GENETIC INTERACTION BETWEEN *H2* AND NKC RECEPTOR GENES CONFERS INNATE RESISTANCE TO CYTOMEGALOVIRUS INFECTION

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ABSTRACT

In mice, *H2* and *Ly49h* genes determine natural resistance to cytomegalovirus infection by alternate mechanisms. We are interested in MA/My, an inbred strain that is resistant to MCMV infection despite the absence of Ly49H receptor and the presence of a haplotype highly related to the MCMV-susceptible strains 129 and FVB/N. Therefore, it is interesting to study its genetic basis of resistance to MCMV infection. In this study, we have identified $Cmv1^{rm}$, a new resistance allele conferring resistance in MA/My. We have demonstrated by statistical analyses that both *H2* and NKC genes are important and that their genetic interaction is necessary to confer resistance in MA/My. By the characterization of the *Ly49* gene repertoire of MA/My, we identified 3 potential activating *Ly49* gene candidates. Finally, we have confirmed the presence of an important additive effect of *H2* and NKC in a FVB/N x BALB.K cross, indicating that $Cmv1^{rm}$ resistance mechanism may be present in other inbred strains.

RÉSUMÉ

Chez les souris, la résistance à l'infection par le cytomégalovirus est déterminée par les gènes du *H2* et *Ly49h*. Chez la souris MA/My, on dénote une résistance au MCMV malgré l'absence du récepteur Ly49H et la présence d'un haplotype semblable au souris sensibles 129 et FVB/N. Il est donc intéressant d'étudier la base génétique de la résistance à l'infection par le cytomegalovirus chez cette souris. Grâce à cette étude, nous avons identifié un nouvel allele de résistance chez MA/My, nommé $Cmv1^{rm}$. De plus, nous avons démontré que les gènes du *H2* et du NKC sont importants et que leur intéraction génétique est nécessaire pour conférer la résistance chez MA/My. Par la charactérisation du répertoire des gènes *Ly49* chez MA/My, nous avons identifié 3 récepteurs activateurs candidats. Finalement, nous avons confirmé la présence d'un effet additif important entre les gènes du *H2* et du NKC chez FVB/N x BALB.K, indicant que le mécanisme de résistance conferré par $Cmv1^{rm}$ pourrait être aussi présent chez d'autres lignées pures.

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CONTRIBUTIONS OF AUTHORS

Dr. J-C Loredo Osti performed the analysis of variance and Sonia Girard Adam did the haplotype analysis.

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LIST OF ABBREVIATIONS

A_2M	Alpha 2 Macroglobulin
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BAC	Bacterial Artificial Chromosome
bp	Base Pairs
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
CEN	Centromere
CHO	Chinese Hamster Ovary
CID	Cytomegalic Inclusion Disease
Clr	C-type lectin-related
cM	Centimorgan
CMV	Cytomegalovirus
CTL	Cytotoxic Lymphocyte
DAP	DNAX Activation Protein
DMEM	Dulbecco Minimal Essential Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
ER	Endoplasmic Reticulum
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Compartment
FBS	Fetal Bovine Serum
gp	Glycoprotein
H2	Histocompatibility-2

HAART	Highly Active Antiretroviral Therapy
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HEPES	N-2-hydroxethylpiperazine-N-2-ethane-sulphonic acid
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HTLV	Human T cell Leukemia Virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Ig-like transcript
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer cell Immunoglobulin-like Receptor
LB	Luria-Bertani Medium
LIR	Leukocyte Immunoglobulin-like Receptor
LOD	Logarithm of odds ratio
Log	Logarithm
LRC	Leukocyte Receptor Complex
LRS	Likelihood Ratio Statistic
mRNA	Messenger Ribonucleic Acid
MCMV	Murine Cytomegalovirus
MEF	Mouse Embryonic Fibroblasts

MHC	Major Histocompatibility Complex
MIC	MHC class I-related chain molecule
MIP	Macrophage Inflammatory Protein
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear Factor-κB
NK	Natural Killer
NKC	Natural Killer Gene Complex
NKD	Natural Killer Domain
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
pOVA	Ovalbumin-derived peptide
QTL	Quantitative Trait Loci
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TAP	Transporters associated with Antigen Processing
TBE	Tris-borate electrophoresis buffer
TEL	Telomere
TNF	Tumor Necrosis Factor
ULBP	UL16-binding proteins

CHAPTER ONE: INTRODUCTION

1.1 Cytomegalovirus infection in humans

Human cytomegalovirus (HCMV) infects about 60 % of adults in developed world and more than 90% of the population in developing countries. Following primary infection, HCMV establishes life-long latency with intermittent reactivation (Soderberg-Naucler and Nelson, 1999). Whereas most of these infections remain asymptomatic in immuncompetent patients, HCMV-associated disease represents a major threat to immunocompromised patients. HCMV is responsible for a substantial fraction of the morbidity and mortality that occurs following organ transplantation. The infection encompasses a range of clinical illnesses: in bone marrow transplant recipients, the infection is manifested by interstitial pneunomia that is responsible of 30-48% mortality rate despite adequate treatment (Enright et al., 1993) and is manifested by organ specific disease in 20-40% of solid organ transplant recipients (Tolkoff-Rubin and Rubin, 1998; Stratta, 1993). In 2002, over 2,800 cases of transplantation-associated HCMV have been identified in the United-States, from which 57% had severe disease that caused death in 10% of these infected persons. HCMV diseases, including retinitis, colitis and encephalitis occur in persons with AIDS and are associated with decreased survival after diagnosis of HIV infection (Salmon-Ceron, 2001). A recent study has confirmed that, even with the introduction of highly active antiretroviral therapy (HAART), the presence of CMV in the blood of HIV infected patients is independently associated with an increased risk of AIDS-defining diagnosis and death (Deayton et al., 2004). Additionally, 30,000-40,000 infants are born with congenital CMV infection annually in the United States, making CMV by far the most common and important of all congenital infections (http://www.emedicine.com/PED/topics544.htm). Among infected newborns, 10%

developed illness such as deafness, prematurity, encephalitis and hematological disorders with 1% resulting in death (Reddehase, 2002).

Given the high prevalence of CMV-induced pathology in neonates, AIDS patients and transplant recipients, efficient treatments to cure and prevent the infection are desperately needed. Currently, many antiviral therapies are used for prophylaxis and/or therapy of CMV infection (http://www.emedicine.com/PED/topics544.htm). While acyclovir is considered safe and efficacious, ganciclovir and foscarnet have toxicities associated with them. In addition, the increased and prolonged use of these compounds has led to the emergence of viral resistance against most of these drugs (Villarreal, 2003). While many vaccines against HCMV are under clinical trials, no single vaccine is available. A HCMV vaccine aiming at preventing cytomegalic inclusion disease (CID) is presently being investigated and has been predicted to save lives and prevent life-long disability (Reddehase, 2002).

The absence of efficient therapies against HCMV infection has motivated researchers to unravel the complex interplay of viral and host functions that lead to pathogenesis. By understanding the early immune responses against HCMV infection at the molecular level, it would allow the identification of new targets in order to develop alternative therapeutic strategies that either stimulate or exploit host resistance mechanisms.

1.2 Mouse model of infection

The study of infectious diseases under complex genetic control in humans is confounded by a variety of factors, including complex host/virus/environment interactions (Casanova and Abel, 2004). So far, the mouse model proved to be an excellent tool for genetic dissection of viral diseases such as CMV infection (Lee et al., 2003a). Because CMV is species-specific, infection in mouse is studied using the murine cytomegalovirus (MCMV) model. Indeed, MCMV and HCMV share many similarities in their biological properties and pathogenesis (Britt and Alford, 1996). For example, MCMV infection recapitulates the large spectrum of clinical manifestations observed in HCMV infected patients including high viral titers in target organs associated with pneumonitis (Shanley, 1984), hepatitis (Trgovcich et al., 2000) and retinitis (Hayashi, Suwa, Shimomura and Ohashi, 1995). Models of congenital MCMV infection are also reported in mice (Shellam and Flexman, 1986). Finally, inbred strains of mice differ markedly in susceptibility to MCMV, enabling a genetic approach to the mapping of susceptibility traits and the possible identification of key host response mechanisms and molecules by positional cloning. Consequently, through comparative mapping, human orthologous genes can be identified to study their role in human disease.

1.3 Immune response to cytomegalovirus infection

1.3.1 Innate Immunity

Innate immunity is the first arm of defense against infections. It provides a rapid efficacious mechanism to eliminate pathogens. Upon CMV infection, the innate immune system responds within a few hours by secretion of IFN-α, IFN-β and cytokines TNF-α and IL-12 by infected cells. This cellular response stimulates the activation of natural killer (NK) cells, which is particularly important during herpesviruses infection, including CMV (Biron, Nguyen, Pien, Cousens and Salazar-Mather, 1999).

1.3.1.1 Natural Killer cells

Natural killer (NK) cells are bone marrow-derived lymphocytes, distinct from T and B cells, which recognize and kill abnormal cells without prior sensitization (Yokoyama, Kim and French, 2004). NK cells were initially identified through their ability to kill tumor cells and their role in tumor surveillance (Yokoyama and Scalzo, 2002). Over the years, NK cells have been shown to also participate in the normal host response to pathogenic infections, particularly during herpesviruses infection. This important role was illustrated by the clinical case of a patient identified with a complete lack of NK cells as well as no spontaneous or IL-2-inducible NK cell cytotoxic functions (Biron, Byron and Sullivan, 1989). This adolescent patient presented with an overwhelming Herpes Zoster infection and developed primary life-threatening HCMV infection despite normal antibody and T cell functions. In mice, the role of NK cells in defense against MCMV is as relevant as in humans, particularly during early stages of infection (Biron and Brossay, 2001). In fact, beige mice, which have a defect in natural

killing, have enhanced susceptibility to MCMV infection (Shellam, Allan, Papadimitriou and Bancroft, 1981). Also, Welsh and co-workers have demonstrated that MCMV infection results in a marked viral replication in internal organs (spleen and liver) and increased mortality in mice treated with the monoclonal antibody anti-NK1.1, abolishing the NK cell activity (Bukowski, Woda, Habu, Okumura and Welsh, 1983; Bukowski, Woda and Welsh, 1984; Welsh et al., 1990). In addition, *in vivo* depletion of NK cells with the monoclonal antibodies PK136, recognizing surface antigens preferentially expressed at the surface of NK cells, results in an increased sensitivity to infection with MCMV (Scalzo et al., 1992).

The principal role of NK cells is to limit the infection while the acquired immunity develops. Therefore, NK cells provide rapid specific cytotoxic and cytokine functions against virus-infected cells within 2 to 6 days after infection (Dokun et al., 2001a; Dokun et al., 2001b). NK cell activity is regulated by a balance of inhibitory and activating receptors expressed at their surface. Activating receptors trigger NK cells by binding to a variety of target-cell ligands that are constitutively expressed, encoded by a pathogen, stress-induced or encoded by other host cells (Yokoyama et al., 2004). On the other hand, inhibitory receptors, specific for MHC class I molecules, exert a protective role sparing normal cells from NK cell-mediated lysis. Upon binding to MHC-class I molecules, these receptors deliver signals that suppress, rather than activate, NK cell function (Moretta et al., 2002). The lack of engagement of such MHC-specific receptors results in target cell lysis. As a consequence, NK cells kill those target cells that have lost or express insufficient amounts of MHC-class I molecules, a frequent event in CMV-infected cells.

The immunity conferred by NK cells involves secretion of cytokines, in particular interferon-γ (IFN-γ) as well as direct perforin-dependent cytolysis of virus-infected cells (Biron et al., 1999). Studies undertaken by Tay and Welsh (1997) using perforin-deficient mice, IFN-γ-receptor-deficient mice and mice treated with anti-IFN-γ antibodies, revealed different mechanisms of control in different organs. They demonstrated that NK cells in C57BL/6 mice control MCMV growth via a perforin-dependent cytotoxic mechanism in the spleen, while the IFN-γ produced by NK cells is a major mediator in the regulation of the infection in the liver. The production of IFN-γ by NK cell is important for antiviral response. IFN-γ induces a number of functional effects on macrophages such as induction of MHC class I expression, increased antigen presentation, production of antimicrobial oxygen and nitrogen intermediates, and release of IL-12, which are all important mechanisms to ensure a perfect control of the virus (Boehm, Klamp, Groot and Howard, 1997). NK cell secreting IFN-γ and other cytokines, including IL-4 and IL-13 are important for the modulation of the adaptive immune response responsible for the clearance of the virus (Biron et al., 1999).

1.3.2 Adaptive Immunity

The adaptive immune system is comprised of a diverse repertoire of T and B cells that provide a highly specific response to pathogens. The optimal result takes about 6-10 days but is a curative highly specific adaptive response (Flynn, Riberdy, Christensen, Altman and Doherty, 1999). Although antiviral antibodies created by B cells are not crucially involved in the control of primary MCMV infection (Jonjic et al., 1994), $CD8^+$ T cells are effector cells particularly important during CMV infection.

1.3.2.1 CD8+ T cells

T cells are a subset of lymphocytes defined by their development in the thymus, which recognize foreign antigens presented on the cell surface by MHC molecules (Janeway, Jr., Travers, Walport and Shlomchik, 2001b). Different T cell populations have different functions. A major classification of T cells includes $CD4^+$ T cells or $CD8^+$ T cells. $CD4^+$ T cells, or helper T cells, recognize cell types presenting antigen fragments by MHC class II molecules (e.g. B cells, macrophages, dendritic cells). On the other hand, $CD8^+$ T cells, or cytotoxic T lymphocytes (CTL) because of their ability to kill virus-infected cells and tumor cells, recognize antigen fragments presented by MHC class I molecules expressed by virtually every cell in the body. Because MHC class I molecules present peptides derived from the cytosol, such as viral proteins, $CD8^+$ T are the cells that will recognize viral-infected cells. Upon activation, these cells discharge cytoplasmic granules containing the proteins perforin and granzymes that, once inside the cell, cause self-destruction by apoptosis.

$CD8^+$ T cells are the principal effector T cells in the control of CMV. In fact, HCMV and MCMV are both held in check by $CD8^+$ T cells in their respective immunocompetent host. The protective role of $CD8^+$ T cells against CMV infection was first discovered in a mouse experiment showing that $CD8^+$ T cells sensitized *in vivo* during infection of an immunocompetent host, have an antiviral effect when transferred into an immunosuppressed infected host (Reddehase et al., 1985). Furthermore, an inverse correlation between reconstitution of $CD8^+$ T cells after bone marrow transplantation and HCMV disease was observed in human (Riddell et al., 1992). Although these experiments demonstrated the essential role of $CD8^+$ T cells for virus

clearance, some evidence suggests the participation of $CD4^+$ T lymphocyte subset. In mice, depletion of $CD8^+$ T cells by monoclonal antibodies does not prevent the development of an efficient immune effector function and elimination of the virus from tissues. In addition, mice depleted of $CD4^+$ T cells are unable to eliminate the virus from salivary glands despite a strong influx of $CD8^+$ T cells into this organ. These observations suggest that even in the presence of a functional $CD8^+$ T cell subset, $CD4^+$ T cells activity is essential for an efficient antiviral activity (Jonjic, Mutter, Weiland, Reddehase and Koszinowski, 1989). The role of $CD4^+$ T cells is also important during CMV infection in humans. Recently, a study was undertaken to determine the kinetics and properties of CMV-specific $CD4^+$ T cells in healthy individuals and renal transplant recipients. They demonstrated that, in contrast to asymptomatic individuals where the CMV-specific $CD4^+$ T cells response preceded CMV-specific $CD8^+$ T cells, the CMV-specific effector memory $CD4^+$ T cell response is delayed and only detectable after antiviral therapy in symptomatic individuals. The appearance of disease symptoms in these patients suggests that formation of effector memory $CD4^+$ T cells is necessary for recovery of infection and that functional $CD8^+$ T cells and antibody responses are insufficient to control viral replication (Gamadia, Rentenaar, van Lier and ten, I, 2004).

1.4 CMV immune evasion

Cytomegalovirus is a virus member of the β-subfamily of the herpesvirus group. CMV is a large double-stranded DNA virus with a genome containing approximately 230 kb, with a considerable protein-coding capacity, predicted to be approximately 165 and 170 open reading frames (ORFs) for HCMV (Chee et al., 1990) and MCMV (Rawlinson, Farrell and Barrell, 1996; Fahnestock et al., 1995; Chapman and Bjorkman, 1998) respectively. While the genome contains genes encoding for proteins necessary for the virus metabolism, synthesis and assembly, more than 50% of the total genome consists of genes involved in host interactions in order to favor the infection (Alcami and Koszinowski, 2000). In fact, under the selective evolutionary pressure exerted by cells of the immune system, MCMV and HCMV have acquired sets of genes encoding glycoproteins, called immunoevasins, who's only known function is interference with the host immune defense, especially their most redoubtable enemies, NK cells and $CD8^+$ T cells.

