centimorgans
CMV	Cytomegalovirus
DAP	DNAX Activation Protein
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
Fix/Perm	Fixation and Permeabilization
GFP	Green Fluorescent Protein
HAAT	Highly Active Antiretroviral Combination Therapy
H2	Histocompatibility-2
HCMV	Human Cytomegalovirus
HHV	Human Herpesvirus
HLA	Human Leucocyte Antigen
HSV	Herpes Simplex Virus
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
Kb	Kilobases
KIR	Killer cell Immunoglobulin-like Receptor
mAb	Monoclonal Antibody

Mb	Megabases
MCMV	Murine Cytomegalovirus
MHC	Major Histocompatibility Complex
Myr	Million year
NFAT	Nuclear Factor of Activated T cells
NK	Natural Killer
NKC	Natural Killer Gene Complex
NKD	Natural Killer Receptor Domain
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFU	Plaque Forming Unit
QTL	Quantitative Trait Loci
RAE-1	Retinoic Acid Early Transcript 1
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TBE	Tris-Borate EDTA Buffer
TEL	Telomer

CONTRIBUTION OF AUTHORS

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1. INTRODUCTION

1.1 Pathogenesis of cytomegalovirus

1.1.1 Infection in humans

Human cytomegalovirus (HCMV) infects more than 70% of the world population (Rawlinson, 1999). Person to person contacts appears to be the mode of transmission of HCMV. The virus can be found in urine, cervical and vaginal excretions, semen, breast milk, tears, feces, and blood (Reynolds, Stagno, Hosty, Tiller, & Alford, Jr., 1973; Lang & Kummer, 1975; Stagno, Reynolds, Pass, & Alford, 1980). Following infection, the virus establishes long term latency and intermittent reactivation, depending on the status of the host immune system (Soderberg-Naucler & Nelson, 1999). Primary infections are usually asymptomatic in most adults or children. Some symptomatic individuals experience a mononucleosis-like syndrome, associated with fever, sore throat, swollen glands and fatigue, with no long-term health consequences (Klemola & Kaariainen, 1965; Horwitz, Henle, & Henle, 1979). However, serious consequences and even death may occur in high risk groups, such as neonates and immunocompromised patients.

Primary infections with HCMV in pregnant women may cause severe complications to the fetus. HCMV is the most important cause of congenital viral infection in industrialized countries, with an incidence between 0.15% to 2.0% (Friedman S & Ford-Jones EL, 1999; Gaytant, Steegers, Semmekrot, Merkus, & Galama, 2002). Approximately 10% of these children will present birth defects such as hearing loss, mental retardation and neuromuscular defects, resulting from intrauterine infection (Demmler, 1991; Trincado et al., 2000). Individuals with compromised immune system, including organ transplant recipients, patients with cancer, patients receiving immunosuppressive drugs, and HIV-positive patients are at risk of reactivation of the

dormant virus. Interstitial pneumonia is complication in more than 50% of bone marrow recipients (de Medeiros, Moreira, & Pasquini, 2000), while over 20% of solid organ transplant recipient face organ-specific diseases (Stratta, 1993; Tolkoff-Rubin & Rubin, 1998). Patients with acquired immunodeficiency syndrome (AIDS) often develop HCMV infection causing clinical manifestations such as pneumonitis, retinitis, colitis and encephalitis, which are associated with decreased survival after diagnosis of HIV infection (Wallace & Hannah, 1987; Morgello, Cho, Nielsen, Devinsky, & Petito, 1987; Welch et al., 1998). However, the introduction of highly active antiretroviral therapy (HAART) dramatically reduced the incidence of HCMV-induced diseases in AIDS patients (Drew, 2003). For example, the use of HAART is associated with an 81% lower mortality rate in AIDS patients with cytomegalovirus retinitis (Kempen et al., 2003). However, HCMV-induced diseases remain a problem for patients resistant or intolerant to HAART. Furthermore, the impact of HCMV in the general population might have been underrated. Experimental evidence suggests that HCMV infection and vascular disease might be linked (Demmler, 1991; Trincado et al., 2000). The viral infection is associated with the development of atherosclerotic plaques in the vasculature of the heart (Speir et al., 1994).

1.1.2 Treatment of cytomegalovirus infection

Given the prevalence of CMV-induced pathology in neonates, AIDS patients, and transplant recipients, a vaccine against HCMV is greatly needed. Unfortunately, there is no licensed vaccine available. Despite the development of antiviral drugs, HCMV infections are very difficult to manage (Villarreal, 2003). Presently, treatments are based on nucleoside analogue inhibitors that target the viral DNA polymerase. Acyclovir and

penciclovir antiviral therapies are considered relatively safe and efficacious. However, other drugs such as ganciclovir and foscarnet are associated with many side effects (Zaia et al., 2000). Current therapies attenuate symptoms but do not completely clear the infection. Furthermore, a substantial number of drug-resistant HCMV strains have emerged in marrow, lung, renal and liver transplant recipients (Knox, Drobyski, & Carrigan, 1991; Lurain et al., 1996; Rosen et al., 1997; Bienvenu et al., 2000) as well as other immunocompromised patient populations (Sasaki et al., 1997; Wolf et al., 1998). Treatments to prevent CMV infection following transplantation are associated with additional healthcare cost. A report assessing the economic impact of cytomegalovirus infection after liver transplantation revealed that liver transplant recipients who developed CMV disease had significantly higher medical costs than those who developed asymptomatic CMV infection or experienced no CMV infection (Falagas et al., 1997). CMV disease is associated with a 49% increase in costs in this patient population (Kim et al., 2000). Furthermore, the cost of treating neonatal CMV complications represents over two billion US dollars annually in the USA alone (Daniel, Gull, Peyser, & Lessing, 1995).

The absence of efficient therapeutic strategies and the severity of diseases associated with HCMV infection, motivated researchers to unravel the complex interplay of viral and host functions leading to pathogenesis. A better understanding of the early host response against HCMV infection can provide a better rationale to the identification of novel therapeutic targets.

1.2 Experimental mouse models

1.2.1 Mouse model of CMV infection

The complex host/virus/environment interactions can be best studied in a controlled fashion in mouse experimental models. The mouse has become the prominent model for studying host susceptibility to infectious disease because of the underlying biological similarity between mouse and human, the extensive comparative genetic linkage map and the ability to manipulate the mouse genome. Stringent species specificity prevents the direct study of HCMV in the mouse. However, infection with murine cytomegalovirus (MCMV) presents an excellent surrogate model of human infection. For example, many clinical manifestation of HCMV disease, such as retinitis, pneumonitis and hepatitis, have been observed in mice (Shanley, 1984; Hayashi, Kurihara, & Uchida, 1985; Trgovcich et al., 2000; Krmpotic, Bubic, Polic, Lucin, & Jonjic, 2003). Furthermore, MCMV infection also mimics congenital viral infections, as well as fatal diseases (Shellam & Flexman, 1986; Shanley, Biczak, & Forman, 1993). More importantly, different strains of inbred mice exhibit striking differences in their level of susceptibility to MCMV infection as measured by organ-specific viral replication in the first days after infection, disease severity and survival (Allan & Shellam, 1984; Shanley, 1984). Such differences provide an excellent tool for mapping susceptibility/resistance genes implicated in the host response against MCMV. For this, crosses between resistant and susceptible strains are performed to determine the mode of inheritance and subsequently, linkage analysis is used to correlate the inheritance of susceptibility or resistance with one or more chromosomal regions. Ultimately, comparative mapping can be used to identify human orthologous genes. In recent years, several of the associated genes controlling resistance to viral infection have been isolated and characterized

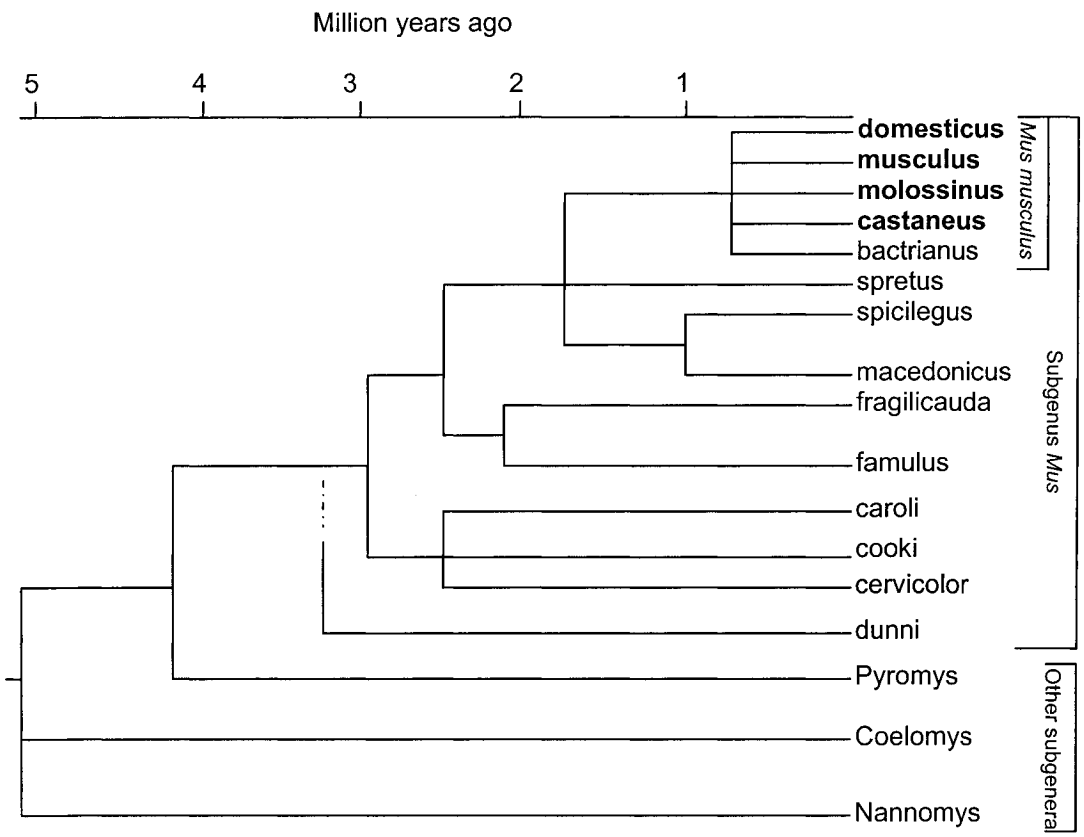
concurrent with the development of genomic technologies spurred by the initiative of the Human Genome Project (Lee et al., 2003).

1.2.2 Classical and wild-derived inbred mice

Modern mouse genetics was initiated by William Castle and Abbie Lathrop, nearly 100 years ago (Silver LM, 1995). The mice used and generated by Lathrop and Castle are the ancestors of many of the classical inbred strains as we know them today. In fact, the *Mus musculus* group of mice can be divided into five subspecies, *domesticus*, *musculus*, *molossinus*, *castaneus* and *bactrianus*, which originated from a common ancestor about 1 Myr ago (Guenet & Bonhomme, 2003) (Figure 1). These subspecies display numerous morphological and molecular differences. However, progenitors of classical inbred strains, were a small hybrid population derived from *M.m. musculus*, *M.m. domesticus*, *M.m. molossinus* and *M.m. castaneus* (Yonekawa et al., 1981; Beck et al., 2000). As a result, classical inbred mouse strains present limited genetic polymorphism. Despite many advantages such as easy breeding, short-generation time (10-12 weeks), tolerated inbreeding, genetically well characterized strains and manipulation of the genome, the absence of genetic variability is a major drawback of classical inbred strains when compared to human populations. To overcome this disadvantage, mouse geneticists decided to take advantage of the diversity existing among wild specimens of the *Mus genus*. New inbred strains of mice, from different *Mus musculus* subspecies, including *domesticus* (WMP/Pas,WLA/Pas), *musculus* (MBT/Pas,MAI/Pas,PWK/Pas) and *casteneus* (CAST/Ei), were derived from progenitors caught in different geographical locations by pioneer investigators (Guenet et al., 2003). The derivation of new inbred strains from other species of the *Mus genus*, such as *Mus*

Figure 1: Phylogenetic tree of the genus *Mus*.

The genus *Mus* emerged from the split of different subgenera, *Nannomys*, *Coelomys* and *Pyromys*, about 5 Myr ago. These subgenera present different karyotype than laboratory mouse and hybrids between these subgenera have never been reported. The next split, around 3 Myr ago, of three related Asian species, *Mus cervicolor*, *Mus cookie* and *Mus caroli* on one side, and a branch leading to the house mouse (*Mus musculus*) and five other congeneric species, *famulus*, *fragilicauda*, *macedonicus*, *spicilegus* and *spretus* on the other side. 2 Myr ago, two related species, *Mus famulus* from India and *Mus fragilicauda* from Thailand, separated. The next step, at 1.5 Myr ago, groups three morphologically very similar Palearctic species with short tails: *Mus spretus*, from the western Mediterranean, *Mus spicilegus* found in central Europe and *Mus macedonicus* from the eastern Mediterranean. The last split, which occurred less than 1Myr ago represents the *Mus musculus* complex species. The species at the origin of the modern laboratory strains are in bold.



Guénet et Bonhomme 2003
TRENDS in genetics

spretus (SEG/Pas, STF/Pas), the most distantly related species of the *genus Mus* was also undertaken. Wild-derived mice are now kept as inbred lines and can be crossed with laboratory strains to produce offspring that carry polymorphisms not available in classical inbred strains (Bonhomme et al., 1984). Wild-derived strains provide new experimental models for the study of human diseases, including infectious diseases, as recently shown by the identification of the Flv1/OAS1 gene, associated with susceptibility to West Nile virus (Guenet & Despres, 2003).

1.3 Immune response to cytomegalovirus infection

In vertebrates, host response to infection is mediated by two interrelated systems: innate immunity and the adaptive immune response. These two systems serve distinct and complementary roles in the protection of the host against infection. Although innate immunity is usually considered to comprise the 'early' response to pathogens, innate immunity alone is often capable of eradicating infection of the host, or, in many instances, may reduce significantly the pathogen load, allowing for a slower spread of the infection, reduced pathology and a more effective clearing of the infection by the induced response.

1.3.1 Innate immunity

Innate immunity is the first arm of defense against infection, providing a rapid (hours to days) and, most of the time, efficacious mechanism to eliminate pathogens. In particular, Natural Killer (NK) cells represent a component of the innate system that is particularly important during virus infection, including human and mouse cytomegalovirus (Orange, 2002; Webb, Lee, & Vidal, 2002). During infection, NK cells

are triggered by other innate immune mechanisms, including cytokines such as IFN-α, IFN-β as well as IL-12 and TNF-α produced by infected cells (Biron, Nguyen, Pien, Cousens, & Salazar-Mather, 1999).

1.3.2 Role of NK cells in cytomegalovirus infection

NK cells are large granular lymphocytes, distinct from B and T cells, capable of killing abnormal targets prior sensitization (Trinchieri, 1989). This population of cells was initially characterized on its ability to recognize and kill tumor cells (Takasugi, Mickey, & Terasaki, 1973; Jondal & Pross, 1975). However, over the years, NK cell were shown to respond to a variety of microorganisms, including bacteria and protozoa (French & Yokoyama, 2003). NK cells are mostly important in viral infections, particularly those of the herpesviruses. This role was demonstrated in a young patient who completely lacked NK cells and IL-2-inducible NK cell cytotoxic function. This patient, presented extreme sensitivity to Herpes Simplex I, Varicella-Zoster virus and HCMV infections, despite normal antibody and T cell function (Biron, Byron, & Sullivan, 1989).

In the early 80s, Shellam and colleagues demonstrated the crucial role of NK cells in controlling primary MCMV infection and reactivation in the mouse model (Shellam, Allan, Papadimitriou, & Bancroft, 1981). They showed that beige mice, which have defects in natural killing, have an enhanced susceptibility to MCMV. In addition, Welsh and coworkers performed adoptive transfer of mature NK cells into newborn and NK cell-deficient mice which are highly susceptible to MCMV infection. Interestingly, mice which received mature NK cells were protected against the virus (Bukowski, Warner, Dennert, & Welsh, 1985; Welsh, Brubaker, Vargas-Cortes, & O'Donnell, 1991).

Furthermore, *in vivo* depletion of NK cells with PK136 monoclonal antibody in the naturally resistant C57BL/6 mouse strain, lead to a 1000-fold increase in viral titers in these mice, once again revealing the importance of NK cells in early infection with MCMV (Scalzo et al., 1992).

The main role of NK cells is to offer immunity against pathogens until the adaptive immune system takes over. NK cell activation may be specifically directed by recognition of virally-infected cells via activating receptors or by innate cytokines in a non-specific manner (Biron et al., 1999; Yokoyama, Kim, & French, 2004).

NK cells express a variety of inhibitory and activating receptors that can respectively deliver negative or positive stimulatory signals (Yokoyama et al., 2004). These receptors are specific for MHC class I or MHC class I like molecules which are encoded by neighboring host cells or by pathogens (Yokoyama et al., 2004). In fact, at the early stage of MCMV infection (4-6 days post infection) NK cells are believed to be stimulated by virally-infected cells via activating receptors (Dorner et al., 2004). Stimulation following recognition of virus-infected cells, delivers positive signals required for direct NK cell-mediates lysis of infected target (Lanier, Corliss, Wu, Leong, & Phillips, 1998). Negative regulation of activated NK cell cytotoxicity is important to prevent damage to normal cells (French & Yokoyama, 2004). Inhibitory receptors, normally restrains NK cell activation by recognition of target-cell expressed MHC class I molecules. In fact, Raulet and colleagues proposed that NK cells express at least one inhibitory receptor to provide NK cell tolerance and to prevent inappropriate NK cell responses directed at normal host tissues (Raulet, Vance, & McMahon, 2001).

