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From *Cmv1* to *Ly49h*: Molecular Genetics, Haplotype Analysis and Transgenesis to Characterize Innate Immunity to Cytomegalovirus

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From *Cmv1* to *Ly49h*: molecular genetics, haplotype analysis and transgenesis to characterize innate immunity to cytomegalovirus

Seung-Hwan Lee

Thesis submitted to the Faculty of Graduate Studies and Research
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ABSTRACT

In mice, natural resistance to infection with murine cytomegalovirus (MCMV) is controlled by a dominant chromosome 6 locus, *Cmv1*, which is expressed at the level of Natural killer (NK) cells. To characterize the molecular mechanism underlying MCMV-resistance, our laboratory initiated a positional cloning approach for the identification of *Cmv1*, and localized the host resistance locus to a 0.35 cM genetic interval, corresponding to 1.6 Mb genomic DNA.

This dissertation presents the results leading to the cloning and characterization of the *Cmv1* gene. First, I constructed a transcription map for the *Cmv1* interval, which included 19 genes comprising the full *Ly49* gene cluster and other novel candidate genes. In an attempt to narrow down the localization of *Cmv1*, I determined haplotypes in the vicinity of the *Cmv1* interval in a panel of 17 inbred mice strains. This approach demonstrated the presence of three major classes of independent haplotypes. Close inspection of allele sharing among those haplotypes excluded *Ly49e*, *Ly49f* and *Ly49d* as *Cmv1* candidates.

As an alternative approach to cloning *Cmv1*, I took advantage of a recombinant inbred strain, the BXD-8 strain. The BXD-8 line was of particular interest because it is susceptible to MCMV infection while harboring an MCMV-resistance C57BL/6 haplotype at *Cmv1*. Using STS content and PFGE analysis, I showed the presence of a 23 kb deletion in BXD-8. Subsequent gene expression analysis of the full *Ly49* gene cluster revealed that the deletion of *Ly49h* is associated with MCMV susceptibility in BXD-8 mice.

Finally, to test the role of *Ly49h* in host resistance to MCMV infection, I introduced the *Ly49h* gene into a susceptible background. Investigation of *Ly49h* gene expression by RT-PCR and FACS analysis demonstrated a strong correlation between the level of *Ly49h* mRNA and protein expression and the control of MCMV replication, providing conclusive evidence of the allelism between *Cmv1* and *Ly49h*.

In conclusion, I identified the *Ly49h* gene encoding a NK activation receptor as *Cmv1*, supporting a specific role for NK cells in antiviral immunity. Transgenic mice expressing Ly49H will be instrumental for understanding of mechanism of NK mediated resistance and possible pleiotropic effects of *Cmv1*.

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PREFACE

The work presented in this thesis has been published as follows:

Chapter 3

Chantal Depatie, **Seung-Hwan Lee,** Amanda Stafford, Philippe Avener, Philippe Gros and Silvia M. Vidal. Long-range physical map, transcription map and construction of a sequence-ready BAC contig of a 2-Mb region overlapping the host resistance locus *Cmv1* on distal mouse chromosome 6. *Genomics* 66, 161-174, 2000.

Chapter 4

Seung-Hwan Lee, John Gitas, Ahmed Zafer, Pierre Lepage, Tom Hudson, Abdelmajid Belouchi and Silvia M. Vidal. Haplotype mapping indicates two independent origins for the *Cmv1s* susceptibility allele to cytomegalovirus infection and refines its localization within the *Ly49* cluster. *Immunogenetics* 53, 501-505, 2001.

Chapter 5

Seung-Hwan Lee, Sonia Girard, Denis Macina, Maria Busa, Ahmed Zafer, Abdelmajid Belouchi, Philippe Gros and Silvia M. Vidal. Susceptibility to mouse cytomegalovirus is associated with deletion of an activating natural killer cell receptor of the C-type lectin superfamily. *Nature Genetics* 28, 42-45, 2001.

Chapter 6

Seung-Hwan Lee, Ahmed Zafer, Yves de Repentigny, Rashmi Kothary, Michel L. Tremblay, Philippe Gros, Pascale Duplay, John R. Webb and Silvia M. Vidal. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus infection in genetically susceptible mice. *The Journal of Experimental Medicine* 197, 515-526, 2003.

In addition, parts on the Chapter 1 (Introduction) and the Chapter 7 (Discussion) have been combined in:

Seung-Hwan Lee, John R. Webb and Silvia M. Vidal. Innate immunity to cytomegalovirus: the *Cmv1* locus and its role in natural killer cell function. *Microbes and Infection* 4, 1491-1503 2002.

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LIST OF ABBREVIATIONS

A2m	Alpha 2 Macroglobulin
Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
bp	Base Pairs
BAC	Bacterial Artificial Chromosome
BSA	Bovine Serum Albumin
°C	Degrees Celsius
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CEN	Centromere
CHO	Chinese Hamster Ovary
cM	Centimorgans
CMV	Cytomegalovirus
CSDA	Cold Shock Domain Protein A
CTL	Cytotoxic Lymphocyte
DAP	DNAX Activation Protein
DC	Dendritic Cells
DMEM	Dulbecco Minimal Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid

dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid disodium salt
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein Isothiocyanate
Fix/Perm	Fixation and Permeabilization
GFP	Green Flurescent Protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
Gps	Glycoproteins
GST	Glutathione S Transferase
H2	Histocompatibility-2
HCMV	Human Cytomegalovirus
HEPES	N-2-hydroxethylpiperazine-N-2-ethane-sulphonic acid
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
Hpi	Hours Post Infection
HSV	Herpes Simplex Virus
IBD	Identical By Descent
IFN	Interferon

Ig	Immunoglobulin
iNOS	Inducible Nitric Oxide Synthase
IL	Interleukin
ILT	Ig-like transcript
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
kb	Kilobases
KIR	Killer cell Immunoglobulin-like Receptor
LAK	Lymphokine Activated Killer Cells
LD	Linkage Disequilibrium
LIR	Leukocyte Immunoglobulin-like Receptor
LRC	Leukocyte Receptor Complex
mAb	Monoclonal Antibody
MagoH	Mago Nashi Homologue
Mb	Megabases
MCMV	Murine Cytomegalovirus
MDV	Marek's Disease Virus
MEF	Mouse Embryo Fibroblasts
MFI	Median of Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MuHV	Murid HerpesVirus
NFAT	Nuclear Factor of Activated T cells
NK	Natural Killer

NKC	Natural Killer Gene Complex
NKT	Natural Killer T
ORF	Open Reading Frame
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PFGE	Pulse Field Gel Electrophoresis
PFU	Plaque Forming Unit
PI3K	Phosphoinositol-3 Kinase
PRP	Proline Rich Protein
RAE	Retinoic Acid Early Inducible
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SSCP	Single Stranded Conformational Polymorphism
SSLP	Simple Sequence Length Polymorphism
STAT	Signal Transducers and Activators of Transcription
STS	Sequence-Tagged Site
TAE	Tris Acetate-EDTA Buffer

TCF	T Cell Factor
TE	Tris-EDTA Buffer
TEL	Telomere
TNF	Tumor Necrosis Factor
UTR	Untranslated Region
YAC	Yeast Artifical Chromosome

CHAPTER 1

GENERAL INTRODUCTION

Cytomegalovirus (CMV) is a widely distributed pathogen that is responsible for severe disease in immunocompromised individuals (Rawlinson et al. 1999) and probably associated with vascular disease in the general population (Levi et al. 2001). There is increasing evidence that cells of the innate immune system play a key role in controlling this important pathogen. This is particularly evident in the experimental murine CMV model of infection which has revealed an important role for Natural Killer (NK) cells in controlling early viral replication after infection with murine cytomegalovirus (MCMV). In this model, different strains of inbred mice exhibit striking differences in their level of susceptibility to MCMV infection. Genetic studies, performed more than 10 years ago, revealed that this pattern of susceptibility/resistance can be attributed to a single genetic locus termed *Cmv1* (Scalzo et al. 1990).

The main goal of this project was to identify and characterize the *Cmv1* gene to provide a better understanding of the role of NK cells in innate immunity against MCMV infection. This chapter provides a general background of CMV and its mouse model of infection. In addition, it introduces the mouse host resistance locus *Cmv1* as well as the initial efforts on the positional cloning of the *Cmv1* gene.

1.1. PATHOGEN: CYTOMEGALOVIRUS

1.1.1 History

The cytomegaloviruses are ubiquitous agents (Weller 1971) that have been recognized as a distinct subgroup of herpesviruses, based on their virion structure and their ability to establish persistent and latent infection (Pass 2001). CMV cytopathology was observed long before it was recognized as a virus and was initially thought to be due to a protozoan parasite. According to their typical cytopathology in salivary glands,

viruses in this group were generally named as salivary gland viruses until the common name cytomegalovirus was proposed by Weller (Weller 1957) to reflect virus-induced cytopathic effect, the characteristic formation of intranuclear inclusions and enlargement of the infected host cell. Virus infection may result in fulminant, life threatening or debilitating acute diseases depending on the host immunological state.

1.1.2. Taxonomy and classification of cytomegaloviruses

The family *Herpesviridae* is a family of DNA viruses having an enveloped virion 150-200 nm in diameter. The family *Herpesviridae* is characterized by four significant biological properties (Roizman and Pellett. 2003): 1) All herpesviruses carry a large array of enzymes involved in their metabolism. 2) The synthesis of viral DNAs and capsid assembly occurs in the nucleus. 3) Production of infectious progeny virus is invariably accompanied by the destruction of the infected cell. 4) All herpesviruses examined to date are able to remain latent in their natural hosts. Herpesviruses also differ with respect to their biologically properties. Accordingly, the members of the family *Herpesviridae* were further grouped into three subfamilies, the Alphaherpesvirinae, the Betaherpesvirinae and the Gammaherpesvirinae (Roizman and Pellett. 2003). Alphaherpesvirinae are characterized by a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells and capacity to establish latent infections primarily in sensory gangalia. This subfamily contains the genera Simplexvirus (HSV), Varicellovirus (VZV) and Marek's disease-like virus (MDV). Betaherpesvirinae exhibit a restricted host range, a long reproductive cycle and slow growth in culture. This subfamily includes the human cytomegalovirus (HCMV), murine cytomegalovirus (MCMV) and Roseolovirus. Finally,

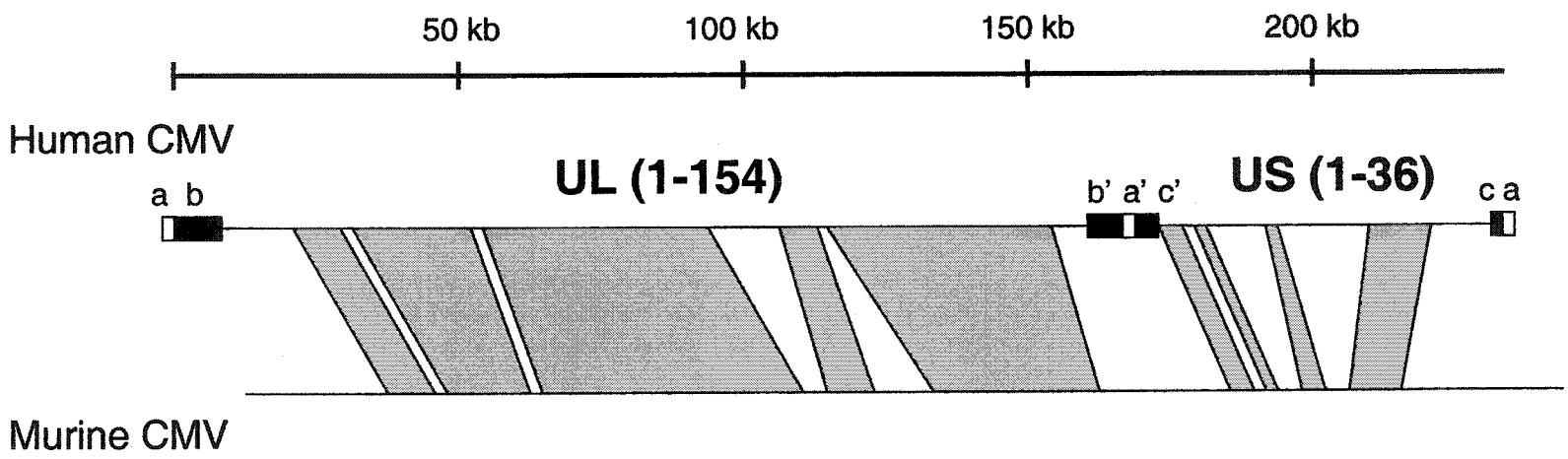
Gammaherpesvirinae show strong tropism for either T or B lymphocytes. As a result, latent virus is frequently demonstrated in lymphoid tissue. This subgroup contains two genera: Lymphocryptovirus, including Epstein Barr Virus (EBV), and Rhadinovirus.

Cytomegaloviruses are the principal members of the Betaherpesvirinae subfamily. All CMVs share common growth characteristics and a cytopathology involving cell enlargement. Besides humans, the distribution of CMVs in nature appears extremely wide, with biologically similar viruses found in other primates, rodents and almost every other animal in which such a virus has been sought. Human CMV has been formally designated as human herpesvirus 5 (HHV-5) by the International Committee on Taxonomy of Viruses whereas mouse CMV was designated as Murid herpesvirus 1 (MuHV-1) (http://www.ncbi.nlm.nih.gov/ICTV/).

1.1.3. CMV genome

The virion of human CMV is composed of a linear DNA genome in an icosahedral nucleocapsid, surrounded by a tegument layer and enveloped in a lipid bilayer membrane (Mocarski 2003). The genomes of the characterized CMVs are all linear DNA molecules ranging in size from 200 to 240 kb, representing the highest potential coding capacity within the herpesvirus family. The genomes of a laboratory-adapted human CMV, the AD169 strain, and the murine CMV Smith strain have been completely sequenced and their similarity compared over at least 200 nonoverlapping open reading frames (ORFs) (Chee et al. 1990a; Rawlinson et al. 1996) (Figure 1.1).

Figure 1.1. Genome structure of HCMV and MCMV. The complete sequences of the AD169 strain of HCMV and the Smith strain of MCMV are compared. The top line is a size scale in kbp. Class E isomerizing genome of HCMV is shown with its characteristic elements in the termini. The high similarity is depicted as grey.


50 kb
100 kb
150 kb
200 kb
Human CMV
UL (1-154)
US (1-36)
a b
b' a' c'
c a
Murine CMV

Gene blocks located in the central 100kb of the HCMV and MCMV genome, encoding 78 ORFs, are closely related to each other, whereas the sequences near the ends of the genomes are less similar and harbor gene families that are arranged in tandem arrays and encode mostly glycoproteins (gps) (Rawlinson et al. 1996) (Figure1.1).

Genomes of herpesviruses are classified by the presence and location of reiterations of terminal sequences greater than 100 bp. According to the scheme, the herpesviruses can be divided into six groups designated by the letters A to F. HCMV includes a class E isomerizing genome, like that of herpes simplex virus (HSV) (Roizman and Pellett. 2003). For example, the termini of HCMV genome consist of two elements. One terminus contains n copies of sequence *a* next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated as *c*. The terminal *ab* and *ca* sequences are also inserted into the genome in an inverted orientation separating the unique sequences into long unique (UL) and short unique (US) sequences (Figure 1.1). ORFs in the human CMV genome are numbered sequentially and designated by their location within the unique (UL, US) sequences and repeated regions of the viral genome, and a nomenclature for naming viral genes and proteins has evolved. Nomenclature of animal CMV ORFs follows that set in place for human CMV ORFs, even though their genomes do not represent the E-type genomes. Therefore, murine or rat CMV ORFs that retain sequence similarity to human CMV ORFs are designated with upper-case letters (e.g., M44, R44) whereas those not conserved in human CMV are represented by lower-case letters (m144, r144). ORFs found in US sequence or the repeated regions of the human CMV genome are not well conserved in other betaherpesviruses (Rawlinson et al. 1996).

As for other herpesviruses, CMV replication is tightly regulated in a multistep process of protein expression. Immediate-early (α), early (β) and late (γ) proteins are synthesized sequentially from corresponding mRNAs whose transcription is regulated in a temporal cascade (Mocarski 2003). Immediate-early proteins are required to regulate transcription from their own promoters and those of subsequently expressed genes. The expression of α genes begins at one hour post infection (hpi) and peaks between 4 and 8 hpi, independently of *de novo* protein synthesis. The transcriptional activation of α genes is influenced by cellular transcriptional factors such as NK-κB, AP-1, Sp1 and CREB/ATF because immediate-early genes have strong promoters, containing a dense collection of host transcription factor binding sites. The cellular basis of permissiveness for CMV replication mainly depends on the efficiency of early viral gene expression and subsequent DNA replication. Expression of early (β) genes depends on the expression of functional immediate-early (α) gene products. Early proteins include many of the enzymes and regulatory factors needed to carry out the synthesis of progeny DNA and proteins. Late (γ) gene transcription starts later than 24 hpi and encodes for most of the structural proteins and surface glycoproteins, proceeding last immediately before virion assembly and eventual lysis of the infected cell.

1.1.4. Immune evasive genes

A characteristic feature of CMV infection in the normal host is the persistence of productive infection, viremia, and virus excretion for months or even years in the presence of the host immune response. The ability of the virus to avoid elimination or termination of active infection by the host's immune system is mediated by CMV gene

products that have the potential to interfere with the immune response (Hengel et al. 1998). For example, several open reading frames have been shown to mediate HCMV and MCMV escape from cytotoxic lymphocyte (CTL) responses. For the recognition of a virus infected cell by CTL, the proper expression of MHC molecules is a prerequisite for antigen presentation. In humans, US3 promotes retention of major histocompatibility complex (MHC) class I in the endoplasmic reticulum. Morevover US2 and US11 have been shown to mediate degradation of MHC class I heavy chain while US6 interferes with antigen presentation (Hengel et al. 1998). In mice, three gene products, m04, m06 and m152 also interfere with MHC class I presentation. The m04 and m06 products attach tightly to mature β2 microglobulin-associated MHC class I molecules in the ER (Kleijnen et al. 1997). The m04 protein does not down regulate MHC class I at the plasma membrane, instead it forms complexes with ternary MHC class I molecules in the ER that are then expressed on the cell surface. The m06 protein binds to MHC class I complexes and redirects their transport to the endocytic route for rapid proteolytic destruction. The m152 gene product accounts for the arrest and accumulation of MHC class I molecules in the ER-Golgi intermediate compartment (del Val et al. 1992; Ziegler et al. 1997).

In addition, a number of other CMV genes encode homologs of cellular gene products that are also thought to play a role in subverting host immune response. Comparison of the complete CMV genome sequence with published host sequences revealed that human and mouse CMV have predicted gene products similar to cellular proteins such as major histocompatibility complex class I antigens, T cell receptor delta chain and chemokine G protein coupled receptors (Chee et al. 1990a; Chee et al. 1990b; Rawlinson et al. 1996). In particular, viral homologs of MHC class I molecules have

been shown to play an important role in interfering with NK cell activity. As NK cells have the ability to spontaneously kill target cells with impaired MHC class I expression, viral MHC class I decoys could inhibit NK cell-mediated killing of virally infected cells. For example, in mouse CMV, deletion of the MHC class I homolog (m144) results in reduced viral titers in susceptible BALB/c mice (Farrell et al. 1997), suggesting that this MHC class I homologue interferes with NK cell cytotoxic activity.

More recently, a bioinformatics approach identified 11 other ORFs in the MCMV genome that encode molecules with putative MHC class I folds (Smith et al. 2002), as determined by structural homology analysis (3D-PSSM), even though they did not show any similarity at the nucleotide sequence level. One of these, m157, was recently identified as a ligand for Ly49 receptors (Arase et al. 2002; Smith et al. 2002) and will be described in the Discussion chapter. The function of others in MCMV infection remains to be identified, but they might also play a role in subverting the host immune response during virus infection.

1.2. CYTOMEGALOVIRUS INFECTION IN HUMANS

Human cytomegalovirus (HCMV) infects more than 70% of the world's population regardless of geographical location or gender (Rawlinson 1999). Following primary infection, HCMV establishes long-term latency with intermittent reactivation (Pass 2001; Soderberg-Naucler and Nelson 1999). In latent infection, viral DNA is present in mononuclear cells derived from peripheral blood or bone marrow of healthy seropositive individuals, but infectious virus is not present (Slobedman and Mocarski 1999). In the latently infected cells, viral gene expression is restricted to latency-associated transcripts and may become productive under proinflammatory conditions

(Kondo et al. 1994). Proinflammatory cytokines are known to stimulate latently infected cells causing differentiation into a permissive cell type that supports viral replication (Soderberg-Naucler and Nelson 1999).

Usually asymptomatic in immunocompetent individuals, HCMV infection may result in severe or even fatal disease in immunocompromised patients (Reusser et al. 1997; Reusser 1998; Soderberg-Naucler and Emery 2001). The most dangerous situation for adults is to undergo a primary infection following blood transfusion or transplantation while also receiving an immunosuppressive regimen. During primary infection, virus is restricted to the salivary gland and kidney in immunocompetent individuals whereas dissemination to a wide range of tissues, with or without specific disease, commonly occurs in immunocompromised individuals. In addition, HCMV is the leading cause of congenital viral infection in humans, with an incidence of 0.2-2.2% amongst live births, of which at least 10% will present with birth defects such as hearing loss or mental retardation (Demmler 1991; Trincado et al. 2000). Interestingly, in the general population the clinical outcome of HCMV infection is non-random as polymorphisms in genes of pro-inflammatory cytokines (TNF-α, IL-1RA) are in association with seropositivity (Hurme and Helminen 1998). HCMV is also responsible for a substantial fraction of the morbidity and mortality that occurs following organ transplantation (Farmer et al. 2001; Kaufman et al. 2001; Nachbaur et al. 2001; Rubin 1999; Singh et al. 2001). In the transplant setting, symptomatic HCMV infection encompasses a range of clinical illnesses including interstitial pneumonia in 50-90% of bone marrow recipients and organ-specific disease in 20-40% of solid organ transplant recipients (de Medeiros et al. 2000; Snydman 1999; Sparrelid et al. 1997; Stratta 1993; Tolkoff-Rubin and Rubin 1998). In transplant recipients susceptibility has been

associated with specific genotypes, in particular with alleles of the HLA locus, also indicating a genetic component (Yamada et al. 2000). HCMV diseases, including retinitis, colitis, and encephalitis, occur in persons with AIDS and have been associated with decreased survival after diagnosis of HIV infection (Murphy et al. 2001; Salmon-Ceron 2001; Welch et al. 1998). Additionally, the impact of HCMV infection in the general population may have been underrated since clinical and experimental evidence is accumulating that there may be a link between HCMV infection and vascular disease (Levi 2001; Valantine 1999; Vercellotti 2001). For example, HCMV seropositivity is a strong risk factor associated with restenosis following coronary atherectomy (Neumann et al. 2001; Zhou et al. 1996) and transplant-associated atherosclerosis in heart transplant patients (Epstein et al. 1996; Koskinen et al. 1999), whereas in animal models, mouse cytomegalovirus (MCMV) infection accelerates formation of atheromatous lesions in $apoE^{-/-}$ mice (Burnett et al. 2001; Hsich et al. 2001). Moreover, a related herpesvirus known as Marek's disease virus (MDV) was found to cause the formation of atherosclerotic lesions in the aortas of chickens and led to accumulation of cholesterol in the aortic wall of MDV-infected chickens (Fabricant and Fabricant 1999).