1.4.1 CMV evasion from $CD8^+$ T cells

In order to evade CTL-mediated destruction, CMV have found ways to down-regulate, by different mechanisms, MHC class I molecules that are efficient at presenting viral peptides to CD8+ T cells. HCMV genome contains at least 4 genes encoding immunomodulatory proteins, named glycoprotein (gp) US2, gpUS3, gpUS6 and gpUS11, which interfere with the normal MHC class I expression by deviating the complex from its normal progression from endoplasmic reticulum (ER) to the cell surface. The HCMV US3 protein binding to MHC class I molecules causes their arrest in the ER (Jones et al.,

1996). On the other hand, US6 binds to the peptide transporter associated with peptide loading of MHC class I molecules (Lehner, Karttunen, Wilkinson and Cresswell, 1997). In addition, US2 and US11 cause proteosomal degradation of MHC class I by redirecting them from the ER to the cytosol (Wiertz et al., 1996b; Wiertz et al., 1996a).

MCMV also encodes proteins which alter MHC class I expression. To date, three MCMV proteins have been identified, which significantly interfere with expression of MHC class I molecules: m152, m04 and m06 (Wagner, Gutermann, Podlech, Reddehase and Koszinowski, 2002). The gene *m152* encodes a type I glycoprotein of 40 kD (gp40). m152/gp40 retains assembled MHC class I complexes in the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and thereby prevents the presentation of MCMV peptides to CD8+ T cells at an early stage of infection (Ziegler et al., 1997). The MCMV gene *m06* encodes for a 48 kD type I transmembrane glycoprotein (m06/gp48). m06/gp48 binds to properly folded MHC class I molecules and redirects their transport to lysosomes for degradation (Reusch et al., 1999). As a consequence, m06- and m152-expressing cells are not able to present antigens to CD8+ T cells. Finally, the MCMV gene *m04* encodes for a 34 kD type I transmembrane glycoprotein (gp34), which binds to MHC class I complexes in the ER and remains associated throughout their transport to the cell surface (Kleijnen et al., 1997). Contrary to the other proteins, m04/gp34 does not interfere with MHC class I transport but rather with the interaction between MHC class I and T cell receptor. However, the cooperation between m152 and m04 is necessary for the efficient escape of MCMV from CTL activity (Kavanagh, Gold, Wagner, Koszinowski and Hill, 2001).

1.4.2 CMV evasion from NK cells

Cytomegalovirus has found ways to avoid recognition and activation of the adaptive immune system by down-regulating MHC class I molecules on infected cells. However, down-regulation of MHC class I renders the infected cell susceptible to recognition by NK cells expressing inhibitory receptors, in accordance with the missing self hypothesis postulated by Karre, Ljunggren, Piontek and Kiessling (1986). Viruses such as HCMV and MCMV escape the innate immune system by deploying MHC class I homologues that inhibit NK cell activity.

HCMV and MCMV genomes encode for MHC class I heavy chains homologue, named UL18 and m144 respectively (Farrell et al., 1997). The HCMV and MCMV molecules UL18 and m144 are 348- and 383- residue type I transmembrane glycoprotein whose extracellular region shares ~25% amino acid sequence identity with the extracellular region of respective MHC class I molecules (Farrell et al., 1997; Rawlinson et al., 1996; Beck and Barrell, 1988). These molecules are thought to function as a mimic of host MHC I molecules and acts as a decoy for NK cells. MHC class I homologues have the ability to engage inhibitory receptors to block NK cell cytotoxicity. Therefore it was proposed that they can protect infected cells from NK cell-mediated lysis (Farrell et al., 1997; Fahnestock et al., 1995; Reyburn et al., 1997). However, this is a controversial hypothesis. The role of m144 and UL18 as inhibitors of NK cell activity was in part demonstrated using cell line transfections. In fact, UL18-transfected fibroblasts are susceptible to lysis by NK cells lines, and fibroblasts infected with wild-type HCMV are lysed more efficiently than those infected with an UL18-deficient HCMV (Leong et al., 1998). In addition, *in vivo* NK cell-mediated rejection of m144-transfected RMA-S cell

line lacking MHC class I expression, is reduced compared to rejection of non-transfected RMA-S cells indicating the ability of m144 to control NK cell-mediated responses (Cretney et al., 1999). On the other hand, it was demonstrated that human fibroblasts infected with HCMV strains AD169 lacking UL18 are killed at similar levels as UL18-containing strains by NK cells (Leong et al., 1998). While m144 binds to a unknown receptor (Cretney et al., 1999), the receptor binding to UL18 is LIR-1, an inhibitory receptor expressed by all monocytes and macrophages but only a minority of NK cells (Cosman et al., 1997), suggesting that UL18 may have only a minor effect on NK cell activity. Contrary to UL18 and MHC class I molecules, m144 does not associate with endogenous peptides due to a deletion within the counterpart of its $\alpha 2$ domain, and is still thermally stable (Chapman et al., 1998). These structural differences suggest that the receptors, mechanisms and functions of the two homologues may be distinct.

Finally, there is also evidence that HCMV encoded UL16 has the capacity to down-regulate ligands for the activating receptors NKG2D (Cosman et al., 2001). NKG2D is an NK cell activating receptor that initiates killing upon recognition of MICA, MICB and ULBPs. Ligands of NKG2D are expressed on the surface of cells undergoing a stress, like transformation or infection. Thus, by preventing the expression of NKG2D ligands, it prevents the recognition and killing of infected cells by NK cells. UL16 is a type I membrane glycoprotein expressed by CMV-infected cells which blocks NKG2D NK cell activation by binding to NKG2D ligands ULBP1-, -2 and MICB (Cosman et al., 2001). A soluble form of UL16 is able to block the NK cell cytokine and chemokine production through its binding to ULBPs ligands. Through a different mechanism, MCMV also down-regulates NKG2D ligands. In fact, gp40 encoded by the *m152* gene,

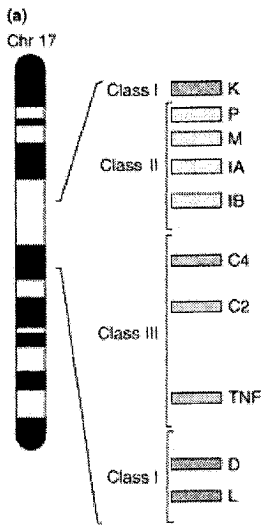
downregulates H-60, a high affinity ligand for mouse NKG2D activating receptor, and thereby inhibit NK cell activation *in vivo*. In the normally MCMV-susceptible BALB/c strain, infection with *m152* (gp40)-deleted mutant virus improves the antiviral NK cell control. This result was shown to be MHC class I-independent indicating the presence of a different role for the MCMV *m152* gene than the one mentioned above (Krmpotic et al., 2002).

1.5 Major Histocompatibility Complex

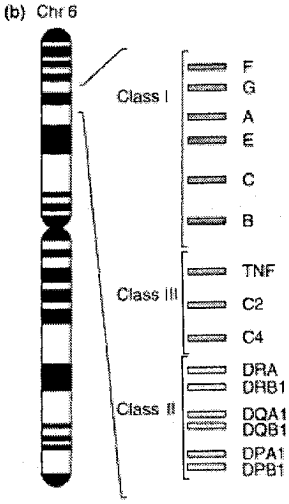
The major histocompatibility complex (MHC) is a set of genes encoding proteins, some of which are implicated in immunological functions. The MHC is located on chromosome 17 in the mouse and chromosome 6 in human. These genes are known as human leukocyte antigen (*HLA*) in humans and as *H2* genes in mouse (Figure 1.1.). MHC genes are grouped into MHC class I, MHC class II and MHC class III according with their function in immunity. MHC class III includes genes encoding complement components (C2, C4) cytokines (TNF-α), lymphotoxin (TNF-β), and proteins involved in antigen processing (Lmp2, Tap1). MHC class I and II encode highly polymorphic glycoproteins which are involved in the presentation of peptide antigens to T cells. MHC class II molecules bind peptides derived from proteins in intracellular vesicles and display peptides derived from pathogens living in macrophage vesicles or internalized by phagocytic cells and B cells to $CD4^+$ T cells. On the other hand, MHC class I molecules are responsible to collect peptides derived from proteins synthesized in the cytosol and to display fragments of viral proteins on the cell surface to $CD8^+$ T cells.

Figure 1.1. Major histocompatibility complex (MHC) genes and their role in virus infection.

(a) Mouse MHC genes (*H2*) on chromosome 17 and their association with viral disease. The *H2-D* region of the MHC class I is associated to resistance to MCMV infection. (b) Human MHC genes (*HLA*) on chromosome 6 and their association with viral infection. Genes encoding class I are depicted in red, class II in yellow and class III in green (Lee et al., 2003a).



MHC class	Virus	Alleles	Consequence	Refs
Class I	Theiler's virus	*H2-D*^{b}	Efficient CTL response to Theiler's virus (*Tmevp1*)	[33]
	Mousepox virus	*H2-D*^{b}	Resistance to lethal Mousepox virus (*Rmp3*)	[34]
	Friend leukemia virus	*H2-D*^{b}	Recovery from Friend leukemia virus (*Rfv1*)	[35]
	Moloney leukemia virus	*H2*	Genetic control of sensitivity to Moloney leukemia virus (*Rmv*)	[36]
	Murine cytomegalovirus	*H2*	Susceptibility to Murine CMV infection	[37]



MHC class	Virus	Alleles	Consequence	Refs
Class I	HIV	*HLA-B*35-Cw*04*	Rapid progression of AIDS	[38]
		*HLA-B*27* and *B*57*	Better prognosis of AIDS	[39]
		HLA-B Bw4-80Ile	Epistatic interaction with *KIR3DS1* in delayed progression to AIDS	[32]
	HTLV-I	*HLA-A*02*	Reduced risk of HTLV-1-induced disease	[40]
Class III	HBV	*TNF α-238*	Development of chronic HBV infection	[41]
Class II	HBV	*HLA-DRB1*1301* and **1302*	Protection against chronic hepatitis B infection in both African and European populations	[42,43]
		*HLA-DQA*0501* and *DQB*0301*	Persistence of HBV infection	[44]
	HCV	*HLA-DRB1*1101* and *HLA-DQB1*0301*	Self-limiting HCV infection	[45–47]
		*HLA-DRB1*04*, *DQA1*03* and *DQB1*0301*	Spontaneous clearance of HCV viraemia	[48]
		*HLA-DQB1*0401-DRB1 *0405*	Progression of liver disease caused by HCV	[49,50]

TRENDS in Genetics

From Lee et al. 2003a

MHC class I molecules play an important role in viral infection. The MHC class I molecules are heterodimers of a membrane-spanning α chain bound non-covalently to β2-microglobulin (β2m) which does not contain a transmembrane domain. The α chain consists of three polymorphic α chain domains, α1, α2, α3, which are encoded by *H2-K*, *H2-D* and *H2-L* genes in mouse and *HLA-A*, *-B* and *–C* genes in humans. The β2m molecule is not polymorphic and not encoded in the MHC locus. For a peptide to be presented by MHC class I molecules, cellular proteins are degraded by the proteasome. Resulting peptides are translocated by transporters associated with antigen processing (TAP) molecules into ER, where they contribute to the assembly of MHC class I molecules (Janeway, Jr., Travers, Walport and Shlomchik, 2001a).

Many disease resistance or susceptibility loci have been mapped to the MHC class I region of both human and mouse (Figure 1.1.). In mouse, *Rfv1* and *Rfv2*, the Friend leukemia virus infection loci, map on the *H2-D* and *IA* subregion respectively (Britt and Chesebro, 1983). Studies on congenic mice have led to the discovery that *H2-D* region is also responsible for the different level of resistance or susceptibility to mousepox infections (Brownstein, Bhatt, Gras and Budris, 1992). The association between MHC and viral diseases is also observed in humans. For example, *HLA-B*27* and *HLAB*57* alleles are associated with a better prognosis of AIDS (Kaslow et al., 1996) in contrast to *HLA-B*35-Cw*04* haplotype, which is associated with the rapid progression to AIDS in Caucasian populations (Carrington et al., 1999).

1.6 Natural killer complex

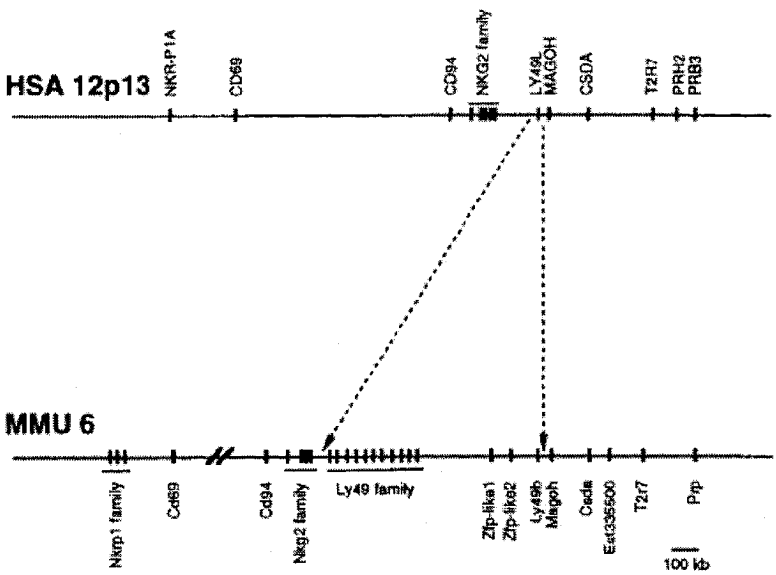
In mice, the natural killer complex (NKC) is a 4 Mb genomic region located on the distal portion of mouse chromosome 6 (Trowsdale et al., 2001). A feature of the NKC is the presence of a remarkable number of phenotypic loci associated with immune function or susceptibility to disease (Figure 1.2.a) (Webb, Lee and Vidal, 2002). In particular, genetic studies identified the gene *Cmv1* as the major determinant of resistance to MCMV infection and localized it in the NKC region (Scalzo, Fitzgerald, Simmons, La Vista and Shellam, 1990). Following this discovery, many studies have demonstrated the association of the NKC region to other phenotypes dependent upon NK cell-mediated immunity. Genetic mapping studies have revealed linkage between the locus influencing Chinese hamster ovary (CHO) target cell killing, termed *Chok*, and loci encoded within the NK gene complex (NKC) (Idris, Iizuka, Smith, Scalzo and Yokoyama, 1998). These results suggest that *Chok* encodes an NK cell receptor specific for CHO cells, responsible for NK cell-mediated cytotoxicity of tumor cells. In addition, loci contributing to susceptibility to cutaneous leishmania (Beebe, Mauze, Schork and Coffman, 1997), Insulin-dependent Diabetes Mellitus (Melanitou, Joly, Lathrop, Boitard and Avner, 1998) and ectromelia virus (Delano and Brownstein, 1995) in mice are also localized to this chromosomal region.

Figure 1.2. Maps of the natural killer complex (NKC) and the leukocyte receptor complex (LRC) in humans and mice.

(a) Genetic organization of the NKC on human chromosome 12p13 (HSA 12p13) and the syntenic region on mouse chromosome 6 (MMU 6). Note the presence of only *Ly49l* pseudogene in human. (b) Genetic organization of the LRC on human chromosome 19q13 (HSA 19q13) and the syntenic region on mouse chromosome 7 (MMU 7). *KIR* genes in humans are functionally homologous to *Ly49* genes in mice (Webb et al., 2002).

a)

The Natural Killer Complex

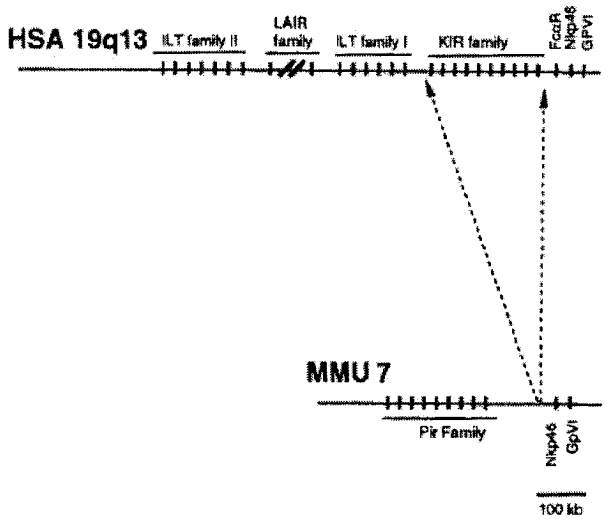


Phenotypic loci linked to murine NKC

Cmv1: Resistance to Murine Cytomegalovirus
Chok: Killing of CHO cell
Idd6: Susceptibility to insulin-dependent diabetes mellitus
Rmp1: Resistance to mousepox virus
Scl: Th1 response to *Leishmania* infection
Nka: Rat NK cell lysis of allogeneic lymphocytes
Oia2: Rat oil induced arthritis susceptibility locus

b)

The Leukocyte Receptor Complex



From Webb et al. 2002

The NKC contains genes and gene families encoding a variety of surface receptors expressed on NK cells and some T cells which can be considered perfect candidates for resistance to these diseases (Figure 1.2.a) (Brown et al., 1997). Among the NKC-encoded receptors, three families are preferentially expressed on NK cells: the NKRP1 (A-F) receptors, of which NK1.1 (NKRP1C) is the first known member and the most specific serologic marker on C57BL/6-derived NK cells; NKG2 (A-D)/CD94 receptors, heterodimers which bind non-classical MHC I Qa-1b (Vance, Kraft, Altman, Jensen and Raulet, 1998); and Ly49 receptors, which bind MHC class I molecules. In addition, a new family of genes, named *Clr* (C-type lectin-related) has been identified using a strategy involving random sequencing of bacterial artificial chromosome (BAC) clones from the centromeric part of the NKC (Plougastel, Dubbelde and Yokoyama, 2001). Among the seven members identified so far (Clra-g), Clrg is the ligand for the activating receptor Nkrp1f while Clrb is recognized by the inhibitory receptor Nkrp1d (Iizuka, Naidenko, Plougastel, Fremont and Yokoyama, 2003).