Soluble cytokines such as IFN-α/β, γ and IL-12 are important for controlling viral replication (Biron et al., 1999). IFN-α/β secreted by virus-infected cells promotes a

"non-specific" activation of NK cells at an early stage after MCMV infection (1-2 days post infection), triggering NK cell response and cytotoxic functions characterized by IFN-γ production and NK cell proliferation (Dorner et al., 2004). The role of IFN-α/β as potent inducers of NK cell cytotoxic function against MCMV was demonstrated in mice. Mice deficient of IFN-α/β (-/-) are characterized with poor response against MCMV infection (Orange & Biron, 1996) and die shortly after infection (Salazar-Mather, Lewis, & Biron, 2002). In addition, IFN-α/β affect NK cell trafficking. In MCMV infection NK cell migrate to the site of infection in response to IFN-α/β (Salazar-Mather, Ishikawa, & Biron, 1996). Furthermore, virus-induced IL-12 which promotes NK cells IFN-γ release is important for protection against MCMV. Depletion of IFN-γ and IL-12 with monoclonal antibodies in mice is characterized by an increase viral replication in the liver, suggesting that the control of MCMV infection in that organ is dependent upon IFN-γ production (Orange et al., 1996; Tay & Welsh, 1997). IFN-γ is believed to exert its antiviral effects through different number of functional effects on macrophages: induction of MHC class I expression, increased Ag presentation, production of nitrogen intermediates and release of IL-12 (Boehm, Klamp, Groot, & Howard, 1997). In contrast to the liver, the control of MCMV replication in the spleen is predominantly governed by a perforin-dependent, IFN-γ-independent mechanism (Tay et al., 1997). In addition to the production of antiviral cytokines, NK cells can produce certain chemokines important for pro-inflammatory functions (Biron et al., 1999). For example, macrophage-inflammatory 1 α (MIP1-α) is important for promoting inflammatory responses in liver by recruiting NK cells, following MCMV infection (Salazar-Mather, Orange, & Biron, 1998). The production of cytokines and chemokines may influence the initiation and development of acquired immunity which ensure control of viral infection at a later stage (Kos, 1998).

1.3.3 Adaptive immunity

Adaptive immunity is mediated by a selected repertoire of B and T antigen-specific lymphocytes. In contrast to innate immunity, the acquired immune system is characterized by a slow, but yet highly specific response to pathogens, which involves immunological memory (Janeway CA Jr, 2001b). Whereas humoral immunity mediated by B cells is not crucial for the control of CMV infection, T cells are key players for sustained immune surveillance (Welsh et al., 1991) (French et al., 2004).

1.3.4 Role of T cells in cytomegalovirus infection

T cells are a subset of lymphocytes defined by their development in the thymus and their recognition of specific antigen presented at the surface of cells by the major histocompatibility complex (MHC) molecules (Janeway CA Jr, 2001a). CD4+T and CD8+T cells are associated with distinct functions. CD4+T cells or helper T cells recognize peptides presented by MHC class II molecules. MHC class II molecules are loaded with peptides generated within intracellular vesicles (Janeway CA Jr, 2001a). Activation of helper T cells leads to the activation of other cell types. In contrast, CD8+T cells or cytotoxic cells recognize peptides derived from pathogens residing in the cytosol and presented in the context of MHC class I molecules (Janeway CA Jr, 2001a). Activation of CD8+T cells mediates direct destruction of infected target cells.

T cells play an important role against several viral infections, including influenza virus (Yap, Ada, & McKenzie, 1978), choriomeningitis virus (Byrne & Oldstone, 1984), herpes virus simplex (Larsen, Feng, Horohov, Moore, & Rouse, 1984) and cytomegalovirus (Starr & Allison, 1977; Ho, 1980). CD8+T cells are the principal effector T cell in the control of mouse and human cytomegalovirus infections. The

protective role of CD8+T cells was best characterized in the mouse model by Reddehase and colleagues. Adoptive transfer of CD8+T-cells into immunocompromised mice confers protection against lethal MCMV (Reddehase, Jonjic, Weiland, Mutter, & Koszinowski, 1988). In addition, depletion of CD8+T cells from the transferred lymphocyte population is characterized by a reduced antiviral activity (Reddehase et al., 1985). These observations suggest that CD8+T cells are essential for MCMV viral clearance.

Several studies in mice revealed that CD4+T cells are not essential for initiating a CD8+T mediated immune response against several viral infections such as LCMV, HSV and MCMV (Reddehase, Mutter, Munch, Buhring, & Koszinowski, 1987; Nash et al., 1987; Ahmed, Butler, & Bhatti, 1988). Jonjic and colleagues showed that mice, long-term depleted of CD4+T cells, generated protective CD8+T effector cells and restricted persistent virus multiplication, suggesting that CD4+T cells are dispensable for the control of MCMV replication. However, clearance from particular organs, for example salivary glands, depends on the presence of CD4+T cells rather than on CD8+T cells (Jonjic, Mutter, Weiland, Reddehase, & Koszinowski, 1989). Similarly in humans, a study to determine the kinetics and properties of CMV-specific CD4+T cells in healthy individuals and renal transplant patients revealed a CMV-specific CD4+T cell response preceding a CMV-specific CD8+T cell response in asymptomatic individuals. In contrast, the CD4+T cell response was delayed in symptomatic individuals. These observations suggest that functional CD8+T cells alone are insufficient to control HCMV replication and that formation of effector memory CD4+ T cells are mandatory for recovery of infection in humans (Gamadia, Rentenaar, van Lier, & ten, I, 2004).

1.4 Cytomegalovirus genes of immune evasion

Cytomegaloviruses are members of the *Herpesviridae* family. They are enveloped viruses with a nucleocapsid of icosahedral symmetry containing a double-stranded DNA genome of about 200 kb (Rawlinson, Farrell, & Barrell, 1996). Annotation of full-length genomic sequences of human AD169 strain (HCMV) and murine Smith strain (MCMV) indicated the presence of 165 and 170 open reading frames (ORFs), respectively (Chee et al., 1990) (Rawlinson et al., 1996). Many of these ORFs, are predicted to encode functions important in DNA replication, metabolism function and in virion maturation or structure. In addition, over 50% of the proteins encoded by CMVs genomes are predicted to participate in host-pathogen interactions, favoring viral replication (Mocarski ES, 1996). These predictions are not surprising since CMVs, capable of latency and reactivation must be able to avoid the host's immune response to establish life-long infection. Among the strategies employed by CMVs to escape the innate and adaptive immune systems, mimicry and downregulation of MHC class I molecules are best characterized.

1.4.1 Evasion of CD8+T cells

Multiple genes from cytomegaloviruses viral genomes interfere with the MHC class I pathway of antigen presentation. MCMV and HCMV encode viral polypeptides, collectively known as immunoevasins, which provide independent mechanisms to interfere with MHC class I antigen presentation, and therefore prevent efficient recognition of the infected cells by CD8+T cells. Table 1 is a summary of the immunoevasin gene products encoded by both MCMV and HCMV.

Table 1: MHC class I targeting immunoevasins of cytomegaloviruses

ORF	protein	Proposed mode of action	References
MCMV			
m04	gp34	Binds and escorts MHC class I molecules to the cell surface	(Kleijnen et al., 1997; Kavanagh, Koszinowski, & Hill, 2001)
m06	gp48	Reroutes MHC class I complexes for lysosomal degradation	(Reusch et al., 1999)
m152	gp40	Triggers retention of MHC class I complexes in the ERGIC	(del et al., 1992; Ziegler et al., 1997)
HCMV			
US2	gp24	Reroutes MHC class I α -chains for proteasomal degradation	(Wiertz et al., 1996b; Gewurz et al., 2001)
US3	gp23	Retains MHC class I complexes in the ER	(Jones et al., 1996; Ahn et al., 1996)
US6	gp21	Blocks TAP-mediated translocation of peptides into the ER	(Ahn et al., 1997; Hengel et al., 1997; Lehner, Karttunen, Wilkinson, & Cresswell, 1997)
US11	gp33	Reroutes MHC class I α - chains for proteasomal degradation	(Jones et al., 1995; Wiertz et al., 1996a; van der Wal, Kikkert, & Wiertz, 2002)

The mouse CMV genome, express three major proteins, m04/gp34, m06/gp48 and m152/gp40, which downmodulate cell surface expression of MHC class I proteins via distinct post-translational mechanisms. In fact previous studies involving triple mutants; m04, m06 and m152 completely fail to downmodulate MHC class I molecules, suggesting the importance of these genes for MHC class I modulation in escape from immune clearance (Wagner, Gutermann, Podlech, Reddehase, & Koszinowski, 2002). However, a recent study claims that the interference of murine cytomegalovirus gene products, m04/gp34, m06/gp48 and m152/gp40 with antigen presentation has little effect on the size or the effector memory phenotype of the CD8+T cell response. In fact, infection of C57BL/6 mice with wild-type MCMV and MCMV virus lacking *m04*, *m06* and *m152* revealed very little differences between the two viruses with respect to viral

replication and clearance during the acute and chronic phase of infection (Gold et al., 2004). These results are in opposition with the notion that *m04*, *m06* and *m152* are necessary for CMV persistence in the host (Wagner et al., 2002).

The *m152* gene product gp40 is expressed early after infection. Hengel and colleagues revealed that cells infected by *m152* mutated viruses are associated with an increased susceptibility to CD8+T cells control during infection in mice (Hengel et al., 1999). In relation with this work, Gutermann et al. recently proposed that gp40 retains MHC class I complexes in the ER—Golgi intermediate compartment (ERGIC), therefore preventing peptide presentation by MHC class I molecules at the surface of infected cells. On the other hand, m04/gp34 binds to MHC class I in the ER, and the gp34-MHC class I complex is exposed at the cell surface (Gutermann et al., 2002). In this case, CD8+T cells receptors might not recognize the gp34-MHC class I complex (Kavanagh et al., 2001). Similar to m04/gp34, m06/gp48 protein also forms a complex with the loaded MHC I in the ER. However, m06/gp48 targets the complex to the endosome/lysosome for degradation (Reusch et al., 1999).

The human CMV genome comprises at least four immunoevasin genes: US2, US3, US6, and US11. As opposed to MCMV, HCMV controls the MHC class I pathway of antigen presentation at earlier checkpoints. Early after infection, the 32 kDa US3/gp23 protein retains MHC class I complexes in the ER (Ahn et al., 1997). In contrast, US2 and US11 gene products cause proteosomal degradation of MHC class I by redirecting them from the ER to the cytosol (Wiertz et al., 1996a). Finally, in the late phase of infection, US6 encoded protein gp21 blocks the translocation of peptides into the ER (Lehner et al., 1997).

1.4.2 Evasion of NK cells

Cytomegaloviruses avoid detection and activation of the adaptive immune system by downregulation of MHC class I molecules on infected cells. However, lack of MHC class I should render the infected cell susceptible to NK cell killing (Karre, 2002). NK cells sense aberrant expression of MHC class I through the presence of inhibitory MHC class I receptors. To ensure evasion of NK cells, HCMV and MCMV deploy MHC class I homologues which inhibit NK-cell mediated attack.

Human CMV encodes the human MHC class I heavy chain homolog, UL18 gene product, which is predicted to alter the immune response (Warren, Ducroq, Lehner, & Borysiewicz, 1994). Several experiments demonstrated that glycoprotein UL18 associates with the host β2-microglobulin and is expressed on the cell surface. It is proposed that UL18 gene product, might serve as a "decoy MHC" molecule, capable of presenting endogenous peptides and inhibiting NK lysis by binding to LIR1 receptor present on the NK cell (Stannard & Hardie, 1991; Warren et al., 1994; Fahnestock et al., 1995). However, the precise functional consequence of gpUL18/LIR1 interaction is still unknown (Diefenbach & Raulet, 2003). MCMV also contains a mouse MHC class I homologue, m144, which is however evolutionary distinct from human CMV gpUL18 (Farrell et al., 1997; Beisser et al., 2000). Similar to the immunomodulatory role proposed for UL18 in humans, m144 protein is suggested to inhibit NK activity in infected mice. In fact, infection of susceptible mice with a mutant virus lacking *m144* results in a significant viral titer reduction (Farrell et al., 1997; Kubota, Kubota, Farrell, vis-Poynter, & Takei, 1999).

In addition to mimicry, the interaction of viral proteins with non-classical MHC gene products modifies the NK cells response to virally infected cells. Evidence suggests

that virally encoded UL16 type I membrane glycoprotein interacts with ligands for the activating receptors of the NKG2 family (Cosman et al., 2001). For example, NKG2D activating receptor is activated upon crosslinking of MICB, ULBP1 and ULBP2, which triggers killing of infected cells (Sutherland et al., 2002). Downregulation of these ligands by UL16 gene product is found to block NKG2D NK cell activation (Dunn et al., 2003). Similar to HCMV, MCMV *m152* gene product downregulates retinoic acid early (RAE) proteins (Lodoen et al., 2003) while, m155 downregulates H60 (Lodoen et al., 2004). RAE proteins and H60 are ligands of mouse NKG2D, and thereby inhibit NK cell activation *in vivo*. In fact, BALB/c mice infected with *m152*-deleted mutant virus revealed an improved antiviral NK cell control. Interestingly, m152 counters both innate and adaptive immune control in BALB/c mouse strain (Krmpotic et al., 2002).

1.5 Genetic control of mouse cytomegalovirus infection

As indicated above, while some inbred strains of mice are able to control MCMV infection, other strains allow uncontrolled virus replication in target organs and may even succumb to MCMV challenge (Allan et al., 1984; Shanley, 1984). These observations indicate that the genetic constitution of the host influences susceptibility or resistance to MCMV infection. A large body of data indicate that both, *H2* and *non-H2* (*Cmv1)* loci, play a role for MCMV resistance.

1.5.1 The *H2* locus

The major histocompatibility complex (MHC) encodes highly polymorphic genes implicated in immune function, including the MHC molecules involved in presentation of antigen to T cells. The human MHC, HLA, is located on chromosome 6, whereas the

mouse MHC, also known as *H2* maps on mouse chromosome 17 (Janeway CA Jr, 2001a). The role of *H2* genes in MCMV resistance has been appreciated for more than 20 years. In a seminal study by Chalmer and colleagues (Chalmer, Mackenzie, & Stanley, 1977; Grundy, Mackenzie, & Stanley, 1981) survival to MCMV infection of classical BALB/c ($H2^d$) and their congenic counterparts at *H2*, BALB.B ($H2^b$) and BALB.K ($H2^k$) was used to demonstrate the influence of *H2*. Mice carrying the $H2^k$ alleles were 10 times more resistant than those carrying the $H2^b$ or $H2^d$ alleles (Chalmer et al., 1977). The association of *H2* with MCMV-resistance was confirmed using mice of B10 genetic background, carrying congenic and sub-congenic regions of *H2* (Chalmer et al., 1977). These studies also demonstrated the existence of two distinct regions involved in the control of infection: One the K/IA sub-region and the D sub-region. These domains encode the *H2K* and *H2D* molecules involved in peptide presentation (Grundy et al., 1981). Consistent with the results of Chalmer and colleagues, infection of peritoneal macrophages from mice of different *H2* revealed that $H2^k$ cells are more resistant to MCMV, while $H2^b$ and $H2^d$ cells are permissive to the virus (Price, Gibbons, & Shellam, 1990). These results suggest that in addition to their role in peptide presentation, classical *H2* molecules are also implicated in regulation of viral binding or entry into the cell (Price et al., 1990; Wykes et al., 1993).

The effect of *non-H2* gene (s) was observed in mouse strains of the C57BL genetic background. In these strains, *non-H2* gene (s) exert an influence on the level of MCMV replication in target organs, in particular the spleen (Allan et al., 1984). The resistance to MCMV infection in F1 hybrids between C57BL ($H2^b$) and BALB/c ($H2^d$) was markedly enhanced compared to what was predicted from their *H2* haplotype. These results suggested that a gene (s) in the C57BL background could override the effect of *H2*

genes (Grundy et al., 1981). The *non-H2* gene(s) was found to play a role early during MCMV infection. In particular, it appeared to regulate the non-adaptive host responses mediated by IFN-γ (Grundy, Trapman, Allan, Shellam, & Melief, 1982; Allan & Shellam, 1985), inflammatory cells (Price, Winter, & Shellam, 1987) and NK cells (Shellam et al., 1981; Bukowski, Woda, & Welsh, 1984).

1.5.2 The *Cmv1* locus

Using segregation analysis in populations of mice issued from MCMV-susceptible (BALB/c) and MCMV-resistant (C57BL/6) progenitors, Scalzo and colleagues, determined that a single autosomal dominant locus, named *Cmv1*, is responsible for the control of replication of MCMV in mice (Scalzo, Fitzgerald, Simmons, La Vista, & Shellam, 1990). Two allelic forms of *Cmv1* are observed: $Cmv1^r$ and $Cmv1^s$. C57BL/6 mice characterized with low levels of MCMV in the spleen, have the $Cmv1^r$ allele. In contrast, mouse strains such as BALB/c, DBA/2J, A/J carry the susceptibility allele, $Cmv1^s$ and exhibit a 10^3 to 10^4 increase in splenic viral titers (Scalzo et al., 1995). Beside controlling the replication of MCMV in the spleen, *Cmv1* is associated with the restriction of the virus in the bone marrow and in the thymus, but not in the liver (Scalzo et al., 1990; Price, Olver, Gibbons, Teo, & Shellam, 1993). *In vivo* depletion of CD4+T and CD8+T cells had no effect on the phenotype, suggesting that T cells are unlikely to be involved in the control of MCMV replication. However, depletion of NK cells using NK1.1 monoclonal antibody rendered C57BL/6 mice MCMV-susceptible, indicating that the effect of the *Cmv1* gene is mediated by NK cells (Scalzo et al., 1992). Furthermore, analysis of the inheritance pattern of MCMV-resistance in CXB and BXD recombinant inbred strains localized *Cmv1* on distal mouse chromosome 6, tightly linked to *Nk1.1*

(Scalzo et al., 1992). This region of the mouse chromosome was defined as the natural killer gene complex (NKC) because of the presence of multiple genes and gene families involved in NK cell function (Yokoyama & Seaman, 1993).