There is currently no vaccine available against HCMV and benefits of treatment with available drugs -foscarnet, gancyclovir and cidofovir- are undermined by toxicity and emergence of drug resistant strains (Chou 1999; Emery 2001; Villarreal 2001; Zaia et al. 2000) indicating the need for additional therapeutic approaches. The serious medical problems associated with HCMV infection have thus stimulated research aimed at understanding the complex interplay of viral and host functions that lead to pathogenesis. In this respect, understanding, at the molecular level, the early host

resistance against HCMV infection could provide a rationale for developing alternative therapeutic strategies that either stimulate or exploit host resistance mechanisms.

1.3. MOUSE MODELS OF CMV INFECTION

Generally the mouse has provided the ideal model for studying host susceptibility to infectious disease because of the underlying biological similarity between mouse and human (Blackwell 2001; Cooke and Hill 2001). The ability to generate hundreds of meioses from a single set of genetically defined parents and well-defined experimental conditions for mouse infection studies alleviates the problems of genetic heterogeneity and environmental effects inherent to human studies. In addition, reverse genetics technologies enabling targeted mutagenesis and transgenesis helped to define key molecules participating in host defense against various pathogens.

Although cytomegaloviruses characteristically exhibit strict host species specificity, infection of mice with murine cytomegalovirus (MCMV) has served as a useful model for HCMV infection since these viruses share many similarities in their biological properties and pathogenesis (Pass 2001). Thus, infection of mice with MCMV recapitulates the large spectrum of clinical manifestations observed in virus-induced disease in humans, from congenital infection (Fitzgerald and Shellam 1991) to fatal disease (Shanley et al. 1993). For example, high viral titres in target organs are associated with pneumonitis (Shanley 1984), hepatitis (Trgovcich et al. 2000) and retinitis (Hayashi et al. 1985; Hayashi et al. 1995). Moreover high viral titres in the spleen or liver may lead to fatal infection (Shanley et al. 1993). There are also models of congenital viral infection by MCMV (Fitzgerald and Shellam 1991; Shellam and Flexman 1986).

More importantly, different strains of inbred mice exhibit striking differences in their level of susceptibility to MCMV infection, as measured by organ-specific viral replication in the first days after infection, disease severity and survival, indicating host genetic factors controlling the susceptibility (Allan and Shellam 1984). Therefore, experimental models of infection of mice with MCMV have provided an excellent tool for the *in vivo* dissection of the individual components of the host response that are important in mediating resistance to MCMV and HCMV infection.

1.4. IMMUNE RESPONSE TO CMV INFECTION

To date, all available evidence suggests that the cellular response to cytomegalovirus infection is strikingly similar in humans and mice (Pass 2001). Therefore most of available data generated in mice might be informative for our understanding of the immune response to HCMV infection. The immune mechanisms involved are complex and include both innate and adaptive immune responses.

1.4.1. Innate immunity to CMV infection

Innate immunity constitutes the first line of defense, providing a rapid response by the expression of germ-line encoded proteins that pre-exist or are induced within hours of infection. Cytokines, chemokines, complement, phagocytes and natural killer cells represent key participants in innate immunity. The innate immune system is comprised of a battery of so-called pattern recognition receptors (Janeway, Jr. and Medzhitov 2002) that stand at the ready to immediately detect and promote an appropriate response against incoming pathogens prior to the activation of the adaptive

immune system. The increased susceptibility to CMV in humans and mice lacking innate components such as various cytokines or NK cells indicated that innate immunity is crucial in the resistance to MCMV infection.

1.4.1.1. Cyokines and chemokines

Cytokines are soluble protein factors produced by cells to act in the cells bearing corresponding cytokine receptors. Cytokines mediate mainly autocrine and paracrine effects. Cytokines are generally pleiotropic and activate multiple distinct pathways, but may also have overlapping functions (Biron 2001a). Importantly, cytokines are involved in both innate and adaptive immunity.

Various cytokines including Interferon (IFN)-α, β, γ, IL12 and IL18 are important in MCMV infection as viral susceptibility is increased in mice lacking those cytokines or their receptors (Table 1.1.). It has been shown that IFN-α, β produced by MCMV infected cells activate NK cell responses, such as NK blastogenesis and activation of their cytolytic function, which provide protective immunity during the early phases of MCMV infection (Welsh 1978; Biron 2001b). Mice depleted of IFN-α, β with neutralizing antibodies or by homologous recombination (IFN-α, β -/- mice) presented a poor NK cell response against MCMV infection (Orange and Biron 1996a) and succumbed shortly after infection (Salazar-Mather et al. 2002).

Table 1.1. Natural and induced mutations of genes affecting susceptibility against MCMV

Gene name	*Expression*	*References*
Cytokine, chemokine-associated genes		
IL2/IL15Rβ -/-	T, NK	(Tsunobuchi et al. 2000)
IL-12 -/-	B, Macrophage	(Carr et al. 1999; Pien et al. 2000)
IL-18 -/-	Macrophage	(Pien et al. 2000)
IFN-α/βR -/-	NK, Dendritic Cell	(Dutia et al. 1999)
IFN-γ -/-	T, NK	(Ellison et al. 2000; Fernandez et al. 2000)
IFN-γR -/-	Macrophage	(Fernandez et al. 2000)
IRF-1 -/-	T, NK	(Dutia et al. 1999; Fernandez et al. 2000)
MIP-1α (CCL3) -/-	T, Monocyte, Mast cell	(Salazar-Mather et al. 1998)
Cytotoxicity associated genes		
Beige (Lyst)	T, NK, Macrophage	(Ellison et al. 2000; Rager-Zisman et al. 1987)
Perforin -/- (PKO)	T, NK	(Fernandez et al. 2000; Ghiasi et al. 1999; Riera et al. 2000)
Granzyme-A -/-	T, NK	(Riera et al. 2000)
Granzyme-B -/-	T, NK	(Riera et al. 2000)
Recombination associated genes		
SCID	T, B	(Ellison et al. 2000; Welsh et al. 1991)
RAG-2 -/-	T, B	(Riera et al. 2000)
Leukocyte receptor-associated genes		
Ly49h (Klra8)	NK	(Brown et al. 2001a; Lee et al. 2001a)

Several cytokines produced by NK cells during MCMV infection are important in protection. Depletion of IFN-γ or IL-12, an inducer of IFN-γ production, with monoclonal antibodies increased the incidence of MCMV-induced hepatitis and viral replication in the liver (Orange et al. 1995; Orange and Biron 1996a; Tay and Welsh 1997). A role for NK cell mediated regulation of MCMV infection was further demonstrated in IFN-γ receptor null mice. These mice present higher viral titers in the liver, suggesting that NK cell-produced IFN-γ is responsible for control of viral replication in the liver (Tay and Welsh 1997). Probably IFN-γ exerts its antiviral effects

via induction of inducible nitric oxide synthase (iNOS) in cells such as macrophages, Kupffer cells, and hepatocytes. In fact, mice treated with an iNOS inhibitor resulted in enhanced MCMV synthesis in the liver (Tay and Welsh 1997), while mice lacking iNOS developed significantly higher titers of infectious MCMV in the lung (Fernandez et al. 2000). In addition, chemokines normally expressed early during infection, such as MIP1-α (a mediator of inflammation), are important in promoting inflammatory responses in liver by recruiting NK cells. Mice deficient in MIP1-α failed to evoke both inflammatory and protective liver responses, resulting in high MCMV susceptibility (Salazar-Mather et al. 1998) (Table 1.1.).

1.4.1.2. Monocytes and macrophages

Monocyte-derived macrophages are key elements both in latent and acute infection with HCMV, suggesting that these cells play a major role in the pathogenesis and latency of HCMV (Fish et al. 1996). It has been demonstrated that differentiation of monocytes into macrophages renders these cells permissive to productive HCMV infection. Immunohistochemistry analysis of tissue sections of various infected organs confirmed the presence of viral proteins representing all stages of permissive HCMV infection in macrophages, suggesting that these cells play an important role in the spread of HCMV in solid organs (Sinzger and Jahn 1996).

In MCMV infection, monocytes and macrophages also play a central role in pathogenesis, providing functions beneficial to both the virus and the host. Differentiated macrophages are targets for MCMV infection within tissues. They harbor latent MCMV DNA, support viral replication both *in vitro* and *in vivo* and present

foreign antigen to CD4+ T cells in the context of MHC class II (Hayashi et al. 1985). These CD4+ T-cells might contribute to the protection against MCMV infection by producing IFN-γ and thereby activating macrophages. For example, activated macrophages produce nitric oxide and other cytokines that mediate inflammatory responses, such as IL-1α and TNF-α (He et al. 1995). Depletion of splenic macrophages significantly enhances replication of MCMV in the spleen (Hanson et al. 1999). Thus, viral replication in splenic macrophages may protect other highly permissive cell types from infection by filtering accessible virus.

1.4.1.3. Natural killer cells

NK cells are a population of lymphocytes that were originally characterized in terms of their ability to kill tumor cells. They are so named because of their propensity to kill, without prior sensitization, certain neoplastic and virus infected cells as well as normal allogenic bone marrow-derived cells (Trinchieri 1989; Biron et al. 1999). Although NK cells originate in the bone marrow and overlap functionally with T cells in many aspects, they mature in a thymus-independent manner and can be distinguished from T and B lymphocytes by their lack of variant surface antigen receptor molecules. However, it is becoming increasingly apparent that NK cells are anything but homogeneous, indeed the NK population is comprised of a complex arrangement of subsets expressing overlapping repertoires of invariant surface receptors specific for MHC class I and class-I like molecules (Lanier 1998). There is also increasingly compelling evidence that NK cells play a crucial role in host defence against infection, particularly herpesvirus infection, including CMV (Cerwenka and Lanier 2001).

The crucial role of NK cells in controlling primary CMV infection and reactivation, in humans and in experimental infections in mice, has been recognized for more than 20 years (Shellam et al. 1981). The most direct evidence supporting a role for NK cells in defence against herpesviruses comes from a patient identified with a complete lack of NK cells as well as no spontaneous or IL-2-inducible NK cell cytotoxic function (Biron et al. 1989). This deficiency of NK cell responses despite normal antibody and memory T cell functions resulted in a primary life-threatening HCMV infection. In addition, low NK cell activity is associated with HCMV reactivation and poor prognosis in graft recipients (Slavin et al. 1993; Venema et al. 1994).

Mouse studies have also provided evidence supporting an important role for NK cells in combating viral disease, particularly during the very early stages of infection (Biron and Brossay 2001c). Increased susceptibility to infection with MCMV, characterized by splenic viral titers 10^3 to 10^4 fold higher than in controls, was observed after *in vivo* depletion of NK cells with the monoclonal antibodies PK136 or anti-asialoGM1 (Scalzo et al. 1992), which recognize surface antigens preferentially expressed on NK cells. Moreover, the identification of spontaneous mutations as well as the characterization of models of targeted mutagenesis helped to define NK cells as the major participants in host defense against herpesviruses. For example, a link between resistance to MCMV infection and NK cell function was established by studies that showed that beige mice (genetically deficient in functional NK cells) have increased susceptibility to murine CMV (Shellam et al. 1981). Similarly, mice carrying targeted mutations within genes involved in the cytolytic function of NK cells, including those encoding perforin (Ghiasi et al. 1999; Fernandez et al. 2000; Riera et al. 2000) and granzyme A/B (Riera et al. 2000), also demonstrate high susceptibility to the early

stages of MCMV infection. As shown in the Table 1.1, although natural and induced mutations determining susceptibility to MCMV have an effect on several immune cell populations, the majority affect either the activation or function of NK cells.

1.4.2. Adaptive immunity to CMV infection

Adaptive immunity is a slower, yet highly specific response mediated by T and B lymphocytes that confers effective and long-lasting protection against infection. Adaptive immunity is based on the generation of a large repertoire of antigen-recognition receptors by somatic gene rearrangement. Adaptive immunity is characterized by specificity, immunological memory and self/nonself recognition.

1.4.2.1 T cells

Generally the severity of disease due to CMV infection parallels the degree of impairment of T-cell responses. T cell-mediated immunity against CMV infection involves contributions from CD8+ and CD4+ T cells (Pass 2001). Following prior immune maturation, CD8+ T cells recognize viral epitopes in the context of self MHC class I molecules on infected cells and kill these infected cells, whereas CD4+ T cells produce cytokines that activate the antiviral activities of NK cells and CD8+ T cells. In HCMV infection, adoptive transfer of CMV-specific cytotoxic T lymphocyte clones provides a benefit to the host in bone marrow allograft recipients and demonstrates that MHC class I restricted CTL response to structural proteins (ppUL83 and ppUL32) is sufficient for adaptive immunotherapy (Jin et al. 2000).

In MCMV infection, in fact, the cellular response by CD8+ T cells is sufficient to clear infection from most tissues and for survival (Koszinowski et al. 1990). Adoptive

transfer of CD8+ cytotoxic T cells protected mice from lethal challenge. However, clearance from particular organs such as salivary glands is dependent on CD4+ T cells response rather than on CD8+ cells. It has been known that specific adaptive cellular immunity is important for control of acute infection and maintenance of latency. For example, early investigations using mouse models of infection showed that suppression of T cell function led to reactivation and dissemination of natural infection (Reddehase et al. 1987).

1.4.2.2. B cells and antibody

Antibodies to approximately 10 immunogenic human CMV proteins can be detected in sera of seropositive humans (Britt 1991). The role of antibody in controlling CMV infections in immunocompetent adults is not clear. Humoral immunity is less important than cellular immunity in controlling CMV infections in immunocompromised patients, however immune gammaglobulin prophylaxis is effective at prevention of CMV disease (Snydman et al. 1987). Furthermore, humoral immunity appears to be important in preventing symptomatic infection in the fetus, in the breast fed newborn and in the premature infant who receives seropositive blood (Yeager et al. 1981). In a murine CMV model, passive immunization and active immunization against the envelope glycoprotein B protect mice from lethal challenge (Rapp et al. 1992).

1.5. HOST GENETIC CONTROL OF MCMV INFECTION

The distinct phenotypes of MCMV susceptibility exhibited by different inbred strains of mice have stimulated investigation of the genetic components of resistance. In laboratory mice, resistance to MCMV is under multigenic control, with contribution of both *H2* and non-*H2* genes (Allan and Shellam 1984). Whereas *H2* genes modulate the infectivity of individual target cells, non-*H2* genes regulate host resistance by cell-mediated defense mechanisms.

1.5.1. *H2* genes

The MHC (histocompatibility-2 (H2) in mice and human leukocyte antigen (HLA) in humans) is a set of genes, present in all vertebrates, with immunological and non-immunological functions. Resistance of adult mice to acute lethal infection with MCMV is controlled by genes linked to the H2 complex (Chalmer et al. 1977; Grundy et al. 1981). Studies in H2 congenic mouse strains demonstrated that animals possessing the $H2^k$ haplotype, particularly at the K/I-A region, are 10 times more resistant than mice of the $H2^b$ or $H2^d$ haplotypes. The main function of MHC molecules is the binding and presentation of antigenic peptides to T lymphocytes. However, subsequent studies indicated that the alleles of class-I MHC molecules affect the ability of MCMV to infect fibroblasts or macrophages but do not affect antigen presentation to T cells, suggesting that H2 molecules are receptors for MCMV. In fact, peritoneal macrophages from $H2^b$, $H2^d$, $H2^v$ and $H2^r$ mice are very permissive to MCMV infection, while macrophages from $H2^k$ mice remain relatively uninfected (Price et al. 1990). More specifically, *in vitro* infection of cells transfected with sequences encoding H2 molecules showed that,

while the $H2^k$ allele precludes MCMV infection of transfected cells, the $H2^b$ or $H2^d$ allele allow efficient infection by MCMV (Wykes et al. 1993; Price 1994).

1.5.2. Non-*H2* genes

So far, several non-*H2* genes that determine susceptibility to MCMV have been shown to affect NK cell function, confirming the major role of this cell population in the control of MCMV infection.

1.5.2.1. The *Beige* locus

In mice, the *Beige* mutation is a recessive trait that causes hypopigmentation, bleeding and generalized dysfunction of immune cells including NK cells. Mice with the *beige* mutation are the homologues of the Chédiak-Higashi syndrome in humans, which is characterized an increased susceptibility to cytomegalovirus (Shellam et al. 1981; Spritz 1998). Early studies demonstrated a deficiency in lysosomal activity in NK cells (Haliotis et al. 1980) in the *Beige*/Chédiak-Higashi syndrome, resulting in reduced cytolytic activity against tumors or virally infected cells. The sensitivity of *Beige* mice to MCMV provided the first evidence for the importance of NK cells in early infection.

Cloning of the *Beige* gene was achieved by positional cloning and confirmed by *in vitro* genetic complementation (Perou et al. 1996). In mice, its product is a ubiquitously expressed protein involved in the routing of proteins to the lysosomes. Because NK cells use their lysosomal compartment in the cytolytic process to transport reactive serine proteases, known as granzymes, and perforin to the cell surface, the deficiency in the lysosomal protein would explain the impaired NK cell cytolytic activity observed in *beige* mice. Although the function of the human counterpart is not

yet known, it is also thought to be involved in the regulation of intracellular protein traffic.

1.5.2.2. The *Cmv1* locus

Enumeration of MCMV viral titers in the spleens and livers of different inbred mouse strains identified highly resistant (C57BL/6) and highly susceptible strains (BALB/c, A/J and DBA) in terms of viral replication in target organs (Allan and Shellam 1984). Genetic studies using these MCMV resistant and susceptible strains as parental strains to generate segregating F2 and backcross populations identified a single locus, *Cmv1*, which determines early viral replication (Scalzo et al. 1990).

1.6. THE *Cmv1* LOCUS AND ITS GENETIC CONTEXT

1.6.1. Summary of the *Cmv1*locus

Mendelian analysis of the progeny of crosses between MCMV resistant and susceptible strains indicated that *Cmv1* controls early viral replication in a dominant manner (Scalzo et al. 1990). The effect of this locus is observed in the spleen, bone marrow and thymus, with only a minor effect in the liver at early time-points (Scalzo et al. 1990; Price et al. 1993) (Figure 1.2). *Cmv1* presents two allelic forms in inbred mouse strains, either the dominant $Cmv1^r$ (low virus titer, resistant) or the recessive $Cmv1^s$ (high virus titer, susceptible). In the spleen, $Cmv1^s$ mouse strains exhibit splenic MCMV titers that are 10^3 to 10^4 times higher than $Cmv1^r$ mice at day 3 post-infection (Scalzo et al. 1990). Studies of MCMV replication in *H2* compatible irradiation bone marrow chimeras indicated that *Cmv1* mediates its effect through an irradiation-sensitive, bone marrow-derived cell population. Depletion of T cells by intraperitoneal

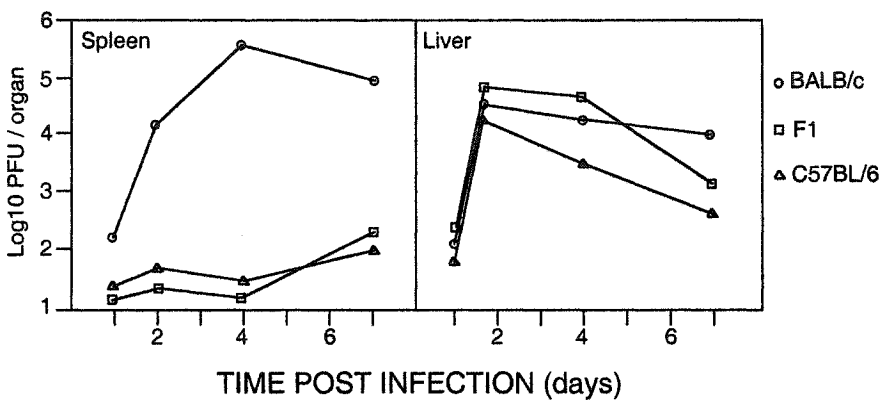
administration of anti-CD4 or anti-CD8 monoclonal antibodies had no effect on virus titers in the spleens of C57BL/6 mice at day 3 post-infection. In contrast, *in vivo* depletion of NK cells using a monoclonal antibody directed towards the NK1.1 alloantigen expressed on NK cells resulted in increased MCMV growth in spleen indicating an NK cell mediated mechanism of resistance (Scalzo et al. 1992). The mechanisms by which NK cells bearing the *Cmv1*^{r} allele are able to control early MCMV replication in the spleen appear to be IFN-γ-independent and perforin-dependent (Tay and Welsh 1997). Interestingly, initial mapping of the *Cmv1* locus by Scalzo and colleagues using recombinant inbred strains (Scalzo et al. 1990; Scalzo et al. 1999) and a small backcross panel (Forbes et al. 1997) indicated that *Cmv1* was located on the distal part of chromosome 6 linked to the NK cell gene complex (NKC).

1.6.2. The Natural Killer Gene Complex (NKC)

The NKC is a region on distal mouse chromosome 6 so named because of the presence of numerous genes important for NK cell function (Yokoyama and Plougastel 2003). Most NKC-related genes code for cell surface receptors of the C-type lectin superfamily expressed in endothelial, dendritic and NK cells. Many of these genes belong to gene families that share high amino-acid identity and are clustered in approximately 2 megabases of genomic DNA between the alpha-2-macroglobulin (*A2m*) gene and the proline rich protein (*Prp*) gene cluster (Figure 1.3a) (Brown et al. 1999; Depatie et al. 1999; Renedo et al. 2000; Brown et al. 2001a; Plougastel et al. 2001; Sobanov et al. 2001).

Figure 1.2. MCMV replication in spleen and liver of *Cmv1*^{r} and *Cmv1*^{s} strains

(From Scalzo et al., 1990). Mice were inoculated by the intraperitoneal route with 2×10^4 PFU of MCMV.


Spleen
Liver
Log10 PFU / organ
6
5
4
3
2
1
2
4
6
2
4
6
TIME POST INFECTION (days)
BALB/c
F1
C57BL/6

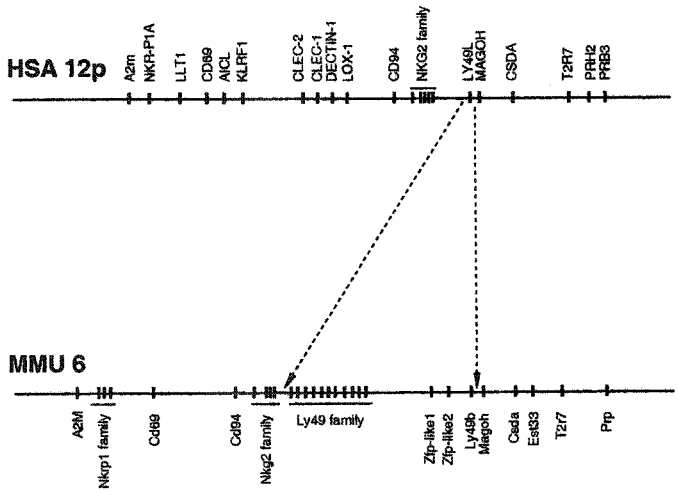
Among the NKC-encoded receptors, members from three families are preferentially expressed on NK cells: the NKRP1(A-F) receptors, of which NK1.1 (NKRP1C) was the first known member and turned out to be the most specific serologic marker on C57BL/6-derived NK cells; Ly49(A-N) receptors, which bind MHC class I molecules; and the NKG2(A-D)/CD94 receptors, heterodimers which bind non-classical MHC I Qa-1^b (Vance et al. 1998). Individual NK cell receptors elicit either inhibitory or activating signals that control the cytotoxic activity of NK cells. Inhibitory receptors contain immunotyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain that, upon ligand binding, recruit intracellular tyrosine phosphatases to mediate inhibition. In contrast, activating receptors lack intrinsic signaling capacity but are non-covalently linked to adaptor molecules that signal activation. Together these complex arrays of receptors mediate detection of suitable targets by NK cells through a balance of inhibitory and activating signals.