In human, the NKC is a 2 Mb region located on chromosome 12p13.1 (Suto et al., 1997) (Figure 1.2.a). This region contains genes encoding type II transmembrane C-type lectin proteins. While the majority is expressed on NK cells, some others are expressed on a broader range of cells. It is interesting to observe a certain difference of organization of the NKC in mice and humans. While the mouse contains 5 forms of *Nkrp1* genes, the human NKC contains only one form, *NKRP1A*, which shares 45% amino acids identity with mouse *Nkrp1c* (Lanier, Chang and Phillips, 1994). No ligand for this receptor has been identified yet. For the *Nkg2* family, in addition to contain the genes present in mice (*Nkg2a-d*), the human NKC contains *NKG2E* and *NKG2F*. The most striking difference

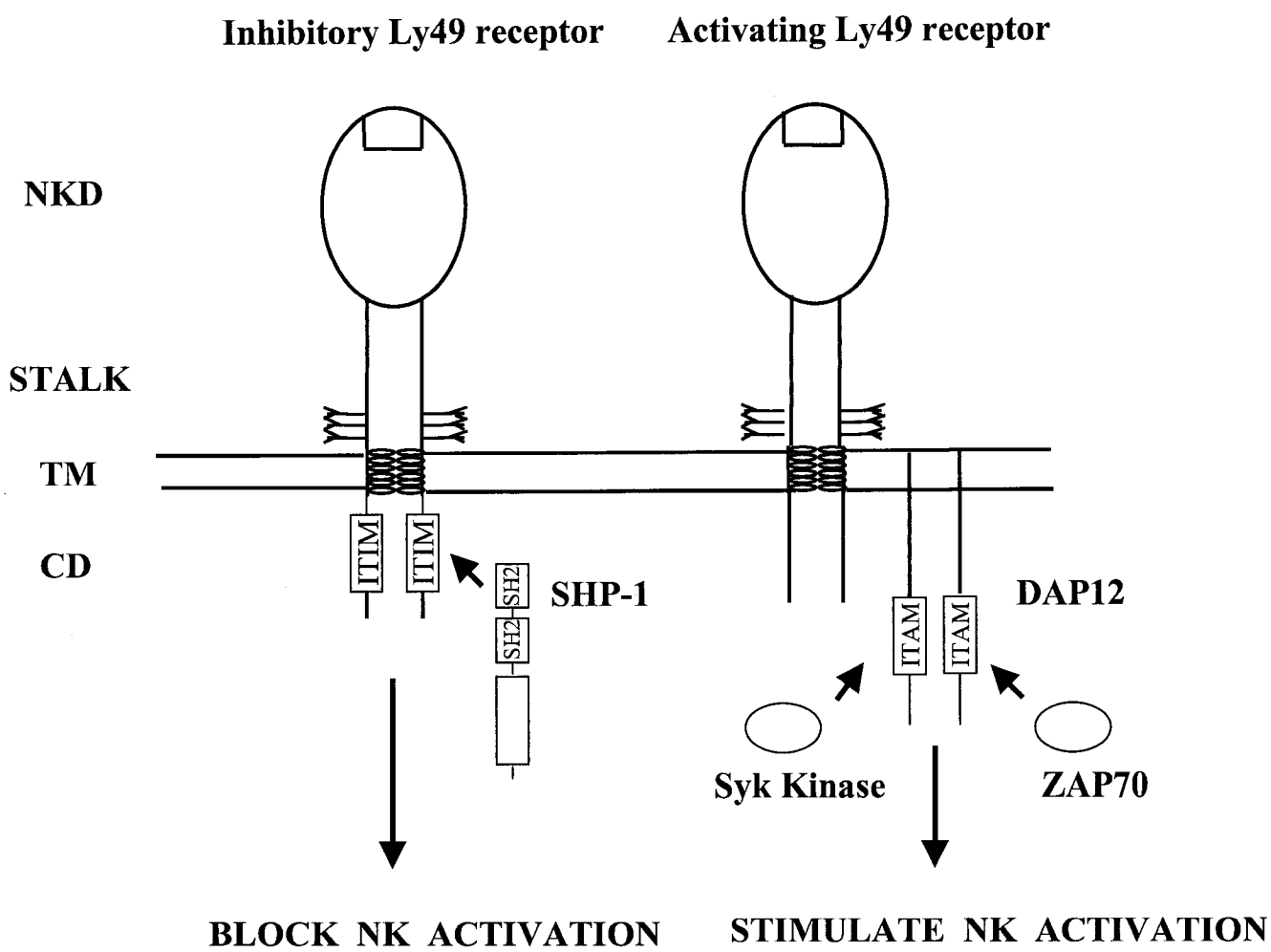
between the two species is the *Ly49* gene family. In fact, only a single gene in humans, known as *LY49L*, is translated but encodes an apparently non-functional molecule resulting from a point mutation (Lanier et al., 1994). However, mouse Ly49 receptor family appears to be functionally equivalent to human killer cell immunoglobulin-like receptors (KIR) which genes are found on the human chromosome 19q13.42 in a region named leukocyte receptor complex (LRC) (Wende, Colonna, Ziegler and Volz, 1999) (Figure 1.2.b). As Ly49 receptors, KIR receptors recognize MHC class I molecules (HLA-I) and control the NK cell activity by sending either inhibitory or activating signals depending on the presence or absence of ITIM motif in the cytoplasmic tail (Vely and Vivier, 1997).

1.6.1 *Ly49* gene family

The *Ly49* gene family is the best-characterized mouse NKC family. Ly49 are highly polymorphic type II transmembrane glycoproteins belonging to the C-lectin type superfamily. The Ly49 proteins are expressed as disulfide-linked homodimers, with each chain composed of an extracellular NK receptor domain (NKD) connected via a stalk region of 25-75 residues to transmembrane and cytoplasmic domains (Sawicki et al., 2001). Ly49 receptors fall into two distinct categories based upon stimulatory or inhibitory signaling following ligand binding (Figure 1.3.). Signaling via inhibitory Ly49 receptors involves the tyrosine phosphorylation of a cytoplasmic immunoreceptor tyrosine inhibiting motif (ITIM; I/VxYxxL/V) and its subsequent association with the tyrosine phosphatase SHP-1 (Mason et al., 1997; Nakamura et al., 1997).

Figure 1.3. Schematic representation of inhibitory and activating Ly49 receptors expressed on the surface of NK cells.

Ly49 receptors are disulfide-linked homodimers, with each chain composed of an extracellular NK domain (NKD) connected via a stalk region to transmembrane (TM) and cytoplasmic (CD) domains. Signaling via inhibitory receptors involves tyrosine phosphorylation of a cytoplasmic ITIM sequence and its subsequent association with the tyrosine phosphatase SHP-1. Signaling via activating receptors involves the tyrosine phosphorylation of a cytoplasmic ITAM sequence on the DAP12 adaptor molecule, which in turn leads to the recruitment and activation of Syk and ZAP70.


Inhibitory Ly49 receptor
Activating Ly49 receptor
NKD
STALK
TM
CD
ITIM
ITIM
SH2
SH2
SHP-1
ITAM
ITAM
DAP12
Syk Kinase
ZAP70
BLOCK NK ACTIVATION
STIMULATE NK ACTIVATION

The identification of key substrates for this phosphatase has been difficult; however it is clear that its recruitment to the inhibitory receptor blocks signaling activities of spatially proximal signaling molecules (Billadeau and Leibson, 2002). Activating Ly49 signals involve the tyrosine phosphorylation of a cytoplasmic immunoreceptor tyrosine-activating motif (ITAM; YXXL/I(X_{6-8})YXXL/I) on the Ly49 receptor-associated adaptor molecule DAP12, which in turn leads to the activation of the tandem SH2 domain-containing Syk and ZAP70 tyrosine kinases. This event results in phosphorylation of multiple signaling molecules involved in linking the engaged receptor to its downstream signaling pathways and subsequent effector functions (Mason et al., 1997; Nakamura et al., 1997).

Mapping of genomic clones indicated that, except for the most distantly related *Ly49b*, *Ly49* genes are located in a 620 kb cluster telomeric to *Cd69* (Brown et al., 1997; Wilhelm, Gagnier and Mager, 2002). *Cd69* encodes a lectin-like molecule marking early NK- and T-cell activation (Ziegler et al., 1994). Southern blot analysis revealed that different inbred mouse strains show not only a high degree of polymorphism but also different number of *Ly49* genes (Takei, Brennan and Mager, 1997). The *Ly49* gene repertoire is characterized extensively in two particular inbred strains: C57BL/6 and 129 mice. In C57BL/6 mice, 10 genes, *Ly49a* to *Ly49j*, are producing mRNAs with a complete coding region while *Ly49k*, *Ly49m* and *Ly49n* represent transcribed pseudogenes and *Ly49l* does not produce a transcript (McQueen, Freeman, Takei and Mager, 1998; McQueen, Lohwasser, Takei and Mager, 1999). In 129 mice, screening of a cDNA library led to the discovery of 10 distinct full-length *Ly49*-related coding

sequences: *Ly49e*, *g*, *i*, *o*, *p*, *r*, *s*, *t*, *u* and *v*. Among them, only *Ly49e* is identical between the two strains, while others are unique or allelic forms (Makrigiannis et al., 1999).

While most Ly49 receptors bind MHC class I molecules, they differ in ligand specificities. Ligand specificity of many C57BL/6 Ly49 inhibitory receptors has been confirmed using soluble MHC tetramers (Hanke et al., 1999). Some receptors have broad range of tetramer binding. For example, $Ly49A^{C57BL/6}$ recognizes the class I molecules $H2\text{-}D^{d,k,p}$, and $Ly49C^{C57BL/6}$ recognizes tetramers $H2\text{-}K^{b,d}$, $\text{-}D^{b,d,k}$, all resulting in inhibition of lysis (Dam et al., 2003; Daniels, Karlhofer, Seaman and Yokoyama, 1994; Hanke et al., 1999; Olsson-Alheim, Sundback, Karre and Sentman, 1999). In contrast, $Ly49G2^{C57BL/6}$ binding to $H2\text{-}D^{d}$ is very specific. Surprisingly, two activating receptors recognize and bind to MHC class I molecules. $Ly49D^{+}$ NK cells interacts with $H2\text{-}D^{d}$ target cells, leading to DAP12 phosphorylation and IFN-γ production (Mason, Willette-Brown, Mason, McVicar and Ortaldo, 2000; Nakamura et al., 1999a). A recent study using nonobese diabetic mice has shown that Ly49P, an activating receptor related to Ly49A in its extracellular domain (Makrigiannis et al., 1999), induces NK cytotoxicity by recognizing $H2\text{-}D^{d}$-bearing targets (Silver et al., 2000).

Alternate ligands may also be responsible for Ly49 activation. Transgenic transfer of $Ly49d^{C57BL/6}$ into BALB/c NK cells has been shown to confer cytotoxic activity against CHO cells (Idris et al., 1999). This experiment demonstrated that Ly49D is the *Chok* gene product, indicating that alternate Ly49D ligands may flag tumor cells for destruction, or not, by Ly49D-expressing NK cells. As Ly49D is conferring resistance to tumor cells in C57BL/6 mice, other Ly49 receptors, either inhibitory or activating, may confer resistance to infectious disease dependent on NK cell immunity.

1.7 Genetic control of MCMV infection

In mice, genetically determined resistance to MCMV infection is under complex genetic control, with both *H2* and non-*H2* gene effects (Chalmer, Mackenzie and Stanley, 1977; Grundy, Mackenzie and Stanley, 1981).

1.7.1 *H2* genes

The resistance of adult mice to acute infection with MCMV is controlled by genes linked to the *H2* complex (Chalmer et al., 1977; Grundy et al., 1981). Strains such as C3H and CBA carry the protective $H2^k$ haplotype, while mice with the $H2^b$ (C57BL/6) or $H2^d$ (BALB/c) haplotypes are more susceptible. Assessment of the relative 50% lethal dose and virus titers in *H2* congenic strains of mice with the B10 genetic background demonstrated that the $H2^k$ haplotype is approximately 10 times more resistant than the $H2^b$ or $H2^d$ haplotypes, confirming the *H2* association of resistance to MCMV infection (Grundy et al., 1981). In this case, susceptibility is inherited as a dominant trait. Also, this study reported that there are as least two genes within the *H2* complex that are involved; one mapping to the *K / IA* sub-region and the other to the *D* sub-region of the MHC class I cluster. Subsequent studies on *in vitro* infection of macrophages and fibroblast lines from different inbred and *H2* congenic mice indicated that the MHC class I allelic composition affects the ability of MCMV to infect the cells, suggesting that H2 molecules are acting as a receptor for MCMV (Price, Winter, Nikoletti, Hudson and Shellam, 1987; Price, Gibbons and Shellam, 1990). In fact, less than 10% of the macrophages from resistant $H2^k$ strains were affected, whereas 90% of $H2^d$ cells and approximately 80% of $H2^b$ and $H2^a$ cells became infected. A β2m-deficient cell

demonstrated the requirement for MHC class I molecules in MCMV infection. In addition, experiment with MHC class I transfected cell lines suggests that H2-D^d is particularly efficient in promoting infection (Wykes et al., 1993).

1.7.2 Non-*H2* genes

Non-*H2* linked genes are also involved in determining the outcome of MCMV infection, particularly in strains of the C57BL genetic background, where non-*H2* gene effects override susceptibility determined by *H2* loci. In fact, increased resistance to MCMV infection is associated with non-*H2* genes in the C57BL/6 inbred mouse strain, which has a susceptible $H2^b$ haplotype (Grundy et al., 1981). Genetic studies by the group of Scalzo et al. (1990) identified a single locus, *Cmv1*, as the major determinant of MCMV-resistance in the C57BL/6 strain. *Cmv1* is an autosomal dominant trait that restricts viral replication in the spleen but not in the liver, which are two major target organs during acute MCMV infection. As defined by the strain distribution pattern of splenic MCMV replication in CXB recombinant inbred strain, the location of *Cmv1* is on chromosome 6 suggesting NK cell involvement in the resistant phenotype. Moreover, genetic and physical mapping demonstrated the close linkage of *Cmv1* to the NKC and suggested that *Cmv1* encodes an NK cell receptor (Scalzo et al., 1995; Depatie, Muise, Lepage, Gros and Vidal, 1997; Brown et al., 1999; Depatie et al., 2000).