Fine mapping to define the genetic interval delineating *Cmv1* precisely was mandatory, since all members of the NKC were considered potential candidates for *Cmv1*. Segregation and haplotype analysis allowed to refine the localization of *Cmv1* to the *Ly49* gene cluster (Lee et al., 2001b; Brown et al., 2001b). Finally, over 10 years after the identification of *Cmv1* locus, three independent groups identified *Cmv1* as being allelic to a member of the Ly49 cluster, Ly49H (Daniels et al., 2001; Brown et al., 2001a; Lee et al., 2001a). Transgenic expression of Ly49H receptor conferred resistance to MCMV in genetically susceptible mice, confirming allelism between *Cmv1* and *Ly49h* (Lee et al., 2003).

1.6 The natural killer gene complex

In the mouse, the NKC is located on chromosome 6, and spans a region of approximately 4Mb in size (Brown et al., 1997). An interesting feature of the NKC is that several loci play a role in controlling the immune function. Among the described phenotypes associated with these loci, several are linked with susceptibility to infection. Different phenotypic loci linked to the murine NKC are represented in table 2.

Table 2: Phenotypic loci associated to the mouse NKC

Loci	Described phenotype	References
Cmv1	Resistance to mouse cytomegalovirus	(Scalzo et al., 1992)
Chok	Killing of CHO cells(Tumor killing)	(Idris, Iizuka, Smith, Scalzo, & Yokoyama, 1998)
Idd6	Susceptibility to Insulin-dependent Diabetes Mellitus	(Melanitou, Joly, Lathrop, Boitard, & Avner, 1998)
Rmp1	Resistance to mousepox virus	(Delano & Brownstein, 1995)
Scl	Th1 response to Leishmania infection	(Beebe, Mauze, Schork, & Coffman, 1997)

Idris and colleagues determined that NK cell-mediated cytotoxicity against the Chinese hamster ovary (CHO) tumor cell line, the *Chok* locus, is identical to Ly49D. Identity of other *NKC* loci implicated in susceptibility to insulin-dependent diabetes Mellitus (Idd6), mousepox virus (Rmp1) and Leishmania (Scl) infection remains to be determined. It is interesting to note, however, that these susceptibility traits all involve NK-cell-mediated immunity and that the C57BL/6 mouse presents the active, "resistance" allele.

The proximal domain of the NKC contains genes coding for structurally unrelated proteins including the hematopoietic cell phosphatase, SHP-1 (Yi, Gilbert, Jenkins, Copeland, & Ihle, 1992), the lymphocyte activation protein 3, LAG3 (Triebel et al., 1990; Miyazaki, Dierich, Benoist, & Mathis, 1996) and the lymphotoxin beta receptor, LTBR (Nakamura et al., 1995). In contrast, most of the genes on the distal portion code for cell surface receptors of the C-type lectin superfamily expressed in endothelial, dendritic and NK cells. Many of these genes belong to gene families that share high amino-acid identity and are clustered in about 2Mb of genomic DNA between the alpha-2-macroglobulin (*A2m*) gene and the proline rich protein (*Prp*) gene cluster (Brown, Scalzo, Matsumoto, &

Yokoyama, 1997; Yokoyama & Plougastel, 2003). The *Nkrp1*, *Clr*, *Nkg2* and *Ly49* cluster, are the main gene families found in the mouse NKC (Figure 2a).

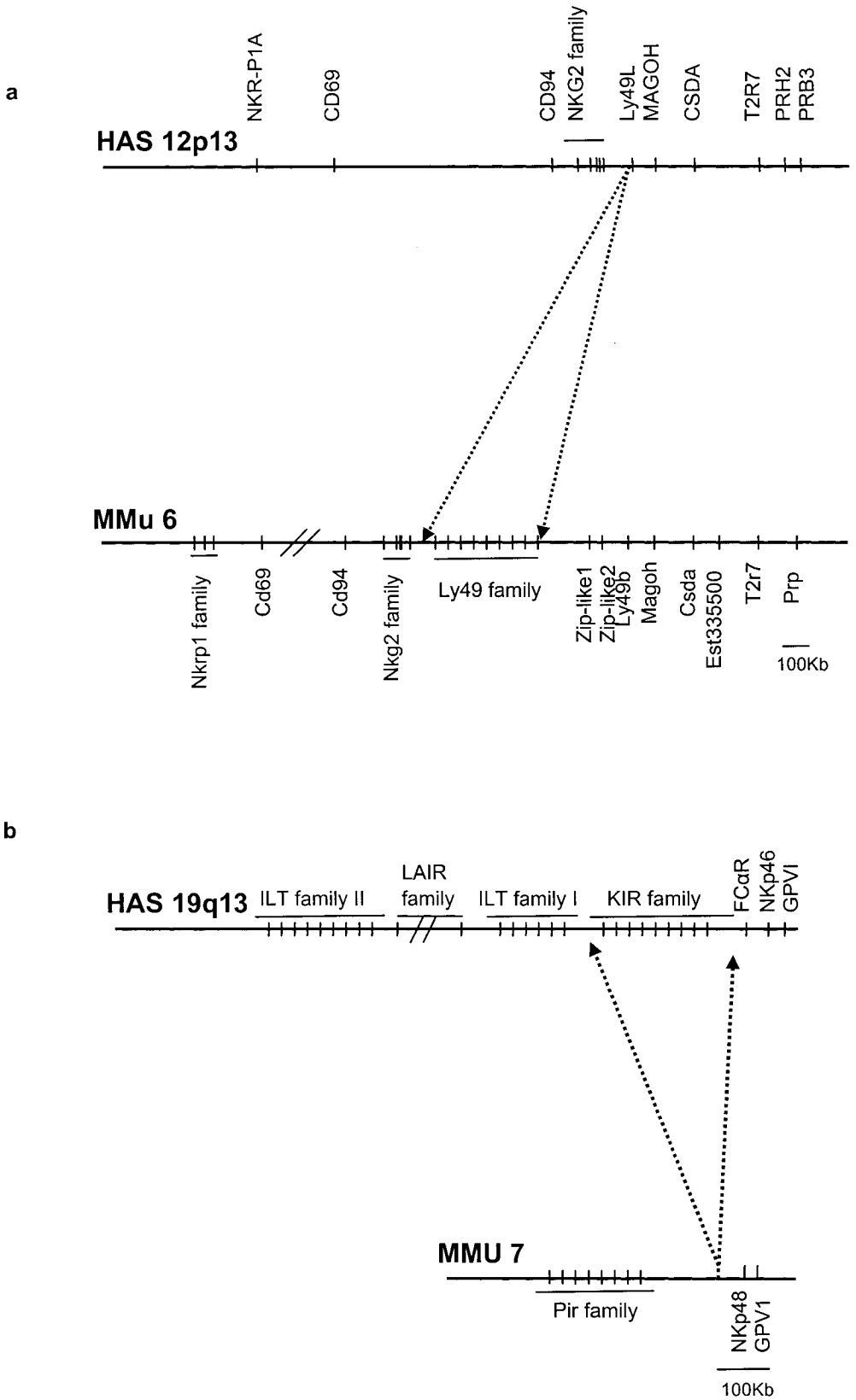
The *Nkrp1* gene family is clustered in a locus close to CD69, a cell surface molecule early induced, during lymphocyte activation (Cebrian et al., 1988). In the C57BL/6 mice, four transcripts of the *Nkrp1* gene cluster were identified: *Nkrp1a*, *c*, *d* and *f*) (Plougastel, Matsumoto, Dubbelde, & Yokoyama, 2001). *Nkrp1a* codes for the NK1.1 antigen expressed on NK cells and certain T cell populations. Recently, seven members of the *Clr* (C-type lectin-related) gene family, (*Clra-g*) were identified (Plougastel, Dubbelde, & Yokoyama, 2001). Among these genes *Clrg and Clrb, were* identified as ligands for the activating NKRP1F and the inhibitory receptor NKPR1D respectively (Iizuka, Naidenko, Plougastel, Fremont, & Yokoyama, 2003) . Members of the *Nkg2 (a-d)* and *Ly49* multi-gene family are predominant in the mouse NKC. NKG2 molecules are expressed on all NK cells whereas Ly49 receptors are expressed on NK cell subpopulations. With the exception of NKG2D, which form homodimers, NKG2 molecules are expressed as heterodimers with the invariant CD94 molecule (Lazetic, Chang, Houchins, Lanier, & Phillips, 1996). A remarkable characteristic of NKG2D receptors is the variety of ligands that they recognize, including the minor histocompatibility molecule (H60) and several members of the RAE-1 (retinoic acid early transcript 1) family (Cerwenka et al., 2000; Diefenbach, Jamieson, Liu, Shastri, & Raulet, 2000). Other members of the NKG2 family recognize Qa-1, which is a mouse non-classical MHC molecule. In contrast to classical MHC class I molecules involved in peptide presentation, Qa-1 presents leader MHC sequences and has limited polymorphism (Vance, Jamieson, & Raulet, 1999). The *Ly49* cluster is the best characterized gene

family in the NKC. Ly49 receptors bind classical MHC class I and MHC class I like molecules. A detailed description of Ly49s is presented in section 1.6.1.

In humans, the NKC is located on chromosome 12p13.1 and spans a region of approximately 2Mb in size (Yokoyama et al., 2003).The organization of the human NKC is relatively different from the one observed in the mouse (Figure 2a). In contrast to the mouse NKC, only one member of the NKRP1 family is observed in humans, NKRA1, which shares 45% amino acid identity with its mouse counterpart (Lanier, Chang, & Phillips, 1994). *NKG2 (A-D)* are both present in mouse and human. However, *NKG2E* and *NKG2F* are specific to the human genome (Yokoyama et al., 2003). Surprisingly, only one member of the *Ly49* cluster, *Ly49l* pseudogene, is present in the human NKC (Westgaard, Berg, Orstavik, Fossum, & Dissen, 1998). Ly49 receptors are considered the functional homologues of human killer-cell immunoglobulin-like receptors (KIRs) of the immunoglobulin superfamily. Despite being structurally unrelated to Ly49s, KIR receptors are similarly expressed in overlapping subsets of NK cells, recognize HLA class I determinants (Long & Rajagopalan, 2000), and signal through the same intracellular pathways as Ly49 receptors (Colucci, Di Santo, & Leibson, 2002a). However, *KIR* genes are not encoded in the NKC. They are rather clustered on human chromosome 19q26 within a genetic region known as the leukocyte receptor complex (LRC), which is characterized by the presence of multiple immunoreceptor gene families. While some LRC members are well conserved, no rodent *Kir* gene has been detected on the syntenic region of mouse chromosome 7 (Webb et al., 2002) (Figure 2b). However, the first close rodent homologue of the NK receptor KIR family, KIRL1 was recently identified on mouse chromosome X (Welch, Kasahara, & Spain, 2003).

Figure 2: Natural Killer complex (NKC) and the Leukocyte Receptor complex (LRC) in humans and mice.

a) Genetic organization of the NKC on human chromosome 12 and mouse chromosome 6. Genes of the NKC code for C-type lectin receptors. Note the presence of a single *Ly49* gene in humans as opposed to the *Ly49* mutigene family in mice. b) Genetic organization of the LRC on human chromosome 19 an on mouse chromosome 7. Genes of the LRC encode for receptors of the immunoglobulin superfamily. Note the absence of the KIR domain in the mice LRC.



Webb et al. 2002
Genes and Immunity

1.6.1 Ly49 receptor genes and proteins

Ly49 genes are characterized by the presence of a high degree of allelic polymorphism, different copy numbers and genomic organization in inbred strains. In the C57BL/6 mouse, the *Ly49* gene cluster presents 11 active genes (*a-j* and *q*) and five pseudogenes (*k-n* and *v*) (McQueen, Freeman, Takei, & Mager, 1998; Wilhelm, Gagnier, & Mager, 2002). The *Ly49* gene cluster of 129/J strain comprise: *Ly49e, g, i, o, p, r, s, t, u,* and *v*. *Ly49e*, *g* and *i* were found to be allelic forms of the respective C57BL/6 genes (Makrigiannis et al., 1999; Anderson, Ortaldo, & McVicar, 2001). Furthermore, the presence of chimeric *Ly49* genes, such as *Ly49u/i*, *p/d* and *l/r* was observed (Makrigiannis et al., 2002). The NOD strain was the only one found to have a number of activating receptor genes exceeding the number of genes coding inhibitory receptors. Four of the six *Ly49s* described in this strain are activating (*Ly49d*, *m*, *p* and *w*) (Silver, Gong, Hazes, & Kane, 2001).

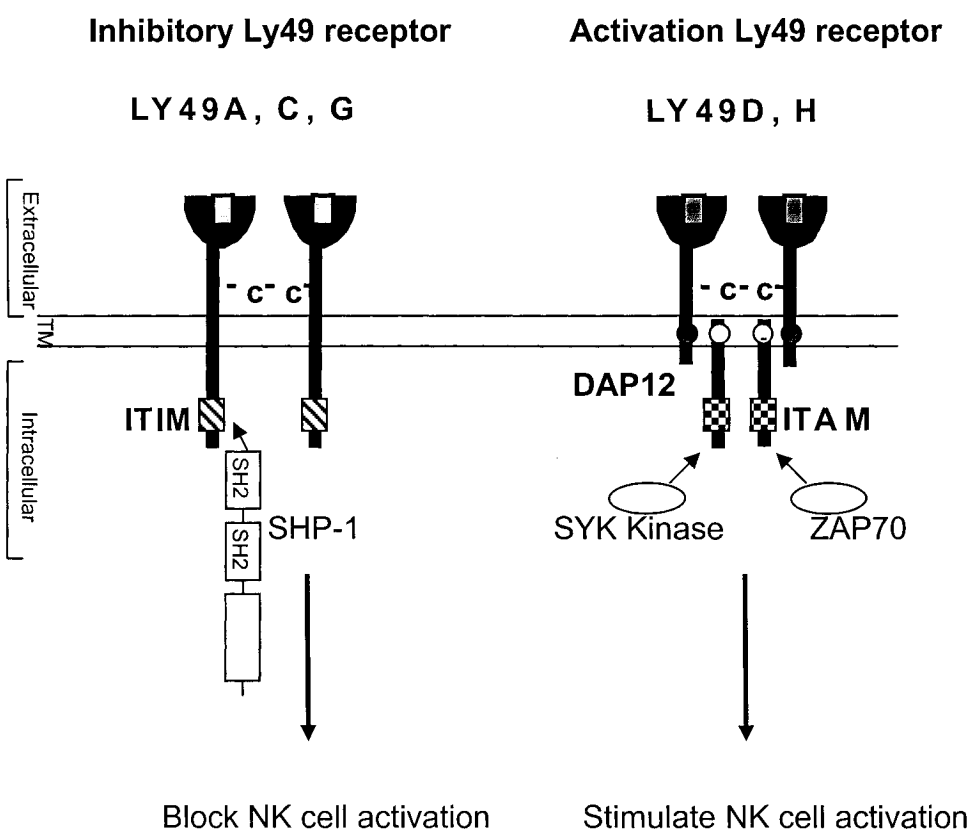
Ly49 receptors are mostly expressed on NK and NK T cells, and also on some specialized T-cell subpopulation (Ortaldo, Winkler-Pickett, Mason, & Mason, 1998; Coles, McMahon, Takizawa, & Raulet, 2000). The Ly49 proteins form homodimers which are linked by disulfide bonds. The extracellular domain of the Ly49 receptor family is comprised of a C-type lectin motif (Anderson et al., 2001). This domain is known as natural killer domain (NKD) because, in contrast to lectins, Ly49s do not recognize sugar moieties. Two categories of Ly49 receptors, either inhibitory or activating, are recognized based on the structural organization of their intracellular domains (Figure 3).

The cytoplasmic domain of inhibitory receptors is distinguished by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) characterized by the consensus sequence (I/VxYxxL/V). Following ligand binding, the ITIM motif is tyrosine phosphorylated, which allows recruitment of the phosphatase SHP-1 to attenuate intracellular signaling (Mason et al., 1997; Nakamura et al., 1997). The cytoplasmic domain of activating receptors lacks the ITIM motif. Activating receptors, in contrast, are characterized by the presence of a charged amino acid, usually arginine, in their transmembrane-spanning domain. The positively charged amino acid is mandatory for the formation of a non-covalent interaction between the receptor and the DNAX activation protein 12 (DAP12) which is necessary for the initiation of an activating signal (Lanier et al., 1998). The DAP12 cytoplasmic tail contains a single immunotyrosine base activating motif (ITAM) composed of two tyrosine residues followed by three hydrophobic residues. The phosphorylation of DAP12 via Src-family kinases, leads to the recruitment and activation of SYK/ZAP70 kinase SYK. It as been shown, that SYK mediates all the downstream events, leading to target cell lysis (Mason, Willette-Brown, Mason, McVicar, & Ortaldo, 2000) (Colucci et al., 2002b) (Figure 3).

The high level of homology between inhibitory and activating receptors may suggest that one might have evolved from the other by selective pressure imposed by pathogens (Arase & Lanier, 2002). Interestingly, several laboratories demonstrated that pseudogenes from one strain can be found as an active gene in another strain (Silver et al., 2001). These studies revealed that the number and the type of Ly49 receptors expressed on subset of NK cells vary from one strain to the other (Ortaldo et al., 1999). This phenomenon also occurs in *KIR* genes found in humans (Uhrberg et al., 1997). It has been shown, that the percentage of particular Ly49 molecules on the surface of a given subset

Figure 3: Diagram of Ly49 inhibitory and activating receptors.

Inhibitory receptors are characterized by ITIM motifs in their intracellular domain. Upon ligand recognition SHP-1 associates with the phosphorylated ITIM motif and block NK cell activation. Activating receptors have a positively charged amino acid in their transmembrane domain, which allows interaction with adaptor molecules, such as DAP12. SYK and ZAP70 tyrosine kinases associate to DAP12 ITAM motif which activates NK cells following ligand recognition.


Inhibitory Ly49 receptor
LY49A, C, G
Activation Ly49 receptor
LY49D, H
Extracellular
TM
Intracellular
ITIM
SH2
SH2
SHP-1
DAP12
ITAM
SYK Kinase
ZAP70
Block NK cell activation
Stimulate NK cell activation

of NK cells may be regulated by the expression of MHC class I molecule in that strain (Kase, Johansson, Olsson-Alheim, Karre, & Hoglund, 1998) A balance between inhibitory and activating receptors mediates the cytotoxic function of NK cells. This balance might have an influence on the tendency of a particular strain to develop autoimmunity. Consistent with this hypothesis, congenic NOD mice containing C57BL/6 NK complex were shown to have a reduced incidence in diabetes (Carnaud, Gombert, Donnars, Garchon, & Herbelin, 2001).