A remarkable number of phenotypic traits associated with immune function or susceptibility to disease have been mapped to the NKC, supporting the importance of the region for natural immunity and NK-cell cytotoxicity. In addition to the *Cmv1* gene, the murine NKC region has been linked with phenotypes associated with NK cell-mediated immunity such as *Chok*, a mouse locus controlling tumor killing by NK cells (Idris et al. 1998), loci contributing to susceptibility to cutaneous leishmaniasis (Beebe et al. 1997), Insulin-Dependent Diabetes Mellitus (Melanitou et al. 1998) and ectromelia virus (Delano and Brownstein 1995). In rats, *Nka*, an autosomal dominant locus that controls NK cell lysis of allogeneic lymphocytes (Dissen et al. 1996) and *Oia2*, a locus controlling susceptibility to oil-induced arthritis have been mapped to the NKC (Jansson et al. 1999).

Figure 1.3. Comparative maps of the Natural Killer Complex (NKC) and the Leukocyte Receptor Complex (LRC) in humans and mice. The NKC and the LRC are two major clusters of genes encoding activating or inhibitory receptors preferentially expressed on NK cells (see text) (a) Genetic organization of the Natural Killer Complex on human chromosome 12p13 and the syntenic region on mouse chromosome 6. Genes of the NKC code for C-type lectin receptors. Note the expansion of the genomic region in the vicinity of the *LY49L* pseudogene on the mouse chromosome domain harboring the *Ly49* gene family. *MAGOH* (Mago nashi homologue) (Newmark and Boswell 1994), *CSDA* (cold shock domain protein A) (Coles et al. 1996), *T2R* (taste receptor) (Adler et al. 2000) and *PRP* (proline-rich protein) (Maeda et al. 1985) are tightly linked to the NKC although functionally unrelated. (b) Genetic organization of the Leukocyte Receptor Complex on human chromosome 19q13 and the syntenic region on mouse chromosome 7. Genes of the LRC encode for receptors of the immunoglobulin superfamily. Note the absence of the KIR domain on mouse chromosome 7.

a

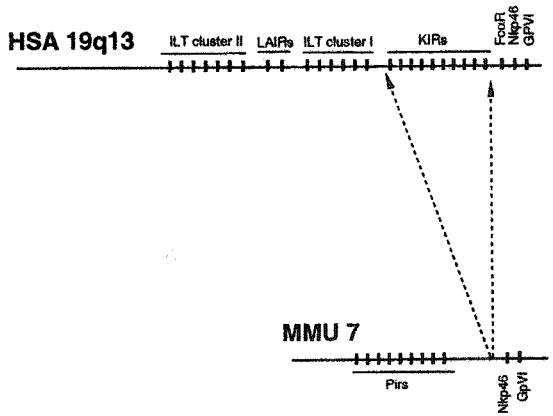
The Natural Killer gene complex



Phenotypic loci linked to murine NKC
Cmv-1: Resistance to Murine Cytomegalovirus
Chok: Killing of CHO cell
Idd-6: Susceptibility to insulin-dependent diabetes mellitus
Rmp-1: Resistance to mousepox virus
Scl: Th1 response to *Leishmania* infection
Nka: Rat NK cell lysis of allogeneic lymphocytes

b

The Leukocyte Receptor gene Complex



The human NKC is located on human chromosome 12p13.1 (Figure 1.3a) (Trowsdale et al. 2001). Despite the presence of obvious orthologues maintained between species, such as CD69 and CD94, there are important differences between the human and mouse NKC. For example, in humans there is a single *NKRP1A* and 5 *NKG2* genes, as opposed to 5 and 4 genes respectively, in mice. It is also remarkable that the different NKG2 loci are more similar within a species than between humans and mice. This probably reflects the very active duplication, divergence, and either functional or total loss of genes since separation of rodent and primate lineages. However, the most striking difference in the human NKC is the absence of the *Ly49* gene cluster (Figure 1.3a). In fact, humans possess a single *Ly49* gene that only produces aberrantly spliced non-functional transcripts (Westgaard et al. 1998). Interestingly, human NK cells express a family of receptors known as the killer cell immunoglobulin-like receptors (KIR) that are functionally analogous to the murine Ly49 receptors (Figure 1.3b). The KIR also bind directly to classical MHC class I molecules, are present as activating or inhibitory isoforms and signal through identical pathways as do the Ly49s. However, *KIR* genes are not encoded in the NKC, rather they are clustered on human chromosome 19q26 within a genetic region known as the leukocyte receptor complex (LRC). Whereas *Nkp46* and the gene cluster of paired-immunoglobulin like receptor family (*Pir*), likely orthologues of Ig-like transcript (*ILT*)s, are localized to a syntenic region on mouse chromosome 7, *KIR*s are completely absent from rodents (Figure 1.3b) (Kubagawa et al. 1999). Not surprisingly, the KIR in humans has been associated with loci controlling innate or autoimmune responses such as tumor killing (Ikeda et al. 1997) and rheumatoid arthritis (Yen et al. 2001).

1.6.3. Molecular genetics of the *Cmv1* locus

Because of the genetic complexity of the NKC and the absence of a reliable *in vitro* assay to assess susceptibility/resistance to MCMV, at least two groups initiated a positional cloning approach to isolate *Cmv1* (Depatie et al. 1997; Forbes et al. 1997; Scalzo et al. 1999; Depatie et al. 2000). This approach is based strictly on the knowledge of the chromosomal location of a specific gene to achieve its molecular cloning, after chromosome walking towards the gene from the most closely linked genetic markers on that chromosome.

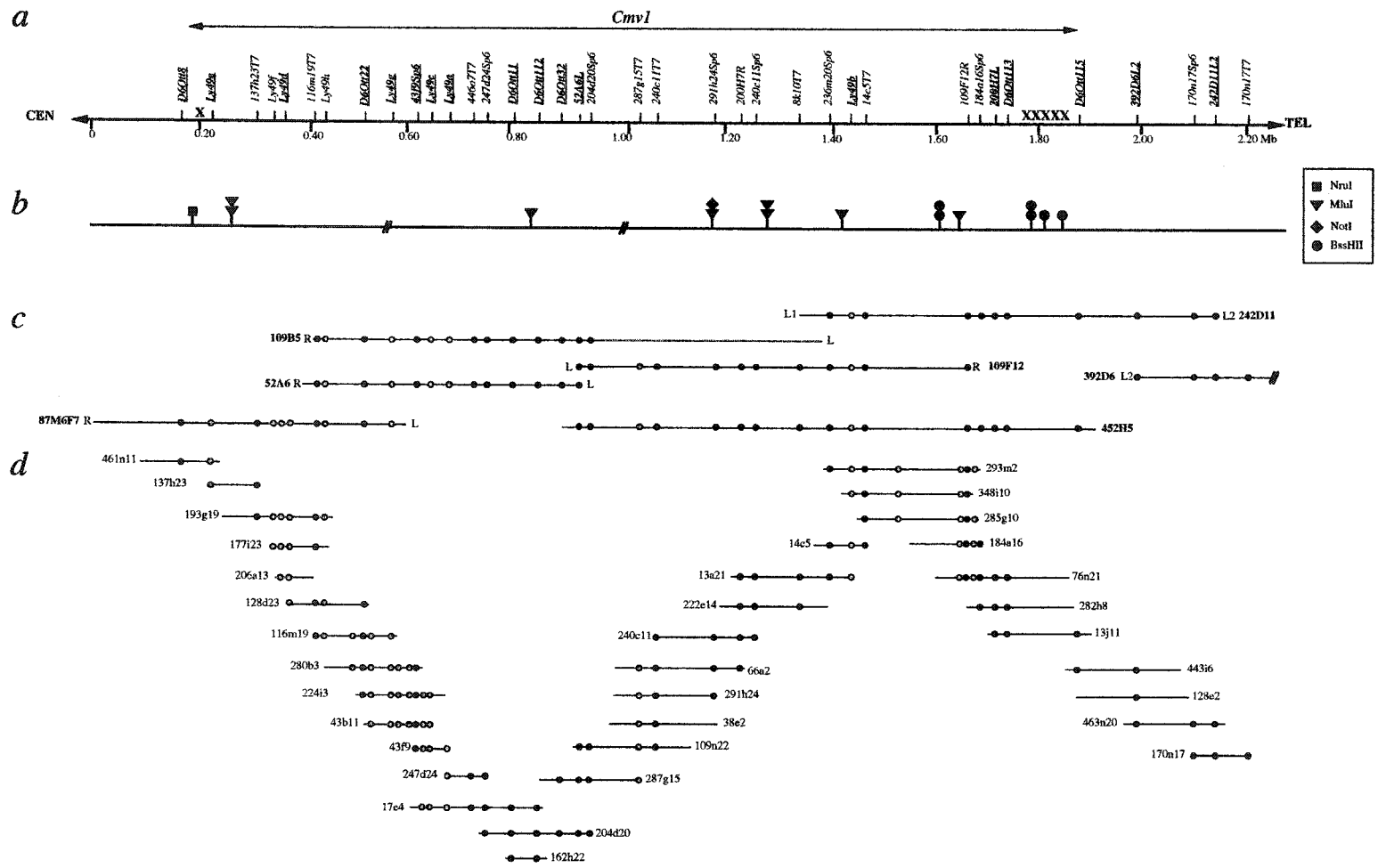
Several mapping panels totalling more than 3500 informative meioses from crosses between the resistant C57BL/6 and various susceptible strains (including A/J, BALB/c and BXD-8) were produced for genetic mapping (Depatie et al. 1997; Forbes et al. 1997; Lee et al. 2001a). The inheritance of MCMV susceptibility as measured by viral titer 3-4 days post-infection was compared with the inheritance of informative markers dispersed through the NKC, generating a high-resolution linkage map in the vicinity of *Cmv1*. This allowed the localization of *Cmv1* on the distal region of the NKC tightly linked to the *Ly49* gene cluster. Our group produced a map in the vicinity of *Cmv1*, with the gene order and intergene distance (in cM) as follows: *Nk1.1* –(0.3)- *Cd94/Nkg2d* –(0.05)- *Ly49a-n/Cmv1* –(0.3)- *Prp/Kap* (Depatie et al. 1999). This approach was successful in that it excluded most genes from the NKC, such as the *Nkrp* and *Nkg2* families. To further study this 0.35 cM region delineated by markers *D6Ott8* and *D6Ott115*, the critical genetic interval was represented by 24 yeast artificial chromosomes (YAC) and 75 bacterial artificial chromosomes (BAC). Based on a BAC-based physical map, Fluorescence In Situ Hybridization (FISH) analysis and sizing of the interval by pulse-field electrophoresis, the *Cmv1* critical domain was estimated to

span 1.6 Mb of genomic DNA, including the whole *Ly49* cluster (Depatie et al. 2000) (Figure 1.4). Moreover, potential candidates *Ly49a*, *Ly49c* and *Ly49g* genes in the critical interval were assessed for their contribution to MCMV resistance by performing selective *in vivo* antibody depletion of NK cell populations expressing these specific receptors. In the experiment, although Fluorescence Activated Cell Sorting (FACS) analysis demonstrated specific depletion of subsets of NK cell populations expressing the individual receptors, depleted *Cmv1*^{r} mice were able to control viral replication in target organs at the same level as control mice, demonstrating that none of these 3 genes are likely to be responsible for mediating resistance (Depatie et al. 1999). However, other *Ly49* genes still remained as strong candidates for *Cmv1* locus.

1.6.4. The *Ly49* gene family

As stated previously, the *Ly49* gene family contains at least 14 members in MCMV resistant C57BL/6, however, only a handful have been extensively studied to date (predominantly Ly49A, C, D and G2). Sequence alignments indicate that all Ly49 proteins are highly related and all are likely to be comprised of disulfide linked type II membrane spanning homodimers with an extracellular domain comprised of a C-type lectin motif (Smith et al. 1994; Anderson et al. 2001). Ly49 proteins are expressed predominantly on cells of the NK and NK T lineages although a small percentage of CD8 T cells also express Ly49 molecules.

Figure 1.4. Genetic, physical, BAC contig and transcription map of *Cmv1*. Schematic representation of the *Cmv1* candidate region. (a) STS content mapping of markers along the chromosome. The order of markers was established based on the YAC and BAC contig. Markers in bold have also been mapped genetically. The chromosomal orientation is indicated as centromeric (CEN) and telomeric (TEL). The "×" indicates the localization of the closest proximal and distal cross-overs defining the minimal *Cmv1* interval. (b) Restriction map of the *Cmv1* region. Restriction sites for 4 enzymes, *Mlu I*, *Not I*, *Nru I* and *BssH II*, are indicated and represented by different symbols (see legend). (c) The physical and STS content map of the YAC contig. Orientation of YAC inserts in the contig is indicated when possible. Black and gray circles represent markers anchored to the genomic clones. Position of some markers with respect to the restriction sites may not be accurate. (d) The physical and STS content map of the BAC contig as described above.


a
Cmv1
CEN
TEL
XXXXX
0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20 Mb
b
NruI
MluI
NotI
BssHII
c
L1
L2 242D11
109B5 R
L
L
R 109F12
52A6 R
L
392D6 L2
87M6F7 R
L
452H5
d
461n11
137h23
193g19
177i23
206a13
128d23
116m19
280b3
224i3
43b11
43f9
247d24
17e4
293m2
348i10
285g10
14c5
184a16
13a21
76n21
222e14
282h8
240c11
13j11
66n2
443i6
291h24
128e2
38e2
463n20
109n22
170n17
287g15
204d20
162h22

Sequence alignments of cDNA sequences together with Ly49-specific monoclonal antibody staining of NK cells from different inbred mouse strains indicates a high degree of polymorphism within the *Ly49* family (Smith et al. 1994; Makrigiannis and Anderson 2000; Anderson et al. 2001) (Figure 1.5). The recent elucidation of the genetic organization of the *Ly49* cluster in C57BL/6 and 129 strains underscores this characteristic (Makrigiannis et al. 2002). The 129 strain possesses at least 19 *Ly49* genes spanning a genomic region of about 600 kb. While it was possible to establish some allelic correlations between *Ly49* genes from C57BL/6 and 129, many, such as $Ly49r^{129}$ and $Ly49p^{129}$ or $Ly49c^{C57BL/6}$ and $Ly49m^{C57BL/6}$ do not have obvious orthologues in these mouse strains.

Ly49 proteins fall into two distinct categories based upon stimulatory or inhibitory signalling following ligand binding (Figure 1.6). These divergent functional activities are the consequence of the structural organization of their intracellular domains. Inhibitory members of the Ly49 family contain an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) (VXYXXV/L) which becomes tyrosine phosphorylated upon ligand binding leading to recruitment of the SHP-1 phosphatase (Mason et al. 1997; Nakamura et al. 1997). The exact target of the SHP-1 phosphatase in NK signaling is not yet clear, however, it is likely that it interferes with the signaling activities of spatially proximal signaling molecules. Most known ligands of Ly49 proteins are MHC class I molecules however there are distinct binding specificities for each Ly49 molecule (Table 1.2.). For example, the inhibitory molecule Ly49A binds to $H2D^{b,d,k,p}$ whereas Ly49C bind to $H2K^{b,d}$ and $H2D^{b,d,k}$ (Anderson et al. 2001). The Ly49G2 inhibitory receptor seems to be specific for $H2D^{d}$ (Mason et al. 1995).

Figure 1.5. Allelic polymorphisms of Ly49 receptors. Amino acid substitutions in Ly49A, Ly49C and Ly49G between resistant C57BL/6 and susceptible BABL/c are shown with their positions. IC indicates intracellular; TM, transmembrance; and CRD, carbohydrate recognition domain, respectively.

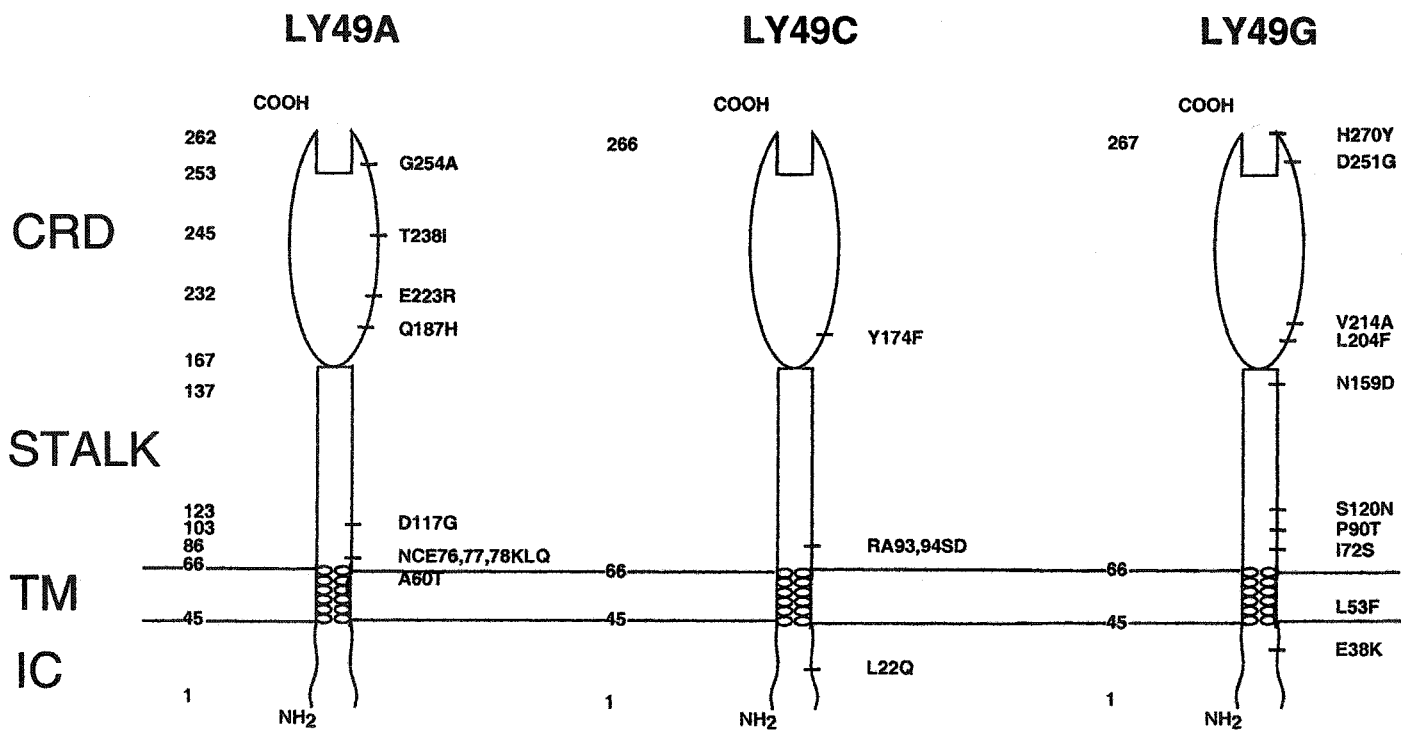

LY49A
LY49C
LY49G
COOH
COOH
COOH
CRD
STALK
TM
IC
262
253
245
232
167
137
123
103
86
66
45
1
266
66
45
1
267
66
45
1
G254A
T238I
E223R
Q187H
D117G
NCE76,77,78KLQ
A60T
Y174F
RA93,94SD
L22Q
H270Y
D251G
V214A
L204F
N159D
S120N
P90T
I72S
L53F
E38K
NH2
NH2
NH2

Figure 1.6. Schematic diagram of Ly49 receptors expressed on the surface of NK cells. Inhibitory receptors have an ITIM in their cytoplasmic tail that becomes tyrosine phosphorylated and associates with phosphatase SHP-1 to attenuate intracellular signalling. Activating receptors have a charged arginine residue in their transmembrane domain that enables binding to neighboring molecules such as DAP12. DAP12 possesses an ITAM in its cytoplasmic domain that serves as a docking site for Syk and Zap70 protein tyrosine kinases, thereby triggering a positive signalling cascade to activate NK cell cytolytic function upon ligand binding.

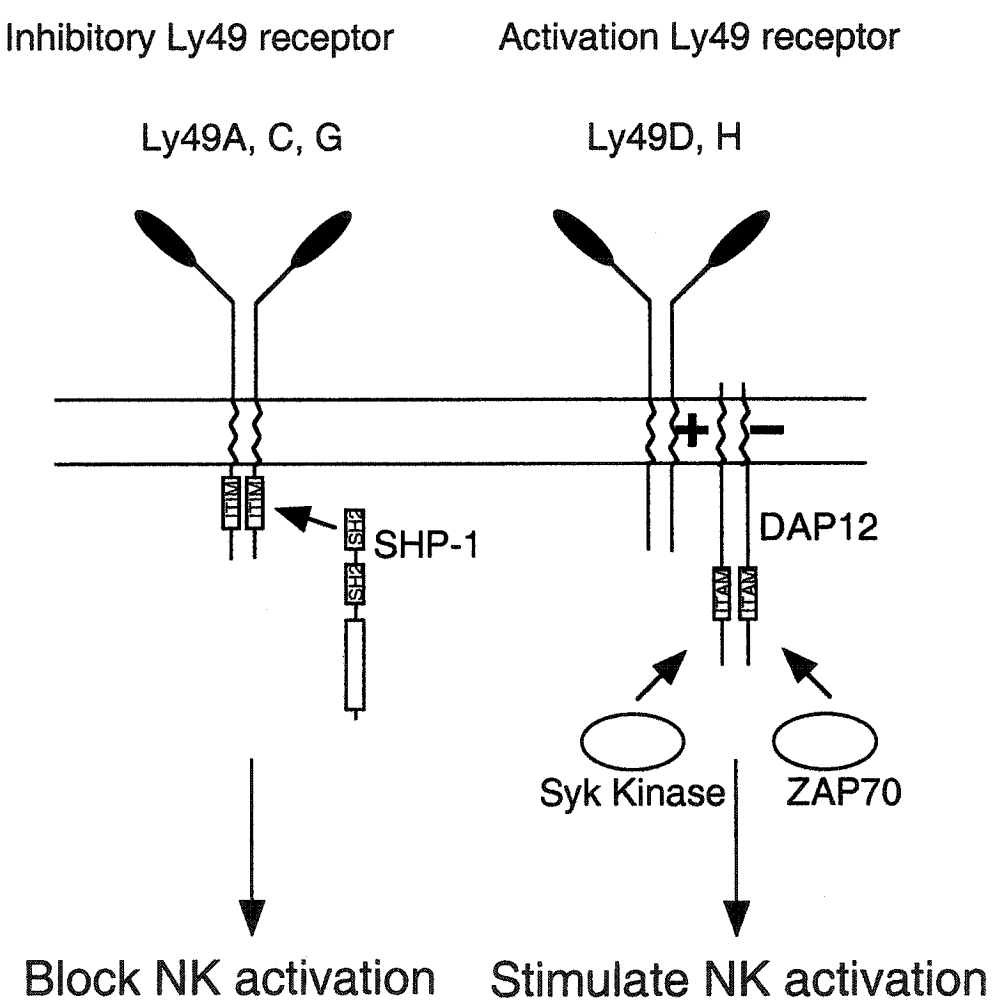

Inhibitory Ly49 receptor
Activation Ly49 receptor
Ly49A, C, G
Ly49D, H
ITIM
ITIM
SH2
SH2
SHP-1
+
−
DAP12
ITAM
ITAM
Syk Kinase
ZAP70
Block NK activation
Stimulate NK activation

Table 1.2. Functions and ligands of Ly49 receptors.