In common inbred mice, *Cmv1* presents two alleles: a dominant resistant allele, $Cmv1^r$, and a recessive susceptibility allele, $Cmv1^s$. $Cmv1^r$ mouse strains are 5-fold more resistant to lethal MCMV infection and exhibit 3-4 Log_{10} lower levels of viral replication in the spleen than $Cmv1^s$ strains (Scalzo et al., 1990). Haplotype studies using genetic

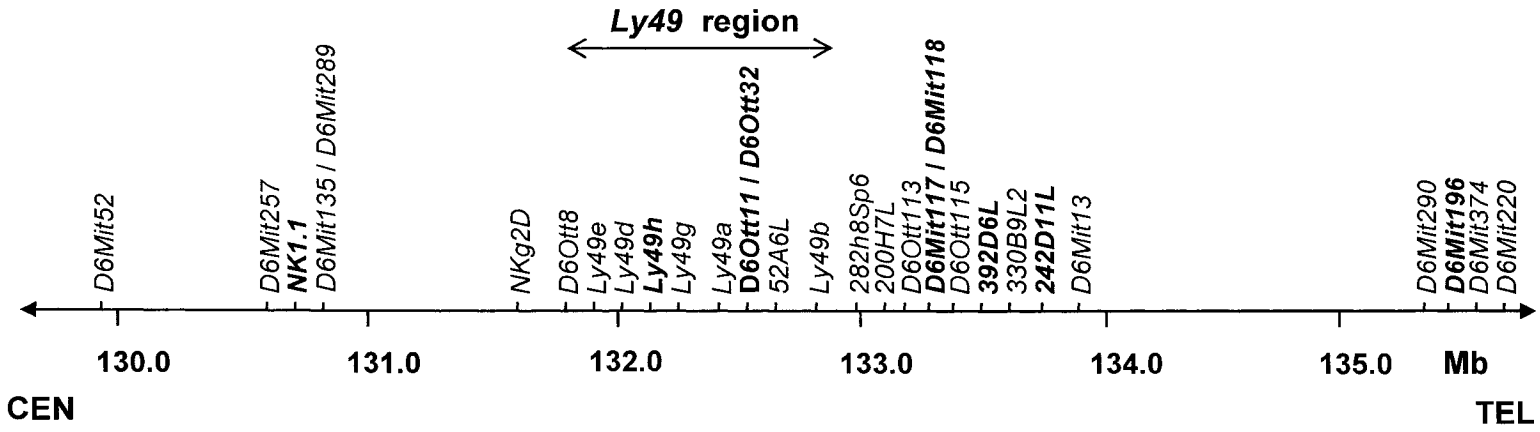
markers in the vicinity of *Cmv1* demonstrate that, while the *Cmv1*^{r} haplotype is unique to C57BL strains, there are two independent origins for MCMV-susceptibility in mice. Based on haplotype relatedness, MCMV-susceptible strains of mice are clustered in two groups; mice similar to 129 forming one group and mice similar to BALB/c forming a second unrelated subset (Figure 1.4). This observation indicates the possibility of genetic heterogeneity at this locus, i.e., the presence of alternate genes determining susceptibility in each group of susceptible mice.

Cmv1 encodes an activating NK cell receptor Ly49H (Brown et al., 2001; Daniels et al., 2001; Lee et al., 2001a) associated with an ITAM-bearing DAP12 adapter protein (Smith, Wu, Bakker, Phillips and Lanier, 1998). Ly49H is present in strains demonstrating resistance to MCMV (e.g. C57BL/6), but is absent in susceptible strains (e.g. BALB/c, 129) (Lee et al., 2001b). The role of Ly49H-bearing NK cells in these animals was validated by restoring MCMV-resistance in genetically susceptible mice through transgenic expression of Ly49H (Lee et al., 2003b). Recent studies have revealed that Ly49H specifically recognizes MCMV-infected cells via direct interaction with the *m157* MCMV gene product, which has a structural homology to MHC class I molecules (Arase, Mocarski, Campbell, Hill and Lanier, 2002b; Smith et al., 2002b). Loss of the *m157* gene is associated with gain of virulence in Ly49H^{+} but not in Ly49H^{-} mouse strains, indicating that m157 is the only MCMV-encoded protein that activates Ly49H^{+} NK cells (Bubi et al., 2004). (Arase et al., 2002b) demonstrated that m157 also binds to an inhibitory receptor, Ly49I, expressed on NK cells in 129 mice. This data suggest

Figure 1.4. Haplotype mapping in the vicinity of *Cmv1*.

(a) Physical map of markers used for haplotype mapping. The DNA markers are clustered in the *Ly49* region or distributed within the NKC genomic domain, covering a physical distance of 5 Mb. The position of markers was obtained from ensembl website (www.ensembl.org). Markers in bold were arbitrarily positioned between well-defined markers. (b) Haplotype map of chromosome 6 with 30 polymorphic markers. Numbers indicate the relative size of PCR products (1 is the shortest) for microsatellite markers. Letters indicate PCR-RFLP. np = no product.

a



b

	MCMV Titer	D6Mit52	D6Mit257	Nk1.1	D6Mit135	D6Mit289	Nkg2d	D6Ott8	Ly49e	Ly49d	Ly49h	Ly49g	Ly49a	D6Ott11	D6Ott32	52A6L	Ly49b	282h8Sp6	200H7L	D6Ott113	D6Ott117	D6Ott118	D6Ott115	392D6L	330B9L2	242D11L	D6Mit 13	D6Mit290
								Ly49 region																				
C57BL/6	2.06 ± 0.6	3	4	1	3	1	A	1	A	1	1	1	4	3	1	1	1	A	A	3	3	1	3	2	1	1	3	3
MA/My	2.24 ± 0.15	3	3	1	1	1	A	1	A	1	np	3	2	2	np	np	np	A	A	2	3	1	4	2	1	1	4	2
129/J	4.8 ± 0.05	2	1	np	4	2	A	1	A	1	np	3	2	4	np	np	1	A	A	2	3	1	3	2	1	1	3	2
FVB/N	5 ± 0.3	1	2	1	2	5	A	1	A	1	np	3	2	2	np	np	3	A	A	2	3	1	2	2	1	1	5	3
BALB/c	4.67 ± 0.18	2	1	np	6	3	B	1	B	np	np	2	1	1	np	2	2	B	B	1	1	2	1	1	np	np	1	1

that MCMV susceptibility in BALB/c mice could be explained by the lack of an activating receptor for m157, whereas in the 129 strain it is caused by m157 binding to an inhibitory receptor; therefore supporting genetic heterogeneity at *Cmv1* locus. Based on the studies mentioned above, it has been proposed that m157 and Ly49H/Ly49I do not represent the only mechanism where MCMV, or other viruses, influence NK cell response, and that other activating NK receptors may have evolved to deal with pathogens (Arase and Lanier, 2002).

The MA/My inbred strain is MCMV-resistant despite the absence of *Ly49h* mRNA and protein. The MA/My strain origins can be traced to the years 1903-1915 in the Lathrop-Loeb colony, the same producer of C57BL strains (Beck et al., 2000). In our laboratory, haplotype analysis in the *Cmv1* region was undertaken in order to understand the molecular basis of resistance in MA/My. Thirty informative DNA markers that are either clustered in the *Ly49* region or distributed within the NKC genomic domain were used for the analysis (Figure 1.4.a). The distribution of haplotypes demonstrates that MA/My is genetically divergent from C57BL/6 mouse strain (14/30 markers in common) (Figure 1.4.b). In particular, markers across the *Ly49* gene cluster are polymorphic between these two strains, indicating the presence of a different *Ly49* gene repertoire. On the other hand, MA/My haplotype is more similar to that of the MCMV-susceptible strains 129 and FVB/N. This resemblance is noteworthy at the *Nkg2D – D6Mit13* interval, where 18/20 markers are shared with both susceptible strains versus 11/20 shared with C57BL, indicating that this allele sharing may be the result of a common origin.

1.8 Genetic dissection of complex traits

Many diseases, including susceptibility to infection, are controlled by multiple genes and are therefore referred as multigenic or genetically complex traits, as opposed to phenotypes that are controlled by single genes, called monogenic or Mendelian traits (Glazier, Nadeau and Aitman, 2002). The localization and the identification of loci underlying such complex traits, named quantitative trait loci (QTLs), are facilitated by using informative crosses of inbred mouse strains, where the parental genetic effects are segregating.

1.8.1 Experimental crosses

In mice, QTLs are typically mapped using F2 or backcross populations from parental strains presenting extremes phenotypes, for example, MCMV-resistance and MCMV-susceptibility. For both populations, the parental strains are first intercrossed to generate the F1 generation. Breeding of F1 mice to one another produces F2 mice and breeding of F1 mice with one of the parental strain produces backcross mice (N2). N2 have two possible genotypes at each locus (ex: AA, AB) in contrast to F2, which have three possible genotypes at each locus (ex AA, AB, BB). Those two types of cross present advantages and disadvantages, which might have an impact on the breeding scheme for mapping experiments. First, the fact that informative meiotic events will occur in both parents of an F2 cross, less F2 mice are needed to have as much recombination information on a per animal basis as in a backcross approach. However, the data obtained are more complex and more difficult to analyze and localize new marker loci. Also, if there are many genes at play, the population will be less informative

in a sense that too many F2 mice will present intermediate phenotypes. On the other hand, more N2 are needed to have the same recombination information because each N2 mice are viewed as a single meiotic event. Nevertheless, because of the presence of only two possible genotypes in the N2 generation, the localization of new markers is facilitated and the analysis is less complex (http://www.informatics.jax.org/silver/). The choice of breeding depends on the phenotype in question and the mode of inheritance of the QTL. The F2 population is more appropriate when both parental strains have the phenotype under examination but differ in magnitude, and analysis of the F1s shows an intermediate phenotype (Moore and Nagle, 2000). On the other hand, a backcross population is generally used when the trait of interest is directed by major dominant locus.

The size of the population required for QTL analysis depends on many factors such as the overall phenotypic variance of the generation, the number of QTL and the variance of each QTL, and consequently, is difficult to predict. However, the more mice analyzed, the easier a single-gene trait is differentiated from a polygenic trait based on the distribution of the phenotype. In fact, the presence of a major gene effect will become more likely apparent in the second generation of either a backcross or F2 cross, which will present different characteristic distributions, depending on the mode of inheritance of the QTL (Moore et al., 2000). If the QTL having the major effect is co-dominant, F2 mice phenotypes will be distributed among three classes (in a 1:2:1 ratio) that will recapitulate those expressed in the F1 hybrid and each of the two progenitor strains. Phenotypes of N2 mice, in contrast, will fall into two equally populated classes with separable distributions that parallel those found in the F1 hybrid and in the inbred strain used in the backcross. On the other hand, if the QTL is recessive, F2 and N2 mice will be

distributed in two classes, albeit with a 1:3 ratio and 1:1 ratio, respectively. However, as the number of loci increases, the distribution looks increasingly like a normal (Gaussian) curve (http://www.informatics.jax.org/silver/).

1.8.2 QTL mapping

In order to localize a QTL, we need to determine the phenotype (the trait value) and genotype at a number of genetic markers for each mouse of the progeny. If we have an idea on possible candidate genes, the genotyping will be done using markers located in potential candidate region. However, if no potential candidate genes can be hypothetically proposed, we generally choose to cover the genome uniformly by using markers 10–20 centiMorgans (cM) apart. The objective is to identify genomic regions for which there is an association between the phenotype of interest and the marker allele. To date, statistical methods, such as analysis of variance and interval mapping which are discussed below, were developed to identify QTLs.

1.8.2.1 Analysis of variance

The simplest method for QTL mapping is analysis of variance (ANOVA, sometimes called marker regression) at the marker loci. For each marker used, mice progeny are separated according to their genotypes at the marker, and the phenotype distributions of the different genotypic classes are compared. If the marker analyzed is not linked to the phenotype, a random distribution of phenotype across genotypic classes will be observed. In contrast, if there is linkage, phenotypes will be non-randomly distributed across genotypes or, in other words, significant genotype/phenotype

associations will be observed. This method allows estimating the amount of the total trait variance that would be explained by a QTL at this marker. The advantage of using ANOVA is that a genetic map for the markers is not required, and the method may be easily extended to account for multiple loci. However, the ANOVA approach for QTL mapping has important weaknesses. First, individuals whose genotypes are missing at the marker are eliminated. Second, estimates of QTL location and QTL effect are indistinguishable. In fact, QTL location is indicated only by looking at which markers give the greatest differences between genotype group averages, and the apparent QTL effect at a marker will be smaller than the true QTL effect if recombination between the marker and the QTL occurred. Third, when the markers are widely spaced, the QTL may be quite far from all markers, and so the power for QTL detection will decrease (Broman, 2001).

1.8.2.2 Interval mapping

The interval mapping method was developed to overcome the disadvantages of analysis of variance at marker loci and is currently the most popular approach for QTL mapping in experimental crosses. First, it calculates the probability that a mouse has a certain genotype at every chosen position of the interval, which depends on the flanking marker's genotypes and their distance. By assuming a putative QTL gene at several estimated positions and given the probable genotypes, the LOD (logarithm of odd ratio) score is calculated: logarithm of the odds of the likelihood for the presence of the gene over the likelihood for the absence of the gene (null hypothesis) (Broman, 2001). (Lander and Kruglyak, 1995) proposed that a suggestive linkage is expected to occur one time at

random in a genome scan and has an estimated minimum LOD score of 2.0. A significant linkage is expected to occur 0.05 times at random in a genome scan and has an estimated minimum LOD score of 3.4. The probability of obtaining a LOD score as large as or larger than that which was observed if there were no QTLs is called the P value. Small P values indicate that the null hypothesis is false i.e. that there really is a QTL (Broman, 2001). To calculate a P value, sample data are collected and the appropriate test statistic for the test you are performing is calculated, such as t-statistic for testing means and Chi-Square for testing variances. Using the theoretical distribution of the test statistic, the area under the curve (for continuous variables) is determined using a look up table or integral calculus. By convention, a P value < 0.05 means that there is less than 5% probability that the finding (null hypothesis rejected) was due to chance alone and is often considered significant (http://www.informatics.jax.org/silver/).

Although the interval mapping provides improved estimates of QTL effects and takes proper account of missing data, the disadvantage is that it requires intense computation and the use of specially designed software. However, many statistical packages, such as Map Manager QT and MapMaker, are available to perform these statistical analyses and detect QTL. While the association between the phenotype and the marker allele is determined by the LOD score method in MapMaker program, the Map Manager QT program uses the likelihood ratio statistics (LRS), which relates to LOD score by dividing the LRS by 4.6 (define 4.6) (Moore et al., 2000).

1.9 Thesis objective

The main objective of my M.Sc. project was to identify novel genes of MA/My background responsible for resistance to MCMV infection, thereby allowing a better understanding of the different host defense mechanisms against MCMV infection.

Specific objectives were:

1) Determine the mode of inheritance of MCMV-resistance in MA/My by scoring viral titers in F2 populations issued from crosses between MA/My and the MCMV-susceptible mouse strains BALB.K or BALB/c, and between the susceptible strains BALB.K and FVB or 129.

2) Test the candidacy of *H2* and NKC regions in MCMV-resistance by applying ANOVA to determine if there is a significant correlation between *Ly49e* (linked to NKC) and *IAA1* (linked to *H2*) genotypes and viral titers in the populations indicated above.

3) Follow the MCMV infection kinetics in MA/My to determine if the protective effect is present throughout the course of the infection.

4) Characterize the MA/My repertoire of *Ly49* genes through cDNA cloning and DNA sequence analysis to identify potential *Ly49* receptor candidate genes for the resistance to MCMV infection.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Mice

The inbred mouse strains MA/My, BALB/c, BALB.K, 129 and FVB/N were purchased from either The Jackson Laboratory (Bar Harbor, ME) or Charles River (Wilmington, MA). F1 and F2 populations were produced from the crosses between (MA/My x BALB/c), (MA/My x BALB.K), (FVB/N x BALB.K) and (129 x BALB.K). Mice were bred and maintained at the animal facility of the University of Ottawa and McGill University in agreement with guidelines and regulations of the Canadian Council on Animal Care.

2.2 MCMV phenotype assessment

The Smith strain of murine cytomegalovirus (MCMV) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The virus stock was produced by salivary gland propagation in 3-week-old BALB/c mice as described in the literature (Depatie et al., 1997). Mice were intraperitoneally infected at 6 to 8 weeks of age with 5 x 10^3 PFU of MCMV. The viral titers were determined on MA/My (n=16), BALB.K (n=25), BALB/c (n=15), C57BL/6 (n=22), 129 (n=18), FVB/N (n=20), (MA/My x BALB.K) F1 (n=6), (MA/My x BALB/c) F1 (n=10), (FVB/N x BALB.K) F1 (n=6), (129 x BALB.K) F1 (n=5), (MA/My x BALB.K) F2 (n=226, (MA/My x BALB/c) F2 (n=119), (FVB/N x BALB.K) F2 (n=137) and (129 x BALB.K) F2 (n=66) mice by determining the number of MCMV plaque forming units (PFU) by plaque assay method (Scalzo, Farrell and Karupiah, 2000). Briefly, BALB/c mouse embryonic cells (MEFs; see below) were grown at 37 °C for 3 days in DMEM medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 mM Hepes and 10% heat

10% heat inactivated FBS and plated in 24-well tissue culture plates to obtain a final concentration between 1.6 x 10^5 to 2.0 x 10^5 cells/well. After 24 hrs of incubation, MEF cells were washed with DMEM (same as before but containing 2% FBS) and infected with 0.2 ml of organ homogenates from infected mice. After 60 min. incubation at 37 °C in 5% CO_2 incubator, cells were overlayed with DMEM medium containing 2% low-melting agarose (GIBCO-BRL) and 13.5% FBS. After 3 days of culture at 37 °C, infected cells were fixed with 10% formalin and MCMV plaques were visualized by staining with 1% methylene blue in 70% ethanol. Viral load was determined by counting the number of MCMV plaques and expressed as Log_{10} PFU. Phenotype is determined by viral titre in the spleen, with low and high viral titers indicating resistance or susceptibility to MCMV, respectively. Liver viral loads were determined to ensure that mice were properly infected. Mice with liver viral load lower than 1.69 Log_{10} PFU, were excluded from the analysis

2.3 MEF cells preparation

Mouse embryo fibroblast (MEF) cells were prepared from strain BALB/c as previously described (Scalzo et al., 2000), with modifications. Briefly, embryos were obtained at 14-16 days of pregnancy and dissected out of the embryonic sacs with livers and heads removed. The embryonic tissue was then finely minced with two forceps, washed with PBS, and incubated at 4 °C with trypsin overnight. The following day the cells were dissociated into a single cell suspension and plated on T175 flasks in complete DMEM (10% FBS, 25 mM Hepes, penicillin, streptomycin, and glutamine).