All known ligands for Ly49 receptors are MHC class I or MHC-like molecules. Cell assay staining, crystallography and site directed mutagenesis have been used to study the MHC class I binding characteristics of known NK cell receptors. Distinct binding specificities for each Ly49 molecule are observed. Some Ly49 protein can bind to more than one ligand, whereas others are specific for one particular MHC or MHC-like molecules. Table 3 is a summary of different Ly49 receptors and their ligands, identified in different mouse strains.

Table 3: Functions and ligands of Ly49 receptors

Gene	Receptor	Function	Ligand specificity	References
Ly49a$^{C57BL/6}$	Ly49A	Inhibitory	H2D^{d}	(Karlhofer, Ribaudo, & Yokoyama, 1992)
Ly49c$^{C57BL/6}$	Ly49C	Inhibitory	H2K^{b,d} , H2D^{k,b,d}	(Stoneman et al., 1995; Hanke et al., 1999)
Ly49g$^{C57BL/6}$	Ly49G	Inhibitory	H2D^{d}	(Mason et al., 1995; Brennan, Mahon, Mager, Jefferies, & Takei, 1996; Hanke et al., 1999)
Ly49d$^{C57BL/6}$	Ly49D	Activating	H2D^{d}, Hm1-C4	(Nakamura et al., 1999; George, Mason, Ortaldo, Kumar, & Bennett, 1999; Furukawa, Iizuka, Poursine-Laurent, Shastri, & Yokoyama, 2002)
Ly49h$^{C57BL/6}$	Ly49H	Activating	m157	(Arase, Mocarski, Campbell, Hill, & Lanier, 2002; Smith et al., 2002)
Ly49v$^{129/J}$	Ly49V	Inhibitory	H2D^{b,d,k} ,H2K^{b,d,k} ,H2L^{d}	(Anderson et al., 2001; Makrigiannis et al., 2001)
Ly49o$^{129/J}$	Ly49O	Inhibitory	H2D^{d}, H2L^{d}	(Anderson et al., 2001; Makrigiannis et al., 2001)
Ly49i$^{129/J}$	Ly49I	Inhibitory	H2D^{k}, H2K^{b,d,k}, m157	(Anderson et al., 2001; Makrigiannis et al., 2001; Arase et al., 2002)
Ly49g$^{129/J}$	L49G	Inhibitory	H2D^{k} H2D^{d}	(Anderson et al., 2001; Makrigiannis et al., 2001)
Ly49r$^{129/J}$	Ly49R	Activating	H2D^{d,k}	(Anderson et al., 2001; Makrigiannis et al., 2001)
Ly49w^{NOD}	Ly49W	Activating	H2D^{d,k}	(Silver et al., 2001)
Ly49p^{NOD}	Ly49P	Activating	H2D^{d}	(Silver et al., 2000)

*Arbitrarily, a new name was assigned to receptors displaying less than 95% identity while those showing more than 95% identity but yet different are given a superscript with the mouse strain.

Through engagement of self-MHC class I, the role of Ly49 inhibitory receptors is thought to prevent the lysis of healthy autologous cells. Interestingly, it was reported that MHC class I antigen binding is not restricted to inhibitory receptors. Ly49D$^{C57BL/6}$ activating receptor and its homologue inhibitory receptor Ly49A$^{C57BL/6}$ both bind class I MHC molecule, H2D^{d} (Karlhofer et al., 1992; Nakamura et al., 1999). In contrast, Ly49G2$^{C57BL/6}$ shows very fine specificity toward H2D^{d} (Mason et al., 1995). Ligation of Ly49D with H2D^{d} expressing target cells results in both an increase in cytotoxicity and IFN-γ production (Mason et al., 2000). Furthermore, activating Ly49R$^{129/J}$ and Ly49W^{NOD} receptors interact with both H2D^{d} and H2D^{k} ligand, whereas Ly49P^{NOD} seems to be specific to H2D^{d} (Silver et al., 2000; Silver et al., 2001). Similar to Ly49D$^{C57BL/6}$, a study

using nonobese diabetic mice revealed that Ly49P induced NK cytotoxicity by recognizing $H2D^d$-bearing targets (Silver et al., 2000). Consequently, these studies indicate that NK cells can be activated rather than inhibited following interaction of activating NK cell receptors with MHC class I antigens. The biological significance of these interactions is not fully clarified. Some evidence suggests that Ly49D activating receptor may have alternate ligands for mediating resistance to tumor cells in C57BL/6 mice (Nakamura et al., 1999; George et al., 1999; Furukawa et al., 2002). Similar to Ly49D, other Ly49 alternative inhibitory or activating receptors may be involved in the control of infectious diseases. Unraveling novel members of the Ly49 receptor repertoire and their ligands will continue to shed light on their role in regulating immunity.

1.6.2 Role of Ly49H against murine cytomegalovirus

The crucial role of activating Ly49 receptors in host defense against viruses was demonstrated by the genetic analysis of the response against MCMV, and the subsequent identification of Ly49H in mediating natural resistance to MCMV in C57BL/6 mice as described in section 1.5.2 (Daniels et al., 2001; Brown et al., 2001a; Lee et al., 2001a). However, C57BL/6 mice are the exception among inbred mice, as other strains do not possess *Ly49h* and are susceptible to MCMV (Lee et al., 2001b). Arase and colleagues revealed that an early virally encoded class I homologue, known as m157, activates NK cells via Ly49H receptor, allowing specific destruction of MCMV-infected cells (Arase et al., 2002) (Figure 4a). Interestingly, Ly49H became the first NK cell receptor involved in direct recognition of a pathogen-encoded protein. In addition, these results demonstrated that NK cells possess a unique role in host defense that it is distinct from other lymphocyte populations.

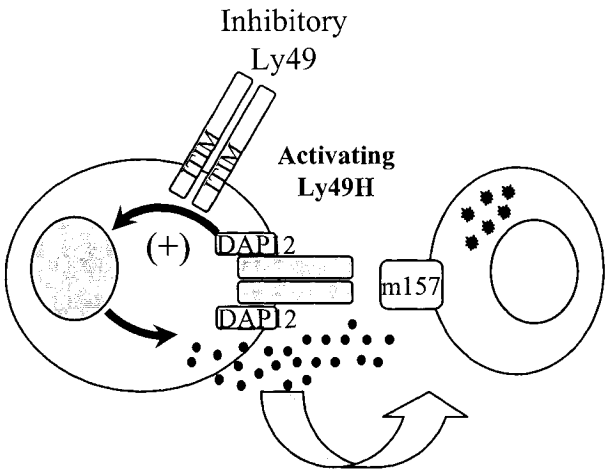
It is intriguing however, that MCMV would maintain in its genome a non-essential gene that targets infected cells for NK cell killing. Moreover, other Ly49 receptors exist that can bind to m157, but the outcome of this interaction is thought to facilitate viral replication. One such receptor is the Ly49I inhibitory receptor on NK cells of the susceptible 129/J strain, where m157 may in fact switch off NK cells upon viral recognition (Arase et al., 2002) (Figure 4b). On the other hand, most MCMV strains express m157 variants that do not trigger NK cell activation (Voigt et al., 2003) and even resistant C57BL/6 mice become highly permissive to viral replication when infected with a mutant MCMV strain that lacks m157 (Bubi et al., 2004). Thus, although m157-Ly49H remains the first evidence of direct recognition between a virus and NK cells, its relevance in nature during NK cell activation against viral infection is not obvious (Colucci & Vidal, 2004). Consequently, we hypothesized that possible alternative mechanisms other than Ly49H/m157, might exist.

Figure 4: Mechanisms of MCMV-resistance/susceptibility in mice.

Inbred strains of mice exhibit different patterns of resistance and susceptibility to MCMV. a) Natural resistance to MCMV in C57BL/6 mice is mediated by binding of NK cell activating receptor Ly49H to the pathogen-encoded glycoprotein m157. Ly49H receptor sends an activating signal to the cell via DAP12 adaptor protein, resulting in perforin release and killing of infected cells. Ly49I inhibitory receptor from C57BL/6 does not bind m157, allowing a dominant stimulatory signal from Ly49H receptor. b) The 129/J mouse strain lacks Ly49H receptor, but possesses an Ly49I inhibitory receptor which binds m157. Ly49I/m157 interaction results in the release of an inhibitory signal by Ly49I receptor via its ITIM motif and infected cells survive.

a

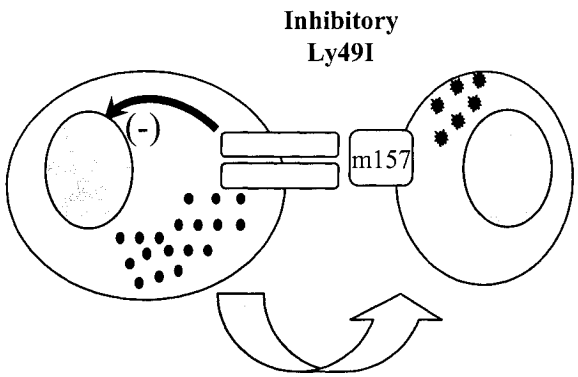
MCMV-Resistant C57BL/6



Perforin release and killing of MCMV-infected cell

b

MCMV-Susceptible 129/J



No perforin release Infected cell survives

Lee SH et al. 2002
Microbes and Infection

2. STATEMENT OF OBJECTIVES

Interesting questions emerged with the identification of Ly49H as the mediator of resistance against MCMV. Among these are whether other NK receptor genes are involved in host defense and how critical such alternative recognition mechanisms might be. To address these issues in the mouse model, other *Cmv1*-resistance alleles should be studied. However, the use of classical laboratory mouse stocks sets serious limitations, as most of these stocks carry susceptibility alleles. We reasoned that novel resistance genes to MCMV infection might be found in inbred strains of mice derived from wild specimens of the genus *Mus*. Interestingly, PWK/Pas wild-derived inbred strains is MCMV-resistant despite the absence of Ly49H mRNA and protein. Therefore, the main objective of my research project was to identify novel resistance alleles of PWK/Pas background responsible for resistance to MCMV infection, thereby improving our understanding on alternative natural defense mechanisms mediated by natural killer cells against MCMV infection.

Specific objectives:

1) Assess MCMV-resistance/susceptibility in wild-derived mice
2) Study the role of m157 in PWK/Pas defense mechanism against MCMV
3) Segregation analysis of the PWK/Pas resistance trait
4) Study the effect of the *NKC* and *H2* loci in PWK/Pas resistance
5) Haplotype analysis at *Cmv1* in a panel of wild-derived mice
6) Characterize the PWK/Pas Ly49 repertoire to identify and test potential candidates

3. MATERIALS AND METHODS

3.1 Mice

Classical inbred strain BALB/c, C57BL/6J and 129/J were purchased from the Jackson Laboratory (Bar Harbour, ME). Wild-derived inbred strains PWK/Pas, SEG/Pas, STF/Pas, MBT/Pas, MAI/Pas, WLA/Pas, WMP/Pas and CAST/Ei were maintained at the Institut Pasteur, Paris. Resistant (*Cmv1*^{r}) PWK/Pas and susceptible (*Cmv1*^{s}) BALB/c inbred strains were used to produce 75 [(PWK/Pas x BALB/c) F1 x BALB/c] segregating backcross mice. Since F1 males were sterile, most probably due to Haldane's rule predicting male infertility in hybrid species (Guenet et al., 2003), backcross N2 progeny were generated by coupling F1 females to parental BALB/c males. Animals were kept at the Central Animal Facilities of the Institut Pasteur and used for experiments at six to twelve weeks of age. All protocols for animal experiments were reviewed by the Central Animal Facilities of the Institut Pasteur and were done in accordance with guidelines approved by the French Ministry of Agriculture.

3.2 MCMV phenotype

The Smith strain of MCMV was obtained from the American Type Culture Collection (ATCC) and propagated by salivary glands as previously described (Depatie, Muise, Lepage, Gros, & Vidal, 1997). The tissue culture grown Δm157 MCMV and wild-type (WT) MCMV were kindly provided by Stipan Jonjic (Bubi et al., 2004). At 7 weeks of age, mice were infected intraperitoneally (i.p.) with $5X10^3$ MCMV plaque forming units (PFU). The degree of infection was assessed by determining the viral load in the spleen and the liver 3 days post-infection by plaque assay on BALB/c mouse embryonic

fibroblasts as described (Depatie et al., 1997). Viral titers were expressed as LOG_{10} of MCMV PFU per organ.

3.3 Tail DNA extraction

Genomic DNA was extracted from various mouse strains following the modified alkaline method (Truett et al., 2000). Mouse tail tip (2mm) was surgically excise and added to 600ul of 50mM sodium hydroxide (NaOH). The NaOH solution containing the tail was placed on a heating block at 95 degree Celsius for 20 minutes and vortexed every 5 minutes. Following the incubation, 50ul of 1M tris (pH7.0) was added to obtain a pH of 8.3 which is ideal for PCR (polymerase chain reaction). Following neutralization, centrifugation at room temperature (RT) for 5 minutes allowed the pellet to be separated from the aqueous phase. The aqueous phase containing the genomic DNA was transferred to a fresh tube. An aliquot of 2ul was used for PCR.

3.4 Genotyping

A total of 70 [(PWK x BALB/c)F1 x BALB/c] backcross mice were genotyped. Genotypes in the *NKC* and *H2* loci were determined by PCR-RFLP. The *NKC* was amplified using *Ly49e-F* (5'-GAGAGTCAATGAGGGAATTTATCC-3') *and Ly49e-R* (5'-CCCAAGATGAGTGAGCAGGAGG-3') primers followed by *Hinc*II enzyme digestion, as previously described (Brown et al., 1997). The amplification of BALB/c and PWK/Pas genomic DNA with *Ly49e* marker generated a product size of 1100bp. Discrimination between heterozygous (PWK/Pas x BALB/c) and homozygous (BALB/c x BALB/c) animals was determined by *Hinc*II restriction enzyme digest.

The *H2* locus was genotyped with the MHC class II specific primers for IAA1 by taking advantage of polymorphisms present in the α chain of the murine MHC class II antigen I-A. IAA1-F (5'-GAAGACGACATTGAGGCCGACCACGTAGGC-3') and IAA1-R (5'-ATTGGTAGCTGGGGTGGAATTTGACCTCTT-3') primers generated PCR product of 263bp. Based upon the presence and the absence of *Hind*III or *Pst*I sites, discrimination between the d(BALB/c) and f, q, u or b (PWK/Pas) alleles is possible (Peng & Craft, 1996). Following digestion with *Pst*1 enzyme, homozygous (BALB/c x BALB/c) animals were distinguished from heterozygous (PWK/Pas x BALB/c) mice. All polymorphisms were observed by ethidium bromide staining following electrophoresis in 0.5 X TBE buffer on regular 2% agarose gels.

3.5 Statistical analysis

The contribution of PWK/Pas alleles at *NKC* and *H2* to the segregation of the phenotype (i.e, the LOG_{10} of the number of PFU in the spleen) in [(PWK/Pas x BALB/c)F1 x BALB/c] backcross mice was estimated under the linear model " phenotype = m + nkc + h2 + e "; where 'nkc' and 'h2' are used to represent the number of PWK/Pas alleles at each locus, 'm' is the common mean value and 'e' are the usual independent normally distributed random deviations. LOD scores for linkage were calculated by taking the LOG_{10} of the likelihood ratio of the model.

3.6 Genetic markers

We carried out haplotype mapping on genomic DNA using a set of 20 polymorphic markers including 14 that were previously localized to the minimal genetic interval of *Cmv1* (Depatie et al., 1999; Depatie et al., 2000; Lee et al., 2001a; Lee et al.,

2003) . In addition, we used 6 new markers (*SV175*, *Ly49h(15R)*, *SV50*, *SV151*, *SV168* and *SV169)* derived from the *Ly49h* genomic DNA sequence (Wilhelm et al., 2002). The molecular characteristics of these markers are presented in table 4. PCR reactions were performed using 20 ng of genomic DNA in a 20-μL volume reaction containing 10 pmol of each primer, 0.2 units of Taq DNA polymerase (Boehringer Mannheim Biochemica)], and 100 nM dNTPs under previously described conditions (Depatie et al., 1999; Depatie et al., 2000; Lee et al., 2001a; Lee et al., 2003). Simple sequence and restriction fragment length polymorphisms were visualized by ethidium bromide staining following electrophoresis in 0.5 X TBE buffer on either 1% regular agarose or 7% acrylamide gels. Products obtained with markers within the *Ly49h* gene, *SV175*, *Ly49h (15/R)* and *SV50*, were sequenced to confirm their identity in individual mouse strains.

Table 4: Genetic markers for haplotype analysis

LOCUS	PRIMERS	PRIMER SEQUENCE (5'-3')	PRODUCT SIZE (bp)
Ly49h	*SV175-F*	ATGCTCTCCCAATAAAGTTGTTA	290
	SV175-R	TGTACAAGAGATCAGGAAATTGAG	
Ly49h	*Ly49h-15*	GTTCAAAATTTACTAGTTGC	348
	Ly49h-R	TGTCAAGATAGATAGGAGAGG	
Ly49h	*SV50-F*	GGAAGGTATTTCCATATTGG	236
	SV50-R	TTACAATTACAGACGATATT	
Ly49h	*SV151-F*	GTGCTACCACTGAAAACCATTG	204
	SV151-R	CTGTCTCTTGAGTCACCTGCAC	
Ly49h	*SV168-F*	TTAGCAGAGATAAGTATGCAAGGA	195
	SV168-R	TTTTCTAAGTGTGTAGGTGTGTGG	
Ly49h	*SV169-F*	CAACATATAAAGAAACAGGACTTG	318
	SV169-R	AAGTGGTCTCAGTGTCTCAGTGT	

3.7 PWK/Pas cDNA cloning

Total RNA from PWK/Pas natural killer cells was isolated with Trizol reagent (Life Technologies) and reverse transcribed using SuperScript II polymerase (Life Technologies) with an oligo(dT) primer (Amersham). NK cell receptor cDNAs were amplified with gene specific oligonucleotide primers for *Ly49s* and *Nkg2d*. Oligonucleotide sequences are presented in table 5. Amplified products were analyzed by gel fractionation, purified with QIAEX II Gel Extraction Kit (Qiagen) and directly ligated into pGEM-T easy Vector (Promega). A minimum of three identical clones from two independent PCR reactions were sequenced for each PWK/Pas novel genes. DNA sequence analysis of these clones was performed using standard nucleotide-nucleotide BLAST (blastn) and standard protein-protein BLAST (blastp) found on NCBI website (www.ncbi.nlm.nih.gov). The alignment program Clustalw was used for multiple sequence alignments (www.ebi.ac.uk/clustalw).