Mouse strain	Receptor	Function	Ligands	References
C57BL/6	Ly49A	Inhibition	$H2D^b$, $H2D^d$, $H2D^k$, $H2D^p$	(Karlhofer et al. 1992)
	Ly49C	Inhibition	$H2K^b$, $H2D^b$, $H2K^d$, $H2D^d$, $H2D^k$	(Stoneman et al. 1995; Hanke et al. 1999)
	Ly49D	Activation	$H2D^d$, Hm1-C4	(Nakamura et al. 1999; George et al. 1999 ; Furukawa et al. 2002)
	Ly49G	Inhibition	$H2D^d$	(Mason et al. 1995; Brennan et al. 1996 ; Hanke et al. 1999)
	Ly49H	Activation	m157	(Arase et al. 2002; Smith et al. 2002)
129/J	Ly49G	Inhibition	$H2D^d$, $H2K^d$	(Makrigiannis and Anderson 2001; Anderson et al. 2001)
	Ly49I	Inhibition	$H2D^k$, $H2K^b$, $H2K^d$, $H2K^k$, m157	(Makrigiannis and Anderson 2001; Anderson et al. 2001; Arase et al, 2002)
	Ly49O	Inhibition	$H2D^d$, $H2L^d$	(Makrigiannis and Anderson 2001; Anderson et al. 2001)
	Ly49R	Activation	H2Dd, $H2D^k$	(Makrigiannis and Anderson 2001; Anderson et al. 2001)
	Ly49V	Inhibition	$H2D^b$, $H2D^d$, $H2D^k$, $H2K^b$, $H2K^d$, $H2K^k$, $H2L^d$	(Makrigiannis and Anderson 2001; Anderson et al. 2001)

Different subsets of NK cells appear to express overlapping sets of Ly49 molecules such that all cells express at least two different inhibitory receptors (Anderson et al. 2001). The percentage of cells expressing a given Ly49 molecule varies between different strains of mice and appears to be regulated by the expression of the cognate MHC class I molecule in that strain (Andersson et al. 1998; Wilhelm et al. 2001). This process has been termed 'receptor calibration' and although the precise mechanism behind this regulated pattern of expression has been the subject of intense scrutiny we

still know little about the process. Recently, the crystal structure of an inhibitory Ly49 molecule, Ly49A, complexed with its ligand $H2D^d$ was reported (Tormo et al. 1999). Interestingly, the Ly49A homodimer was found to simultaneously contact two MHC class I molecules, one at the $\alpha 2$ domain of the peptide-binding site and the other at a site overlapping the CD8 binding site. The authors suggest that Ly49A dimers may in fact bind to one MHC class I molecule on a putative target cell using the former site, and a second neighboring MHC class I molecule on the NK cell itself using the latter site. Indeed it has been shown that an MHC class I/peptide complex can be simultaneously bound by both a TCR and an inhibitory Ly49A molecule suggesting a potential role in the activation of TCR-expressing NK T cells (Natarajan et al. 1999).

The second category of Ly49 molecules, the activating Ly49 molecules, has intracellular domains which are structurally distinct from those of the inhibitory Ly49 molecules. In contrast to the inhibitory Ly49 molecules, activating Ly49 receptors lack tyrosine residues in their intracellular domain and are thus incapable of signaling themselves. Rather, activating Ly49 receptors signal through an adapter molecule called DNAX activation protein 12 (DAP12) which associates with Ly49 through a non-covalent interaction using a charged residue in the membrane-spanning domain (Lanier et al. 1998; Mason et al. 2000). DAP12 is a transmembrane protein with a very short extracellular domain containing a cysteine residue required for homodimer formation. The intracellular domain of DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) which becomes phosphorylated upon ligand binding and recruits the cytoplasmic protein tyrosine kinases Syk and ZAP70. These in turn activate downstream signaling pathways that result in mobilization of intracellular calcium stores and activation of the cell. In addition to complexing with activating Ly49 molecules,

DAP12 functions as an adapter molecule for a number of activating surface receptors including mouse and human CD94/NKG2C, activating human KIR proteins, and the myeloid cell-specific factors MDL-1, TREM-1 and TREM-2 (Lanier and Bakker 2000). Consequently, DAP12 deficiency have diminished Ly49-mediated killing of certain tumors as well as other immune defects in mice. Surprisingly, humans with DAP12 deficiency have neurological symptoms characterized by progressive presenile frontal lobe dementia (Paloneva et al. 2000) indicating that DAP12 plays an important role in human brain function. However, no obvious immune defects have been reported associated with DAP12 deficiency in humans.

Of the 14 Ly49 molecules encoded in the Ly49 locus of mouse chromosome 6, only Ly49D and Ly49H have been identified as activating receptors. Ly49D is known to recognize the MHC class I molecule $H2D^d$ (Nakamura et al. 1999; George et al. 1999) although the physiological significance of this recognition is not yet clear. Ligation of Ly49D with cross-linking antibodies or by $H2D^d$-expressing target cells results in both increased cytotoxicity and IFN-γ production however both can be down-regulated by a concurrent signal from the inhibitory receptor Ly49G2 (also $H2D^d$–specific) (Mason et al. 2000). This latter finding suggests that signals from inhibitory Ly49 receptors are dominant over signals derived from activating Ly49 receptors. Although the shared presence of both positive and negative signaling receptors for the same ligand on a single NK cell presents a confusing picture in terms of NK function, recent evidence suggests that inhibitory or activation Ly49 receptors have different affinities for MHC class I and that this may be influenced by the peptide bound to MHC class I (Nakamura and Seaman 2001).

Among many phenotypic loci located in the NKC complex, *Chok* locus controls tumor killing by NK cells, showing the profound differences between NK cells from C57BL/6 (B6) and BALB/c mice with respect to killing of Chinese hamster ovary (CHO) cells. Subsequent studies demonstrated that Ly49D activation receptor on C57BL/6 NK cells recognizes CHO cells and triggers natural killing. In addition, transgenic transfer of $Ly49d^{C57BL/6}$ into BALB/c NK cells was recently shown to confer cytotoxic activity against CHO cells, establishing that Ly49D is the *Chok* gene product (Idris et al. 1999) and indicating that alternate Ly49D ligands may flag tumor cells for destruction by Ly49D-expressing NK cells. Consistent with this notion, a recent study demonstrated that a Chinese hamster classical MHC class I molecule is the ligand for Ly-49D using a reporter gene assay system as well as NK cell killing assays (Furukawa et al. 2002). It still remains to be elucidated why a murine NK cell receptor recognizes a hamster MHC class I molecule. Gene chip analyses have indicated that NK cells activated through cross-linking of Ly49D surface receptors up-regulate a number of soluble mediators of inflammation, in particular the chemokines MIP-1α and MIP-1β, suggesting that NK cells may play a key role in initiating inflammation and recruitment of cells of the adaptive immune response (Ortaldo et al. 2001).

Ly49H, another activation Ly49 receptor, is an activating receptor specifically expressed on 56% of prototypical $NK1.1^{+}CD3^{-}$ NK cells (Smith et al. 2000). Crosslinking of Ly49H by monoclonal antibodies results in calcium mobilization, cytokine production and redirected lysis of target cells (Gosselin et al. 1999; Smith et al. 2000), suggesting its involvement of direct killing of virus infected cells as a possible mechanism of resistance to infection. Unlike most other Ly49 family members, however, Ly49H does not bind to any known MHC class I molecule.

1.7. STATEMENT OF OBJECTIVES

In the absence of an *in vitro* system to identify *Cmv1*, the main objective of my Ph.D. project was to identify and characterize the *Cmv1* locus using positional cloning and functional complementation, thereby allowing a better understanding of the role of NK cells in innate immunity to MCMV infection.

Specific objectives were;

1) Generation of a transcription map in the *Cmv1* interval (Chapter 3)

2) Haplotype analysis of *Cmv1* region in a panel of 17 inbred mice (Chapter 4)

3) Investigation of the susceptibility of the recombinant inbred strain BXD-8 (Chapter 5)

4) *In vivo* complementation analysis by BAC transgenic mice expressing Ly49H to formally demonstrate the allelism between *Ly49h* and *Cmv1* (Chapter 6)

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1. VIRUS

The Smith strain of murine cytomegalovirus (MCMV) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and passaged twice in mouse salivary glands to restore virulence. Briefly, 3 week old weaning BALB/c female mice were infected by an intraperitoneal injection with 5×10^3 plaque forming units (PFU) of virus. Three weeks later, the mice were sacrificed and the salivary glands removed and pooled. The tissue was homogenized in a polytron in 5 volumes of Dulbecco minimal essential medium (D-MEM; Gibco/BRL) containing 10% heat inactivated Fetal Bovine Serum (FBS) (Gibco/BRL), before being clarified by low speed centrifugation, aliquoted and stored in liquid nitrogen. For virus titration, one vial to be titrated was thawed rapidly in a 37 °C water bath and serially diluted 10-fold in D-MEM containing 2% of FBS. PFUs of these diluted viruses were determined in triplicate by plaque assay as described below (section 2.4).

2.2. MICE

All inbred mouse strains were purchased from either The Jackson Laboratory (Bar Harbor, ME) or Charles River (Wilmington, MA). The congenic strains C.C57BL/6-Ly49e-D6Mit15 and C.C57BL/6-D6Mit230-D6Ott113 were produced from progeny 118 and 236 issued from the backcross panel (BALB/c × C57BL/6)F1 × BALB/c previously described (Depatie et al. 1997). Both founder animals were

determined to be resistant to MCMV (*Cmv1*^{r}) by progeny testing. Progeny 118 carried a recombination event between *Cd94/Nkg2d* and *Ly49e* whereas 236 possessed a crossover between *D6Ott113* and *D6Mit13*. Marker selected animals were back-crossed to the BALB/c strain over 4 generations. Heterozygous animals identified at the 4th generation were selected for brother-sister mating. All mice were bred and maintained at the animal facility at the University of Ottawa in agreement with guidelines and regulations of the Canadian Council on Animal Care.

2.3. MOUSE EMBRYONIC FIBROBLAST (MEF) PREPARATION

Embryos were obtained from BALB/c mice at 14-16 d of pregnancy and transferred into sterile 80 mm Petri dishes containing phosphate buffered saline (PBS). Organs such as heart and liver were removed and the remaining embryo was minced and finely chopped with sterile scissors to about 3-mm diameter followed by three washes with PBS. Then embryos were incubated in 0.25% Trypsin-EDTA (Gibco BRL) at 4 °C overnight (6-18 hr). The next day, after removing as much 0.25% Trypsin-EDTA as possible, embryos were incubated at 37 °C for 20-30 min for full trypsinization. The activity of trypsin was inhibited by adding DMEM containing 10% FBS. The MEF cells were gently dispersed by pipetting and collected by centrifugation for 10 min at 500g. Cells were resuspended in DMEM containing 10% FBS, seeded at 1×10^5/ml on 14 cm tissue culture dishes and grown for 2-7 days until heavily confluent. The confluent cells were removed by trypsinization and resuspended in DMEM containing 20% FBS and

10% Dimethyl Sulfoxide (DMSO) at 1-2 million cells/ml and then stored in -80 °C or in liquid nitrogen.

2.4. MCMV PHENOTYPE ASSESSMENT

Mice were challenged at 6 or 7 weeks of age with a virulent stock of the MCMV strain Smith. Mice were infected intraperitoneally with 0.2 ml of PBS containing 5×10^3 PFU of MCMV. The degree of infection was assessed by determining the number MCMV PFU in the spleen and the liver by plaque assay in MEFs. Whereas the *Cmv1* locus determines viral replication in the spleen, it does not significantly affect viral load in the liver. Therefore, to ensure whether mice were properly infected, the level of MCMV replication in the liver was determined. Briefly, MEF cells were seeded in 24-well tissue culture plates at 1.6×10^5 cells/well in D-MEM medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin and 10% heat inactivated FBS. After incubation for 24 h at 37 °C in a 5% CO_2 incubator, the monolayers were washed once with adsorption medium (D-MEM medium supplemented as before but containing 2% FBS) and were infected with 0.2 ml of serial tenfold dilutions of organ homogenates in adsorption medium. Virus was allowed to adsorb for 60 min. at 37 °C in a 5% CO_2 incubator before overlaying the plates with DMEM medium containing 0.25% low-melting agarose (Gibco-BRL) and 10% FBS. Three days later, infected cells were fixed for 10 min. with 10% formalin and MCMV plaques were revealed by staining with 1% methylene blue in 70% ethanol.

2.5. TAIL DNA EXTRACTION

Mouse tail DNA was extracted using the modified alkaline lysis method (Truett et al. 2000). Briefly, a 2 mm tail biopsy was incubated in 600 µl of a 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 solution was then centrifuged to remove any remaining debris. Aliquots of 2 µl of this solution were directly used for PCR reactions.

CHAPTER THREE

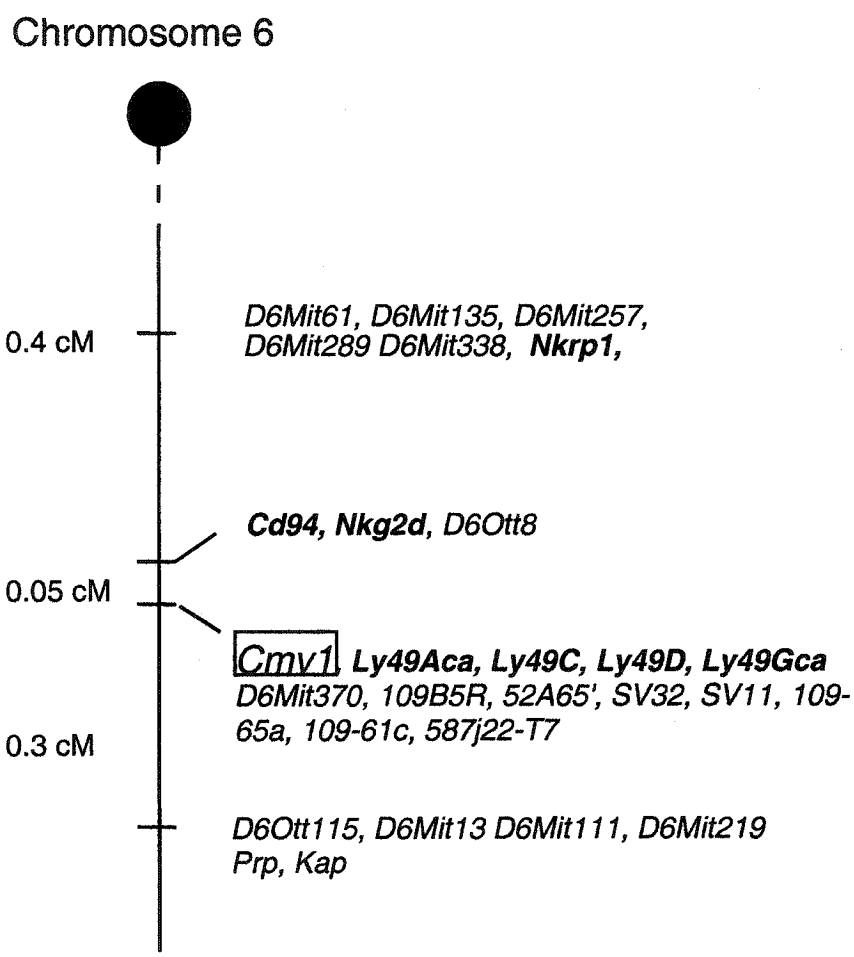
CONSTRUCTION OF A TRANSCRIPTIONAL MAP IN THE *Cmv1* INTERVAL

3.1. INTRODUCTION

The host resistance locus *Cmv1* controls viral replication of murine cytomegalovirus (MCMV) in the spleen of infected mice (Scalzo et al. 1990). To understand the mechanism of action of *Cmv1* and its relationship with innate immunity loci, a positional cloning approach to isolate the gene was initiated in the lab. Using segregation analysis in populations of informative backcross mice, *Cmv1* was localized to a 0.35-cM interval distal mouse chromosome 6 (Depatie et al. 1999), tightly linked to the *Ly49* gene family (Figure 3.1). As the second stage for the positional cloning of *Cmv1*, a high-resolution physical map of the *Cmv1* genetic interval was generated, based on long-range restriction mapping by pulsed-field gel electrophoresis (PFGE), fluorescence in situ hybridization (FISH) analysis of interphase nuclei, and the assembly of a cloned contig of 24 YACs and 75 BACs (Figure 1.3b, c, d). The initial genetic mapping analysis excluded *Nk1.1*, *Cd94* and *Nkg2d*, while retaining *Ly49* gene family as candidates for *Cmv1* (Figure 3.1) (Depatie et al. 1999). The full complexity of the *Ly49* family remains to be understood: to date 14 *Ly49* genes (designated *Ly49a-n)* clustered on mouse chromosome 6 have been cloned and other, as yet uncharacterized, *Ly49* genes may be localized within the *Cmv1* interval.

Using *in vivo* antibody depletion experiments targeted against Ly49A, Ly49C, Ly49G2 and Ly49I expressing cell subsets, our group (Depatie et al. 1999) and others (Tay et al. 1999) have shown that these cell populations are unlikely to be involved in MCMV resistance during early infection.

Figure 3.1. Schematic representation of a 0.35cM *Cmv1* minimal genetic interval. The gene order and the mapped loci were determined by pedigree analysis, and the intergenic distances are given as estimates of recombination frequencies with backcross animals. Markers in bold originated from transcribed genes. The centromere of the chromosome is shown as a black circle. Recombination frequencies are shown to the left of the chromosome.


Chromosome 6
0.4 cM
D6Mit61, D6Mit135, D6Mit257,
D6Mit289 D6Mit338, Nkrp1,
Cd94, Nkg2d, D6Ott8
0.05 cM
Cmv1 Ly49Aca, Ly49C, Ly49D, Ly49Gca
D6Mit370, 109B5R, 52A65', SV32, SV11, 109-
65a, 109-61c, 587j22-T7
0.3 cM
D6Ott115, D6Mit13 D6Mit111, D6Mit219
Prp, Kap

However, other family members are likely to be localized to the *Cmv1* interval and *Ly49d* has been identified recently as the gene product of the cytotoxicity locus *Chok* (Idris et al. 1999). Therefore, the candidacy of this gene family for *Cmv1* cannot be disregarded at this point.

In positional cloning, genetic and physical mapping of a candidate region is followed by the identification and localization of transcribed sequences or candidate genes within a limited genomic region defined by the genetic linkage analysis. The extraction of transcribed sequences from genomic sequences has been one of the rate-limiting steps in positional cloning. As the next step in the positional cloning of *Cmv1*, I constructed a transcriptional map of the *Cmv1* critical genetic interval, including at least 19 transcription units. The availability of a comprehensive transcriptional map in the *Cmv1* region is essential for the identification of *Cmv1* by positional cloning.

3.2. MATERIAL AND METHODS

3.2.1. Direct BAC sequencing

Target BAC clones within the *Ly49* gene cluster were chosen for direct sequencing using *Ly49* gene-specific oligonucleotides (Table 3.1) based on their approximate localization in the BAC contig in the *Cmv1* interval. The BAC DNA was isolated by the akaline lysis method with gentle handling to prevent the breakage of BAC DNA. At least 1 μg of BAC DNA was used for sequencing using ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences) following conditions specified by the manufacturer. The *Ly49* gene-specific

oligonucleotides were designed to anneal exclusively to a single specific member of the *Ly49* family by having unique sequences in the 3' end of the oligonucleotides.

Table 3.1. Oligos used in transcription mapping

Locus name	Oligo sequence 5' to 3'	Location
Ly49c	CAGGGTTGCAGAAACAAGTA	Exon 2
Ly49d-5'	GTCATCTCGGGAGTATGTAG	5' UTR
Ly49d-3'	AATAATTACTGTGATCAATC	3' UTR
Ly49f	CCTCAAGGTTGCAGAAACTT	Exon 2
Ly49h	CTATCACAATGAGCTGCCAA	Exon 4
Ly49i	(McQueen et al. 1998)	Exon 4
Ly49j	ATAGTATTGGTTTCACTATT	Exon 4
Ly49k	CTGTTCTCTGTTGAGGGATC	Exon 4
Ly49l	AATGATTTATCACATTTATC	Exon 4
Ly49m	TGCCAAGATAAGTGCAGCAC	Exon 7
Ly49n	CTTTAAGTCTATAGGATGTT	Exon 4
Magoh-like	CTTAGATATGCCAACAACAG	Position 6-22[a]
Y-box-like	CAGTTTCTCCATCCCCCACA	Position 336-354[b]

[a]Corresponding sequence in Genbank Accession No. L35549.

[b]Corresponding sequence in Genbank Accession No. AF035939.

3.2.2. Exon Amplification

Exon amplification to identify coding sequences in genomic DNA was performed on BAC clones overlapping the *Cmv1* candidate region. Briefly, purified BAC DNA was digested with *BamHI*/*Bgl*II and fragments were introduced into *BamHI*-digested and dephosphorylated pSLP3 cloning vector (Gibco BRL), followed by transformation into *E. Coli* strain DH5α. Transformed cells were grown overnight in 10 ml of LB broth with ampicillin (100 μg/ml) and plasmid DNA was isolated by the alkaline lysis method. COS-7 monkey cells were transiently transfected by

electroporation using 5-10 μg of each DNA pool, followed by incubation for 24 hrs at 37 °C, at which point total RNA was isolated using TRIzol® reagent (Gibco BRL). For first-strand cDNA synthesis, 1-3 μg of RNA was incubated with 20 pmol of pSPL3 specific oligonucleotide SA2 and incubated with 200 U of SuperScript II™ Reverse Transcriptase (Gibco BRL), followed by RNase H (Gibco BRL) digestion. Trapped genomic exons were PCR amplified from cDNAs with a primary PCR using oligonucleotide SA2 and SD6 and followed by *BstX*I digest and a second PCR with oligonucleotide primers dUSA4 and dUSD2 according to manufacturer's instructions (Gibco BRL). PCR-amplified exons were purified by gel electrophoresis on low-melting agarose gels and cloned directly into a dT-tailed *EcoR*V-digested pBluescipt II KS (+) plasmid vector (Marchuk et al. 1991). Inserts were sequenced as described above.