2.4 Tail DNA extraction

Genomic DNA was extracted from mouse tail using the modified alkaline lysis method (Truett et al., 2000). Briefly, a 2 mm tail was incubated in 600 µl of 50 mM NaOH solution at 95 °C for 20 min. with mixing every 5 min. At the end of the incubation, the DNA solution was vortexed for 30 sec. and neutralized by adding 50 µl of 1M Tris (pH 7.0). The DNA was then separated from debris by centrifugation for 5 min. Aliquots of 2 µl of this solution were directly used for PCR reactions.

2.5 QTL analysis

Mouse genomic DNA was obtained as described above. The genotypic analysis of the *H2* and NKC regions was performed on 119 (MA/My x BALB/c) F2, 137 (FVB/N x BALB.K) F2, 66 (129 x BALB.K) F2. The 226 (MA/My x BALB.K) F2 mice were only genotyped at the NKC region since MA/My and BALB.K have the same $H2^k$ allele. All PCR reactions were performed using 96-wells PCR plates and PTC-100 Programmable Thermal Controller PCR machine (MJ Research, Inc.). The *H2* allelic composition was determined using marker *IAA1* which amplifies the α1 exon of the murine MHC class II antigen I-A α chain. This exon contains three RFLPs, based upon the presence or absence of *Hind* III or *Pst* I sites, distinguishing between the $H2^b$, $H2^d$, and $H2^k$ haplotypes. When no enzyme digestion site is present, such as in the MA/My allele ($H2^k$), the PCR reaction yields a fragment of 203 bp. On the other hand, the 129 allele ($H2^b$) yields a 167 bp/96 bp doublet following *Hind* III enzyme digestion and the BALB/c allele ($H2^d$) yields a 206 bp/57 bp doublet following *Pst* I enzyme digestion. Primer sequences, PCR conditions and enzyme digestion reactions were as described in

the literature with modifications (Peng and Craft, 1996). Briefly, PCR amplification was performed in a 20 µl reaction with 2 µl of mouse tail genomic DNA, 0,5 µl of each primer *IAA1R* and *IAA1F* (10-µM), 2 µl of 10X PCR buffer containing $MgCl_2$ (25 mM), 0,6 µl dNTPs (2,5 mM) and 0,25 µl taq polymerase. PCR conditions were as described in literature. PCR reactions were followed by the addition of 4 U of appropriate enzymes in a 20 µl total reaction composed of NEB3 buffer and ddH_2O and incubation at 37 °C for 2h00. Digestion products were separated on a 2,0 % high-resolution gel agarose containing 5X TBE at 100 V for 2h30. The FVB allele ($H2^q$) was identified using the microsatellite marker *D17MIT28* (Mouse Mappairs, Research Genetics) mapping in the *H2K* region of the MHC class I. PCR reagents and conditions are as described in Research Genetics protocol. PCR products were separated on a 2,2% high-resolution agarose containing 0,5% TBE buffer at 110 V for 1h30.

The NKC region was amplified using 0,5 µl of each *Ly49eF* (5'-GAGAGTCAATGAGGGAATTTATCC-3') and *Ly49eR* (5'-CCCAAGATGAGTGAGC AGGAGG-3') primers (10 µM). Conditions for PCR consisted of an initial 95 °C, 1 min. denaturation step, then 34 cycles of 95 °C for 30 sec., 57 °C for 30 sec., 72 °C for 1,5 min., followed by a final 5 min. at 72 °C. PCR products were digested using 4 U of *Hinc* II enzyme in 10X NEB3 buffer, 10X BSA completed to 20 µl of ddH_2O. Digestion generated 950 bp fragment for MA/My ($Ly49e^m$), FVB/N ($Ly49e^f$) and 129 ($Ly49e^j$) allele and 800 bp fragment for BALB/c ($Ly49e^c$) and BALB.K ($Ly49e^c$) allele which were all separated on a 1,5% agarose gel at 100V for 60 min.

2.6 Interval mapping

Mouse genomic DNA was obtained as described above. The genotypic analysis was performed on 226 (MA/My x BALB.K) F2 mice. Mice were genotyped using *NKg2D* RFLP's marker and microsatellite markers *D6MIT300*, *D6MIT52*, *D6MIT135* and *D6MIT291* (Mouse MapPairs) purchased from Research Genetics (Huntsville, AL, USA). For *NKg2D*, 4 µl of mouse tail genomic DNA was used for amplification with 2,0 µl of 10X PCR buffer containing $MgCl_2$ (25 mM), 1,5 µl of dNTPs (2,5 mM), 1.0 µl of taq polymerase and 0,5 µl of each primers (10 µM) in a total volume of 20 µl. PCR conditions consisted of an initial 95 °C, 1 min. denaturation step, then 34 cycles of 95 °C for 30 sec., 55 °C for 30 sec., 72 °C for 1,0 min., followed by a final 3 min. at 72 °C. PCR products were then incubated at 37 °C for 2h00 with 4 U of *Xba* I enzyme in 1,6 µl 10X NEB2 buffer, 10X BSA completed to 16 µl with ddH_2O. Digestion reaction generated fragments of 350 bp for MA/My allele and 700 bp for BALB.K allele which were electrophoresed on 1,5 % agarose gel containing 0,5% TBE at 85 V for 1h00. Genotyping with microsatellite markers was performed as described in Research Genetics protocols. Microsatellites were identified by $\alpha[^{32}P]$-dATP labelled PCR run on a 6% acrylamide gel, exposed overnight, and detected by autoradiography.

2.7 RT-PCR and sequencing of cDNAs

Total RNA was prepared from the spleen using TRIzol reagent (Invitrogen, Life Technologies) following the manufacturer's protocol. Enrichment for $polyA^+$ mRNA from total RNA was performed using Oligotex mRNA Batch Protocol (Qiagen). Briefly, 1 µg sample of mRNA was reverse-transcribed into cDNA with the Advantage cDNA

polymerase (Clontech). For PCR reaction, 20 ng of the cDNA sample was used for amplification with 1,0 µl of *Ly49* universal primers *Ly49-F* (5'-CCCAAGATGAGRGAGCAGGAGG-3') and *Ly49-R* (5'-GAGAGTCAATGAGGGAA TTTATCC-3'), 5,0 µl 10X PCR buffer containing $MgCl_2$, 3,0 dNTPs (2,5 mM) and 0,5 µl taq polymerase in a total volume reaction of 20 µl. The PCR conditions were as follow: 94 °C for 3 min. followed by 35 cycles of 94 °C for 30 sec., 55 °C for 1 min., and 68 °C for 1 min. and a final step of 3 min. at 68 °C. PCR products were run at 100 V for a 1h00 on a 0,5% agarose gel containing 0.5X TBE buffer. Bands corresponding to *Ly49* transcripts (850-bp) were purified with QIAEX II Gel Extraction Kit (Qiagen). Following purification, *Ly49* transcripts were directly ligated into pGEM-T Easy Vector (Promega) with a ratio of 3:1 (insert:vector) according to manufacturer's conditions. The ligation reaction was transformed into chemically competent DH5α cells according to manufacturer's recommendations (Invitrogen, Life Technologies). Clones covered with nylon membrane were grown overnight at 37°C on LB agar plates. Positive clones, identified by *M13* primer PCR amplification, were sequenced in order to characterize the MA/My repertoire. DNA sequencing was performing using SequiTherm EXCEL™ II DNA Sequencing Kits-LC (Epicentre) with Licor automated system. A total of 20 clones were sequenced for MA/My mouse using *M13-F* (5'-CGCCAGGGTTTTCCCAGTCAC GAC-'3) and *M13-R* (5'-TCACACCACGGAAACAGCTATGAC-'3) fluorescent primers. The analysis of these clones was performed using standard parameters of nucleotide-nucleotide BLAST (blastn) and standard protein-protein BLAST (blastp) found on NCBI website (www.ncbi.nlm.nih.gov). Clustalw was used for multiple sequence alignments (www.ebi.ac.uk/clustalw)

2.8 Statistical analysis

The Log_{10} of the number of PFU in the spleen was analyzed as a quantitative trait. For chromosome 6 markers, genotype data of 226 (MA/My X BALB.K) F_2 was correlated with their respective MCMV Log_{10} PFU counts in the spleen using MapManager QTb (24). This program based on multiple regressions allows identification of loci affecting the quantitative trait. The contribution of NKC and *H2* alleles to the segregation of the phenotype in 119 (MA/My X BALB/c) F_2, 66 (129 x BALB.K) F_2 and 137 (FVB/N x BALB.K) F_2 mice was estimated under the linear model " phenotype = m + nkc + h2 + nkc:h2 + e ". Here, nkc and h2 are used to represent the number of MA/My, 129, or FVB/N alleles at each locus, m is the common mean value, nkc:h2 denotes the interaction term, and e is 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. Significance of differences observed in reference to control groups was assessed using the two-tailed Student's t-test.

CHAPTER THREE: RESULTS

3.1 Genetic analysis of MCMV-resistance in MA/My.

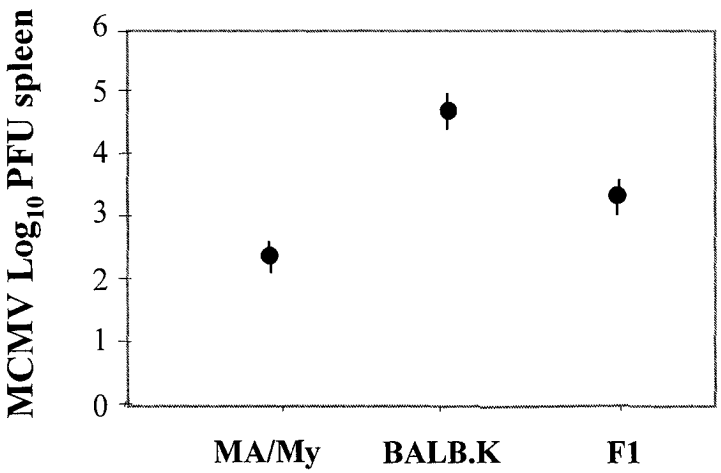
The mode of inheritance of the MA/My resistance trait was investigated in F1 and F2 progenies issued from crosses between MCMV-resistant MA/My and MCMV-susceptible BALB.K. These strains share the same $H2^k$ haplotype, thus facilitating the localization of non-*H2* loci in this cross. The read-out phenotype was spleen viral load measured in Log_{10} PFU counts recovered. As shown in figure 3.1.a, (MA/My x BALB.K) F1 mice have a mean viral titer of Log_{10} 3.35 ± 0.05 PFU, intermediate between MA/My (average Log_{10} PFU in the spleen of 2.24 ± 0.15, $P < 2.8e-6$) and BALB.K (average Log_{10} PFU in the spleen of 4.43 ± 0.17, $P < 2.3e-6$) suggesting that resistance is controlled by co-dominant alleles. In addition, the phenotypic distribution of F2 progeny is discontinuous, with three peaks at Log_{10} PFU of 2.5, 3.2 and 4.0, consistent with the presence of a major co-dominant gene effect (Figure 3.1.b).

Although the MA/My strain does not express the Ly49H receptor, we did not exclude the possibility that a member of the Ly49 family or another NK cell receptor gene might contribute to MCMV-resistance in MA/My. Therefore, the 226 MCMV-infected F2 progeny were individually genotyped with *Ly49e*, a marker present in all inbred strains, and 5 additional markers spanning 7 centimorgans (cM) in the NKC. Inspection of the empirical frequencies of the genotypic classes for *Ly49e* demonstrates a clustering of mice homozygous for alleles inherited from BALB.K ($Ly49e^k$) towards the susceptible end of the distribution. In contrast, animals having inherited the MA/My allele ($Ly49e^m$) are clustered towards the resistant end of the phenotypic spectrum (Figure. 3.1.b). The logarithm of odds (LOD) score profile for the MCMV-resistance trait shows the strongest association with markers *Ly49e* and *Nkg2D* (Table 3.1.), with LOD score

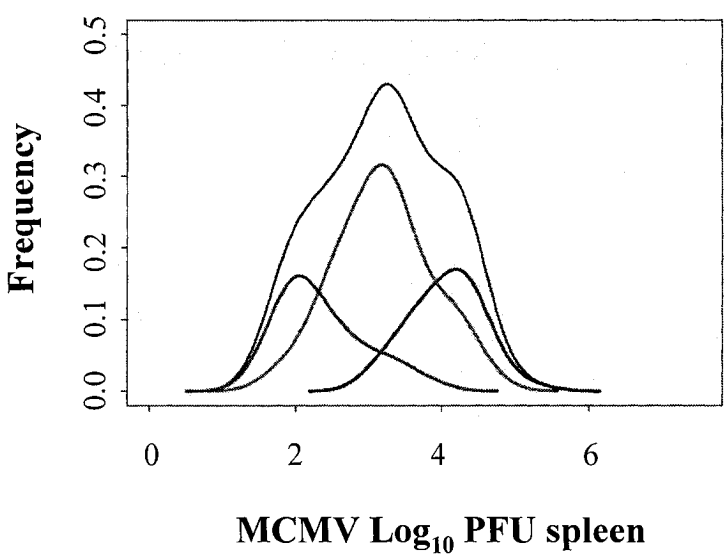
Figure 3.1. Genetic analysis of MCMV-resistance in (MA/My x BALB.K) populations.

(a) MCMV viral titers in the spleen of MA/My (n=16), BALB.K (n=25) and F1 (n=10) population. Viral loads were determined at 3 days after an intraperitoneal (ip) injection of 5 x 10^3 PFU MCMV Smith strain (salivary gland virus) for 5-8 mice of the indicated mice. The data are expressed as the mean Log_{10} of the PFUs in the spleen. Bars show standard errors. (b) Phenotypic distribution of (MA/My x BALB.K) F2 population (n=226). (c) Empirical density (black line) of Log_{10} spleen PFU counts and NKC genotypes (colors) in (MA/My x BALB.K) F2 population (n=226). Colored lines indicate the empirical density of genotypes at *Ly49e* (NKC). In the right end (blue), distribution of homozygous BALB.K genotypes (n=55); in the left end (red), distribution of homozygous MA/My genotypes (n=53); and in the middle (purple), heterozygous genotypes (n=118) at *Ly49e* (NKC).

a)


MCMV Log10 PFU spleen
0
1
2
3
4
5
6
MA/My
BALB.K
F1

b)


Frequency
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
MCMV Log10 PFU spleen

peak value of 22.7 (P < 2.2e-16) under an additive mode of inheritance. The LOD score at the flanking markers *D6MIT135* (proximal) and *D6MIT291* (distal) was 20.8 and 18.7 respectively (Table 3.1.). These linkage analyses located a novel resistance locus that we named *Cmv1*^{rm}. According to the 1-LOD support interval, *Cmv1*^{rm} is located in a region of approximately 3 cM, excluding NKC genes proximal to *D6Mit135*, such as the *Nkrp* and *Clrb* gene families, while retaining *Ly49* genes as strong candidates for the resistance trait.

Table 3.1. Interval mapping in (MA/My x BALB.K) F2 population

Model			QTL analysis		
Locus	**cM**[a]	**Mode**[b]	**LRS**[c]	**LOD**[c]	**% variance**[d]
D6MIT300	59.2	Additive	85.6	18.63	32
D6MIT52	61.4	Additive	96.1	20.9	35
D6MIT135	62.3	Additive	95.7	20.8	35
Nkg2D	62.53	Additive	104.4	22.7	37
Ly49e	62.62	Additive	104.4	22.7	37
D6MIT291	66.0	Additive	86.0	18.7	32

The analysis was performed on 226 (MA/My x BALB.K) F2 mice. [a]The position of each markers was obtained from the Jackson Laboratory website (www.jax.org). [b]The mode indicates the regression analysis model used to evaluate the significance of a putative QTL. [c]The likelihood ratio statistic (LRS) and the logarithm of the odds ratio (LOD) are used to measure the significance of each potential association of the trait with a locus. [d]The amount of total trait variance explained by a QTL at this locus is expressed as a percentage.