Table 5: Summary of PCR primers used for cDNA amplification

LOCUS	PRIMERS	PRIMER SEQUENCE (5'-3')	PCR PRODUCT SIZE(bp)
NKG2d	NKG2d-F	ACAACCTGGATCAGTTTCTGAAG	779
	NKG2d-R	TCTGGTTGTTGCTGAGATGG	
Ly49d	Ly49d-F	CACAGAAATCACTCAAGGAC	1001
	Ly49d-R	CAAAATCTTCCAGATTGTCT	
Ly49h	Ly49h-1	AGCCTCTTAGGGGATACAGAC	1042
	Ly49h-R	TGTCAAGATAGATAGGAGAGG	
Ly49n	Ly49n-F	TTCCCAACTATGAGATTCCAC	770
	Ly49n-R	GCTTTAGATAAAAATAAACATCCTA	
Ly49k	Ly49k-F	GATGGGTGAGCAGGAAGTCG	707
	Ly49k-R	CCACAAATACAGTAGTAGGGAA	
Ly49i	Ly49i-F	CCGATAGAGACACAGAGAACA	924
	Ly49i-R	GGGACTGTACTCTCCTATCTA	
Ly49b	Ly49b-1	CGAGGCCACATTTTAATACA	1220
	Ly49b-b	GAGGTAGGAGAATTAACCTGAAAAT	

3.8 Cell reporter assay

The 2B4 T cell hybridoma transfected with NFAT-GFP (Nuclear Factor of Activated T cells-Green fluorescent protein) reporter gene and FLAG-DAP12 (DNAX Activation Protein 12) was transduced with either *Ly49pk3* or *Ly49h* cDNA, cloned into the pGMX-T vector as described (Arase et al., 2002). Mouse NIH3-T3 cells were infected with MCMV (Smith strain) at an MOI of 1.0, incubated for 24 hours, and used as target cells for the reporter assay. Reporter cells were co-cultured overnight with either the infected NIH3-T3 or the Ba/F3 transduced with m157 (Ba/F3-m157) (Voigt et al., 2003) target cells, at a 2:1 ratio. Activation of reporter cells was monitored by Fluorescence Activated Cell Sorting (FACS) analysis of GFP expression on a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson).

3.9 Flow cytometric analysis

Natural Killer (NK) cells were prepared from mouse spleen by magnetic separation using anti-CD3 micro beads following the manufacturer's protocol (Ademtech). IL-2 activated NK cells were stained for surface expression of CD122 (IL-2R) using (PE)-conjugated murine anti-CD122 and CD3 using (APC)-conjugated anti-CD3 (Pharmingen). For intracellular staining, the cells were fixed, permeabilized (Pharmingen) and stained for cytoplasmic domain of Ly49H receptor using a polyclonal anti-Ly49H antibody stained by a secondary Fluorescein Isothiocyanate (FITC)-conjugated anti-rabbit IgG (Pharmingen) as previously described (Lee et al., 2003).

4. RESULTS

4.1 Cytomegalovirus infection in wild-derived mouse strains

We have measured early immune responses to MCMV in eight wild-derived inbred strains of mice derived from either the *Mus musculus* (PWK/Pas, MBT/Pas, MAI/Pas, WLA/Pas, WMP/Pas and CAST/Ei) or the *Mus spretus* (SEG/Pas and STF/Pas) species. The C57BL/6 resistant and BALB/c susceptible classical inbred strains were used as controls. Mice were infected with a sub-lethal dose of MCMV and viral titers from the spleen and the liver were determined by plaque assay, 3 days post infection (Figure 5). Five (MBT/Pas, MAI/Pas, WLA/Pas, WMP/Pas and CAST/Ei) out of the six *Mus musculus*-derived strains, are susceptible and present high viral titers in the spleen and liver, comparable to those observed in the susceptible strain BALB/c (LOG_{10} PFU spleen 4.0 and liver 4.3). In contrast, PWK/Pas strain is resistant with viral titers of LOG_{10} PFU of 1.9 in the spleen and 3.8 in the liver, which are comparable to the resistant C57BL/6 mouse strain. SEG/Pas and STF/Pas mouse strains derived from the *Mus spretus* present very low titers, below LOG_{10} PFU of 1.1 in both the spleen and liver, likely reflecting the incapability of MCMV to replicate in the *M. spretus* cells.

4.2 Staining of wild-derived mice NK cells with anti-Ly49H

Preliminary experiments revealed that PWK/Pas leukocytes reacted with most monoclonal antibodies used to detect leukocytes of classical laboratory strains, thus allowing FACS analysis (Girard Adam et al., 2004) (document in preparation). A polyclonal anti-Ly49H antibody recognizing the cytoplasmic domain of Ly49H receptor was used to stain NK cells from C57BL/6, BALB/c and PWK/Pas (Figure 6). 51.1% of the C57BL/6, IL-2 activated NK cells stain positive with this antibody, whereas in

Figure 5: Survey of wild-derived mouse strains for MCMV-resistance.

MCMV titers in the spleen (black) and in the liver (white) were determined by plaque assay 3 days after infection. The dashed line indicates the level of detection of our assay (LOG_{10} PFU > 1.69). Statistically significant differences in comparison with observed viral titers in MCMV-susceptible BALB/c mice at P values < 0.05 (*) and < 0.001 (**) are indicated. Bars indicate standard deviations.

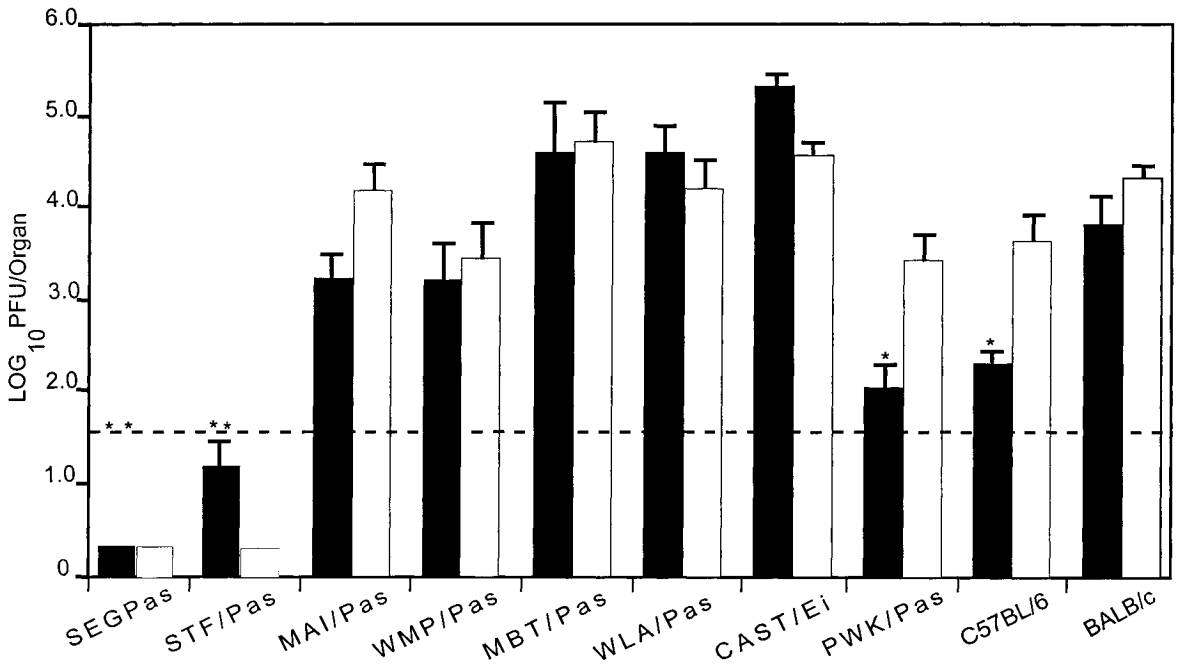
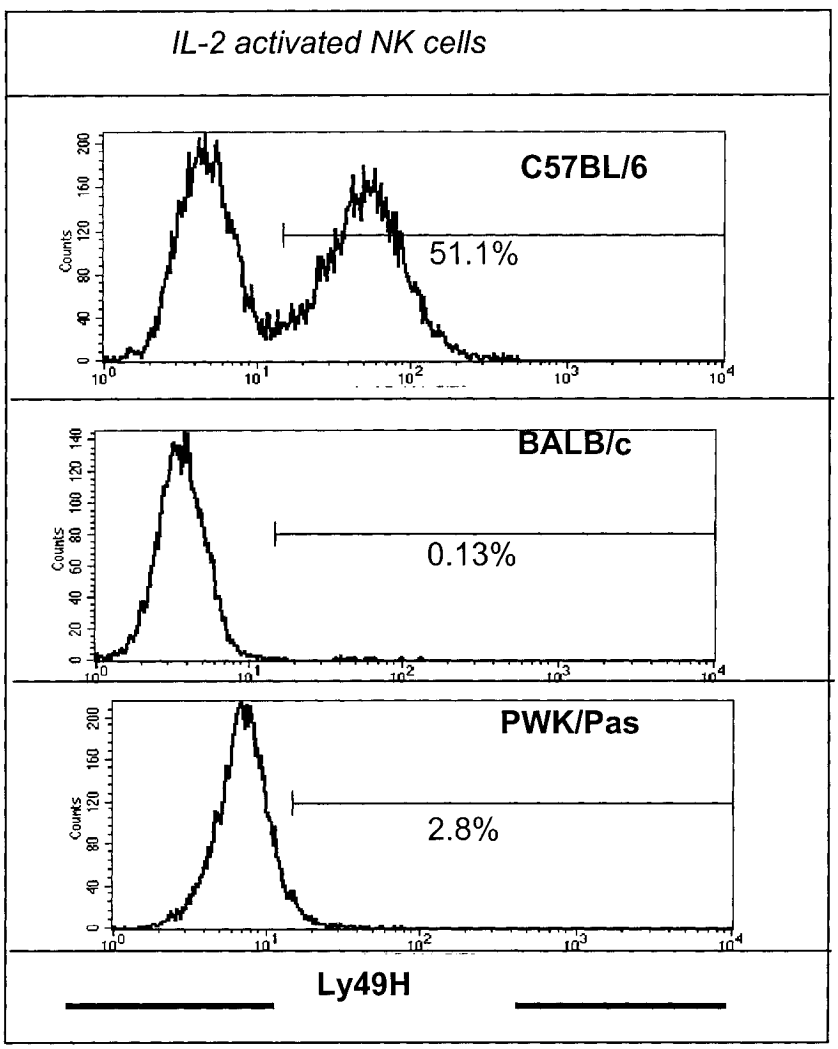

6.0
5.0
4.0
3.0
2.0
1.0
0
LOG10 PFU/Organ
SEGPas
STF/Pas
MAI/Pas
WMP/Pas
MBT/Pas
WLA/Pas
CAST/Ei
PWK/Pas
C57BL/6
BALB/c

Figure 6: Ly49H intracellular staining.

IL-2 activated NK cells were stained with mAb specific for CD3, CD122, then permeabilized and stained intracellularly with polyclonal Ab specific for Ly49H. CD3-CD122+ NK cells were electronically gated. The histogram shows Ly49H intracellular staining against a negative control on gated NK cells for the indicated mice. Numbers in histograms represent percentages of cells staining positive.


IL-2 activated NK cells
C57BL/6
51.1%
Counts
BALB/c
0.13%
Counts
PWK/Pas
2.8%
Counts
Ly49H

BALB/c and PWK/Pas the percentages are 0.13% and 2.8%, respectively. These results suggest that while PWK/Pas NK cells do not express a *bona fide* Ly49H molecule, anti-Ly49H antibody may weakly bind other receptors in this strain.

4.3 PWK/Pas mice are resistant to Δm157 mutant MCMV

To test whether PWK/Pas mice respond to viruses that fail to activate NK cells through m157, we used Δm157 MCMV, a mutant virus which does not express m157 because of a targeted mutation (Bubi et al., 2004) (Figure 7) . The C57BL/6 control strain present average, viral titers of LOG_{10} PFU of 3.0 and 4.3 in the spleen when infected with wild-type MCMV and Δm157 MCMV respectively. In contrast PWK/Pas mice are resistant when infected with either virus, presenting comparable average viral titers in the spleen of LOG_{10} PFU of 3.0. These results suggest that an m157 independent mechanism of resistance is important to activate PWK/Pas NK cells.

4.4 Segregation analysis of MCMV-resistance

Crosses between MCMV-resistant PWK/Pas and MCMV-susceptible BALB/c mice were set to study the PWK/Pas resistance trait. (BALB/c x PWK/Pas)F1 progeny show a fully resistant phenotype, since 6 out of 6 mice have splenic viral loads comparable to or lower than resistant PWK/Pas and C57BL/6 mice (Figure 8). The phenotype in the [(PWK/Pas x BALB/c) F1 x BALB/c] N2 backcross progeny (n = 70) spans from low to high viral loads in the spleen indicating that the allele (s) controlling this trait are segregating in this cross (Figure 8). The 70 N2 progeny present a bimodal distribution consistent with a major locus effect. Means of each mode are LOG_{10} PFU 2.2 and 5.8, which are very similar to the values of the MCMV-resistant and MCMV-

Figure 7: Infection of wild-derived mice with WT MCMV and Δm157 MCMV.

Groups of 4 mice were infected with WT MCMV or mutant MCMV lacking m157. Viral titers were determined in the spleen (circles) and liver (squares) by plaque assay 3 days post infection with 5.0×10^5 PFU tissue cultured viruses.

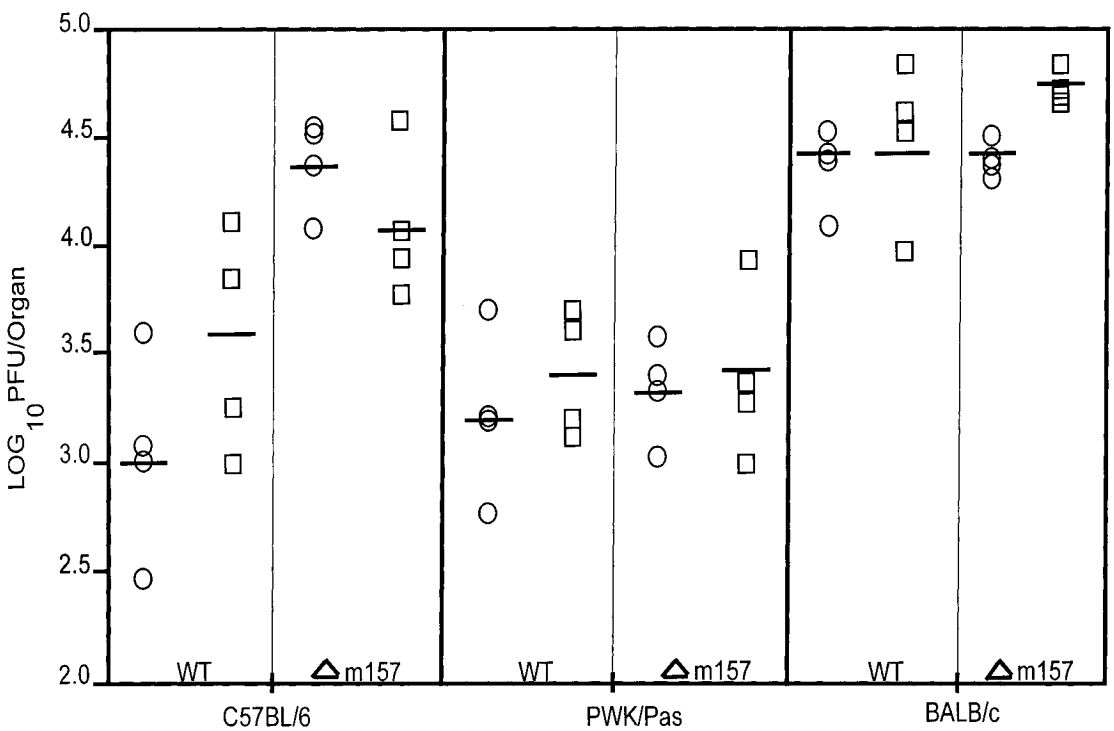
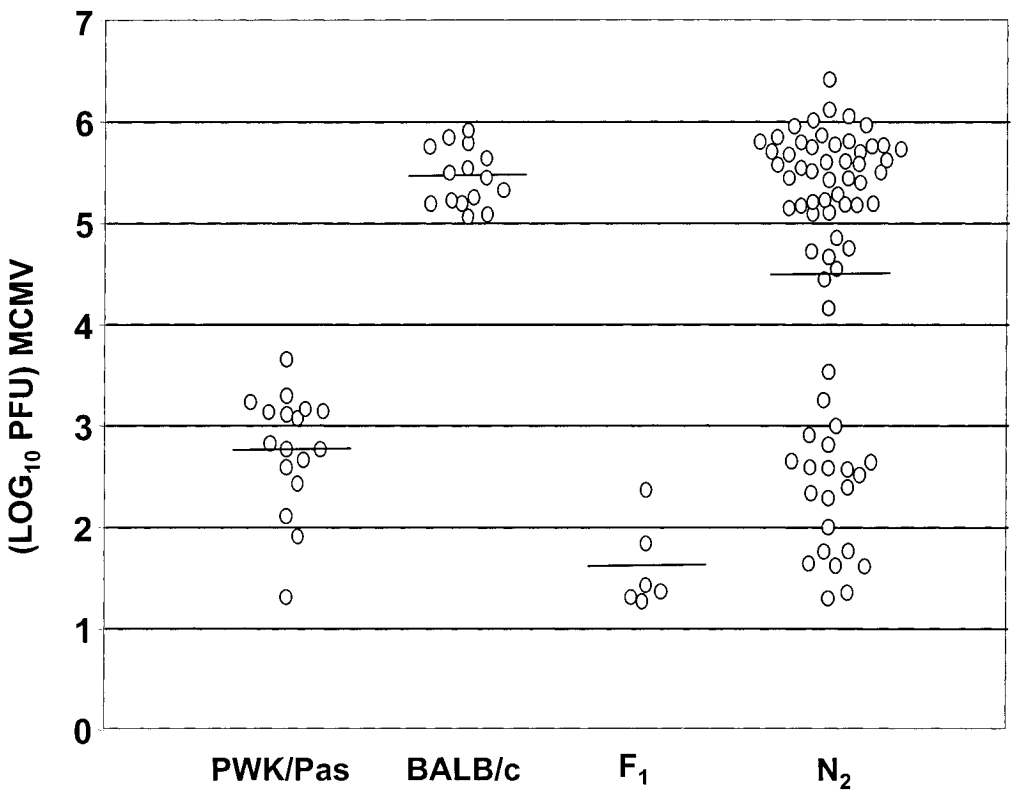

5.0
4.5
4.0
3.5
3.0
2.5
2.0
LOG 10 PFU/Organ
WT
m157
WT
m157
WT
m157
C57BL/6
PWK/Pas
BALB/c

Figure 8: Segregation analysis of MCMV-resistance in PWK/Pas.