3.2.3. Analysis of the sequences of the full-length insert of BAC 13J11

As an alternative approach for transcript identification, the BAC clone 13J11 was shotgun-sequenced in collaboration with Dr. Ben Koop at the University of Victoria. An average 1 kb insert sub-clone library of BAC 13J11 was constructed in pBluescript. End clone sequences were determined with the BigDye terminator cycle sequencing chemistry (Perkin-Elmer) and analyzed using an ABI377. Out of 1118 clones, a total of 526,636 bp of sequence were entered representing 2.8 × coverage for the 201 kb BAC insert. Sequences from each clone were assembled into overlapping contiguous sequences using the Sequencher package (http://www.genecodes.com/sequencher/). These generated contigs were refined by masking repeat sequences using RepeatMasker. The unique sequences were compared to

the public DNA sequence databases using various algorithms and significant similarities to existing genes or ESTs noted. Simultaneously, these contigs were screened using GRAIL (http://compbio.ornl.gov/Grail-1.3/) to predict the presence of exons. Furthermore in collaboration with the Montreal Genome Center, comprehensive homology and GENESCAN (http://genes.mit.edu/GENSCAN.html/) analyses were performed.

3.2.3. RT-PCR and sequencing of cDNAs

Total spleen and liver RNA sample were prepared using the TRIzol reagent (Life Technologies) following the manufacturer's recommendations. Briefly, a 1 ug sample of total RNA was reverse-transcribed into cDNA, and one tenth of the cDNA sample was then used as template for PCR amplification using oligos specific for *Ly49b, Csda, EST335500 and Gapdh.* Gene specific oligonucleotides for the PCR were, *Ly49b*-F: TAACTGGGTCAGTGTTGGTG and *Ly49b*-R: CTGTTCTCTGTTGAGGTAGTG; *Csda*-F: GAGAGCGGAGAGGCGCATGA and *Csda*-R TGTTGGTAAGGTCGTGGGTGT; *EST335500*-F: 5'-GGCCGTGGTCGCAGTGAACT-3' and *EST335500*-R: 5'-GCTGGCTTCTCTGGCCACCT-3'; *Gapdh*-F: 5'-ACCACAGTCCATGCCATCAC-3' and *Gapdh*-R: 5'-TCCACCACCCTGTTGCTGTA-3'. PCR products were gel-purified and sequenced using the ThermoSequenase radiolabeled terminator cycle sequencing kit (Amersham Life Sciences) following conditions specified by the manufacturer.

3.3. RESULTS

3.3.1. Construction of a transcriptional map

To initiate the construction of a transcriptional map of the *Cmv1* domain, I employed four parallel approaches: (1) systematic BLAST searches of newly cloned sequences, (2) direct BAC sequencing and STS content mapping with *Ly49* gene specific oligonucleotides, (3) exon amplification, and (4) sequencing of the full-length insert BAC 13J11. Using BLAST searches, it was determined that the sequences derived from 137H23Sp6 corresponded to exon 4 of *Ly49e*, that of 247D24T7 contained exon 6 of *Ly49a* and 43F9T7 presented the 3' untranslated region (UTR) of *Ly49a*. This information enabled the accurate placement of these transcription units within the BAC contig and assigned a transcription orientation of telomeric to centromeric for the two genes. Moreover, the end sequence of 287G15T7 was found to be homologous to a zinc finger motif characteristic of the C2H2-type proteins (Pieler and Bellefroid 1994) suggesting the presence of a transcription unit. Highest homology (54/70 aminoacids) was obtained with mouse Zfp35 protein (Cunliffe et al. 1990a). Out of the 14 *Ly49* family members identified to date, 10 genes were analyzed by direct BAC sequencing with a panel of gene specific oligonucleotides (Table 3.1), whereas *Ly49a*, *-b*, *-g* and *-e* were analysed by STS content mapping. By analyzing the retention pattern of specific sequences across the contig, it was determined that all 14 members were localized to the BAC contig with the following transcript order: centromere- *Ly49e- Ly49f- Ly49d- Ly49k- Ly49h- Ly49n- Ly49i- Ly49g- Ly49l- Ly49j- Ly49m- Ly49c- Ly49a- Ly49b-* telomere (Figure 3.2).

Figure 3.2. Fine localization of the *Ly49* gene family. Precise localization of Ly49 genes were determined by either direct BAC sequencing or STS content mapping with Ly49 gene specific oligos. Black fills indicate the presence of specific sequence.

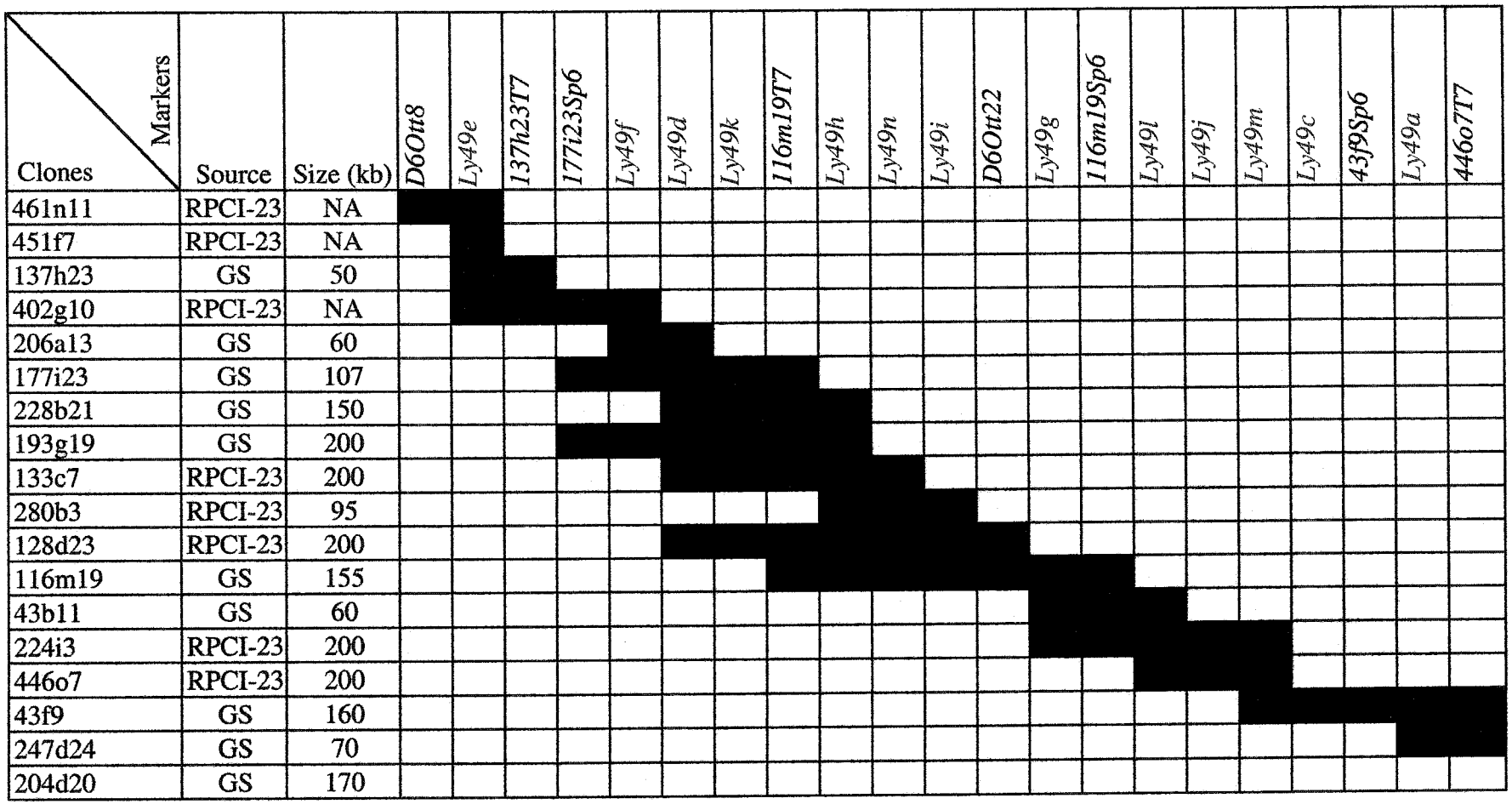
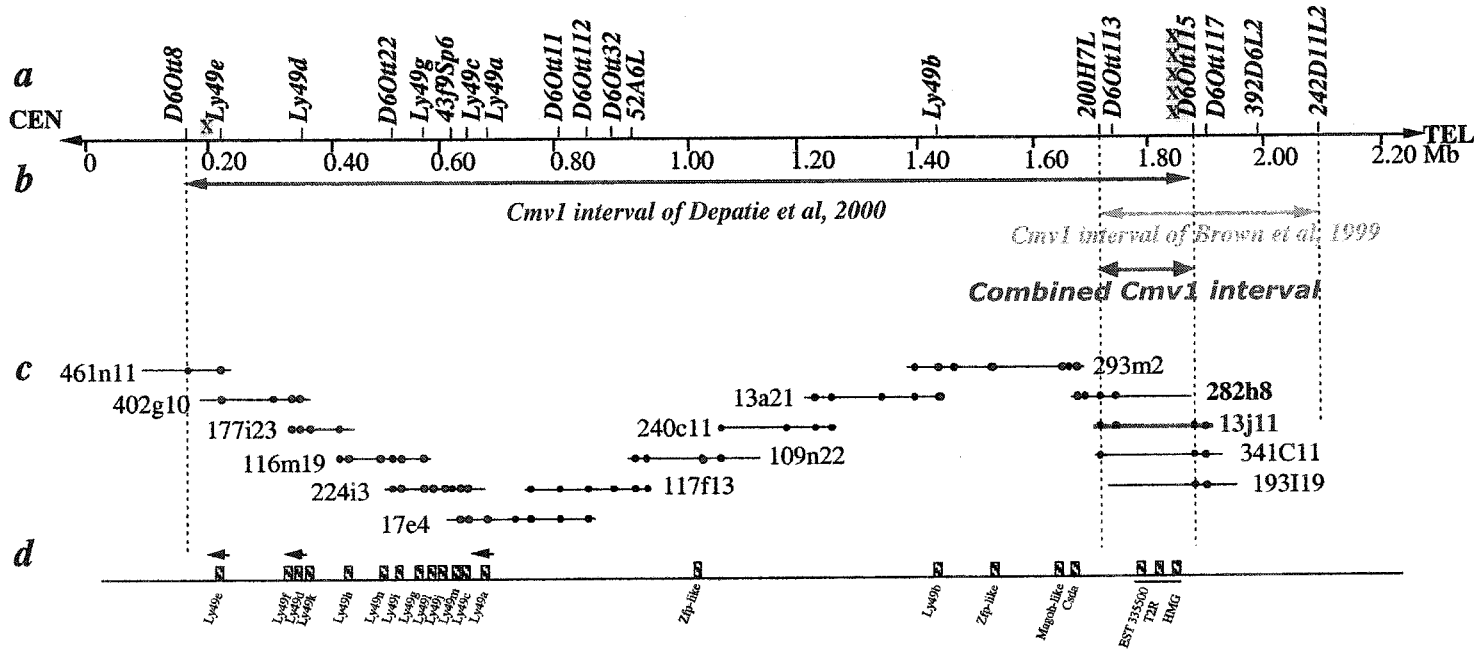

Markers
Clones
Source
Size (kb)
D6Ott8
Ly49e
137h23T7
177i23Sp6
Ly49f
Ly49d
Ly49k
116m19T7
Ly49h
Ly49n
Ly49i
D6Ott22
Ly49g
116m19Sp6
Ly49l
Ly49j
Ly49m
Ly49c
43f9Sp6
Ly49a
446o7T7
461n11 RPCI-23 NA
451f7 RPCI-23 NA
137h23 GS 50
402g10 RPCI-23 NA
206a13 GS 60
177i23 GS 107
228b21 GS 150
193g19 GS 200
133c7 RPCI-23 200
280b3 RPCI-23 95
128d23 RPCI-23 200
116m19 GS 155
43b11 GS 60
224i3 RPCI-23 200
446o7 RPCI-23 200
43f9 GS 160
247d24 GS 70
204d20 GS 170

Figure 3.3. Physical and transcriptional map of the *Cmv1* interval. (a) Long-range physical map of the *Cmv1* interval. The location of polymorphic markers used for the genetic analysis is indicated. The "X" indicates the localization of the closest proximal and distal cross-overs defining the minimal *Cmv1* interval. (b) Two independent genetic minimal intervals for *Cmv1* (1.6 Mb for Depatie et al. 2000 and 390 kb for Brown et al. 1999, respectively). The 200 kb combined *Cmv1* interval is also shown. (c) Minimal tiling path for the BAC contig developed in our laboratory. Circles indicate genes (pink) and STS's (black) used for contig assembly. BAC clones overlapping the combined genetic interval for *Cmv1* and used for transgenic experiments are shown in red. (d) Transcription map of the *Cmv1* candidate region. The direction of *Ly49a*, *Ly49d* and *Ly49e* is indicated. All *Ly49* genes are clustered within a 420 kb region with the exception of *Ly49b* which resides 750 kb telomeric to the cluster.


a
CEN
D6Ot8
Ly49e
Ly49d
D6Ot22
Ly49g
43fpSp6
Ly49c
Ly49a
D6Ot11
D6Ot112
D6Ot32
52A6L
Ly49b
200H7L
D6Ot113
D6Ot115
D6Ot117
392D6L2
242D11L2
TEL
0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
2.00
2.20 Mb
b
Cmv1 interval of Depatie et al, 2000
Cmv1 interval of Brown et al, 1999
Combined Cmv1 interval
c
461n11
402g10
177i23
116m19
224i3
17e4
13a21
240c11
109n22
117f13
293m2
282h8
13j11
341C11
193I19
d
Ly49e
Ly49f
Ly49l
Ly49k
Ly49h
Ly49n
Ly49i
Ly49p
Ly49g
Ly49j
Ly49m
Ly49c
Ly49a
Zip-like
Ly49b
Zip-like
Mango-like
Cuda
EST 335500
T2R
HMG

All genes are clustered within a 420 kb region of genomic DNA anchored to *D6Ott8* with the exception of *Ly49b* that lies ~750 kb telomeric to the cluster. Interestingly, sequence analysis of *Ly49d* fails to detect the 3' end of this gene in the BAC 206A13, whereas BAC 177I23 contains the entire coding sequence, indicating that the transcription orientation of *Ly49d* is telomeric to centromeric. The orientation is consistent with the orientation that has been determined for other members of the *Ly49* cluster (McQueen et al. 1998) (Figure 3.3d).

To identify potential transcripts in the cloned domain, BAC clones in the *Cmv1* interval were systematically analyzed for the presence of transcription units using the technique of exon amplification (Buckler et al. 1991). This method is devised to screen large genomic DNA fragments for the presence of splicing competent exons. Except for the region covered by BACs 224I3 and 17C4, that together contain 8 *Ly49* genes, 13 BACs covering the entire *Cmv1* domain were analyzed (Figure 3.3c). A total of 24 putative exons ranging from 58 to 250 bp were recovered and their nucleotide sequence was determined and compared to publicly available databases for homology searches (Table 3.2).

Table 3.2. Exons identified by Exon Trapping approach

24 putative exons	Gene identity
9 *Ly49* genes	*Ly49b, Ly49d, Ly49e, Ly49f, Ly49m, Ly49k, a new Ly49*
2 known genes	*Csda (Cold Shock Domain protein A), Magoh*
3 ESTs	*EST335500*, two zinc finger binding proteins
10 suspected exons	No significant similarity in GenBank database

Using this approach, 11 homologous sequences were identified of which 8 correspond to known *Ly49* exons (*Ly49e* from BAC 402G10; *Ly49d*, *Ly49k* and *Ly49f* from BAC 177I23; *ly49m* from 224I3 and *Ly49b* from 293M2) and one, a 148 bp exon from BAC 177I23, was found to be highly homologous to *Ly49d* exon 3, possibly defining a new member of the family. In addition, unrelated sequences to *Ly49* were identified indicating the presence of three supplementary transcription units. A 58-bp exon from 293M2 exhibited 90% homology to the serum-inducible protein gene *Magoh* (Zhao et al. 1998). An exon of 185 bp, recovered from BAC 282H8 exhibited 100% homology to mouse *Csda* gene (Sakura et al. 1998) while also displaying significant homology to other DNA binding proteins. This would suggest that this exon might by part of a gene encoding a DNA binding protein. Lastly, a 137-bp exon, amplified from BAC 13J11, displayed 99% homology to *EST335500*, which was originally identified from a mouse embryo brain cDNA library and presented no homology with known genes in publicly available databases. Exons were precisely anchored to the YAC/BAC contig using specific oligonucleotides (Table 3.1) for STS content mapping or direct BAC sequencing.

Lastly, 80% of the 210 kb BAC 13J11 sequence was determined in collaboration with the sequencing facility at the University of Victoria. 13J11 was chosen because it represented the region common in two independent genetic minimal intervals of *Cmv1* published by two different groups. As shown in the Figure 3.3b, the combination of our 1.6 Mb interval with the other group's 390 kb interval generated a 200kb shared region and this region was encompassed by one single BAC 13J11 (Brown et al. 1999; Depatie et al. 2000). A total of 67 independent contigs (201,777 bp) were assembled from BAC 13J11 sequence using the assembly program, Sequencher. Altogether the sequencing

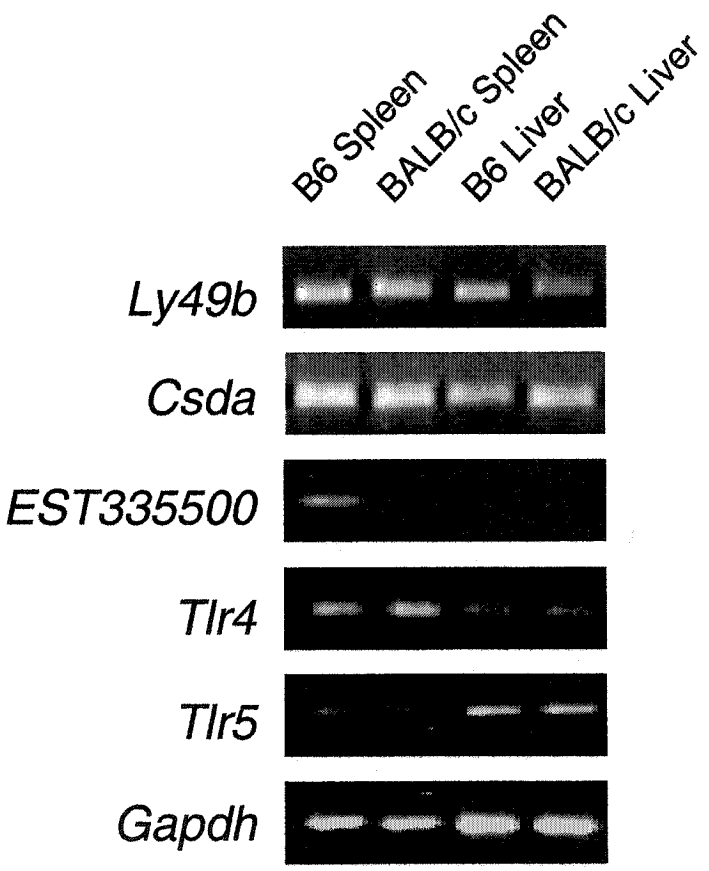
approach of BAC 13J11 allowed the identification of the *Hmg* (high mobility group) gene, the taste receptor gene *T2r*, and at least 5 copies of *EST335500* (see below). A schematic representation of the transcript map for the 19 potential genes localized in this study is presented in Figure 3.3d.

3.3.2. RT-PCR and cDNA sequence analysis of candidate genes for *Cmv1*

A minimum of 19 transcription units reside in the minimal *Cmv1* interval, including 14 members of the *Ly49* gene family, the *Csda* gene (cold-shock domain a protein gene) and the gene defined by a mouse *EST335500* (GeneBank Accession No: W18806) (Figure 3.3d). Initially, I investigated the candidacy of *Ly49b*, *Csda* and *EST335500* using RT-PCR and mutation analysis. *Ly49b* is the most distantly related member of the *Ly49* gene family and lies at about 750 kb distal of the *Ly49* cluster (Figure 3.2d). In contrast to other members of the family, *Ly49b* expression can be detected by RT-PCR in the spleen and the liver of adult mice (Figure 3.3). Sequence analysis of these cDNAs demonstrated 14 nucleotide differences between *Ly49b* of C57BL/6 and that of BALB/c resulting in 13 amino acid substitutions.

The deduced 342-amino acid sequence of the CSDA protein has a cold-shock domain and a DNA-binding domain that is thought to bind to the repressor element in the human GM-CSF (Granulocyte-macrophage colony stimulating factor) promoter (Cole et al. 1996). Macrophages elicit numerous regulatory signals affecting NK cell function. We reasoned that subtle variations in macrophage maturation might affect NK cell function underlying the $Cmv1^s$ phenotype. RT-PCR show expression of *Csda* in all tissues and mouse strains tested (Figure 3.4). However, it is noteworthy that expression in the spleen, the major site of *Cmv1* expression, is higher than that in the liver.

Figure 3.4. RT-PCR analysis of *Ly49b, Csda and EST335500.* *Ly49b, Csda and EST 335500* expression between C57BL/6 and BALB/c mice in the spleen and liver is analyzed by RT-PCR. *Tlr4* and *Tlr5* are used as controls for their tissue specific gene expression, spleen-specific and liver-specific, respectively. *Gapdh* is used as an internal control.


B6 Spleen
BALB/c Spleen
B6 Liver
BALB/c Liver
Ly49b
Csda
EST335500
Tlr4
Tlr5
Gapdh

Sequence analysis indicated a single nucleotide difference between C57BL/6 and BALB/c, resulting in one amino acid substitution.

EST335500 was identified by BLAST searches with exon-trapped sequences from BAC clone 13J11. *EST335500* was of particular interest because it resides in the region common to two independent genetic minimal intervals of *Cmv1* published by two different groups. Interestingly, RT-PCR analysis of *EST335500* indicated that this EST presents an expression pattern consistent with the *Cmv1* phenotype: it is specifically expressed in the spleen of resistant C57BL/6 while the transcript is reduced in susceptible BALB/c (Figure 3.4). DNA sequencing of a 700 bp cDNA identified a single nucleotide change (G to T) resulting in a Val230Asp substitution for the BALB/c cDNA.

Overall, RT-PCR analysis indicated that all candidate genes were expressed in spleen, a target organ of *Cmv1* phenotype, suggesting their candidacy as *Cmv1*. However, DNA sequence polymorphisms between the resistant C57BL/6 and the susceptible BALB/c were found for all genes, ruling out mutation analysis as a valid approach for the identification of *Cmv1*.

3.4. DISCUSSION

YAC and PAC maps have been established for the *Ly49* cluster, accounting for 14 known genes up to date (Brown et al. 1997a; McQueen et al. 1998). However the determination of the precise location and order of the *Ly49* gene family has been retarded by the high similarity in nucleotide sequence among the members.

Furthermore the region distal to the *Ly49* gene cluster has not been analysed extensively for the presence of transcription units. The BAC contig previously generated provided valuable material to generate the organization of the *Ly49* transcriptional units by enabling the localization of all family members to the same physical map. Moreover these BAC clones were useful for exon amplification and sequencing of interesting domains to uncover unknown transcripts.