3.2 Interaction between *H2* and NKC loci confers resistance to MCMV in MA/My

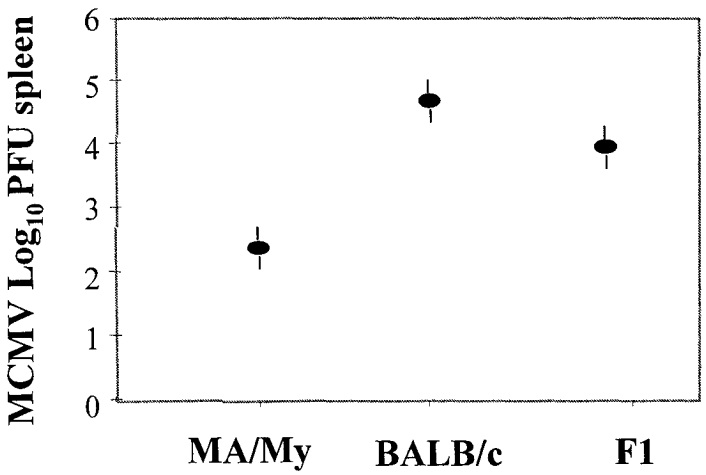
To investigate a possible role of *H2* in MA/My resistance, we studied the segregation of MCMV-resistance in F1 and F2 crosses from MCMV-resistant MA/My ($H2^k$) and MCMV-susceptible BALB/c ($H2^d$) progenitors. Viral load in the spleen at 3 days post-infection was used as read-out phenotype. The mean of the Log_{10} PFU counts recovered from the spleen of (MA/My x BALB/c) F1 population is 4.0 ± 0.15. This value is closer to that of the susceptible parental strain BALB/c (average Log_{10} PFU in the spleen of 4.67 ± 0.19, $P < 0.015$) than the resistant parental strain MA/My (average Log_{10} PFU in the spleen of 2.24 ± 0.15, $P < 7.14e-8$) (Figure 3.2.a). Therefore, MCMV-resistance segregates as a recessive trait in this cross. Further analysis of the frequencies of viral titers in the spleen of 119 (MA/My x BALB/c) F2 showed a normal distribution indicating a more complex genetic control than in the cross involving BALB.K (compare Figure 3.2.b and Figure 3.1.b).

To evaluate the contribution of *H2* and NKC receptor genes to MCMV-resistance, we performed an analysis of variance on spleen viral loads and looked at the association of the different phenotypes of (MA/My x BALB/c) F2 mice to the presence of *IAA1* marker, linked to the *H2*, and *Ly49e* marker, linked to the NKC. The most parsimonious model ($P < 5.3e-09$) accounting for 31% of the phenotypic variance involves a recessive H2 term, an additive NKC component, and their interaction (Table 3.2.). Both, the recessive H2 and the additive NKC components have similar contributions to the variance explained (12%), while their interaction accounts for about 7%. Their respective estimates for the LOD score are 3.81 ($P < 1.41e-05$), 3.58 ($P < 2.44e-05$), and 1.99 ($P < 1.23e-03$). These values indicate significant linkage of *H2* and NKC loci to the trait.

Figure 3.2. Genetic analysis of MCMV-resistance in (MA/My x BALB/c) populations.

(a) MCMV viral titers in the spleen of MA/My (n=16), BALB/c (n=15) and F1 (n=10) population. Viral loads were determined at three days after an intraperitoneal injection of 5×10^3 PFU MCMV Smith Strain (salivary gland virus) for 5-8 mice of the indicate parental or hybrid strains. The data are expressed as the mean Log_{10} PFU in the spleen. Bars show standard errors. (b) Phenotypic distribution of (MA/My x BALB/c) F2 population (n=119).

a)


MCMV Log10 PFU spleen
0
1
2
3
4
5
6
MA/My
BALB/c
F1

b)

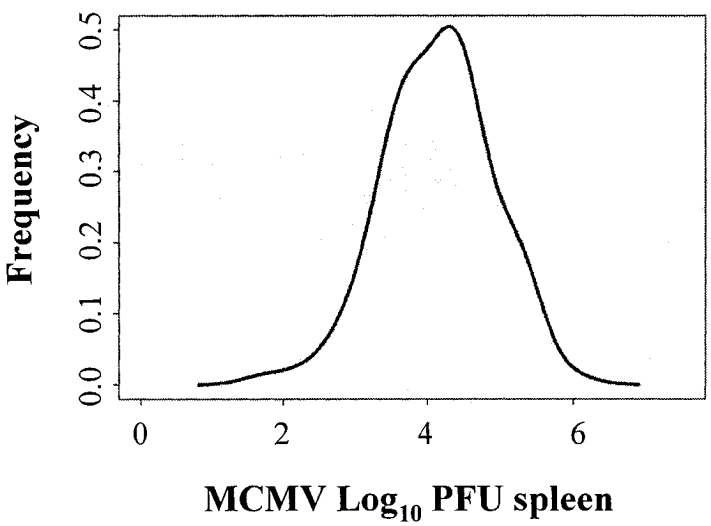

Frequency
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
MCMV Log10 PFU spleen

Table 3.2. Analysis of variance in (MA/My x BALB/c) F2 population

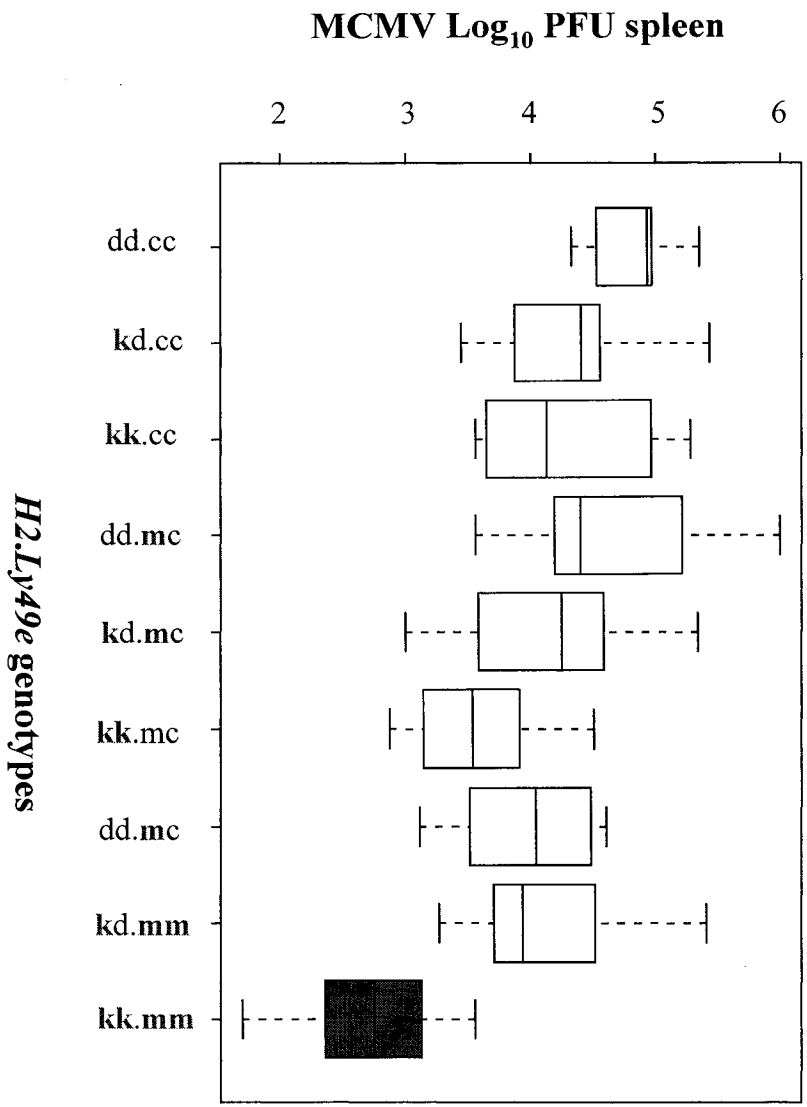
Models	LRS[a]	LOD[a]	P-value[b]	% variance[c]
model	16.53	7.1	5.39e-09	31
H2 recessive	20.58	3.8	1.41e-05	12
NKC additive	19.35	3.6	2.44e-05	12
H2:NKC	10.97	1.9	1.23e-3	7

The analysis was performed on 119 (MA/My x BALB/c) F2 mice. [a]The likelihood ratio statistic (LRS) and the logarithm of the odds ratio (LOD) are used to measure the significance of each potential association of the trait with a locus. [b]A p-value is a measure of how much evidence we have against the null hypotheses. [c]The amount of total trait variance explained by a QTL at this locus is expressed as a percentage.

To study the effect of parental alleles on spleen PFU counts, (MA/My x BALB/c)F2 mice were separated according to their genotype at *Ly49e* (MA/My, $Ly49e^{m}$; BALB/c, $Ly49e^{c}$) and *H2* (MA/My, $H2^{k}$; BALB/c, $H2^{d}$). Results in Figure 3.3. show that $H2^{k}$ alleles are associated with reduced MCMV replication in the spleen. However, the mode of inheritance of $H2^{k}$ is unclear; while it is inherited in a recessive manner in the presence of $Ly49e^{m}$ alleles, it has an additive effect in the presence of $Ly49e^{mc}$ or $Ly49e^{cc}$, supporting more complex gene-gene interactions. Analysis of combined effects of *H2* and *Ly49e* alleles on spleen PFUs suggests a strong interactive effect of the two loci. Mice homozygous for $Ly49e^{m}$ and $H2^{k}$ alleles are fully resistant to MCMV and show average Log_{10} PFU counts of 2.71 ± 0.35, in the range of MA/My controls. On the other hand, mice homozygous for $H2^{d}$ alleles in the presence of either $Ly49e^{mm}$, $Ly49^{mc}$ or $Ly49^{cc}$ are more susceptible to the infection and show Log_{10} PFUs of 3,97, 4,57 and 4,59 respectively; viral titers which are similar to those seen in the susceptible BALB/c controls (4,67 ± 0,19) (Figure 3.2.a and Figure 3.3). Therefore, two unlinked alleles, $H2^{k}$

Figure 3.3. Phenotypic distribution of (MA/My x BALB/c) F2 population according to their *H2* and *Ly49e* allelic combinations.

For the *Ly49e* locus (NKC), m represents the MA/My allele and c represents the BALB/c allele. For the *H2* loci, k represents the MA/My allele and d the BALB/c allele. The shaded box represents resistant mice sharing the $H2^k$ and $Ly49e^m$ alleles at the *H2* and NKC respectively (n=119).


MCMV Log10 PFU spleen
2
3
4
5
6
H2.Ly49e genotypes
dd.cc
kd.cc
kk.cc
dd.mc
kd.mc
kk.mc
dd.mc
kd.mm
kk.mm

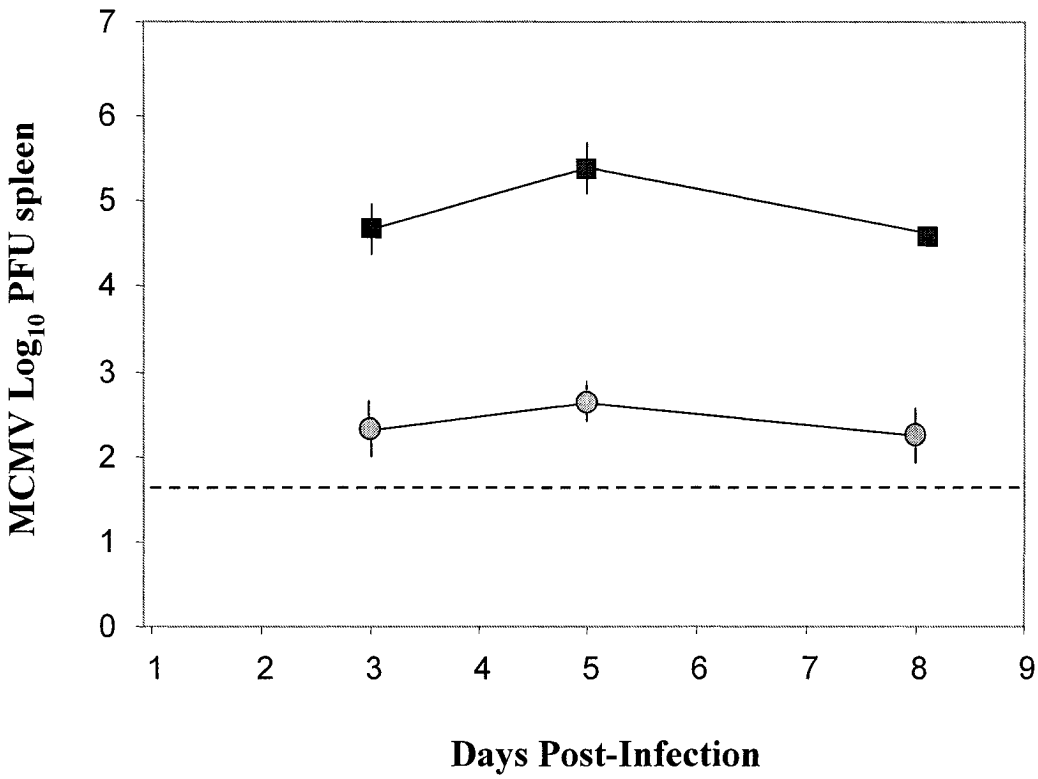
and *Ly49e*^{m}, seem to operate synergically to protect against MCMV infection, supporting the hypothesis of a functional relationship between *H2* and NKC receptor genes.

3.3 Kinetics of MCMV infection in MA/My

To characterize the MCMV-resistance trait, we studied the MCMV infection kinetics in various inbred strains of mice (Figure 3.4.). Group of 3 to 4 MA/My and BALB/c mice were infected with 5 x 10^3 MCMV PFU and sacrificed at different time points (days 3, 5 and 8). We can observe that PFU levels increased from day 3 to day 5 in both strains. At day 3, BALB/c mice have a mean viral titer of Log_{10} 4,67 ± 0.2 PFU that is peaking at Log_{10} 5,39 ± 0.1 PFU on day 5. Thereafter, the mean viral load decreased to Log_{10} 4,65 PFU on day 8. However, because mice were suffering too much from the infection, they had to be humanely sacrificed around this time point of the infection. In contrast, we observe relative control of viral infection in MA/My. In fact, the viral titer in the spleen is Log_{10} 2.31 ± 0.14 PFU at day 3, followed by an increasing viral titer of Log_{10} 2.62 ± 0.12 PFU at day 5. At day 8, the viral titer decreased to Log_{10} 2.23 ± 0.25 PFU, which is even lower than the viral titer at day 3 post-infection. These results demonstrate that in MA/My, the protective effect of the *Cmv1*^{rm} allele is observed over the course of the infection, and also in a model of lethal infection.

Figure 3.4. Kinetics of MCMV infection.

Course of MCMV infection was determined at the indicated time-points in 4-6 MA/My (circles) and BALB/c (squares) mice. Viral titers were determined by plaque assay method after infection with 5×10^3 PFU of MCMV. The dashed line indicates the level of detection of our assay (Log_{10} PFU ≥ 1.69). The data are expressed as the mean Log_{10} PFUs in the spleen. Bars show standard errors.


MCMV Log10 PFU spleen
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
Days Post-Infection

3.4 Characterization of the MA/My Ly49 receptor repertoire

Since *Ly49* receptor genes are strong candidates for the MCMV-resistance trait in MA/My mice, we proceeded to clone the MA/My *Ly49* receptor gene repertoire using degenerate oligonucleotide primers. Sequence alignment of cDNA and predicted amino-acid sequences indicated that the MA/My Ly49 repertoire is composed of at least 3 inhibitory receptors, Ly49G2, Ly49V and Ly49I, and 3 activating receptors, Ly49P, Ly49R and Ly49U (Table 3.3.).

Table 3.3. MA/My Ly49 receptor repertoire

Name	Type	Nucleotide Identity[a]	Amino acid substitution[a]
Ly49G2	Inhibitor	99%	T 91 M
Ly49G2/V	Inhibitor	G2: 100% V: 99%	G2: Identical V: N 222 S
Ly49I	Inhibitor	99%	Identical
Ly49V	Inhibitor	98%	Identical
Ly49P	Activating	99%	N 260 K
Ly49R	Activating	99%	Identical
Ly49U	Activating	99%	K 184 E ; D 266 H

[a]The MA/My Ly49 receptor repertoire was found to be highly homologous to the Ly49 receptor repertoire of the MCMV susceptible strain 129.