Phenotypic distribution of parental PWK/Pas (n=17) and BALB/c (n=11) strains, and of (PWK/Pas x BALB/c) F1 (n=6) and [(PWK/Pas x BALB/c)F1 x BALB/c] N2 progeny (n=70). Data is viral loads in the spleen at 3 days after an i.p. injection of 5 x 10^3 PFU MCMV Smith Strain (Salivary Gland preparation). Mean is indicated for each group by the horizontal bar across the individual symbols. Each symbol represents an individual mouse.


7
6
5
4
3
2
1
0
(LOG10 PFU) MCMV
PWK/Pas
BALB/c
F1
N2

susceptible parental strains (Figure 8). However, 10 % of the N2 cohorts have intermediate values, LOG_{10} PFU 3.8, suggesting that additional genes may influence the phenotype (Figure 8).

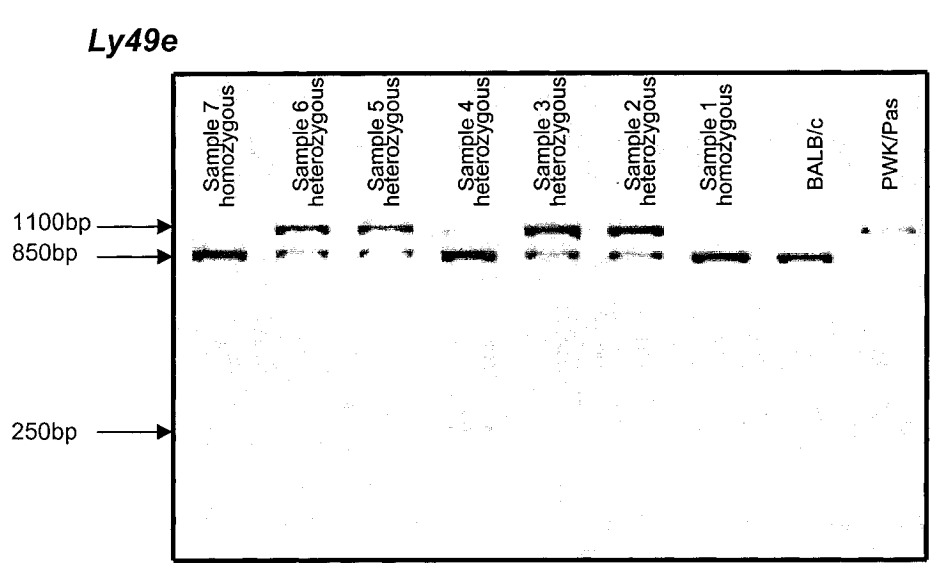
4.5 Genotyping

NK cells are believed to be key players in the natural resistance of wild-derived PWK/Pas mice to MCMV (Girard Adam et al., 2004) (document in preparation). Since preliminary data suggests an Ly49H/m157 independent resistance mechanism, we hypothesize that another gene in the NKC complex, controls the resistance phenotype. Consequently, segregation of the infection phenotype was followed by *Ly49e* marker localized at the extremity of the *Ly49* cluster, at 130.9Mb from mouse chromosome 6, centromere. An additional marker linked to the *H2* locus, *IAA1*, was also used, since the *H2* region has been associated to partial resistance in MCMV in some inbred strains (Chalmer et al., 1977). Genotypes of the 70 [(PWK x BALB/c) F1 X BALB/c] backcross animals were discriminated using these two restriction fragment length polymorphism (RFLP) markers (Figure 9).

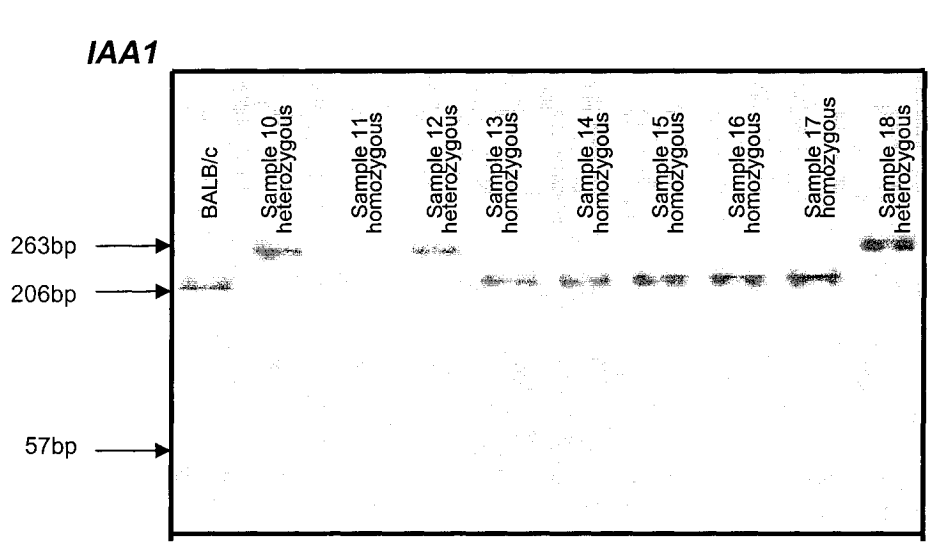
Figure 9: Genotyping of the N2 progeny at the *NKC* and *H2* loci by restriction fragment length polymorphism of PCR products.

Genotypes of the N2 progeny are either BALB/c homozygous or BALB/c x PWK/Pas heterozygous. **a)** Genotyping at the *NKC* locus by RFLP of *Ly49e* marker following *Hinc*II restriction enzyme digest. Amplification of both BALB/c and PWK/Pas genomic DNA with Ly49e primers generates PCR products of 1100bp. Following *Hinc*II digestion, $Ly49e^{BALB/c}$ yields two fragments of 850bp and 250bp, while $Ly49e^{PWK/Pas}$ product remains intact. **b)** Genotyping at the *H2* locus using *Pst*1 digestion of *IAA1* PCR product. Amplification of both BALB/c and PWK/Pas genomic DNA by *IAA1* primers generates PCR product of 263bp in size. Following *Pst*1 digestion, $IAA1^{BALB/c}$ gives two smaller fragments of 206bp and 57bp. In contrast, $IAA1^{PWK/Pas}$ fingerprinting pattern is characterized by three bands of 263bp, 206bp and 57bp in size. Restriction enzyme patterns were visualized by ethidium bromide staining following electrophoresis in 0.5X TBE on a 2% regular agarose gel.

a


Ly49e
Sample 7 homozygous
Sample 6 heterozygous
Sample 5 heterozygous
Sample 4 heterozygous
Sample 3 heterozygous
Sample 2 heterozygous
Sample 1 homozygous
BALB/c
PWK/Pas
1100bp
850bp
250bp

b


IAA1
BALB/c
Sample 10 heterozygous
Sample 11 homozygous
Sample 12 heterozygous
Sample 13 homozygous
Sample 14 homozygous
Sample 15 homozygous
Sample 16 homozygous
Sample 17 homozygous
Sample 18 heterozygous
263bp
206bp
57bp

4.6 Linkage analysis

To evaluate the contribution of the *NKC* and the *H2* loci to the phenotype, we applied the analysis of variance. The statistics supports a two-loci additive model in which both *H2* and *NKC* genes play a significant role in the phenotype determination.

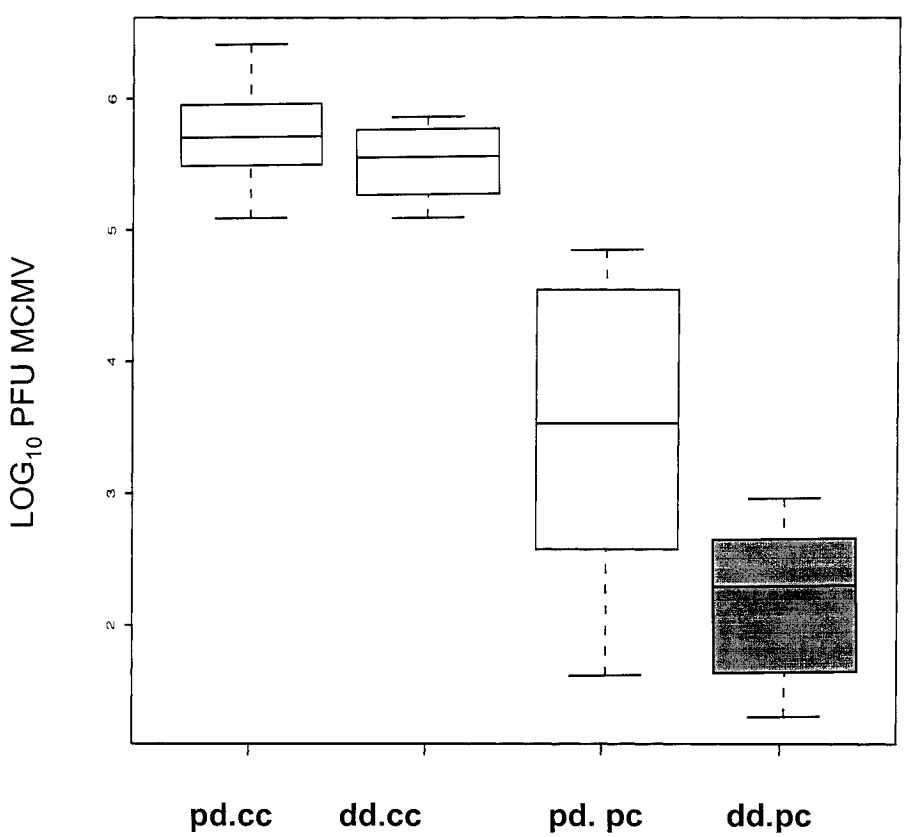
Table 6: Effects of QTLs controlling MCMV infection in [(PWKxBALB/c)F1 x BALB/c] backcross

LOCUS	P-VALUE	LOD SCORE	% VARIANCE
Model	<2.2E-16	25.1	82,1
Ly49e	<2.2E-16	21.9	77,7
IAA1(H2)	1.90E-04	2.7	4,4

The joint LOD score for the model is 25.1 ($P < 2.2e-16$). The proportion of the variation explained by the *H2* locus is estimated to be 4.4% with a LOD score of 2.7 ($P < 1.9e-4$) while for the *NKC* is 77.7% with a LOD score of 21.9 ($P < 2.2e-16$). To visualize the effects of the parental alleles N2 animals were also separated according to their combined *H2* and *NKC* genotypes (Figure 10). Animals carrying the BALB/c allele (cc) at the *NKC* and are either heterozygous (pd) or homozygous (dd) at the *H2* locus are susceptible to infection and present comparable viral loads of LOG_{10} PFU of 5.4 and 5.3, respectively. Results clearly demonstrate that 'p' alleles at the *NKC* are associated with a 2-3 LOG_{10} PFU reduction of viral titers (dd.pc animals). In contrast, it is evident that acquiring a 'p' allele at the *H2* results in an increase of the mean viral titer by more than one LOG_{10} unit. This effect is obvious in animals carrying 'pc' alleles at *NKC* (pd.pc animals) (Figure 10).

Figure 10: Genetic analysis of MCMV-resistance in PWK/Pas.

Box-and-whisker plot per *IAA1.Ly49e* genotype demonstrating the combined effects of *H2* and *NKC* loci on spleen viral titers. Homozygous *NKC* genotypes "cc" ($Ly49e^{BALB/c}$, n=41) are susceptible whereas heterozygous *NKC* genotypes "pc" ($Ly49e^{PWK/pas/BALB/c}$, n=29) are resistant. The two *NKC* genotypes were plotted against homozygous *H2* genotypes "dd" ($IAA1^{BALB/c}$) and heterozygous *H2* genotypes "pd" ($IAA1^{PWK/Pas/BALB/c}$). Bars extend out from a box to the most extreme data point which is no more than 1.5 times the interquartile range from that box. Statistical values are provided in table 6.


LOG10 PFU MCMV
2
3
4
5
6
pd.cc
dd.cc
pd. pc
dd.pc

These results indicated that both *H2* and *NKC* loci are important for MCMV-resistance and that their genetic interaction seems to be implicated in the increase of resistance to the infection.

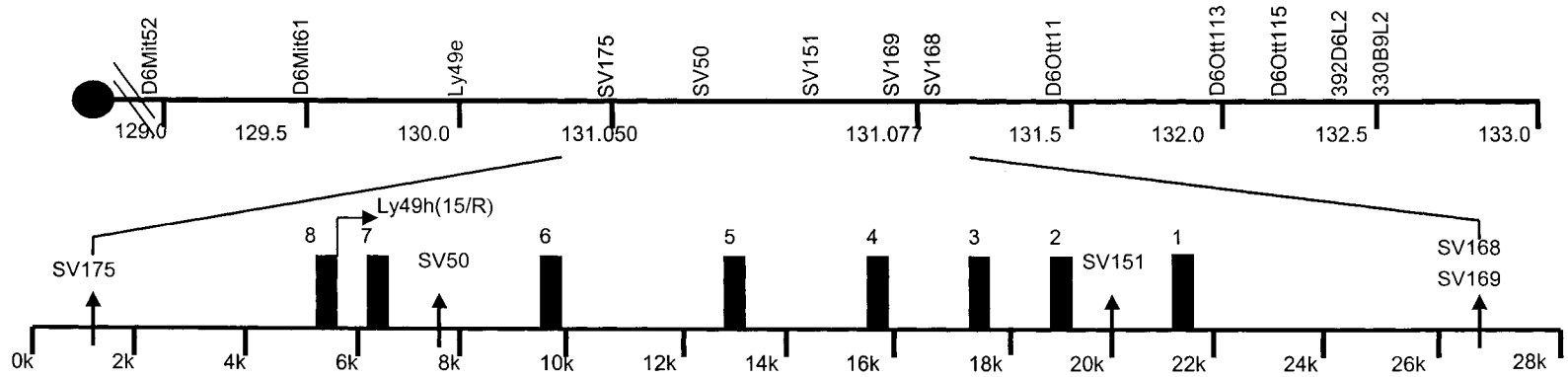
4.7 Haplotype analysis at the *Cmv1* locus

The results of the genetic linkage analysis demonstrated that a gene closely linked to *Ly49e* is responsible for resistance to MCMV infection in PWK/Pas mice. To explore the existence of other *Cmv1*^{r} resistance alleles and to study the genotype/phenotype relationship we determined the allelic composition of a set of linked loci in the vicinity of *Cmv1* and studied their haplotypes in a panel of 11 mouse strains, including the 8 wild-derived inbred strains used in this study, plus C57BL/6, BALB/c and 129/J. The informative DNA markers were either microsatellites (*D6Mit52*, *D6Mit61*, *D6Ott22*, *200H7L*, *D6Ott113*, *D6Ott115*, *392D6L2* and *D6Mit301*) or PCR-RFLPs (*Nkg2d*, *Ly49e*, *D6Ott11* and *282h8Sp6*). We also used three markers that were amplified from C57BL/6J DNA but did not yield a PCR product from BALB/cJ including *330B9L2*, *Ly49n* and 6 novel markers (*SV175*, *Ly49h(15R)*, *SV50*, *SV151*, *SV169* and *SV168*) overlapping the *Ly49h* gene (Figure 11a). Markers *SV175*, *Ly49h(15R)*, *SV50*, previously thought to be C57BL/6 specific, were actually amplified in several wild-derived strains. Sequence DNA analysis of those genomic markers led to the identification of 24 single nucleotide polymorphisms (SNPs), which are included in the study (Figure 11b). Each locus present 2 to 8 alleles that were arbitrarily named 1 to 8 according to the size, 1 being the shortest form. For two-allele systems detected by restriction enzyme digestion, alleles were coded either "A" or "B" (Figure 11b). Remarkably, the haplotypes of PWK/Pas and C57BL/6 are similar in the *Ly49h* region with the highest number of SNPs

Figure 11: Haplotype mapping in the vicinity of *Cmv1* locus.

a) Physical distance of markers used for haplotype mapping. **b)** Haplotype map of chromosome 6 with 20 polymorphic markers. Numbers indicate PCR product size for microsatellite markers. A color was assigned to all mouse strain. PCR-RFLP markers are distinguishable from microsatellite markers, the alleles are scored using letters A and B. SNPs were identified by direct sequencing. They are indicated by their position within the PCR product of origin (*SV175*, *Ly49h(15R)* and *SV50*). Blank squares indicate no PCR product. Individual alleles were arbitrarily color-coded, with pink representing C57BL/6 alleles.

a



b

	D6Mit52	D6Mit61	NKG2D-11	Ly49e	SV175					Ly49h-15/R								SV50										SV151	SV169	SV168	Ly49n	D6Ott22	D6Ott11	SV92	200H7L	D6Ott113	D6Ott115	392D6L2	330B9L2	D6Mit301
					57	77	104	139	231	26	54	58	73	130	184	186	229	25	28	42	65	91	112	188	190	202	204	213												
C57BL/6																																							
PWK/Pas	6	2	B			a		-									g	a				g			t								4	A	6	2	2	2		2
MBT	2	2	B		a	a	g			c		g					g				c	g	a		t	t								A	5	1	5	2		2
CAST/Ei	4	5	A		a	a				c		g					g																6	A	5	1		1		1
MAI	6	1	B		a	a				c		g	a				g	a			c	g	a		t	t							2	A	5	1	3	2		
STF		3	B			a			t	c		g			t	t	g	a		t	c	g	a	a	t	t		c					7	A	1		4	5		7
SEG		1	B							c	c	g	a	t				a	c		c	g	a		t	t							8	A	1		1	3		7
WLA	4	5	B																		c	g			t								7		2	3	2			4
WMP	1	1																a			c	g	a		t	t							7		3	3	8			6
129/J	3	8																															5			3				4
BALB/c	4	6	C	A																													1	A	5	1	5	2		4

conserved between these two strains. C57BL/6 and PWK/Pas shared 17/24 SNPs, with MBT/Pas and MAI/Pas following with 13 and 12/24 common SNPs with C57BL/6. In contrast, the remaining mouse strains (WLA/Pas, WMP/Pas, CAST/Ei, SEG/Pas and STF/Pas) share less than 10 SNPs when compared to C57BL/6 mice. The similarities in haplotypes between PWK/Pas and C57BL/6, supports the idea that PWK/Pas have a Ly49H-related receptor that may be a candidate responsible for the MCMV-resistant phenotype.