Using exon amplification and direct BAC sequencing, *Ly49k* has been anchored on the map in close proximity to *Ly49d* and *Ly49f,* whereas previous studies were not able to link this member to the gene cluster (McQueen et al. 1998). Furthermore, the order of some genes could not be ascertained in the previous maps. Results from this study show that *Ly49f* is located upstream of *Ly49d* and physically linked the *Ly49n/i/g* cluster to *Ly49l/m,* providing an unambiguous orientation. Finally, sequences derived from exon amplification studies suggest the presence of other *Ly49*-related sequences. The *Ly49* cluster spans about 1.2 Mb of genomic DNA where no recombination with *Cmv1* has been detected, indicating that *Ly49* genes stand as attractive candidates. A consensus map of the family can be proposed as follows: cen–*Ly49e– Ly49f– Ly49d– Ly49k/Ly49new– Ly49h– Ly49n– Ly49i– Ly49g– Ly49l– Ly49j– Ly49m– Ly49c– Ly49a– Ly49b.* In addition to *Ly49* genes, four potential transcription units were localized to the *Cmv1* interval, each exhibiting a high degree of homology to the *Zfp35* gene (Cunliffe et al. 1990a) and other zinc-finger protein genes, the *Magoh* gene (Zhao et al. 1998), the *Csda* gene (Cole et al. 1996), or the *EST335500.*

The predicted amino acid sequence from *287G15T7* identifies two zinc-finger motifs of type C2H2 (Cys-X2- Cys-X3-Phe-Sx-Leu-X2-His-X3-His), a motif reportedly involved in DNA binding (Pieler and Bellefroid 1994). The human and mouse genomes

contain hundreds of genes coding for finger proteins of this type; therefore common functional features between a putative protein partly encoded by *287G15T7* and *Zfp35* remain to be determined. *Zfp35* is a mouse chromosome 18 locus (Cunliffe et al. 1990b) expressed in adult testis, where it may play a role in spermatogenesis (Cunliffe et al. 1990a). Translation of exon 285G10 predicts an 18- amino-acid stretch highly conserved in mouse, human, and rat *Magoh* sequences. *Magoh* has been assigned to mouse chromosome 4 (Zhao et al. 1998), indicating that the exon sequences identified probably correspond to a related gene. *Magoh* is the mammalian homologue of the *Drosophila mago nashi* gene (Newmark and Boswell 1994) involved in posterior pole determination of the fly oocyte. Although the function of *Magoh* in mice has not yet been elucidated, mRNA expression is ubiquitous in adult tissues and can be induced by stimulation of quiescent fibroblasts (Zhao et al. 1998). CSDA proteins comprise a family of DNA and RNA binding proteins conserved throughout evolution from *E. coli* to humans (Sakura et al. 1998). Their recognition element is the Y-box, an inverted CCAAT motif contained within the promoter sequence of many genes including all MHC class II genes, histone H2B, and the cystic fibrosis CFTR gene (Duh et al. 1995). Their DNA binding domain spans an 80-amino-acid region near the NH2 terminus overlapping the homology region identified by the 184a16 exon. At this time, it is not known whether *Cmv1* encodes a recognition molecule or whether it is involved in the activation pathway of NK cells. Therefore, detailed characterization of these positional candidates is warranted to assess their candidacy for *Cmv1*. Finally, exon amplification identified 10 exons corresponding to unique sequences with no homology to known genes. Interestingly, most of these exons were isolated from CpG-island-associated BAC clones 240C11, 13A21, and 293M2, suggesting the presence of at least 3 more transcription units in the region.

Lastly, initial analysis of gene expression for several candidate genes showed that all genes tested are highly expressed in spleen. The subsequent sequence analysis of these transcripts from resistant B6 and susceptible BALB/c identified many differences in the nucleotide sequence level, indicating the highly polymorphic nature of the *Cmv1* region. These results exclude mutation analysis as an efficient approach and emphasize the search of alternative approaches to facilitate the cloning of *Cmv1*. For example, the transcription map which precisely localized 19 transcription units, together with the BAC contig previously generated, should provide the means to determine the exact position of *Cmv1* by *in vivo* complementation using BAC transgenesis. Used systematically, this approach should also help to elucidate a possible allelism between *Cmv1* and other natural immunity loci controlling NK cell function.

CHAPTER FOUR

HAPLOTYPE MAPPING INDICATES TWO INDEPENDENT ORIGINS FOR THE *Cmv1*^{S} SUSCEPTIBILITY ALLELE TO CYTOMEGALOVIRUS INFECTION AND REFINES ITS LOCALIZATION WITHIN THE *Ly49* CLUSTER

4.1. INTRODUCTION

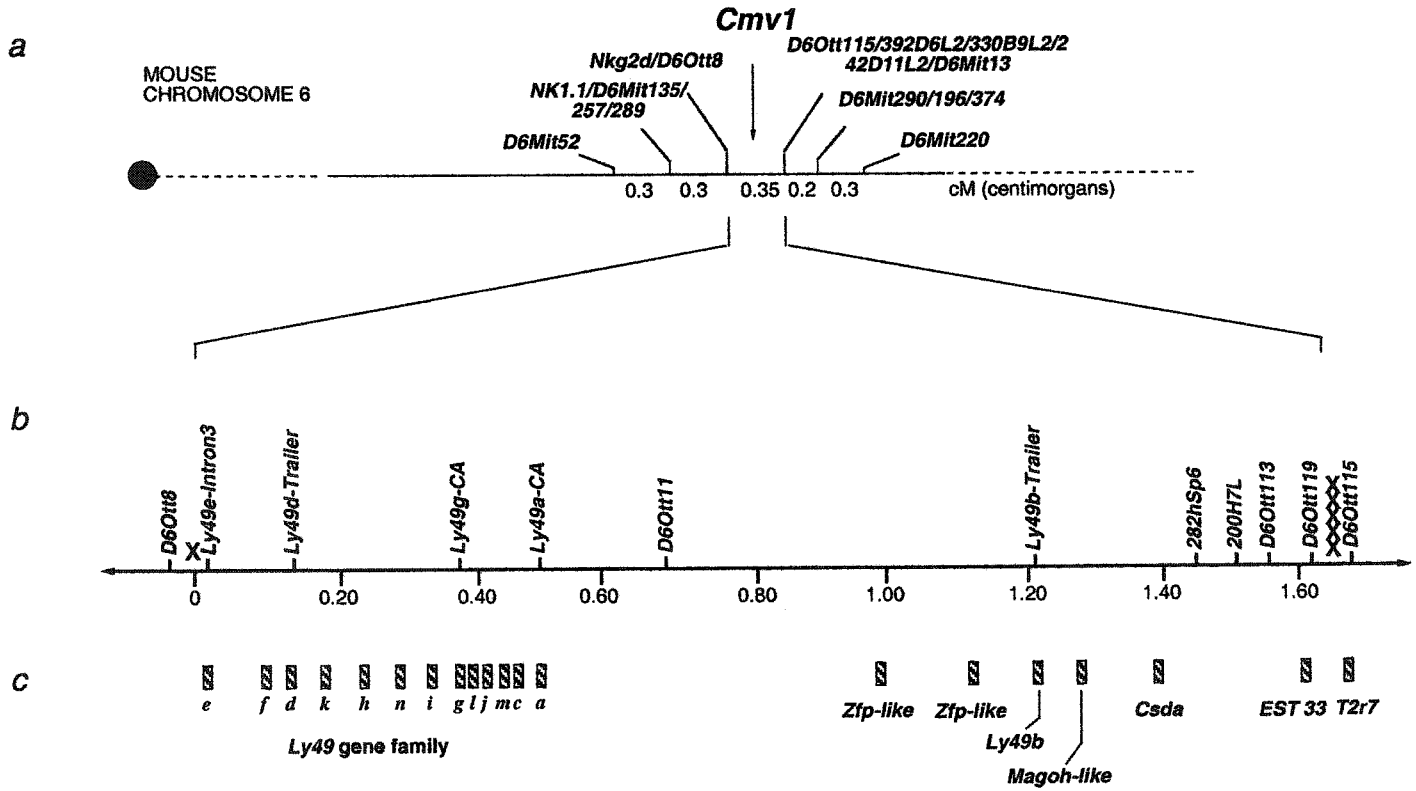
In laboratory mice, the early response to murine cytomegalovirus (MCMV) is determined by an autosomal non-*H2* locus, designated *Cmv1* (Scalzo et al. 1990). *Cmv*1 presents two alleles: the resistant allele $Cmv1^r$, expressed in strains of C57BL origin and the susceptibility allele $Cmv1^s$, expressed in BALB/cJ and A/J strains. Phenotypically, susceptible mice display splenic viral titers 10^3 to 10^4 times higher than those found in resistant mice from day three post-infection. *Cmv1* has been mapped initially to the distal portion of mouse chromosome 6 (Scalzo et al. 1990) within a well defined cluster of NK cell receptor genes termed the Natural Killer gene complex (NKC) (Brown et al. 1999).

To understand the molecular basis of resistance, a molecular genetics approach was initiated, based on genetic mapping and identification of candidate genes, to isolate *Cmv1*. Using segregation analysis in populations of informative backcross mice, *Cmv1* has been localized to a 0.35-cM interval (Figure 4.1a) (Depatie et al. 1999). This interval contains at least 19 transcription units that stand as attractive *Cmv1* candidates, including 14 members of the *Ly49* gene family (Figure 4.1c) (Depatie et al. 2000). Several members of this family have been shown to encode for surface activation or inhibitory *Ly49* receptors that are preferentially expressed on Natural Killer cells (Takei et al. 1997). Sequence comparison between $Cmv1^r$ and $Cmv1^s$ strains indicated allelic differences for transcripts analysed in the *Cmv1* interval, precluding assessment of their candidacy based upon mutation analysis (section 3.3.2; Brennan et al. 1996; Makrigiannis et al. 2000). In addition, the complex structural organization of the *Ly49* gene family further complicated the analysis of the *Cmv1* domain as many of the *Ly49*

genes have different copy numbers and genomic organization in inbred strains, making it difficult to distinguish allelic variants of the same or of distinct *Ly49* gene copies (Makrigiannis et al. 2000).

Thus, as an alternative to genetic linkage analysis, haplotype analysis has been used for fine mapping of candidate region. Haplotype indicates the specific pattern of marker alleles in a certain genome. When the observed frequencies of haplotypes in a population occur more often than expected by chance, it is called Linkage disequilibrium (LD). Linkage disequilibrium mapping is based on the premise that regions adjacent to target genes are transmitted through the generations along with the actual genes (Jorde 1995). Therefore, LD may be defined as the nonrandom association of marker alleles, usually mapping within a short chromosomal region, with a phenotype. Genetic linkage analysis is based only on recombination events that occur in a specific cross whereas LD patterns rely on recombinations that have occurred over generations starting from the origin of the mutated allele in the founder mouse to the fixation of the mutation at homozygosity during inbreeding. Haplotype analysis has been successfully used in humans for the precise location of disease loci and has proved to be an important tool for the positional cloning of several disease genes (Jorde 1995). Although haplotype analysis is mainly used in human populations, it has been used for fine mapping in experimental animals (Malo et al. 1994; Manenti et al. 1999). In an attempt to narrow down the localization of *Cmv1* and to facilitate the characterization of candidate genes, we decided to determine the allelic composition of a set of linked loci in the vicinity of *Cmv1* and study their haplotypes in a panel of 17 inbred strains of mice characterized by their pattern of resistance or susceptibility to MCMV.

Figure 4.1. Location of *Cmv1* and marker loci used in the haplotype analysis (a) The genetic map is shown with recombinational distances in centimorgans (cM). The centromere of the chromosome is represented by the black circle. (b) Long range physical map of the region between the closest proximal (*D6Ott8*) and distal (*D6Ott115*) marker, defining the *Cmv1* critical interval. "X" indicates the localization of crossovers defining the target interval. The physical localization of genetic markers used to construct the haplotype map is shown. (c) Transcriptional map of the *Cmv1* critical interval. Hatched boxes indicate the localization of transcription units.


a
Cmv1
MOUSE
CHROMOSOME 6
Nkg2d/D6Ott8
NK1.1/D6Mit135/
257/289
D6Mit52
D6Ott115/392D6L2/330B9L2/2
42D11L2/D6Mit13
D6Mit290/196/374
D6Mit220
0.3 0.3 0.35 0.2 0.3
cM (centimorgans)
b
D6Ott8
Ly49e-Intron3
Ly49d-Trailer
Ly49g-CA
Ly49a-CA
D6Ott11
Ly49b-Trailer
282inSp6
200H7L
D6Ott113
D6Ott119
D6Ott115
0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
c
e f d k h n i g l j mc a
Ly49 gene family
Zfp-like
Zfp-like
Ly49b
Magoh-like
Csda
EST 33
T2r7

4.2. MATERIAL AND METHODS

4.2.1. Phylogenetic analysis of the *Cmv1* locus

To generate the phylogenetic tree of 17 inbred strains of mouse with polymorphic markers in the vicinity of the *Cmv1* locus, the haplotype data was recoded into binary (0-1) characters by the FACTOR program (http://evolution.genetics.washington.edu/phylip.html) and used to build a cladogram by the Wagner parsimony method with the MIX program. Because the input order of the samples has an effect on the output, the program was run at least three times using different parameters. This yielded seven possible cladograms that were assembled in a consensus tree using CONSENSE. Branch lengths indicate the numbers of replicates that support the branch.

4.3. RESULTS

Twenty-five informative DNA markers that are either flanking the target domain or evenly distributed within the critical interval for *Cmv1* were used for haplotype analysis of 17 inbred strains of mice of diverse ancestral origin (Figure 4.2) (Depatie et al. 1999; Depatie et al. 2000). In addition, previously reported NK1.1 expressions using PK136 antibody was included (www.pharmingen.com) (Scalzo et al. 1995). These strains were placed into two phenotypic groups demonstrating low or high MCMV splenic titers, according to published classification (Scalzo et al. 1990; Scalzo et al. 1995) and this work (Figure 4.2). Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were assessed for splenic MCMV replication at day four after infection

with 2 × 10^4 PFU of the K181 or Smith strain of MCMV. Whereas C57BL/6J and C57BL/10J displayed a splenic viral titer of 10^1-10^2 PFU per organ (low), the other 15 strains including FVB/NJ and AKR/J (not previously tested) had a splenic viral titer of 10^4-10^5 (high).

The informative DNA markers were either microsatellites (*D6Mit52, D6Mit257, D6Mit135, D6Mit289, D6Ott8, Ly49g-CA, Ly49a-CA, D6Ott11, D6Ott113, D6Ott119, D6Ott115, 392D6L2, D6Mit13, D6Mit290, D6Mit196, D6Mit374, D6Mit220*), PCR-RFLPs (*Nkg2d-Intron11, Ly49e-Intron3, 282h8Sp6* and *200H7L*) or detected by SSCP (*Ly49b-Trailer*). Three other markers (*Ly49d-Trailer, 330B9L2, 242D11L2*) that were amplified from C57BL/6J DNA but did not yield a PCR product from BALB/cJ DNA were also included. DNA preparation and genotyping conditions were as previously described (Depatie et al. 1999). The DNA markers span a genetic distance of 1.45-cM (*D6Mit52* to *D6Mit220* interval) (Figure 4.1a) and include 10 markers which were not observed to recombine with *Cmv1* in almost 2000 meiosis (Figure 4.1b) (Depatie et al. 2000). *D6Ott8* and *D6Ott115* are respectively, the closest proximal and distal markers that define the 1.6-Mb critical interval for *Cmv1*, as established by PFGE and FISH (Figure 4.1b).

Each locus presented 2 to 7 alleles that were arbitrarily named 1 to 7 according to the size, where 1 is the shortest form. For two-allele systems detected by restriction enzyme digestion, the most frequent allele was named 1. A core haplotype was defined which consisted of 10 polymorphic markers within the minimal 1.6-Mb interval for *Cmv1*. As shown in Figure 4.2, a total of 8 distinct core haplotypes were identified over the region tested.

Figure 4.2. Haplotype mapping of chromosome 6 in the vicinity of *Cmv1* with 26 polymophic markers All markers have been previously reported with the exception of *D6Ott119* (*Ly49g-CA*, U70367; *Ly49a-CA*, G54745) (Depatie et al. 1999). C57BL/6J alleles are coloured in pink and A/J alleles in yellow. "1" represents the smallest size allele for microsatellites markers, and the most frequent allele for PCR-RFLP markers. "0" indicates that there is not a PCR product. Asterisks (**) indicate the closest proximal and distal markers of the *Cmv1* interval spanning a 1.6-Mb genomic region. The BALB/cJ haplotype involving marker *D6Ott8* shows two alleles, indicating residual heterozygosity at this locus.

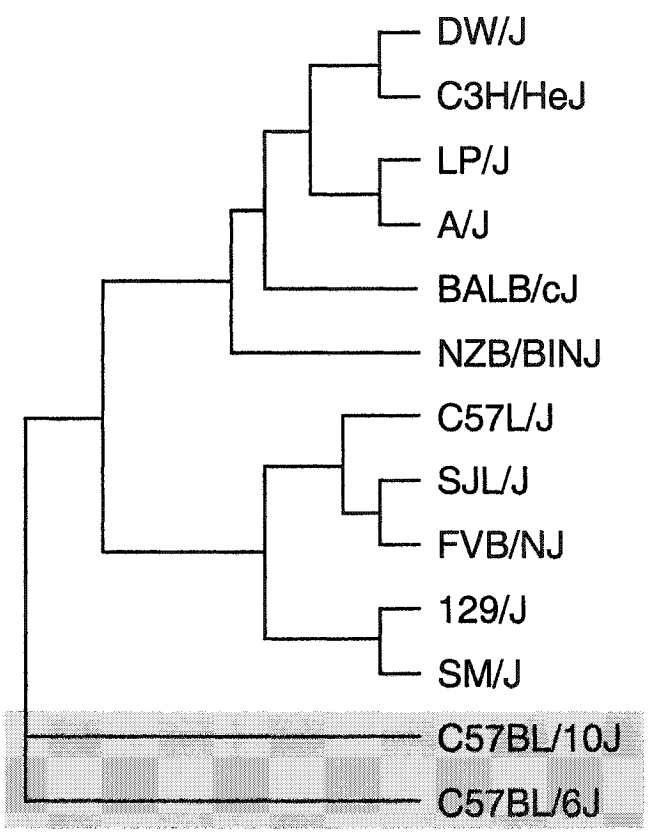
	MCMV titer	*D6Mit52*	*D6Mit257*	*D6Mit135*	*D6Mit289*	*NK1.1*	*Nkg2d-Intron11*	*D6Ott8***	*Ly49e-Intron3*	*Ly49d-Trailer*	*Ly49g-CA*	*Ly49a-CA*	*D6Ott11*	*Ly49b-Trailer*	*282h8Sp6*	*200H7L*	*D6Ott113*	*D6Ott119*	*D6Ott115***	*392D6L2*	*330B9L2*	*242D11L2*	*D6Mit13*	*D6Mit290*	*D6Mit196*	*D6Mit374*	*D6Mit220*
C57BL/6J	Low	3	4	4	1	1	2	1	2	1	1	4	3	2	2	2	3	3	3	2	1	1	3	3	3	4	2
C57BL/10J	Low	3	4	4	1	1	2	1	2	1	1	4	3	2	2	2	3	3	3	2	1	1	3	5	3	4	2
129/J	High	2	1	4	2	0	2	1	2	1	3	2	4	2	2	2	2	3	3	2	1	1	3	2	1	3	7
SM/J	High	2	1	4	2	0	2	1	2	1	3	2	4	2	2	2	2	3	2	2	1	1	3	2	1	3	7
SJL/J	High	1	2	2	5	1	2	1	2	1	3	2	2	0	2	2	2	3	2	2	1	1	5	4	1	4	5
FVB/NJ	High	1	2	2	5	1	2	1	2	1	3	2	2	3	2	2	2	3	2	2	1	1	5	3	3	2	1
C57L/J	High	3	2	2	4	1	2	1	2	1	3	2	3	3	2	2	2	2	4	2	1	1	3	1	1	3	4
NZB/BINJ	High	3	0	1	1	1	2	1	2	1	4	3	2	0	2	1	1	1	1	1	0	1	3	2	3	5	6
C3H/HeJ	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	2	1	2	1	4
DW/J	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	4
BALB/cJ	High	2	1	6	3	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
A/J	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
DBA/1J	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
DBA/2J	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
CBA/CaJ	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
LP/J	High	2	1	5	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3
AKR/J	High	2	1	6	4	0	1	2	1	0	2	1	1	1	1	1	1	1	1	1	0	0	1	1	2	1	3

The most frequent haplotype (1-0-2-1-1-1-1-1-1-1) is present in a group of 9 strains including BALB/cJ, A/J, DBA/2J known to carry the susceptibility allele *Cmv1*^{s} by genetic mapping, as well as in the susceptible strains DBA/1J, C3H/HeJ, CBA/CaJ, DW/J, LP/J and AKR/J. The observed sequence identity at multiple variable loci indicated a common ancestry of this segment of DNA among this group of susceptible strains and identified a susceptibility haplotype that was named S. A second haplotype (2-1-1-4-3-2-2-2-3-3), was exclusively present in the *Cmv1*^{r} strains C57BL/6J and its close relative, C57BL/10J. These data identified the resistance haplotype that is distinct from that found in susceptible strains of the S group at all markers, and that was named R. Five core haplotypes with different combinations of alleles at *D6Ott11*, *Ly49b-Trailer* and *D6Ott119* were characteristic of a group of susceptible strains. For example, (2-1-3-2-3-3-2-2-2-2) is present in C57L/J whereas 129/J, SM/J, SJL/J and FVB/NJ carry (2-1-3-2-2/4/-2/0/3-2-2-2-3). This result suggested that these strains have genotypes distinct at all loci from the S haplotype but, in contrast, they are 50% identical to R alleles. Lastly, there is an eighth haplotype (2-1-4-3-2-0-2-1-1-1) that is unique to the NZB/BINJ strain. Susceptible strains could thus be grouped in two main classes. One class, including strains that were proven by genetic linkage to carry the susceptibility *Cmv1*^{s} allele, presented a haplotype highly unrelated to C57BL/6J. The other class, including FVB/NJ and related strains, presented haplotypes highly related to C57BL/6J (*Cmv1*^{r}). The markers that define the proximal and distal boundaries of the *Cmv1* domain (*Nkg2d-Intron11/D6Ott8* and *D6Ott115/392D6L2/330B9L2/242D11L2*) were added to the core haplotype, extending the analysis over a 2.2-Mb region. With the exception of *D6Ott115*, these markers defined bi-allelic systems that delineated extended haplotypes corresponding to the groups previously defined. Finally, nine

additional SSLP markers (*D6Mit52*, *257*, *135*, *289*, *13*, *290*, *196*, *374* and *220*) were included, allowing the identification of twelve different haplotypes that defined groups closely related to the ones delineated before.

The independent origin of the two groups of susceptible strains was further confirmed by parsimony analysis of the full data using the PHYLYP 3.57c package (Felsenstein J 1989) available at http://www.cbr.nrc.ca/cgi-bin/WebPhypip/index.html. As shown in Figure 4.3, there are three strongly supported branches that are largely congruent with known genealogical relationships. For example C57BL/6J and C57BL/10J derived from the same founding pair form a strong group that is placed as the most deeply divergent among the strains. Similarly, A/J, BALB/cJ, CBA/CaJ and C3H/HeJ, all related to a Bagg albino stock (Atchley and Fitch 1993) form a strong branch as do SJL/J and FVB/NJ, derived from Swiss albino stocks, or 129/J and SM/J, derived from English coat color and chinchilla stocks obtained from W. Castle (Beck et al. 2000). There are also some remarkable discrepancies. For example, although LP/J and 129/J strains are genealogically and phylogenetically related, they are clearly separated in our analysis. In contrast, C57L/J and 129/J in spite of their independent origin, appear to be grouped together in our analysis (Atchley and Fitch 1993; Beck et al.2000). These data support the hypothesis that the observed pattern of genetic divergence in this set of strains can be explained by the segregation of heterozygosity from a small population of highly heterozygous mice and suggest that S and R related haplotypes were present in a diverse set of founders.