In accordance with haplotype analysis, Ly49 receptors cloned from the MA/My strain are highly homologous to those of MCMV-susceptible strain 129, suggesting the presence of a common *Ly49* gene repertoire in the two strains. (Makrigiannis et al., 2002). Allelic forms of Ly49G2, Ly49P and Ly49U were identified. For these receptors in MA/My, all amino acids substitutions from 129 are found in the extracellular domain.

In MA/My Ly49G2, a methionine replaces a threonine at position 91 and for Ly49P, a lysine replaces a asparagine at position 260. In the case of Ly49U, two amino acids are substituted: one lysine for a glutamate at position 184 and one aspartate for a histidine at position 266. However, the three other receptors, Ly49R, Ly49V and Ly49I, are identical at the protein level in MA/My and 129. In addition, we identified a new inhibitory receptor arising from a recombination between Ly49G2 and Ly49V, named here Ly49G2/V. This receptor shares identical amino acid sequence in the cytoplasmic domain, transmembrane domain and part of the extracellular stalk domain (position 67 to 89) with the 129 Ly49G2 receptor. The rest of the protein, from amino acids position 90 to 239, are very similar to Ly49V with a single substitution at position 222 where a serine replaces asparagine in MA/My.

3.5 Determination of *Cmv1* allelic composition in susceptible strains FVB/N and 129

Haplotype analysis at the *Cmv1* region indicated that the MCMV-susceptible strains 129 and FVB/N have inherited many polymorphic markers identical to that of MA/My. Following these observations, we hypothesized that 129 ($H2^b$) and FVB/N ($H2^q$) have the MA/My $Cmv1^{rm}$ allele that is masked by the dominant susceptible effect of the *H2* genes present in those two susceptible strains. In order to test this, we produced two crosses of MCMV-susceptible mice, (FVB/N x BALB.K) and (129 x BALB.K), from which the interaction between $H2^K$ and NKC could be reproduced if in fact, FVB/N and 129 carry $Cmv1^{rm}$. Should this be the case, we expected to observe low viral titers in

mice having inherited the $H2^k$ alleles from BALB.K, and $Cmv1^{rm}$ at NKC from FVB/N or 129.

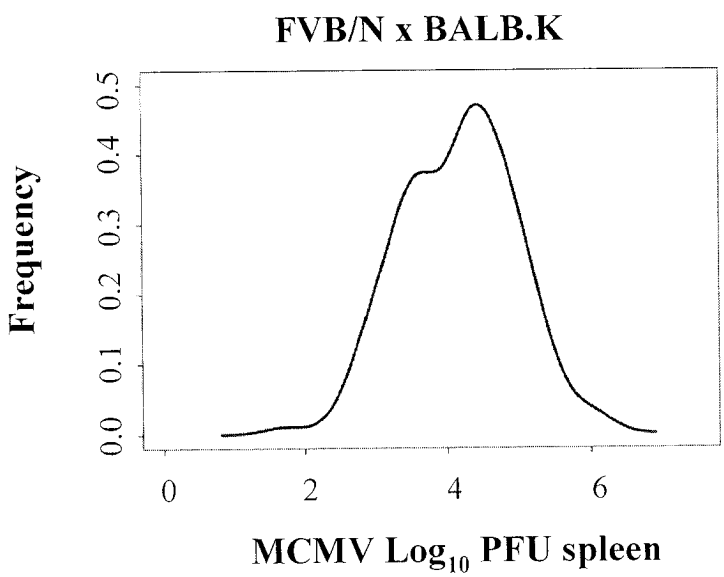
First of all, segregation analysis was performed using 137 (FVB/N x BALB.K) F2 mice and 66 (129 x BALB.K) F2 mice. In both cases, the phenotypic distribution of the population is bimodal (Figure 3.5.a, b). In fact, 2 peaks at Log_{10} PFU of 3.8 and 4.2 for (FVB x BALB.K) F2 and for (129 x BALB.K) F2 are observed, consistent with the presence of at least 2 loci controlling the phenotype. It is interesting to observe the presence of resistant F2 mice in crosses of two susceptible parental strains. In (FVB/N x BALB.K) F2 cross, 28% of mice show viral titers of Log_{10} PFU from 1.6 - 3.6 which is significantly lower to that of FVB/N (P=1.1e-10) or BALB.K (P=2.1e-07) susceptible parental strains. Similarly, in (129 x BALB.K) F2 cross, 21% of mice have titers of Log_{10} PFU from 2.4 - 3.6, also lower that 129 (P =2.1 e-11).

In order to evaluate the contribution NKC and *H2* receptor genes to MCMV-resistance in FVB/N, we performed an analysis of variance on spleen viral loads and looked at the association of the different phenotypes of (FVB/N x BALB.K) F2 population to the presence of *Ly49e* marker, linked to the NKC, and *D17MIT28* marker, linked to *H2* region. For (FVB x BALB.K) F2 population, the most parsimonious model (P < 5.9e-11) accounting for 29.6% of the variance involves additive H2 and NKC terms without interaction (Table 3.4.). While H2 contributes to 8.9% of the variance explained, NKC explains 20.7% of the variance. Their respective estimates for the LOD score are 3.2 (P < 6.4e-05) and 7.2 (P < 4.5e-09) respectively. These values indicate significant linkage of the two loci to the phenotype, the NKC having an effect more important than the *H2*.

Figure 3.5. Phenotypic distribution of (129 x BALB.K) and (FVB/N x BALB.K) F2 populations.

(a) (FVB x BALB.K) F2 population (n = 137) (b) (129 x BALB.K) F2 population (n = 66). Mice have been infected with 5 x 10^3 PFU of virus. MCMV viral titers in the spleen, expressed as average Log_{10} PFU, have been determined by plaque assay method 3 days post-infection.

a)


FVB/N x BALB.K
Frequency
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
MCMV Log10 PFU spleen

b)

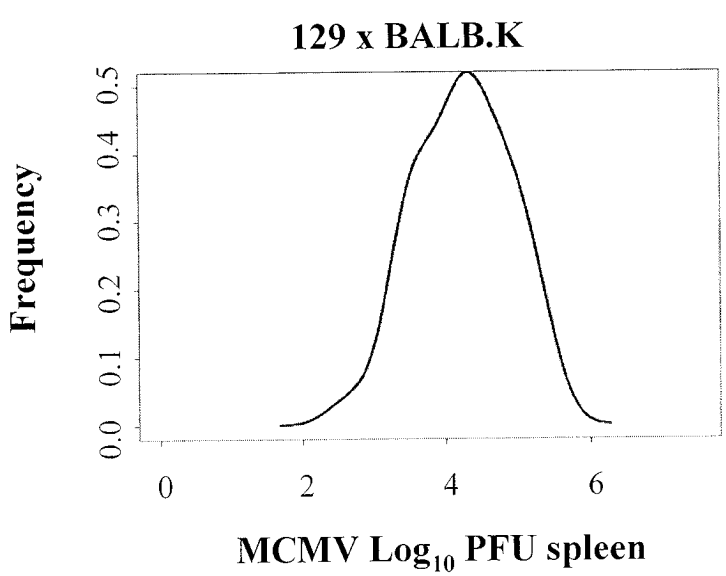

129 x BALB.K
Frequency
0.0
0.1
0.2
0.3
0.4
0.5
0
2
4
6
MCMV Log10 PFU spleen

Table 3.4. Analysis of variance in (FVB/N x BALB.K) F2 population

Models	LRS	LOD	P-value	% Variance
Model	28.210	9.007	5.964e-11	29.6
H2 additive	17.035	3.185	6.412e-05	8.9
NKC additive	39.383	7.177	4.485e-09	20.7

The analysis was performed on 137 (FVB/N x BALB.K) F2 mice. [a]The likelihood ratio statistic (LRS) and the logarithm of the odds ratio (LOD) are used to measure the significance of each potential association of the trait with a locus. [b]A p-value is a measure of how much evidence we have against the null hypotheses. [c]The amount of total trait variance explained by a QTL at this locus is expressed as a percentage.

To study the effect of parental alleles on spleen PFU counts, (FVB/N x BALB.K) F2 mice were divided in groups according to their genotype at *Ly49e* (FVB/N: $Ly49e^{f}$; BALB.K: $Ly49e^{c}$) and *H2* (FVB/N: $H2^{q}$; BALB.K: $H2^{k}$) (Figure 3.6.a). As hypothesized, mice being homozygous for the FVB/N $Ly49e^{f}$ allele and for the BALB.K $H2^{k}$ allele, have the lowest spleen counts in the F2 population (Log_{10} PFU of 3.39 ± 0.21) suggesting the presence of $Cmv1^{rm}$ in FVB/N. However, two differences are noted compared to the MA/My model. First, this viral titer is clearly higher than the viral titer observed in MA/My mice (Log_{10} 2.24 ± 0.15 PFU, P < 3.1e-4) or in mice homozygous at $Ly49e^{m}$ and $H2^{k}$ issued from the (MA/My x BALB/c) F2 cross (Log_{10} 2.71 ± 0.21 PFU, P < 0.03). This result suggests that the protection conferred by the FVB/N $Ly49e^{ff}$ alleles has a lower effect than the protection conferred by the MA/My $Ly49e^{mm}$ alleles. A second remarkable difference resides in the mode of inheritance of the *H2* loci. In the (FVB/N x BALB.K) F2 population, the $H2^{k}$ allele, from BALB.K, is dominant over the $H2^{q}$ allele, from

FVB/N, as mice of $H2^{qk}$ genotype have mean viral titer of Log_{10} 4.09 ± 0.09 PFU, which is similar to that of mice of $H2^{kk}$ (Log_{10} 3.84 ± 0.14 PFU, $P < 0.13$) genotype compared to mice of $H2^{qq}$ (Log_{10} 4.56 ± 0.1 PFU, $P < 0.001$). In contrast, in the (MA/My x BALB/c) F2 population, $H2^{k}$ is recessive with respect to the $H2^{d}$ allele as the mean PFU values of mice of $H2^{kd}$ genotype (Log_{10} 4.20 ± 0.07 PFU) is similar to the mean PFU values of mice of $H2^{dd}$ genotype (Log_{10} 4.43 ± 0.12 PFU, $P < 0.13$) compared to mice of $H2^{kk}$ genotype (Log_{10} 3.65 ± 0.19 PFU, $P < 0.01$).

The contribution of NKC and *H2* receptor genes to MCMV-resistance was also investigated in 129 strain. We performed an analysis of variance on spleen viral loads and looked at the association of the different phenotypes of (129 x BALB.K) F2 population to the presence of *Ly49e* marker, linked to NKC region, and *IAA1* marker, linked to *H2* region. For (129 x BALB.K) F2 population, the most parsimonious model involves only a NKC term without interaction. While the model is not implicating the H2, it does not favour the participation of the NKC. In fact, the NKC is only explaining 7,8% of the variance and its LOD score estimate is 1.168 ($P < 0.02$) which is not significant. Contrary to MA/My and FVB/N, the analysis shows that neither *H2* nor NKC loci are implicated in the phenotype. Additionally, no effect of the parental alleles is observed in that cross. As shown in figure 3.6.b, the possession of either the $Ly49e^{j}$ allele or $H2^{k}$ allele does not have an effect on the level of the resistance.

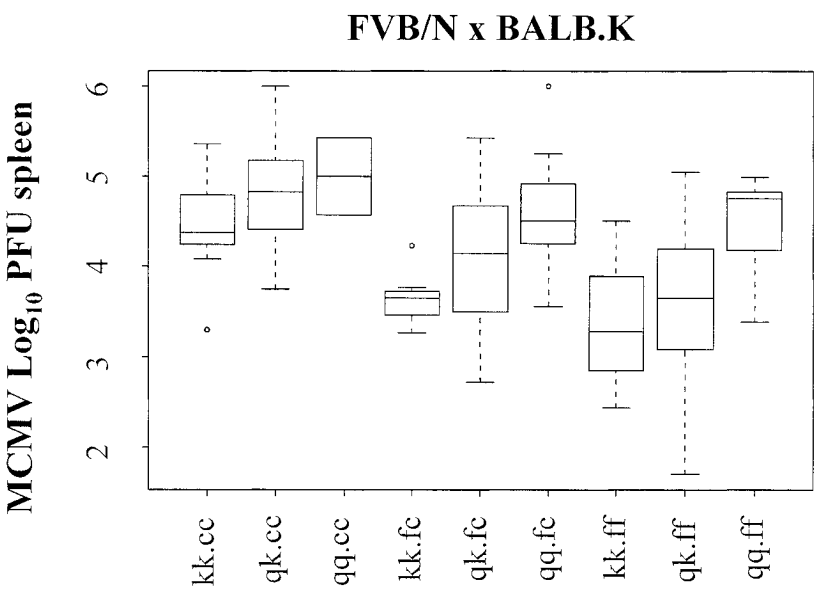
Finally, these results indicate that although the haplotype of FVB/N and 129 is similar to MA/My, they have different effects on the control of CMV infection. We demonstrated that even though it is susceptible, the FVB/N strain possesses an allele conferring resistance to MCMV infection. Contrarily to FVB/N, the allele of resistance

seems to be absent in the susceptible strain 129 since no effect of the NKC was observed. However, this absence of effect might be explained by the presence of dominant background genes hiding the resistance allele. Without further analysis, we cannot decide whether the differences reside at the NKC or somewhere else in the genome.

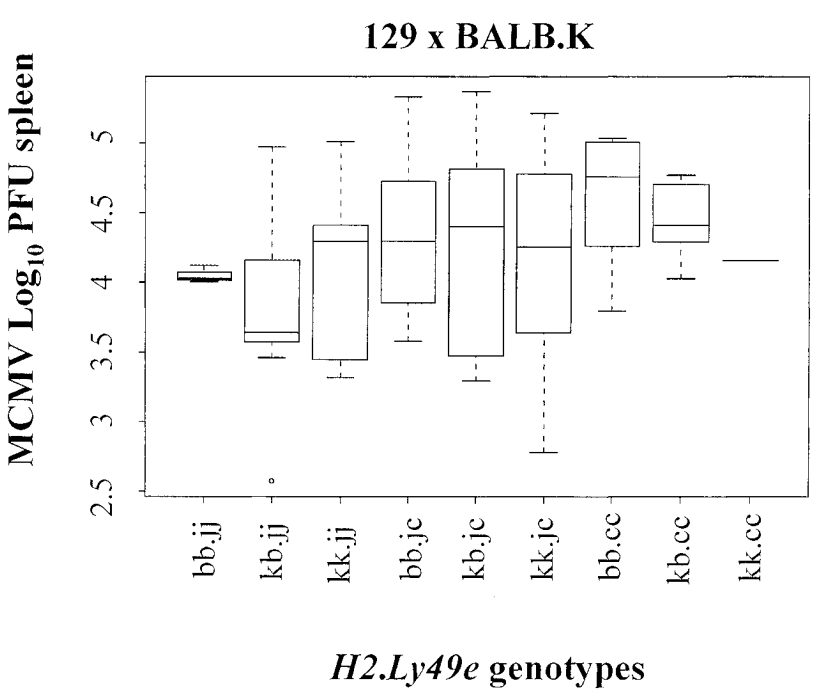
Figure 3.6. Phenotypic distribution of (FVB/N x BALB.K) and (129 x BALB.K) F2 populations according to their *H2* and *Ly49e* allelic combinations.

(a) Distribution of (FVB/N x BALB.K) F2 population (n=137). For the *Ly49e* locus (NKC), f represents the FVB/N allele and c represents the BALB.K allele. For the *H2* loci, q represents the FVB/N allele and k represents the BALB.K allele. (b) Distribution of (129 x BALB.K) F2 population (n=66). For the *Ly49e* locus (NKC), j represents the 129 allele and c represents the BALB.K allele. For the *H2* loci, b represents the 129 allele and k represents the BALB.K allele.

a)


FVB/N x BALB.K
MCMV Log10 PFU spleen
2
3
4
5
6
kk.cc
qk.cc
qq.cc
kk.fc
qk.fc
qq.fc
kk.ff
qk.ff
qq.ff

b)


129 x BALB.K
MCMV Log10 PFU spleen
2.5
3
3.5
4
4.5
5
bb.jj
kb.jj
kk.jj
bb.jc
kb.jc
kk.jc
bb.cc
kb.cc
kk.cc
H2.Ly49e genotypes

CHAPTER FOUR: DISCUSSION AND CONCLUSION

Laboratory mice are excellent tools to study and identify genes of resistance to different infections. In fact, actual inbred strains originate from consanguine crosses between a restricted numbers of probably heterozygous progenitors. Following many years of inbreeding, parental alleles were fixed to the homozygous state. As this process is random, some strains have fixed alleles of resistance whereas other strains have fixed alleles of susceptibility to infection with a variety of pathogens, including CMV. The genetic analysis of crosses between infection resistant and susceptible mice allows the localization of the susceptibility traits, whereas genomic tools allow the isolation of the genes underlying the phenotypes. In C57BL/6 mouse, *Ly49h*, initially named *Cmv1*^{r}, has been identified as the gene responsible for the resistance to MCMV infection. The region of this locus presents different haplotypes that could explain the level of resistance and susceptibility for individual mouse strains. Actually, the *Cmv1*^{r} allele conferring resistance to the infection, and *Cmv1*^{sFVB} and *Cmv1*^{sBALB} alleles, conferring susceptibility to the infection, map to this region. The mouse strain MA/My is resistant to MCMV infection despite the absence of *Ly49h* and the presence of a haplotype similar to the MCMV-susceptible strain 129. In this thesis, we report, the identification of a new resistance allele, *Cmv1*^{rm}, responsible for the resistance of MA/My to MCMV infection and the cloning of 3 potential candidate genes for this allele, the activating Ly49P/R/U receptors. In addition, we provide evidence that a similar MCMV-resistance allele may be present in the MCMV-susceptible strain FVB/N.