4.8 cDNA cloning of the PWK/Pas Ly49 repertoire

To identify alternative *Ly49*genes encoding activating receptors which may be responsible for the PWK/Pas resistance phenotype, we used specific primers to amplify transcripts of activating (Ly49D, Ly49H, Ly49N, Ly49K, and NKG2D) and inhibitory (Ly49I and Ly49B) receptors extracted from RNA of NK cell-enriched splenocytes. The receptors that were cloned and sequenced are represented with their closest relative in table 7.

Table 7: Characterization of the *Ly49* gene cluster in PWK/Pas mouse strain

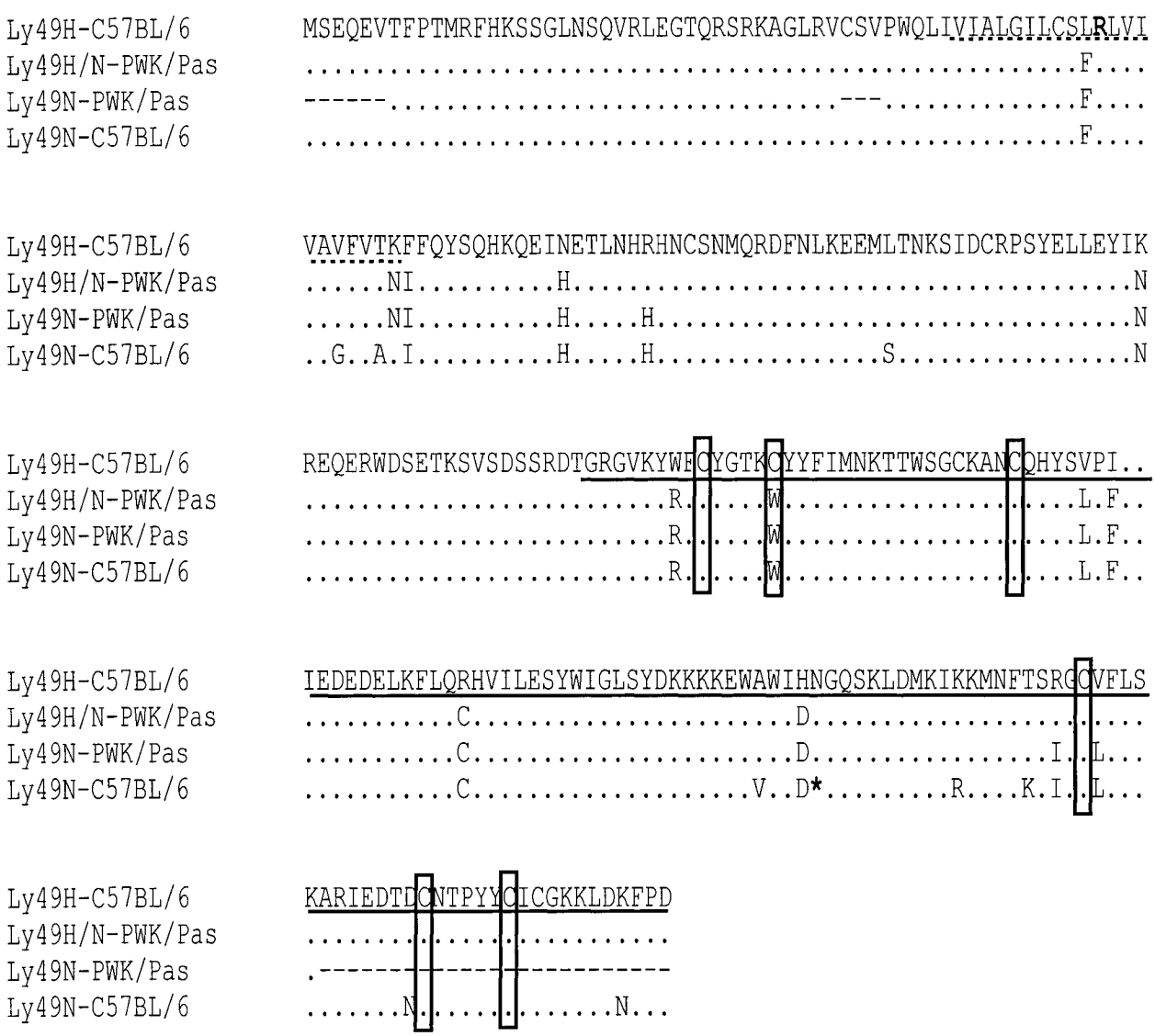
Types of Receptors	Novel PWK Receptors	Receptors Closest Relative	% Identity Nucleic Acid Level	% Identity Amino Acid Level
ACTIVATING				
	Ly49PK2	$Ly49D^{C57BL/6}$	99	99
	Ly49PK7	$Ly49D2^{NOD}$	98	97
	Ly49PK3	$Ly49N/H^{C57BL/6}$	97	96/95
	Ly49PK5	$Ly49N^{C57BL/6}$	97	98
	Ly49PK6	$Ly49K^{C57BL/6}$	88	80
	NKG2D-PK	$NKG2D^{C57BL/6}$	99	99
INHIBITORY				
	Ly49PK1	$Ly49B^{C57BL/6}$	98	100
	Ly49PK4	$Ly49I^{C57BL/6}$	99	99

In line with the haplotype similarity between C57BL/6 and PWK/Pas, the sequences of the PWK/Pas cloned genes share most homology to C57BL/6 genes. In total, 6 activating receptors (Ly49PK2, Ly49PK3, Ly49PK5, Ly49PK6, Ly49PK7 and NKG2D-PK) were sequenced in PWK/Pas. The derived amino acid identity among activating receptors spans from 80% for Ly49K to 99% for Ly49D. In contrast, the 2 inhibitory receptors (Ly49PK1 and Ly49PK4) are allelic to $Ly49B^{C57BL/6}$ and $Ly49I^{C57BL/6}$ and share respectively 100% and 99% identity at the amino acid level. Only one pseudogene, Ly49PK6, closely related to $Ly49K^{C57BL/6}$ was identified.

Interestingly, Ly49PK3 activating receptor amplified by *Ly49h* specific primers shows a very high degree of sequence homology with $Ly49H^{C57BL/6}$ and $Ly49N^{C57BL/6}$ proteins. The proteins are the same length (266 residues), with 95% and 96% identity at the amino acid level, when compared with $Ly49H^{C57BL/6}$ and $Ly49N^{C57BL/6}$ (restored reading frame) respectively. Ly49PK3 and $Ly49H^{C57BL/6}$ receptors differ by eleven amino acids, but only Ly49H is known to bind the MCMV encoded protein, m157. Amino acid sequence alignment of these two receptors revealed that substitutions were not randomly distributed along the length of the protein: six of the eleven substitutions occur within the NKD, three in the stalk extracellular domain and two in the transmembrane domain (TM) (Figure 12). The high degree of sequence and structural homology between Ly49PK3 and $Ly49H^{C57BL/6}$ made Ly49PK3 the most interesting candidate to test. Their similarities may suggest that they play a role in a common functional aspect; the recognition of MCMV virally infected cells.

Figure 12: Alignment of Ly49PK3 a novel member of the Ly49H/N/K cluster.

Amino acid identity with Ly49H$^{C57BL/6}$ is indicated by a dot and gaps are represented by a dash. Transmembrane and natural killer receptor domains are respectively represented by a dash and solid line. Arginine residue characteristic of activating receptors is in bold. Ly49N$^{C57BL/6}$ reading frame was restored. (*) represent stop codon naturally found in Ly49N$^{C57BL/6}$ sequence. Cysteins that are critical for disulfide bond formation are boxed.

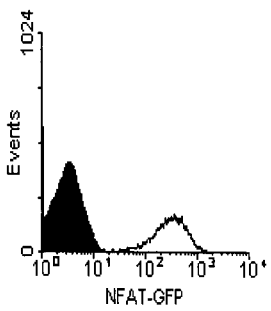
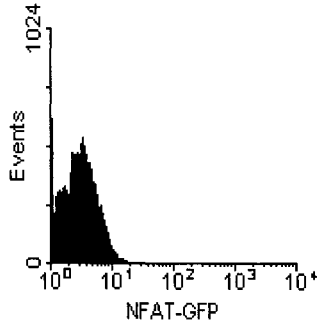
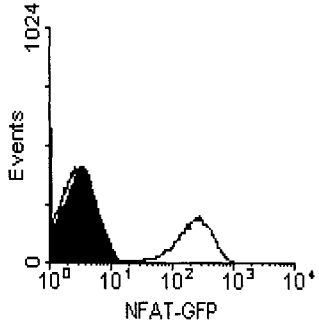
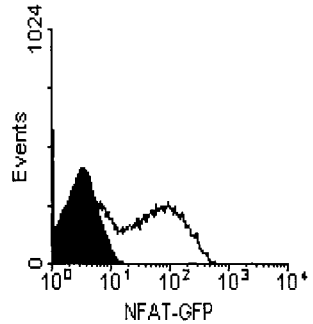
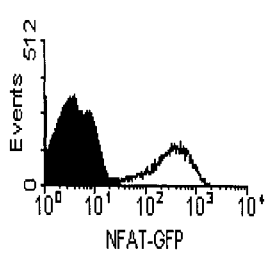
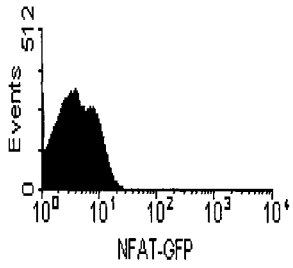
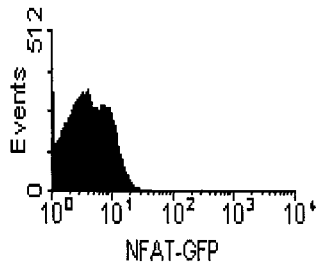
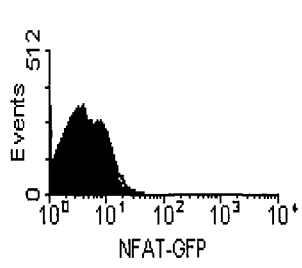

Ly49H-C57BL/6 MSEQEVTFPTMRFHKSSGLNSQVRLEGTQRSRKAGLRVCSVPWQLIVIALGILCSLRLVI
Ly49H/N-PWK/Pas .....F....
Ly49N-PWK/Pas ------.....---.....F....
Ly49N-C57BL/6 .....F....
Ly49H-C57BL/6 VAVFVTKFFQYSQHKQEINETLNHRHNCSNMQRDFNLKEEMLTNKSIDCRPSYELLEYIK
Ly49H/N-PWK/Pas .....NI.....H.....N
Ly49N-PWK/Pas .....NI.....H.....H.....N
Ly49N-C57BL/6 ..G..A.I.....H.....H.....S.....N
Ly49H-C57BL/6 REQERWDSETKSVSDSSRDTGRGVKYWFCYGTKCYYFIMNKTTWSGCKANCQHYSVPI..
Ly49H/N-PWK/Pas .....R.....W.....L.F..
Ly49N-PWK/Pas .....R.....W.....L.F..
Ly49N-C57BL/6 .....R.....W.....L.F..
Ly49H-C57BL/6 IEDEDELKFLQRHVILESYWIGLSYDKKKKEWAWIHNGQSKLDMKIKKMNFTSRGCVFLS
Ly49H/N-PWK/Pas .....C.....D.....
Ly49N-PWK/Pas .....C.....D.....I..L...
Ly49N-C57BL/6 .....C.....V..D*.....R....K.I..L...
Ly49H-C57BL/6 KARIEDTDCNTPYYCICGKKLDKFPD
Ly49H/N-PWK/Pas .....
Ly49N-PWK/Pas .----------
Ly49N-C57BL/6 .....N.....N...

4.9 Functional analysis of a candidate receptor

Similarly to Ly49H, a receptor involved in MCMV-resistance should recognize an MCMV-infected cell. To test this we have expressed the *Ly49pk3* candidate gene in a reporter cell system to be used in an *in vitro* functional assay (Arase et al., 2002). 2B4 cells expressing Ly49H receptor were used as a control. NFAT-GFP synthesis following culture of Ly49H and Ly49PK3 reporter cells on anti-FLAG coated plates confirmed proper intracellular signaling of the reporter gene (Figure 13). 2B4 Ly49H reporter cells recognize both Ba/F3-m157 and MCMV infected cells, demonstrated by GFP expression (Figure 13). In contrast, upon co-culture of Ly49PK3 reporter cells with Ba/F3 cells expressing m157 or MCMV-infected fibroblasts, no GFP signal is detected, suggesting that Ly49PK3 does not bind m157 nor any other MCMV encoded protein (Figure 13). These results suggest that PWK/Pas host defense mechanism against MCMV is independent of Ly49PK3.

Figure 13: Functional analysis of Ly49PK3 receptor.

Surface expression of Ly49PK3 receptor was indirectly confirmed by staining of the adaptor protein DAP-12 with Anti-Flag antibody (not shown). In addition Anti-Flag crosslinking was used to confirm that NFAT-GFP pathway is functional in the receptor expressing cells (i). Overnight co-culture of 2B4-Ly49PK3 reporter cells with target cells Ba/F3-wt (ii), Ba/F3-m157 (iii) or MCMV infected NIH-3T3 cells (iv) did not activate the NFAT-GFP pathway, indicating the absence of the Ly49PK3 ligand. 2B4-Ly49H reporter cells were used as positive control and show activation when co-cultured with Ba/F3-m157 or MCMV infected NIH-3T3 cells.

	(i) FLAG	(ii) Ba/F3-wt	(iii) Ba/F3-m157	(iv) MCMV
2B4 Ly49H				
2B4 Ly49PK3				

5. DISCUSSION

5.1 Resistance to cytomegalovirus in wild-derived strains

The mouse is used as a laboratory animal for more than 300 years (Morse CH, 1978). Classical laboratory mice are excellent experimental models for studying simple phenotypic traits. However, their limited genetic variability is a major drawback in unraveling key players implicated in complex traits. The use of wild mice to investigate the genetic determinism of complex phenotype, including infectious diseases, is becoming the new trend (Guenet, 1998). Wild-derived inbred strains harbors novel genetic polymorphisms, when compared to classical inbred strains which can lead to the identification of several new and potentially interesting genes (Guenet, 1998).

The introduction of various kinds of infectious agents into mice reveals that some strains are more susceptible than others, with wild-derived strains generally more resistant than classical laboratory strains (Gardner, Kozak, & O'Brien, 1991; Sangster, Mackenzie, & Shellam, 1998; Bagot et al., 2002) (Mashimo et al., 2002). In classical inbred strains, genes involved in immunity could be replaced by defective mutant alleles without consequences for the mice, which are kept in protected environments. Surprisingly, most wild-derived strains in our study infected with mouse cytomegalovirus are as susceptible to the virus as most classical inbred strain, including BALB/c. In contrast, two strains derived from *M.spretus*, SEG/Pas and STF/Pas are resistant and present very low viral titers in the spleen as well as in the liver, likely reflecting the incapability of MCMV to replicate in the *M. spretus* cells, rather than successful immune responses in the host. Interestingly, just like members derived from *Mus spretus*, another short-tailed mouse strain, *L. lakedownensis*, is resistant to cytomegalovirus. *L. lakedownensis* and *M.domesticus* are the two only mammals found on Thevenard Island off the northwest

coast of Western Australia. The introduction of the house mouse (*M. domesticus*) on the island, carrying novel source of infection raised concern for the endangered short-tailed mouse, *L. lakedownensis* (Moro, Lloyd, Smith, Shellam, & Lawson, 1999). To assess the threat Moro and colleagues challenged *L. lakedownensis* with various common murine viruses, including cytomegalovirus. As it is suggested for SEG/Pas and STF/Pas, the failure to isolate MCMV from the spleen and the liver from *L. lakedownensis* infected mice, indicates that the virus is unable to replicate in these tissues and therefore, may not be able to infect this host. MCMV infection on Thevenard Island is therefore restricted to its natural house mouse (*M. domesticus*) host (Moro et al., 1999). Taking into account the strict species-specificity of MCMV and the 2 million years divergence between *M. spretus* and *M. musculus*, our results were not unexpected.