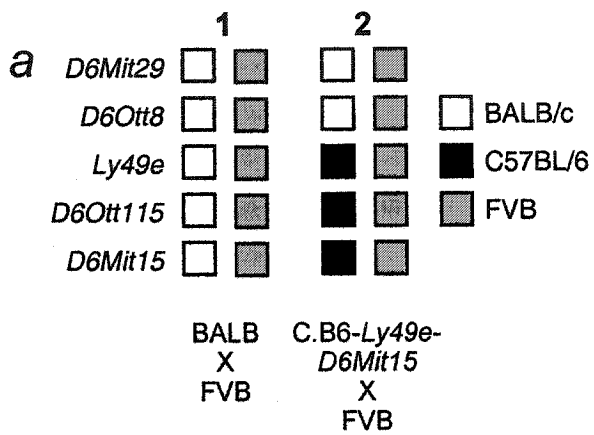
Figure 4.3. Phylogenetic tree of 17 mouse inbred strains in the vicinity of the *Cmv1*. A phylogenetic tree was generated for 17 mouse inbred strains of known susceptibility to MCMV derived by parsimony analysis with polymorphic markers mapped in the vicinity of the *Cmv1* locus. These haplotype data were recoded into binary (0-1) characters by the FACTOR program. To facilitate the analysis, identical haplotypes from DBA/1J, DBA/2J, CBA/CaJ and AKR/J were not used but their position on the tree can be inferred from strain A/J. Branch lengths indicates the numbers of replicates that support the branch. The gray box contains *Cmv1*^{r} strains.

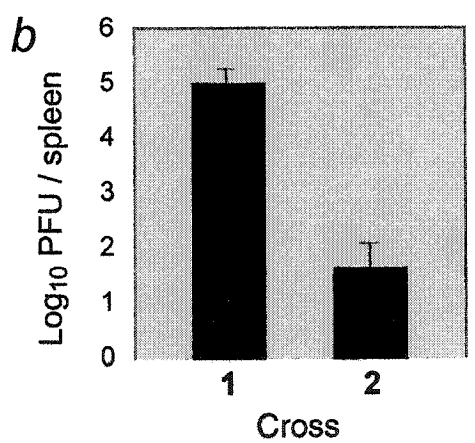

DW/J
C3H/HeJ
LP/J
A/J
BALB/cJ
NZB/BINJ
C57L/J
SJL/J
FVB/NJ
129/J
SM/J
C57BL/10J
C57BL/6J

This analysis however does not elucidate whether strains of the FVB/NJ group carry a susceptibility allele, *Cmv1*^{sFVB}, distinct from the one in the BALB/cJ strain (*Cmv1*^{sBALB}) or, alternatively, a resistant allele, *Cmv1*^{r}, whose expression would be abolished by a modifier locus. To clarify this issue, a genetic complementation test was carried out. The F1 progeny of crosses between FVB/NJ and BALB/cJ or the congenic strain C.C57BL/6-*Ly49e-D6Mit15* were typed for MCMV susceptibility. The congenic strain was produced from progeny 118 issued from the backcross panel (BALB/c × C57BL/6)F1 × BALB/c previously described (Depatie et al. 1997). Progeny 118 presented a recombination between *D6Ott8* (BALB/c) and *Ly49e* (C57BL/6) and carried the *Cmv1*^{r} allele (Figure 4.4a). Marker selected animals were back-crossed to the BALB/c strain over 8 generations. Heterozygous animals identified at the 8th generation were selected for brother-sister mating to produce C.C57BL/6-*Ly49e-D6Mit15*. As shown in Figure 4.4b, whereas (FVB/NJ × BALB/cJ)F1 hybrids present high viral titers in the spleen (Log_{10} = 5), the progeny (FVB/NJ × C.C57BL/6-*Ly49e-D6Mit15*) control MCMV replication efficiently (Log_{10} = 2). This result localizes the susceptibility allele of FVB distal from *D6Ott8* and indicates that FVB/NJ carries a susceptibility allele, *Cvm1*^{sFVB}, distinct but allelic to *Cvm1*^{sBALB}. Given the degree of allele sharing among susceptible strains bearing R related haplotypes, it is assumed that they carry the same susceptibility allele, *Cmv1*^{sFVB}.

Analysis of these strains indicates that there are two regions in disequilibrium with susceptibility: one between *Ly49d-Trailer* and *D6Ott11*, spanning more than 400 kb and the other between *200H7L* and *D6Ott119*, corresponding to about 120 kb.

Figure 4.4. Replication of MCMV in the spleen of hybrid progeny involving the FVB/NJ strain (a) Schematic representation of chromosome 6 pairs in the two crosses tested. The BALB/cJ allele is shown in white, C57BL/6J in black, and FVB/NJ in gray box. (b) Viral titers in the spleen of FVB/NJ×BALB/cJ (1) and FVB/NJ×C.C57BL/6-*Ly49e-D6Mit15* (2) were determined by plaque assay three days after infection with 1×10^4 PFU of Smith strain. Groups of four mice were analyzed by cross.


a
1
2
D6Mit29
D6Ott8
Ly49e
D6Ott115
D6Mit15
BALB/c
C57BL/6
FVB
BALB
X
FVB
C.B6-Ly49e-
D6Mit15
X
FVB


b
Log10 PFU / spleen
0
1
2
3
4
5
6
1
2
Cross

However, inspection of the proximal region of the *Cmv1* genetic interval indicates that they carry a resistant haplotype between the markers *Nkg2d-Intron11* and *Ly49d-Trailer* (Figure 4.2), probably pointing to an ancestral recombination site. These data refine the proximal boundary of *Cmv1* between *Ly49d-Trailer* and *Ly49g-CA*, narrowing down the *Cmv1* domain to a maximum of 1.2 Mb and excluding *Ly49e*, *Ly49f* and *Ly49d* as candidate genes for *Cmv1*.

4.4. DISCUSSION

Identifying the defect in $Cmv1^s$ animals is complicated by the highly polymorphic nature of this chromosomal segment in inbred strains (Takei et al. 1997). As an alternative strategy for mapping, an haplotype sharing approach was used (Lyons et al. 2000; Manenti et al. 1999) together with *in vivo* complementation to refine the localization of *Cmv1*. The most striking observation was that the overall structure of the *Cmv1* region is very similar among mouse strains carrying the S haplotype, further supporting the notion that this allele sharing is identical by descent (IBD) and not merely identical by state. In this group of strains, IBD is maintained for the highly polymorphic microsatellites used for the extended haplotypes in contrast to a greater diversity of haplotype combinations at these loci observed in mice carrying R related haplotypes, indicating a closer relationship among susceptible strains bearing S. As shown in Figure 4.2, it is evident that strains carrying S do not share C57BL/6J alleles at any locus, indicating that over this region strains S and R are distantly related. In contrast, a similar analysis over markers delineating proximally and distally the *Cmv1* interval extended the identity of susceptible strains of the FVB/NJ group and the resistant strain C57BL/6J and supports the content that susceptible strains presenting R related haplotypes and

C57BL/6J share a common ancestral haplotype. Therefore, this study indicated that the *Cmv1*^{s} MCMV susceptibility allele does not result from a recent and unique mutation in a single mouse line but rather presents at least two independent origins giving rise to *Cmv1*^{sBALB} and *Cmv1*^{sFVB}.

The identification of a *Cmv1*^{sFVB}, related to *Cmv1*^{r}, allowed the use of ancestral recombination events to provide additional mapping information excluding three candidate genes from the *Cmv1* interval. Whereas the role of *Ly49e* and *Ly49f* has not yet been elucidated, *Ly49d* encodes for an activator receptor of NK-cell cytolytic activity and it has been identified as the *Chok* gene (Idris et al. 1999). The results reported here thus establish that *Chok* and *Cmv1* are two separate entities, indicating that tightly linked genes control different NK cell functions. Although further studies are required to identify the two sub-regions within the interval in disequilibrium with susceptibility, it cannot be ruled out at this time that two *Cmv1* genes might reside hither. In fact, the existence of a MCMV susceptibility locus distal to *Ly49b* has been suggested by independent linkage studies (Brown et al. 1999). The present characterization of two MCMV susceptibility haplotypes will be relevant to the elucidation of the identity of *Cmv1* by subsequent strain comparison of sequence variations of candidate genes.

CHAPTER FIVE

SUSCEPTIBILITY TO CYTOMEGALOVIRUS INFECTION IS ASSOCIATED WITH DELETION OF THE NATURAL KILLER CELL LECTIN-LIKE RECEPTOR *Ly49h* GENE

5.1. INTRODUCTION

Cytomegalovirus is the leading cause of congenital viral disease and the most important opportunistic infection in immunocompromised patients (Demmler 1991; Ho 1995). We have utilized a murine experimental infection model (MCMV) to study the genetic parameters of host/virus interaction. Susceptibility to infection with MCMV is controlled by *Cmv1,* a chromosome 6 locus that regulates natural killer (NK) cell activity against virally infected targets (Scalzo et al. 1990; Scalzo et al. 1992). We followed a positional cloning strategy to isolate *Cmv1*. *Cmv1* maps within a 0.35 cM interval defined by markers *D6Ott8* and *D6Ott115*, which corresponds to a physical distance of 1.6 Mb (Depatie et al. 1999; Depatie et al. 2000). A transcript map of the region identified 19 genes (Depatie et al. 2000), including members of the *Ly49* family which encode inhibitory or activating NK cell receptors that interact with MHC class I molecules (Brown et al. 1997a; McQueen et al. 1998; Smith et al. 1994). *Ly49* genes have different copy numbers, genomic organization, and are highly polymorphic amongst inbred strains, making it difficult to distinguish between normal allelic variants and distinct *Ly49* genes (Nakamura et al. 1999; Takei et al. 1997), or possible mutations associated with *Cmv1*.

As an alternative model to facilitate the cloning of *Cmv1*, the recombinant inbred strain BXD-8/Ty (BXD-8), derived from $Cmv1^r$ C57BL/6 (resistant) and $Cmv1^s$ DBA/2 (susceptible), is of particular interest. Recombinant inbred strains were derived by brother-sister mating starting at the F2 generation originating from a cross between two standard inbred strains (Taylor et al. 1999). BXD mice are homozygous for all loci, and their genome composition is on average 50% of C57BL/6 (B6) origin and 50% of DBA/2 origin. Each line in the BXD panel will have a unique chromosomal composition

that is a mosaic of chromosomal segments inherited from one or the other parental strain. Interestingly BXD-8 mice were highly susceptible to MCMV infection despite having a C57BL/6 haplotype at *Cmv1* (Scalzo et al. 1990).

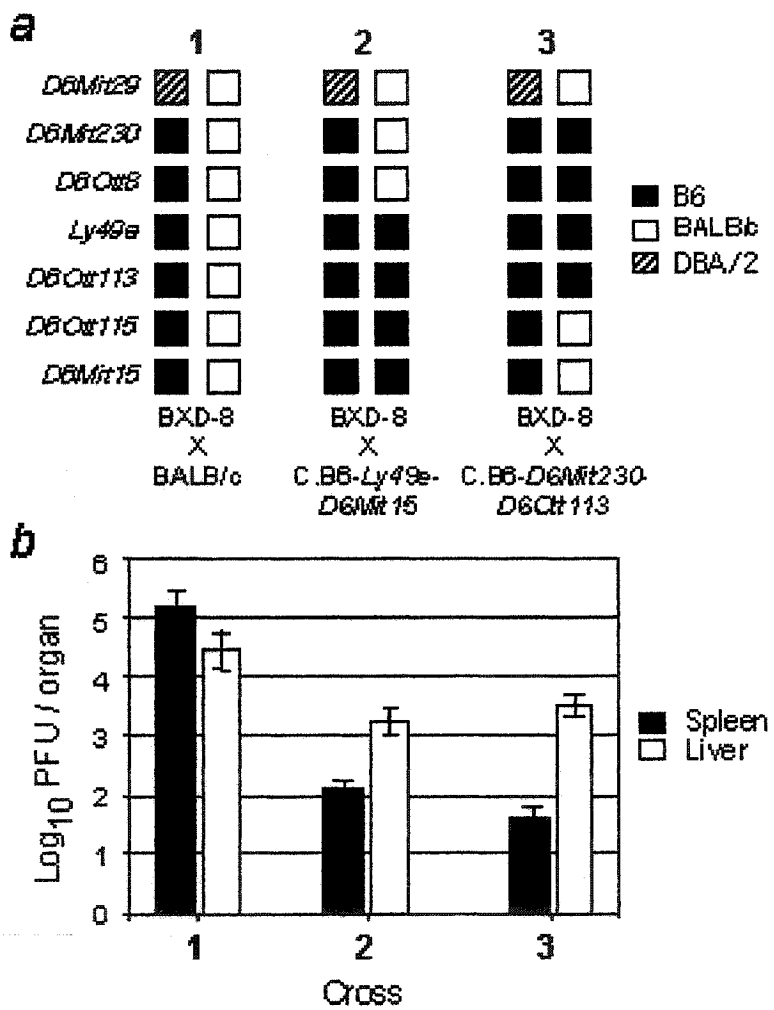
Previously, the mode of inheritance of the BXD-8 susceptibility trait was investigated in F_1 progeny issued from crosses to C57BL/6 and DBA/2 progenitors, demonstrating that MCMV susceptibility in BXD-8 is controlled by a single, autosomal, recessive locus. A genome scan approach in (BXD-8 × C57BL/6)F_2 mice suggested that either *Cmv1* or a closely linked gene is responsible for susceptibility to MCMV infection in BXD-8. Furthermore, *in vivo* complementation analysis with two independent congenic mice containing a recombination over either a proximal or a distal marker indicates that the BXD-8 susceptibility locus is located between *D6Ott8* and *D6Ott115*, the proximal and distal boundaries of the *Cmv1* interval (Figure 5.1). These results indicate that susceptibility of BXD8/Ty mice is determined by a spontaneous mutation at *Cmv1,* thereby suggesting that the BXD/Ty strain is instrumental for identification of strong candidates by mutation analysis.

5.2. MATERIAL AND METHODS

5.2.1. Simple tagged sequence content mapping

Simple tagged sequence (STS) content mapping was performed on genomic DNA using polymorphic and non-polymorphic markers previously localized to the minimal genetic interval of *Cmv1* (Depatie et al. 2000) using PCR conditions described for genetic mapping, except that positive signals for non-polymorphic markers were visualized by agarose gel electrophoresis.

Figure 5.1. *In vivo* complementation of susceptibility to MCMV infection in the BXD-8 strain. (a) Schematic representation of chromosome 6 haplotypes for the three crosses tested. (b) Viral titers in the spleen of (1) (BXD-8XBALB/c)F_1, (2) (BXD-8XC.B6-*Ly49e-D6Mit15*)F_1 and (3) (BXD-8XC.B6-*D6Mit230-D6Ott113)*F_1 progeny were determined by plaque assay 3 days after infection with MCMV.


a
1
2
3
D6Mit29
D6Mit230
D6Ott8
Ly49e
D6Ott113
D6Ott115
D6Mit15
B6
BALB/c
DBA/2
BXD-8
X
BALB/c
BXD-8
X
C.B6-Ly49e-
D6Mit15
BXD-8
X
C.B6-D6Mit230-
D6Ott113
b
Log10 PFU / organ
0
1
2
3
4
5
6
1
2
3
Cross
Spleen
Liver

5.2.2. Gene expression analysis

Spleen cDNA of C57BL/6, BXD-8 and DBA/2 mice strains were used as templates for PCR (35 cycles: 94 °C/30 s, 60 °C/1 min, 72 °C/1 min) using degenerate primer pairs for the consensus sequences of all *Ly49* genes (*a-n*) except for *Ly49b* (Kubota et al. 1999). PCR products were fractionated in agarose gel, blotted to nylon membrane and hybridized to 5'-[γ-^{32}P] end-labeled oligonucleotides specific to each of the *Ly49* genes. Hybridization was performed at 5 °C below the *Tm* of each probe and filters were washed with 3 × SSC at room temperature. Oligonucleotides sequences were either previously described (Kubota et al. 1999), or are: *Ly49e*, 5'-TTGCAGAGACTAGTGAGCCA-3'; *Ly49k*, 5'- CATGAAACTCTCAACAACCA-3'; *Ly49l*, 5'-AATGATTTATCACATTTATC-3'; *Ly49m*, 5'-TGCCAAGATAAGTGCAGCAC-3'; *Ly49n*, 5'-CTTTAAGTCTATAGGATGTT-3'. *Ly49b* expression was analyzed by RT-PCR using specific primers (*Ly49b-1*, 5'-CGAGGCCACATTTTAATACA-3' and *Ly49b-2*, 5'-CTGTTCTCTGTTGAGGTAGTG-3'). For Northern blot analysis of *Ly49h*, a total of 10 µg of poly A^+ RNA was subjected to electrophoresis in formaldehyde-denaturing agarose gel and blotted to a Hybond™-N membrane (Amersham). The filter was hybridized using the full-length *ly49h* probe in a solution containing 1 M NaCl, 10% dextran sulfate and 1% SDS at 65 °C overnight. The membrane was washed with 0.1 × SSC at 68 °C and exposed to Kodak X-ray film with an intensifying screen for 5 days. After stripping the membrane, the mouse *Hprt* cDNA probe was used as an internal control.

5.2.3. Pulse-field gel electrophoresis (PFGE) and Southern analysis

Agarose blocks containing either YAC clones or genomic DNA (Depatie et al. 2000) were digested with either *Sal* I, *Xho* I or both (New England Biolabs). DNA in blocks (1/4 block per lane) was then subjected to pulse-field gel electrophoresis on a 1% agarose gel (CHEF-DR® III, Bio-Rad) under the following conditions: 6 V/cm, 120° angle, 14 °C, 15 s of switching time for 20 h followed by 8 s of switching time for 2 h. Fractionated DNA was blotted to filter using an alkaline transfer solution (0.4 N NaOH, 1.5 M NaCl) for 48 h. Hybridization was performed as previously described (Depatie et al. 2000).

5.2.4. GenBank accession numbers

Ly49d-Exon7, AF346016; *Ly49k-Exon4* , AF346017; *116m19Sp6* , AF346018; *Ly49h-Exon8*, AF346019; *Ly49h-Exon1*, AF346020; *Ly49h-Exon2*, AF346021; *Ly49n-Exon7*, AF346022; *Ly49i-Exon7*, AF346023; *128d23Sp6*, AF346024; *341c11Sp6*, AF346025; *D6Ott117*, AF346026; *D6Ott118*, AF346027.

5.3. RESULTS

5.3.1. STS content mapping and haplotype analysis at the *Cmv1* genetic interval

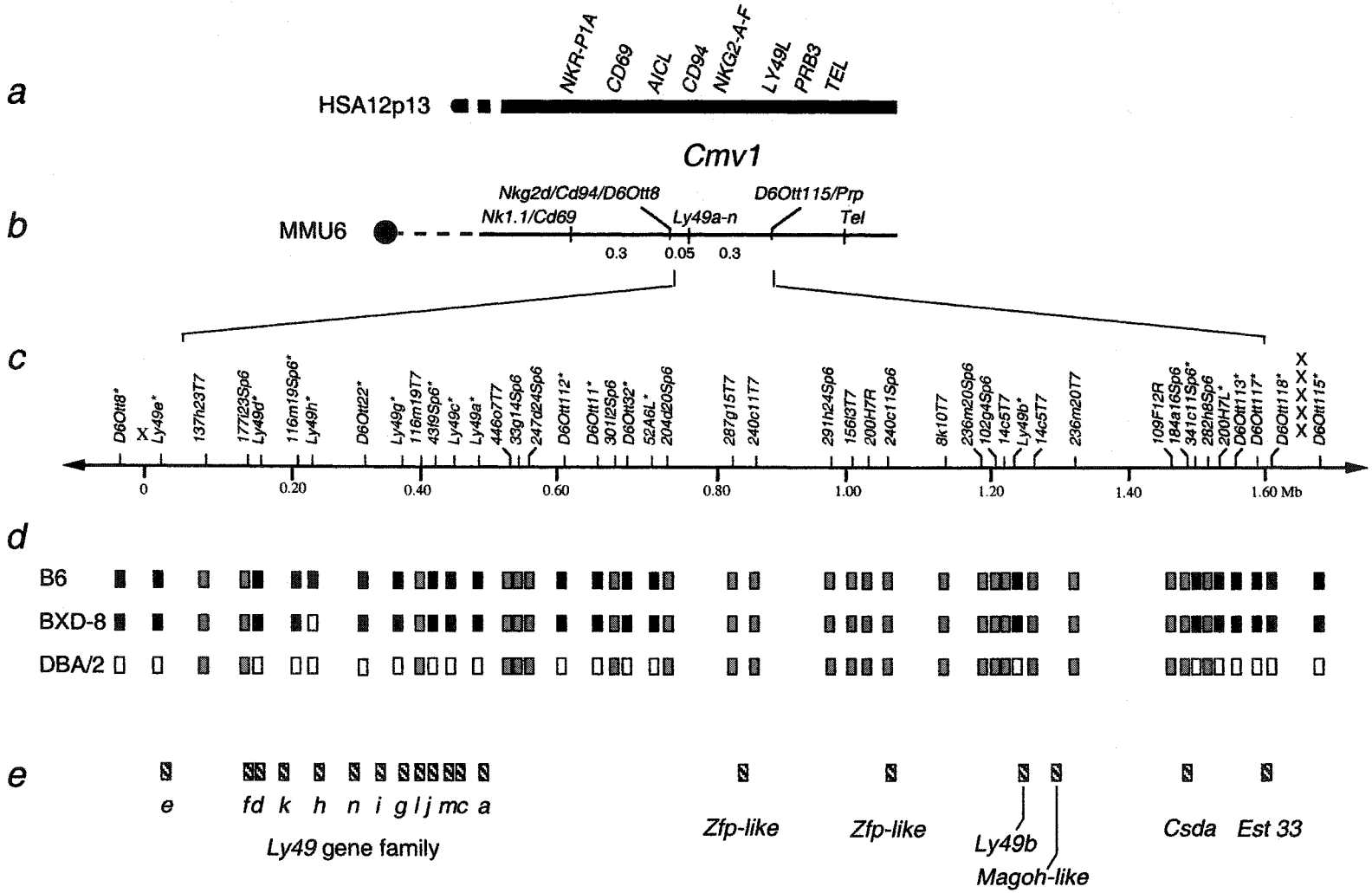
PFGE and FISH were used to construct the physical map of the 1.6 Mb *Cmv1* critical region, and delineated by *D6Ott8* and *D6Ott115* (Depatie et al. 2000). This interval is anchored by 44 markers (average spacing 36 kb), of which 19 were polymorphic between $Cmv1^r$ and $Cmv1^s$ mice (Figure 5.2c). These markers were used for STS content mapping of the *Cmv1* region of BXD-8, C57BL/6 and DBA/2 strains to

determine whether the C57BL/6 haplotype in BXD-8 was intact. The BXD-8 *Cmv1* domain appears to be intact and identical to C57BL/6 for all the loci tested, except for marker *Ly49h (i1)* which maps to intron 1 of the *Ly49h* gene (Figure 5.2d). *Ly49h* maps within the *Ly49* gene cluster, within a 420 kb genomic DNA fragment highly polymorphic between DBA/2 and C57BL/6 (Brown et al. 1997b; Depatie et al. 2000). For example, *Ly49h (i1)* and flanking markers, *116m19Sp6* and *D6Ott22* can only be amplified from C57BL/6, but not DBA/2 DNA. Assigning "1" to C57BL/6 alleles and "0" to absence of PCR product, the haplotype composition for the cluster *116m19Sp6* - *Ly49h(i1)- D6Ott22* is 1-1-1 for C57BL/6, 1-0-1 for BXD-8 and 0-0-0 for DBA/2. These results identify either a recombination event involving a small DBA/2 segment, or alternatively, a possible deletion of *Ly49h* in BXD-8 mice that may underlie susceptibility in the BXD-8.