A new NKC allele is associated with MCMV resistance in MA/My

The genetic basis of host resistance to MCMV infection is well characterized in C57BL/6 mice, carrying the resistance allele *Cmv1*^{r}, which is allelic to gene encoding the activating receptor Ly49H. Ly49H interacts directly with the viral protein m157 (Arase, Mocarski, Campbell, Hill and Lanier, 2002a; Smith et al., 2002a), which shares structural homology with MHC class I molecules and is expressed on the surface of infected cells. The current model proposes that upon recognition of m157, Ly49H elicits signals that activate cytolytic NK cell activity against infected cells that are thus eliminated early after infection, allowing arrest of viral spread in the resistant mouse strains. This mechanism is to date the sole example of direct recognition of a virally encoded protein by an activating NK cell receptor.

Despite the lack of Ly49H, MCMV-infected MA/My mice are phenotypically indistinguishable from C57BL/6. Using linkage analysis, we defined a novel resistance locus linked to the *Ly49* gene cluster: the *Cmv1*^{rm} locus. *Cmv1*^{rm} has several important differences with the previously described *Cmv1*^{r} / *Ly49h* gene. In the study of inheritance, we observed that *Cmv1*^{rm} is co-dominant, rather that dominant as described for *Cmv1*^{r} / *Ly49h.* Another important difference between C57BL/6 and MA/My resistance alleles resides in their sensitivity to *H2* background. While *Cmv1*^{r} / *Ly49h* phenotype is independent of the *H2* environment, results from crosses between MA/My and BALB/c show that only the combination of *Cmv1*^{rm} and *H2*^{k} alleles is associated with MCMV-resistance. As published, *H2*^{k} alleles are associated with reduced MCMV replication in the spleen, with an effect on survival, and are inherited in a recessive fashion (Chalmer et al., 1977). It has been speculated that H2 molecules may be involved in CMV entry in the cells (Wykes et al., 1993). Two alternative models could be

proposed to explain the participation of H2 and non-H2 molecules in CMV infections. In a two-step model, $H2^k$ would limit the entry of the virus into the cells, which would be followed by *Cmv1*^{rm}-promoted killing of infected cells by NK cells, as proposed for Ly49H. In a second model, the physical interaction between NKC and MHC receptors would be required in order to kill MCMV-infected cells. MHC class I molecules are well-characterized ligands of inhibitory and activating Ly49 receptors, which support the second model.

Possible biological functions of genetic interaction between *H2*^{k} and NKC genes

A plausible model would implicate the recognition of $H2^K$ molecules containing viral peptide by a Ly49 receptor expressed at the surface of NK cells. The exact mechanism implicated in this recognition cannot be identified until functional studies are undertaken. However, researchers on CMV infection have brought some evidence that allow us to speculate on possible biological functions that could be responsible of protection against CMV infection.

First, we propose a model where an activating Ly49 receptor expressed on the surface of NK cells recognizes and binds to $H2^K$ molecules containing MCMV-encoded peptides. This model is based on the allelic interaction observed between *KIR* and *HLA* genes in human concerning HIV infection. Mouse *Ly49* and human *KIR* genes are functional homologues, although they come from different structural families. The two sets of genes share some striking features. As well as recognizing MHC class I, they are both expressed in a clonal fashion on different NK cells and they are both polymorphic. They use similar mechanisms for signal transduction. The difference is that *KIR* genes map to the leukocyte receptor complex (LRC), which encodes a cluster of genes encoding

molecules of the immunoglobulin-superfamily expressed on different cell types of the immune system. It was proposed that because they are on separate chromosomes, some combinations of MHC and MHC-class I receptor (KIRs) haplotypes would have particular interactions associated with susceptibility or resistance to infection. In fact, it was reported that the activating *KIR* allele *KIR3DS1*, in combination with *HLA-B* alleles that encode molecules with isoleucine at position 80 (*HLA-B Bw4-80Ile*), is associated with delayed progression to AIDS. The results obtained in this research have led to a model in which binding of HLA-B Bw4-80Ile molecules to KIR3DS1 receptor, leads to activation of NK cells and/or T cells and to elimination of HIV-1-infected cells (Martin et al., 2002). As we found analogous genes in mouse, a genetic combination between *Ly49* and MHC class I genes could be protective against MCMV infection. According to our results, we propose a model where a particular Ly49 activating receptor expressed on MA/My NK cells mediates specific killing of the infected cell through recognition of $H2^K$ molecules in the presence of a viral factor on an MCMV infected cell.

This model requires the discrimination between normal cells and infected cells by the activating receptor. To do so, the activating receptor would need to selectively and specifically recognize and bind to $H2^k$ molecules containing MCMV-derived peptides, i.e it would need to recognize preferably $H2^k$ molecules only if presenting MCMV antigens. So far, these characteristics have been observed only with few human KIR and mouse Ly49 receptors. In human, only one inhibitory receptor, KIR11, was shown to bind to its ligand HLA-B2705 if it was complexes with a subset of all the peptides that bind to it (Peruzzi, Wagtmann and Long, 1996). Research on mice indicated that Ly49C appears also to be a peptide-dependent and –selective inhibitory receptor. In fact, the ability to bind Ly49C-transfected reporter cells and protection from killing by $Ly49C^+$ NK cells is

induced by loading RMA-S cells with $H2K^b$-restricted ovalbumin-derived peptide OVA257-264 (pOVA) compared to other peptides that bind and stabilize H2-K^b equally (Franksson et al., 1999). Three activating receptors also bind MHC class I. In fact, Ly49D, L49P and Ly49W activating receptors are MHC dependent. While Ly49D binds H2-D^d, H2-D^r, H2-D^{sp2} (George, Mason, Ortaldo, Kumar and Bennett, 1999), as well as xenogeneic ligands (Nakamura et al., 1999b), Ly49W interacts weakly with H2-D^d and strongly to H2-D^k (Silver, Gong, Hazes and Kane, 2001). Interestingly, $Ly49P^{129}$ interacts very weakly with H2-D^k and H2-D^d soluble tetramers (Makrigiannis et al., 2001). However, it is still not known if these receptors are selectively binding to these MHC class I molecules in the presence of ligand proteins.

A second possible model would implicate an activating Ly49 receptor directly recognizing a MCMV-encoded protein bound to $H2^k$ molecules. In mice, gp34 encoded by the MCMV *m04* gene, is a MCMV-encoded glycoprotein that form a tight complex with MHC class I molecules (Kleijnen et al., 1997). Contrary to other MCMV-encoded glycoproteins whose main function is to down-regulate MHC class I expression, the gp34 glycoprotein promotes normal MHC class I expression. In fact, gp34 associates with MHC class I molecules in the ER, transports the complex to the cell surface and restores MHC class I surface expression in cells in which the presentation of antigenic peptides is prevented by retention of peptide-loaded MHC class I molecules by the *m152* gene protein. It was demonstrated that gp34 is able to form a complex both with H2-D and H2-K molecules for both haplotypes tested, $H2^b$ and $H2^d$. Unfortunately, the complex formation of this protein with molecules of $H2^k$ haplotype has not been tested. It was proposed that gp34 serves to prevent the attack by NK cells, which would otherwise recognize cells lacking MHC class I expression and that its binding would interfere with

recognition of this complex with $CD8^+$ T cells. However, studies have demonstrated that gp34 might itself be a subject of antigen processing and be recognized by CD8+ T cells (Holtappels et al., 2000). Following these observations, we can postulate that a particular activating Ly49 receptor in MA/My, might recognize $H2^k$ molecules complexed with gp34 in MCMV-infected cells.

Finally, we do not exclude the possibility that MCMV-resistance in MA/My is conferred by the lack of an interaction between an inhibitory receptor and $H2^k$ molecules. In fact, a weak interaction or lack of strong NK cell inhibition would be protective because activating receptors could more easily override the inhibitory effect. In humans, such a mechanism of protection against hepatitis C virus infection has been hypothesized following examination of the synergistic effect of *KIR* and *HLA* allelic combinations (Khakoo et al., 2004). In fact, patients with resolved infection are more frequently associated with homozygous *HLA-C1* alleles than those that have persistent infection. Also, the protective association of homozygous *HLA-C1* is significant only among individuals homozygous for *KIR2DL3* allele encoding for an inhibitory receptor. In this model, the ability of *KIR2DL3* and *HLA-C1* alleles to regulate NK cell activity is effective only in the presence of low-dose of virus, which pointed to an innate immune mechanism. In any case, cloning of $Cmv1^{rm}$ is the obvious first step needed for the molecular dissection of the genetic interaction observed in MA/My.

MA/My Ly49 receptor repertoire

NK cells are well-known players in the MCMV infection in mice. NK cell function depends on their activation by surface activating receptors many of which are encoded at the NKC. It seems that loci exhibiting extreme levels of polymorphism like

MHC, Toll and NK receptors are those whose products interact directly with pathogens, such as herpes viruses and retroviruses, or their products, such as LPS (Trowsdale and Parham, 2004). At the NKC, allelic polymorphism and variation in gene number is most remarkable for *Ly49* genes, prompting them as primary candidates for the MA/My resistance gene. Consistent with this, our mapping efforts have localized *Ly49e* within the 1-LOD-support interval in more than 700 informative meiosis. To present date, 14 members of *Ly49* gene family are identified in the C57BL/6 strain and at least 18 genes are counted in the mouse strain 129 (Makrigiannis et al., 2002). It was therefore important to characterize the MA/My *Ly49* gene repertoire in order to identify possible candidate genes or variants underlying MCMV-resistance.

In accordance to haplotype analysis and Ly49 receptor homology observed between MA/My and 129, we can suppose that MA/My Ly49 repertoire is composed of the same number of similar receptors as the 129 Ly49 repertoire. Certainly, the MA/My repertoire is composed of at least 7 *Ly49* genes from which 3 are identical and 4 could be allelic forms of 129 *Ly49* genes. The MA/My *Ly49* gene repertoire encodes at least 4 types of inhibitory receptors: Ly49G2, Ly49V, Ly49G2/V and Ly49I. The repertoire of this strain presents a new gene coming from the recombination between *Ly49g2* and *Ly49v*. This phenomenon of recombination was seen for the first time in 129 mouse strain (Makrigiannis et al., 2002). This new inhibitory gene receptor, which is for now unique to MA/My strain, increases the level of complexity of the *Ly49* gene family. The MA/My strain possess also three activating genes encoding activating receptors: Ly49P, Ly49R and Ly49U. It has been proposed that *Ly49u* is an allelic form of *Ly49h* (Makrigiannis et al., 2002). Contrary to Ly49H, *in vitro* assays have demonstrated that

cells transfected with Ly49U^{129} are not able to recognize MCMV infected cells, indicating that *Ly49h* and *Ly49u*^{129} are functionally distinct (Arase et al., 2002b).

Similarities between 129 and MA/My *Ly49* gene receptors are remarkable. For example, Ly49U and Ly49R in MA/My are identical to those in 129 at the amino acid level while Ly49U^{129} has 20 amino acids different with its allelic form Ly49H$^{C57BL/6}$. Also, Ly49P^{129} differs by 1 amino acid from Ly49P$^{MA/My}$ while it is absent in C57BL/6 mouse strain. Interestingly, the MA/My *Ly49* gene repertoire is also similar to the repertoire of FVB/N strain. In fact, the inhibitory receptor Ly49I and the activating receptors Ly49P and Ly49U were isolated from FVB/N NK cells. While Ly49I (data not shown) and Ly49P (Makrigiannis personal communication) are identical to MA/My at the amino acid level, Ly49U have few differences (data not shown). These results indicate closer phylogenetic distance between MA/My, 129 and FVB/N at this region than between C57BL/6 and suggest that MA/My has the same *Cmv1* alleles as FVB/N and 129.

Contrary to our expectation, the analysis of variance demonstrated that there is no effect of the NKC loci in 129 x BALB.K cross suggesting the possible presence of an alternative *Cmv1* allele in 129. However, the presence of a similar allele in that strain cannot be excluded, since other background genes may explain the absence of effect of the NKC loci in our sample. We also tested the presence of *Cmv1*^{rm} allele in the susceptible strain FVB/N. Although we were not able to reproduce the genetic interaction between the *H2* and NKC loci, we detected the presence of an important additive effect of the two loci independently. In order to confirm the allelic composition at the NKC for both strains, extensive genotyping in the Ly49 region and comparison with the MA/My

haplotype will be needed. So far, we cannot exclude the presence of *Cmv1*^{rm} resistance mechanism in other inbred strains such as 129 and FVB/N.

Ly49P, Ly49R and Ly49U as potential candidates for *Cmv1*^{rm} in MA/My

To provide further evidence for the function of a candidate gene, we followed the MCMV-infection at three different time-points at peaks of acute infection. During MCMV infection in C57BL/6 mice, evidence of NK cell proliferation associated with control of virus infection was observed. In fact, it was demonstrated that there is a non-specific NK cell proliferation very early between 6-18 hrs post-infection, regardless of NK cell expression of the MCMV-specific Ly49H receptor. Following this early non-specific phase, there is selective proliferation of NK cells expressing the specific activation receptor Ly49H, which recognizes the MCMV-encoded protein m157. Finally, there is a significant contraction of both Ly49H$^+$ and Ly49H$^-$ NK cells which coincides with the onset of the adaptive immune response (Dokun et al., 2001a). Importantly, this proliferation is associated with an enhanced control of viral titers in the spleen and the liver (Lee et al., 2003b). We observed a similar temporal/tissue specific pattern of the control of MCMV infection in spleen and liver in MA/My suggesting a similar mechanism of action, i.e.: our candidate must be also an activating receptor. Therefore, the activating receptors Ly49P, Ly49R and Ly49U are good candidate for the resistance to MCMV infection in the MA/My inbred strains.

The ligand binding specificity to MHC class I of these 3 receptors has been studied in 129 strain (Makrigiannis et al., 2001), and in NOD for Ly49P (Silver et al., 2000). Using labelled MHC class I tetramers, Makrigiannis et al. (2001) have shown that Ly49P and Ly49U have very weak affinity for any of the H-2^k, H2^d or H2^b molecules

tested while Ly49R is clearly stained by D^d tetramers. Using rat RNK-16 cells transfected with Ly-49P, it was shown that Ly49P induced lysis of susceptible targets is dependent on H-$2D^d$-bearing targets. However, none of these studies tested the role of the molecules in the context of MCMV infection, thus the possibility that one of the MA/My Ly49 activating receptors recognize an $H2^k$-infected cell cannot be excluded at this time. It is plausible that ligation of an $H2^k$ molecule depends on the presence of an infection-specific peptide, as described for the inhibitory receptor Ly49C (Franksson et al., 1999). Then, the discrimination between normal and infected cells would be at the level of the conformational change caused by the peptide. If it is also the case for Ly49P, Ly49R or Ly49U, then we can think that these could specifically and selectively recognize and cause the destruction of cells expressing at their surface $H2^k$ molecules containing MCMV-encoded proteins.

Conclusion

In conclusion, the results obtained in this study demonstrate the presence of *Cmv1*^{rm}, a new MA/My resistance allele effective in the presence of $H2^k$ molecules. Although *Cmv1*^{rm} does not seem to have an effect in 129, the allele seems to confer a certain resistance in FVB/N. A possible scenario to account for the differences in the outcome of MCMV infection in the 3 strains could be based in differences at the expression levels of similar inhibitory and activating Ly49 receptors exerting a balanced control of NK cells activity. Whether such differences depend on molecular differences at *Cmv1*^{rm} or on the influence of genes elsewhere in the genome remains to be determined.

Our results indicate that, in addition to Ly49H, there are multiple mechanisms of defense against mouse cytomegalovirus mediated by Ly49 receptors. We would like to propose that this complexity accurately models human cytomegalovirus infection and expect that alternative KIR receptors will influence susceptibility to cytomegalovirus in different populations. The MA/My model reported here provides a novel framework to the molecular dissection of such mechanisms.

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APPENDIX I

1. Research Compliance Certificates

a) Animal research protocol

b) Radioactive materials permit