PWK/Pas, another wild-derived strain, is resistant to cytomegalovirus. Interestingly, this *M. musculus*-derived strain presents an infection pattern very similar to that of resistant C57BL/6 mice, with low viral titers in the spleen and higher in the liver. Such a phenotype is consistent with an NK-cell mediated mechanism of resistance, similar to the one present in C57BL/6 mice. However, contrary to the C57BL/6 strain, PWK/Pas mediated resistance against MCMV is both Ly49H and m157 independent. PWK/Pas cells show some reactivity with anti-Ly49H intracellular antibody compared to Ly49H-negative BALB/c cells. However, the bimodal distribution and bright intensity typical of Ly49H+ NK cells observed in C57BL/6 cells, is absent in PWK/Pas, suggesting the presence of host resistance mechanisms independent of Ly49H receptor in this strain. Furthermore, in contrast to C57BL/6, PWK/Pas mice infected with mutated Δm157 MCMV are resistant to infection, indicating mechanisms also independent of m157. This result is not entirely surprising since a recent study by Voigt et al. has shown that while

m157 is crucial for the activation of Ly49H$^+$ NK cells, most wild isolates of MCMV (~86%) present mutations in m157 (Voigt et al., 2003). Even if the majority of MCMV strains from wild mice fail to activate NK cells via Ly49H, an NK cell response might be mediated by interaction with other activating receptors, such as NKG2D or other activating members of the Ly49 receptor family. Altogether, the pattern of viral growth in target organs, low levels of staining with Ly49H antibody and resistance to Δm157 support our hypothesis that mechanisms other than Ly49H-m157, are important to activate PWK/Pas NK cells.

5.2 The *NKC* harbors multiple resistance mechanisms against MCMV

The implication of NK cells is also supported by the observation that cytomegalovirus resistance in PWK/Pas is linked to the *Ly49* gene cluster at the *NKC*. Similar to C57BL/6, PWK/Pas mediated resistance is controlled by a major dominant gene effect which explains more than 70% of the phenotypic variance. However, it was interesting to detect a minor contribution of the *H2* complex. Viral titers of mice carrying PWK/Pas alleles at the *NKC* and *H2* are significantly higher than those of mice homozygous at *H2*. These results indicate that both *H2* and *NKC* loci are important for MCMV-resistance and that their genetic interaction seems to be implicated in the increase of resistance to the infection. For example, a two-step process could be proposed. *H2* molecules have been shown to play an important role in virus entry, a critical step for successful colonization of the host. D^b transfected macrophages are more susceptible to infection than those transfected with D^d or D^k (Price et al., 1990). Interestingly, the *IAA1* polymorphism for PWK/Pas is identical to that of C57BL/6, $H2^b$, which is the most permissive *H2* haplotype for MCMV infection. Alternatively, a physical interaction

between inhibitory NK cell and *H2* receptor gene products may attenuate NK cell killing activity against infected cells. In fact, the lack of interaction between an inhibitory receptor and an MHC class I molecule has been recently proposed in a study addressing the contribution of KIR-receptors, the functional analogues of Ly49 receptors, and their MHC class I ligands in susceptibility to hepatitis C (Khakoo et al., 2004). Regardless of the role of *H2*, our experiments provided overwhelming evidence of a novel mechanism of MCMV-resistance in PWK/Pas mice, tightly linked to the *Ly49* cluster at the *NKC*.

Despite the presence of a mechanism of resistance distinct of Ly49H, detailed analysis of the NKC domain showed that the haplotypes of PWK/Pas and C57BL/6 are similar in the *Ly49h* region with the highest number of SNPs conserved between these two strains, indicating a possible ancestral relationship at this region. The similarity in the *Ly49h* region is remarkable, in view of the variability observed in a study by Montagutelli and colleagues, revealing marked differences over the whole genome (70% polymorphism in 67 markers tested) between C57BL/6 and PWK/Pas mice (Montagutelli, Serikawa, & Guenet, 1991). NKC haplotype studies in a panel of 22 common inbred strains by Brown et al., reveals that the combination of alleles is highly conserved among genealogically related inbred strains. In contrast, far less similarity is observed among unrelated strains (Brown et al., 2001b). In agreement with this study, wild-derived strains belonging to the same taxa of the *M .musculus*, share the highest number of SNPs in the *Ly49h* region. Apart from PWK/Pas, which shares greater similarities with C57BL/6 strain, the other members of the *M. musculus musculus* subspecies MBT/Pas and MAI/Pas show greater similarities among each other than any other classical inbred or wild-derived strains tested. The same tendency is observed between the members of the *Mus musculus domesticus* subspecies, WLA/Pas and WMP/Pas. Likewise, SEG/Pas and

STF/Pas, which originate from the most distantly related species of the *genus Mus*, *Mus spretus*, share fewer alleles with other mouse strains when their haplotypes are compared in the vicinity of *Ly49h*. Allelic variability at the NKC among wild-derived and classical strains of mice, demonstrated by our work as well as Brown and colleagues is not surprising, since high level of variation is typical of regions containing immune related genes. Just like the *LRC* locus comprising KIR receptors in humans, the *NKC* exhibits extreme levels of polymorphism deploying the possibility of a wide range of defense options against rapidly evolving pathogens.

Haplotype analysis is an informative tool to study the correlation between genotypes and a specific phenotypic trait. For example, Lee and colleagues previously studied the possible genotype/phenotype relationship between *Cmv1* haplotypes and MCMV resistance or susceptibility (Lee et al., 2001b). The haplotype studies in the vicinity of *Cmv1*, which is allelic to *Ly49h*, allowed definition of 3 distinct combinations of alleles in this region: 1) a resistant haplotype, named $Cvm1^{r}$ uniquely present in C57BL/6. 2) a susceptible haplotype, named $Cmv1^{sFVB}$, present in 129/J, SM/J1, SJL/J, FVB/NJ, C57/J and NZB. 3) a second susceptible haplotype, $Cmv1^{sBALB/c}$, present in C3H/HeJ, DW/J, BALB/c, A/J, DBA1/J, DBA1/2J, CBA/CaH, LP/J and AKR. $Cmv1^{sBALB/c}$ is completely different from $Cmv1^{sFVB}$ while transgenic transfer of Ly49H into $Cmv1^{sFVB}$ restores MCMV-resistance, indicating that MCMV susceptibility has two independent origins (Lee et al., 2001b). The presence of different haplotypes at this region raised the possibility that alternative *Ly49* genes may control MCMV resistance or susceptibility in different mouse strains, like PWK/Pas. The highly related haplotypes of both C57BL/6 and PWK/Pas, suggest that these two strains probably have a similar Ly49

repertoire, which supports the idea that an Ly49H-related receptor is a potential candidate for the MCMV-resistance phenotype in PWK/Pas.

5.3 Candidate receptors in MCMV-resistance for PWK/Pas strain

Natural killer cell receptors including *KIR* and *Ly49* multigene families as well as the MHC gene cluster, for which diversity is essential, must evolve to counteract pathogens which attempt to outsmart the immune system and avoid recognition or elimination. The members of a multigene family that are not under selective pressure have a tendency to exchange sequence information with one and other, which lead to a high degree of intra-familial sequence homogeneity (Li, 1991). In contrast, it appears that the *Ly49* multigene family diversifies during evolution in a process driven by their respective ligands. Variation of Ly49 inhibitory receptors is driven by MHC class 1 molecules which are under selective pressure by pathogens. In contrast, variation of Ly49 activating receptors would be driven directly by pathogens. Selective evolution is also predicted for the KIR multigene family in humans. In fact, comparative studies between human and chimpanzees suggest that KIRs evolve at a higher rate than MHC proteins (Khakoo et al., 2000). While some comparison between the MHC and *Ly49* gene clusters can be drawn, the *Ly49* gene family is unique in two ways. As opposed to the MHC, the *Ly49* cluster has not expanded to a multigene family in a large number of species. In fact it appears to be restricted to rodents. In addition, the evolution of activating receptors, which have presumably diverged from their inhibitory counterparts, suggest that the *Ly49* cluster have undergone a functional split. This was also observed within other NK cell receptors, including KIRs (Taylor, Paul, & McVicar, 2000). The evolution of activating receptors appears advantageous to organisms in their fight against microorganisms, as it

was demonstrated for *Ly49h* (Lee et al., 2001a). Activating receptors involved in pathogen recognition must be under the selective pressure of these pathogens. Hence, the characterization of the Ly49 repertoire in MCMV resistant PWK/Pas strain is interesting as an attempt to identify alternative Ly49 activating receptors that could mediate resistance in that strain. We can speculate that resistance allele (s) protecting PWK/Pas from MCMV infection would be positively selected.

The *Ly49* multigene family encodes for inhibitory and activating receptors that vary in number and sequence in different mouse strains. The characterization of the Ly49 repertoire for both C57BL/6 and 129/J respectively revealed the presence of 16 and 19 members of the *Ly49* multigene family in these strains (McQueen et al., 1998; Wilhelm et al., 2002) (Makrigiannis et al., 1999; Anderson et al., 2001). The repertoire of 129/J mouse strain comprises: *Ly49e,g,l,o,p,r,s,t,u* and *v*. Only *Ly49e*, *g* and *i* were found to be allelic forms of the respective C57BL/6 genes, underlying the variability among members of the *Ly49* gene family from one strain to the other. However, in line with the haplotype similarity between C57BL/6 and PWK/Pas, the sequences of the PWK/Pas cloned genes were mostly closely related to the C57BL/6 genes. In C57BL/6 mice five different activating genes have been identified. Two of them, *Ly49h* and *Ly49d* are functional, whereas *Ly49l*, *Ly49m*, *Ly49n*, and *Ly49k* are pseudogenes (Wilhelm et al., 2002). These genes are potential alternative candidate receptors in MCMV-resistance for PWK/Pas strain. In contrast to C57BL/6, PWK/Pas does not express a *bone fide Ly49h* gene or receptor. However, the predicted amino acid sequence of Ly49N/H$^{PWK/Pas}$ (Ly49PK3) receptor, amplified by *Ly49h* specific primers show a very high degree of sequence homology with Ly49H$^{C57BL/6}$ and Ly49N$^{C57BL/6}$, which could have arisen by gene duplication. The conserved intracellular domain between Ly49N/H$^{Pwk/Pas}$ and

Ly49H$^{C57BL/6}$ might explain the weak binding of PWK/Pas NK cells to anti-Ly49H antibodies, which recognize a Ly49H specific intracytoplasmic epitope. In C57BL/6 mice, *Ly49n* transcripts were reported to be altered forms due to frameshifts (McQueen et al., 1998). Interestingly, *Ly49n* specific primers have allowed the identification of a putative functional Ly49N receptor in PWK/Pas (Ly49PK5), which shares high sequence identity with Ly49N from C57BL/6 (restored reading frame). The presence of a functional *Ly49* gene in one strain and not in another was previously demonstrated in NOD mice. For example, *Ly49m* in NOD is functional whereas in the same gene is non-functional in C57BL/6 strain (Silver et al., 2001). Similar to *Ly49k* from C57BL/6, which has a termination codon at the end of exon 4 and a partial deletion of exon 5, *Ly49k* in PWK/Pas is also defective (McQueen et al., 1998) . In agreement with what was observed in C57BL/6, the only pseudogene identified in PWK/Pas encodes a potential activating receptor. It appears that selection has prevented the accumulation of potential harmful mutations in inhibitory receptors in both classical inbred and wild-derived mouse strains. In contrast, mutations appear more predominant in activating receptors. Two other functional activating receptors, respectively related to Ly49D from both C57BL/6 and NOD strains, were identified and are considered potential candidates.

Base on both, linkage and haplotype analysis, Ly49 activating receptors remain primary candidates for MCMV-resistance in PWK/Pas. Another interesting candidate is NKG2D, an activating C-lectin type receptor also located in the NKC, which recognizes several MHC class I like stress induced molecules in response to MCMV infection (Lodoen et al., 2003). Several MCMV-encoded ORFs were reported to downregulate the expression of NKG2D targets, to escape NK cell killing (Krmpotic et al., 2002) (Lodoen et al., 2004). It is possible that PWK/Pas cells are somewhat resistant to evasion strategies

adopted by MCMV. For example, MCMV may fail to downmodulate NKG2D ligands in MCMV-infected PWK/Pas cells. However, linkage analysis would most probably report association to chromosome 10, where the NKG2D ligands are located, rather than the *NKC* as we observed. In addition, analysis of PWK/Pas and C57BL/6 NKG2D receptors indicated 100% sequence identity in the NKD, excluding *Nkg2d* as a potential resistance gene.

Some structural hallmarks of Ly49 activating receptors, namely, the absence of the ITIM motif as well as the presence of the positively charged amino in the transmembrane domain are strictly conserved among all potential Ly49 receptors identified. Proper protein structure partly relies on the presence of key amino acids that allow different types of bonds and interactions. Two adjacent cysteine residues in the 3D structure of a protein can oxidize and form disulfide bonds. These types of bonds are important for the stabilization of the protein, which makes it less sensitive to degradation (Brandon C & Tooze J, 1996). Six invariant cysteine residues (amino acid positions: Cys_1149, Cys_2154,Cys_3171,Cys_4236, Cys_5249 and Cys_6255) were identified in the extracellular domain of all lectin-like NK cell receptors. Pairs of these residues are believed to form intra-chain disulfide bonds (Yokoyama et al., 2003). All the candidate Ly49 receptors identified, except Ly49PK3, are characterized by this structural hallmark. *Ly49pk3* was expressed in a reporter cell system and failed to recognize any MCMV encoded molecules. The substitution of Cys_2154Trp might have an effect on the stability and global structure of the receptor and therefore influence ligand recognition.

Although closely linked to the *Ly49* gene cluster, the newly defined locus mediating PWK/Pas MCMV-resistance was not yet determined as allelic to any

alternative activating Ly49 receptors. However, members of this gene family remain the main prospects, whereas several functional Ly49 activating receptors could still be tested. Known ligands for Ly49 receptors, including Ly49H are MHC class I or MHC class I-like molecules. Consequently, we can speculate that the ligand triggering NK cell-mediated lysis of MCMV-infected cells in PWK/Pas may be an MHC-related molecule encoded by the host or the virus. Interestingly, in their attempt to identify the ligand for Ly49H receptor (m157), Smith and colleagues identified at least 11 other ORFs (m17, m37, m90, m144, m145, m150, m151, m152, m153, m155, m159) encoding molecules with putative MHC class 1-like fold (Smith et al., 2002). As previously proposed for Ly49H in C57BL/6 mice, m144 (Lee et al., 2001a) or any other MHC class I like molecule could serve as a ligand for an unknown PWK/Pas activating receptor. This scenario would promote delivery of a positive signal and subsequent target cell killing. This outcome could be achieved by the absence of an MHC class I-like inhibitory receptor or by competition between a unknown activating receptor and the inhibitory receptor for MHC class I like-molecule binding. In the case of PWK/Pas resistant strain, the ligand would promote an activating signal capable of overriding the MHC class I like molecule-elicited inhibitory signals. Hence, there are two plausible models that may explain MCMV-resistance in PWK/Pas. One would involve the recognition of a virus/host MHC class I-like molecule by a new activating receptor, whereas the other would be based on the absence of an inhibitory receptor MHC class I-related protein binding. Our results revealed many similarities between PWK/Pas and C57BL/6 strains, for example: indistinguishable MCMV infection pattern, highly related haplotype in the vicinity of *Ly49h* and great homology between PWK/Pas Ly49 activating receptors to Ly49H C57BL/6. Base on these observations, similar to the defense mechanism (Ly49H-

m157) encountered in C57BL/6, the presence of a new Ly49 activating receptor is most likely to mediate MCMV-resistance in PWK/Pas.

5.4 Implication for human studies

The functional analogues of Ly49 receptors are Killer Immunoglobulin-like Receptors (KIR) that, much like rodent Ly49, control NK cell functions and show allelic polymorphism. Similar to haplotype variations encountered in mice, KIR haplotypes vary in number and type of genes present from one individual to another (Vilches & Parham, 2002). Because KIR ligands (HLA molecules) map to separate chromosomes than the receptor itself, some individuals will lack specific KIR-HLA receptor ligand pairing. Interestingly, despite the divergence between Ly49 and KIR receptors, their mechanisms of intracellular signal transduction are remarkably conserved (Colucci et al., 2002a). Recent evidence suggests that human KIR may recognize viral pathogens using mechanisms similar to the mouse NK cells. In fact, it was reported that the presence of the activating KIR3DS1, along with alleles of HLA-B, have an epistatic protective effect on AIDS progression (Martin et al., 2002). Until recently only activating KIRs have been associated with disease outcome. Interestingly, inhibitory KIR2DL receptor interaction with HLA-C is associated with resolving hepatitis C virus infection (Khakoo et al., 2004). Similarly, we demonstrated that the MHC in the mouse has an influence in the control of mouse cytomegalovirus. In fact, similar to what is observed in the control of hepatitis C, a mechanism involving an inhibitory receptor was suggested. Our results emphasized that more than one mechanism of defense exists in control of MCMV infection. KIR receptors play a role in HCMV susceptibility (Huard & Fruh, 2000; Lopez-Botet, Angulo, & Guma, 2004). However, it is reasonable to predict that similarly to the mouse model, alternative

KIR mechanisms may have an effect. A better understanding of the mouse model between NK cell receptors and their viral and cellular ligands with which they interact will provide essential information to the identification of human candidate genes. The identification of novel natural killer cell receptors involved in the control of cytomegalovirus infection may eventually lead to manipulation of NK cells in therapeutic strategies for the treatment of this disease.

6. CONCLUSION

We have presented genetic and immunological evidence for a new MCMV resistance trait. NK cells protect wild-derived PWK/Pas mice against MCMV by mechanisms that are independent of the recently described Ly49H-m157 interaction. The resistance trait is linked to the *NKC* and influenced by the *H2*. Our results show that PWK/Pas mice possess unprecedented NK activating receptors that may recognize new virally encoded or virally induced ligands. Laboratory mouse strains, as a result of their unnatural life style, are thought to allow for the accumulation of susceptibility genes that are detrimental for immune responses. On the contrary, some wild-derived mouse strains, like PWK/Pas, may still bear the product of natural selection that under the pressure of pathogens selects for multiple NK activating receptors. The study of these receptors and their inheritance may shed light on human immune resistance mechanisms to infectious diseases.

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8. APPENDIX

I) Research Compliance Certificate-Animal Protocol