4.3.2. Physical mapping in the vicinity of *Ly49h*

We have previously assembled a contig of YAC/BAC clones (of C57BL/6 origin) covering the *Cmv1* region (Depatie et al. 2000). To produce a more detailed map in the vicinity of *Ly49h*, a subset of YAC and BAC clones overlapping *Ly49h* were digested with *Xho* I and *Sal* I, resolved by PFGE and hybridized to a *Ly49h* cDNA probe. In this analysis, the high sequence similarity between *Ly49* family members resulted in appearance of cross-hybridized bands, even under high stringency conditions. The assignment of individual *Ly49* genes to such fragments was obtained by hybridizing BAC digests to gene-specific oligonucleotides (data not shown).

Figure 5.2. Location of *Cmv1* and maker loci used for STS content and haplotype analysis in the BXD-8 strain. (a) Schematic representation of genes within the human natural killer gene complex on 12p13, in the *Cmv1* orthologous region. (b) Schematic representation of the genetic map in the vicinity of *Cmv1*. Recombination frequencies are shown below the chromosome. (c) Physical map of the *Cmv1* critical interval defined by the closest proximal (*D6Ott8*) and distal (*D6Ott115*) markers. The "×" indicates the localization of cross over events defining the target interval. The physical localization of genetic markers is shown. Distances are in kilobases (kb). Polymorphic markers are indicated by an asterisk. (d) STS content mapping within the *Cmv1* interval. Boxes indicate the presence of an individual marker. For polymorphic markers, black boxes represent C57BL/6 alleles and white boxes represent DBA/2 alleles. Haplotypes for C57BL/6, BXD-8 and DBA/2 are shown. (e) Transcript map of the *Cmv1* candidate region. Hatched boxes represent individual transcription units.


a
HSA12p13
NKR-P1A
CD69
AICL
CD94
NKG2-A-F
LY49L
PRB3
TEL
Cmv1
b
MMU6
Nkg2d/Cd94/D6Ott8
Nk1.1/Cd69
Ly49a-n
D6Ott115/Prp
Tel
0.3
0.05
0.3
c
D6Ott8*
X
Ly49e*
137h23T7
177l23Sp6
Ly49d*
116m19Sp6*
Ly49h*
D6Ott22*
Ly49g*
116m19T7
43l9Sp6*
Ly49c*
Ly49a*
446o7T7
33g14Sp6
247d24Sp6
D6Ott112*
D6Ott11*
301l2Sp6
D6Ott32*
52A6L*
204d20Sp6
287g15T7
240c11T7
291h24Sp6
156l3T7
200H7R
240c11Sp6
8k10T7
236m20Sp6
102g4Sp6
14c5T7
Ly49b*
14c5T7
236m20T7
109F12R
184a16Sp6
341c11Sp6*
282h8Sp6
200H7L*
D6Ott113*
D6Ott117*
D6Ott118*
X
X
X
X
X
D6Ott115*
0
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60 Mb
d
B6
BXD-8
DBA/2
e
e
fd k h n i g l j mc a
Ly49 gene family
Zfp-like
Zfp-like
Ly49b
Magoh-like
Csda
Est 33

A summary of the restriction and transcript maps of the region is shown in Figure 5.3. Digestion of C57BL/6 genomic DNA with *Xho* I produced hybridizing fragments identical to those seen in YACs including the 132 kb *Ly49* fragment and the cross-hybridizing 125 kb fragment. Importantly, at high stringency no hybridization signal was detected in similarly digested DBA/2 DNA indicating the absence of *Ly49h* sequences in this strain. On the other hand, BXD-8 genomic DNA produced a hybridization profile distinct from either parental strain, which included a novel *Ly49h* hybridizing *Xho* I fragment of 109 kb, and the 125 kb cross-hybridizing fragment also seen in C57BL/6 (and corresponding to *Ly49j/m/l/c*) (Figure 5.3a). These results suggest the presence of a deletion of about 23 kb in BXD-8, which includes part of, or the entire *Ly49h* gene. Additional hybridization with *Ly49h* probes on *MluI*-digested genomic DNA from C57BL/6 (980kb), and BXD-8 (960kb) confirmed the presence of a 20kb deletion in *Ly49h* of BXD-8 (data not shown).

To define the extent of the BXD-8 deletion, novel markers corresponding, either to BAC ends (*228b21Sp6*, *116m19Sp6-2* and *128d23Sp6*) or to gene specific sequences (*Ly49d-exon7*, *Ly49k-exon4*, *Ly49h-exon8*, *-exon4* and *-exon1*, *ly49n-exon7* and *Ly49i-exon8*) were derived. STS content mapping of the BAC contig, BAC DNA sequencing and hybridization of BAC digests, were used to position all STSs to the physical map (Figure 5.3c). As shown in Figure 5.3d, all loci were amplified from C57BL/6 genomic DNA, whereas none was detected from DBA/2 genomic DNA. Analysis of BXD-8 genomic DNA shows the absence of 4 markers, including the three exons tested for *Ly49h*, as well as *228b21Sp6* which maps to the intron 7 of the same gene. These results confirm the presence of a deletion in BXD-8 that encompasses the entire *Ly49h* gene. The proximal limit of the deletion is within a 10 kb interval delineated by markers

116m19Sp6-2 and *Ly49h-exon8*, whereas the distal end is localized to a 20 kb interval between *Ly49h-exon1* and *ly49n-exon7*.

4.3.3. Expression analysis of the *Ly49* gene cluster

The consequence of the 23 kb deletion on expression of individual *Ly49* genes in BXD-8 was investigated using spleen RNA. For this, degenerate oligonucleotide pairs were used to amplify pools of *Ly49* cDNAs from C57BL/6, BXD-8 and DBA/2 RNAs using RT-PCR, followed by hybridization with gene specific oligonucleotides to detect the individual *Ly49* transcripts (Kubota et al. 1999). As shown in Figure 5.4a, the expression profiles of the *Ly49* genes were strikingly different for C57BL/6 and DBA/2. For example, while *Ly49d* and *h* were absent from DBA/2, *Ly49c, n* appeared up-regulated and *Ly49f, -g, -i* appeared down-regulated. In contrast, expression profiles of C57BL/6 and BXD-8 were very similar for the *Ly49* genes with the noteworthy difference of complete absence of *Ly49h* in BXD-8 strain. This observation was confirmed by standard Northern blot analysis using an *Ly49h* cDNA probe (Figure 5.4b). These results confirm that the 23 kb deletion in BXD-8 abrogates *Ly49h* gene expression, without altering the level of expression of neighboring *Ly49* family members. Together, these findings establish that the *Ly49h* gene and protein plays a key role in host defenses against cytomegalovirus infection.

Figure 5.3. Comparative physical mapping in the region of the *Ly49h* gene (a) Following enzyme digestion, YAC (87M6F7 and 52A6) and mouse (C57BL/6, BXD-8, DBA/2) genomic DNA were separated by PFGE, blotted and hybridized with a *Ly49h* cDNA probe. Arrows on the mouse blot indicate the position of the *Ly49h Xho* I fragment at 132 kb in C57BL/6 and 109 kb in BXD-8. Exposure times were 18 hs for YAC blots and 1 week for mouse genomic DNA blot. (b) The physical map of selected YAC (thick lines) and BAC (thin lines) clones is shown for the restriction enzymes *Xho* I (black triangles) and *Sal* I (white circles). For the YAC clones, only the region of interest is represented. (c) Summary of the restriction and transcription map of the region containing *Ly49h*. The restriction sites for the enzymes *Xho* I and *Sal* I are represented by symbols (see above). The localization of *Ly49 d, -k, -h, -n, -i* genes is indicated by bold letters (above) and thin lines representing exons (below) in the map. The localization of novel markers used for STS content is shown: numbers above the map indicate the position of the corresponding exons used to derived gene specific STSs. Long thin lines indicate the position of BAC end derived STSs. (d) STS content analysis from mouse genomic DNA from C57BL/6, BXD-8 and DBA/2 strains using genetic markers shown in c. Presence of a specific PCR product is indicated (+) while absence is indicated (-).

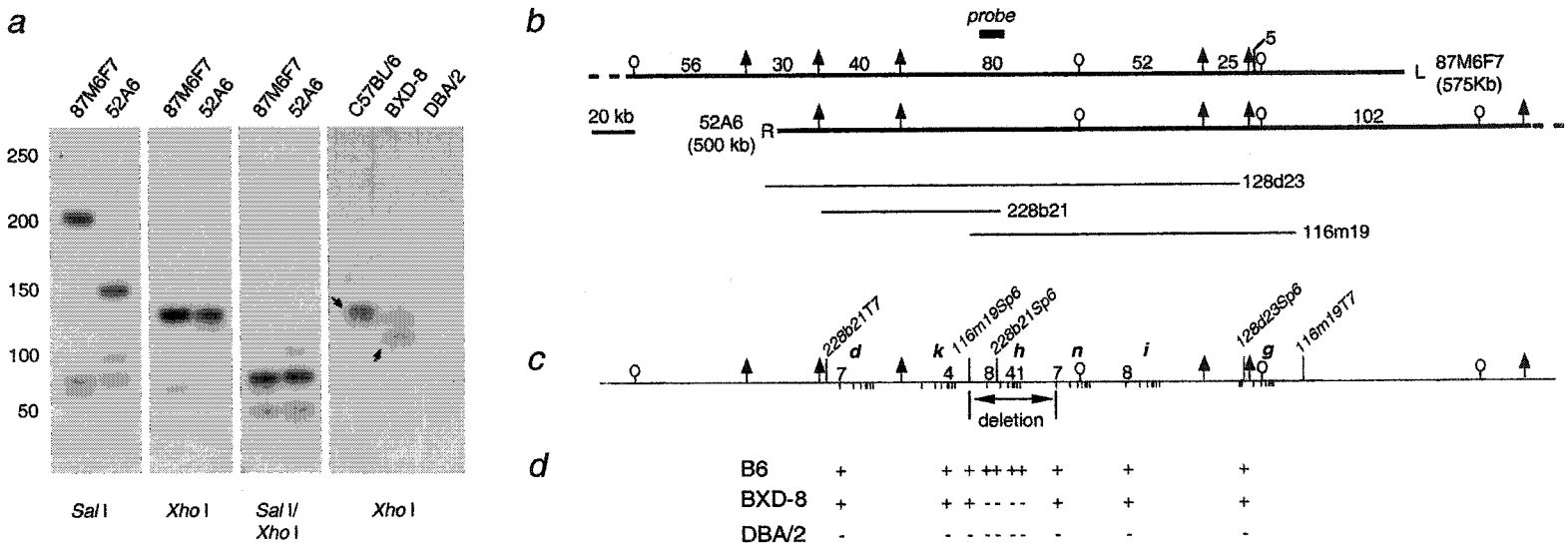
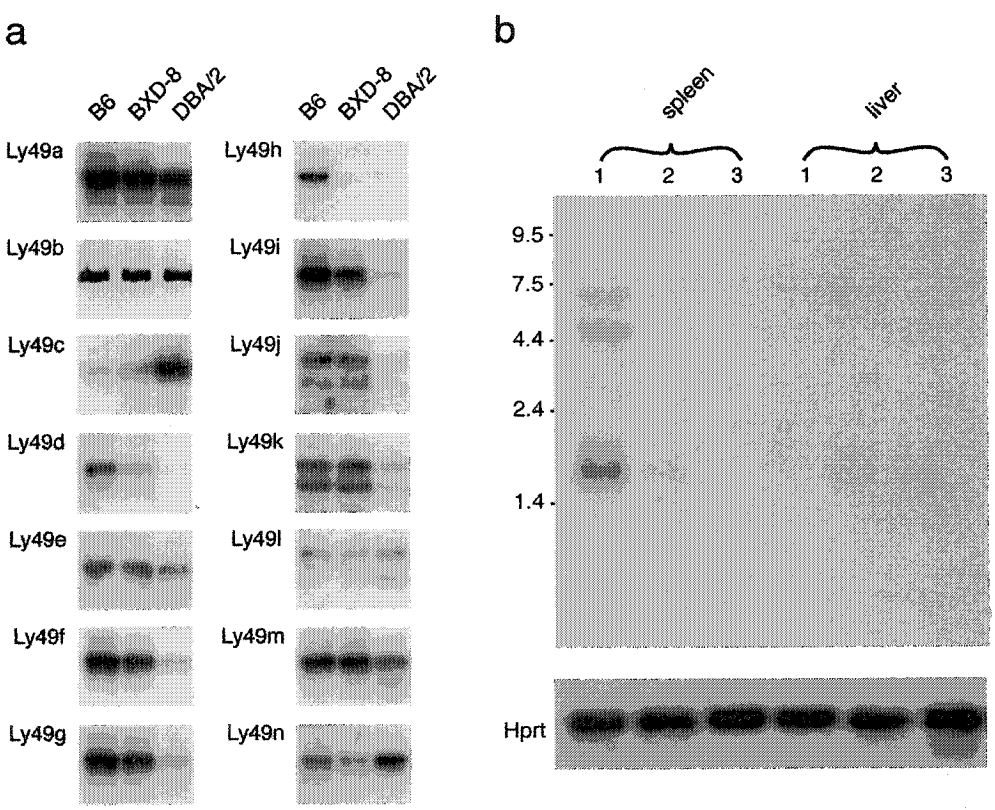

a
87M6F7
52A6
87M6F7
52A6
87M6F7
52A6
C57BL/6
BXD-8
DBA/2
250
200
150
100
50
Sal I
Xho I
Sal I/
Xho I
Xho I
b
probe
56
30
40
80
52
25
5
L
87M6F7
(575Kb)
20 kb
52A6
(500 kb)
R
102
128d23
228b21
116m19
c
228b21T7
d
7
k
4
116m19Sp6
228b21Sp6
h
8
41
7
n
8
i
128d23Sp6
g
116m19T7
deletion
d
B6 + + + ++ ++ + + +
BXD-8 + + + - - - - + + +
DBA/2 - - - - - - - - -

Figure 5.4. Expression analysis of the *Ly49* gene cluster (a) Spleen cDNA was used as template in PCR, and the PCR products were run on agarose gels, blotted and hybridized to *Ly49* gene specific oligonucleotide probes (labeled *a-n*). (b) Northern blot analysis of *Ly49h* transcripts expressed in poly $(A)^+$ mRNA from the C57BL/6 (1), DBA/2 (2) and BXD-8 (3) mouse strains. The position of size markers is indicated in kilobases. Equal loading in each lane was confirmed using *Hprt* as a control probe. Exposure times were 5 days for *Ly49h* and 3 hours for *Hprt*.


a
B6
BXD-8
DBA/2
Ly49a
Ly49b
Ly49c
Ly49d
Ly49e
Ly49f
Ly49g
Ly49h
Ly49i
Ly49j
Ly49k
Ly49l
Ly49m
Ly49n
b
spleen
liver
1
2
3
9.5
7.5
4.4
2.4
1.4
Hprt

4.4. DISCUSSION

Evidence for a role of *Ly49h* in resistance to MCMV infection is compelling. *Ly49h* is located within the 23 kb deletion seen in the *Cmv1* region characteristic of BXD-8, and according to the typical 18-20 kb (McQueen et al. 1998) size of *Ly49* genes the deletion is likely to contain no other genes. In addition, only *Ly49h* mRNA is absent from BXD-8, when compared to the *Cmv1* "isogenic" C57BL/6 parent. Furthermore, LY49H protein is absent from NKT cells but it is expressed on the surface of $NK1.1^{+}$ $CD3^{-}$ splenic NK cells (Smith et al. 2000), the cell population expressing *Cmv1* (Scalzo et al. 1990). Finally, Ly49H is an NK cell activating receptor of the C-lectin type family shown to associate with the ITAM containing DAP12 protein (Gosselin et al. 1999). Activation results in calcium mobilization, cytokine production and lysis of target cells (Gosselin et al. 1999; Smith et al. 2000). These results strongly support a key role for the LY49H receptor in the mechanisms necessary for the destruction of MCMV infected cells and provide the first conclusive evidence of the specific function of NK cells in immunity against infectious agents. Although the ligand of Ly49H remains unknown, the MCMV homolog of MHC class I heavy chains named, gp144 (Farrell et al. 1997), and which is expressed on infected cells, is an excellent candidate. It is proposed that the presence of Ly49H in the resistant mouse and its binding with gp144 initiate the activation cascade leading to cytolysis of infected cells and arrest the spread of the infection; conversely, its absence would cause susceptibility in BXD-8 and DBA/2.

Loci controlling tumor killing by NK cells (*Chok*) (Idris et al. 1998), and susceptibility to ectromelia virus (Delano and Brownstein 1995) (*Rmp1)*, are linked to *Cmv1*. These susceptibility traits involve NK-cell-mediated immunity, and the C57BL/6 mouse presents the active, "resistance" allele (Brown et al. 1997b). It has been

established that Ly49D is the *Chok* gene product (Idris et al. 1999). The results presented here show that *Chok* and *Cmv1* are not allelic, thus expanding the functional diversity of *Ly49* genes in natural immunity. Interestingly another *Ly49* paralog is a likely candidate for *Rmp1*, since BXD-8 mice are resistant to ectromelia virus infection. The proposal that such susceptibility factors be strictly dependent on activating *Ly49* members supports a direct role of these molecules in recognition of foreign antigens. Clinical and epidemiological data indicate a genetic predisposition to cytomegalovirus infection and reactivation in humans (Ho 1994; Hurme and Helminen 1998). The results presented here direct the search for a human functional equivalent to the 12p13 and 19q13 chromosomal regions, encoding killer cell receptors of MHC class I molecules.

CHAPTER SIX

TRANSGENIC EXPRESSION OF THE ACTIVATING NATURAL KILLER RECEPTOR LY49H CONFERS RESISTANCE TO CYTOMEGALOVIRUS IN GENETICALLY SUSCEPTIBLE MICE

6.1. INTRODUCTION

Because of the genetic complexity of the NKC and the absence of a reliable *in vitro* assay to assess susceptibility/resistance to MCMV, at least two groups initiated a positional cloning approach to isolate *Cmv1* (Depatie et al. 1997; Brown et al. 1999; Depatie et al. 1999; Scalzo et al. 1999; Depatie et al. 2000). This approach is based strictly on the knowledge of the chromosomal location of a specific gene to achieve its molecular cloning, after chromosome walking towards the gene from the most closely linked genetic markers on that chromosome (Boehm 1998). Through meiotic and physical mapping using classical inbred strains of mice, a minimal *Cmv1* interval of 0.35 cM was defined (Depatie et al. 1999). This interval translated into 1.6 Mb of genomic DNA and contained a minimum of 20 transcription units (Depatie et al. 2000). Among them, members of the *Ly49* (*Klra)* gene family of NK cell receptors appeared to be the most attractive candidate genes for *Cmv1*. However, an independent mapping effort positioned *Cmv1* within a 390 kb region distal to the *Ly49b* (Brown et al. 1999), which excludes all the members of *Ly49* gene family as a candidate for *Cmv1*. The combination of this interval with a 1.6 Mb genetic interval generated by our group produced a 200 kb overlapping region, suggesting the possibility of modifier genes of MCMV-resistance in the BALB/c or A/J strains used for mapping.

The genetic complexity of this genomic domain was further underscored by haplotype analysis for a set of 25 markers in the vicinity of *Cmv1,* which revealed the presence of different haplotypes in a panel of 17 inbred strains of mice (Lee et al. 2001b). Interestingly, the majority of strains analyzed (15/17) were MCMV susceptible. Accordingly in this group, the MCMV resistance allele, $Cmv1^r$, was associated with a haplotype unique to strains of the C57BL background. Susceptible strains could be

classified in two groups based on their haplotype at *Cmv1*, indicating the presence of two unrelated MCMV susceptibility alleles, named $Cmv1^{sBALB}$ and $Cmv1^{sFVB}$, raising the possibility of genetic heterogeneity at *Cmv1*, such as mutations in alternate *Ly49* members in different inbred strains. While the $Cmv1^{sBALB}$ haplotype is distinct from $Cmv1^{r}$ at every locus, identical alleles are shared at several loci between $Cmv1^{sFVB}$ and $Cmv1^{r}$, indicating that they share a common ancestral haplotype. Close inspection of these haplotypes pointed to two regions in disequilibrium with susceptibility corresponding to the *Ly49d-Ly49a* interval and to the genomic region distal to *Ly49b* (Lee et al. 2001b). A possible interpretation of this result is that tightly linked loci at *Cmv1* contribute to MCMV-susceptibility or resistance.

Recently, three independent groups reported the *Ly49h* gene as an important determinant in MCMV resistance (Brown et al. 2001a; Daniels et al. 2001; Lee et al. 2001a). We took advantage of the recombinant inbred strain BXD-8, derived from $Cmv1^{r}$ C57BL/6 and $Cmv1^{s}$ DBA/2 parents (Taylor et al. 1999). This strain is of particular interest since it is highly susceptible to MCMV infection but harbors a C57BL/6 haplotype at *Cmv1* (Scalzo et al. 1990). Using a combination of linkage analyses in an informative (BXD-8 × C57BL/6)F_2 population, together with genetic complementation *in vivo* using mouse strains congenic for different *Cmv1* segments, physical mapping and expression analysis, we demonstrated that the MCMV susceptibility trait of BXD-8 is associated with a 23 kb deletion encompassing *Ly49h* (Lee et al. 2001a). At the same time, using a panel of monoclonal antibodies, the laboratory of Yokoyama demonstrated that the Ly49H receptor was absent from BXD-8 mice and, moreover, that treatment with the anti-Ly49H monoclonal antibody 3D10 prior to MCMV infection abrogated MCMV-resistance in the C57BL/6 mice (Brown et

al. 2001a). Welsh and co-workers confirmed and extended these results by demonstrating that depletion of Ly49H$^+$ NK cells specifically affects MCMV response but not that to vaccinia virus or lymphocytic choriomeningitis virus (Daniels et al. 2001). The Ly49H protein is an activating receptor expressed in approximately half of the NK cell population in adult C57BL/6 mice (Smith et al. 2000). Ly49H signals through the kinase-associated adapter molecule DAP12 (Gosselin et al. 1999) probably upon recognition of the MCMV-encoded molecule m157 (Arase et al. 2002; Smith et al. 2002), a GPI-linked protein structurally related to non-classical MHC.

While several lines of experiments clearly demonstrate that absence of Ly49H is associated with MCMV-susceptibility, it was important to evaluate the role of the distal 200 kb region during the MCMV response as well as to determine whether expression of Ly49H is sufficient to confer MCMV-resistance in order to provide the formal demonstration of *Ly49h/Cmv1* identity. To achieve this aim we have used an approach of functional *in vivo* complementation by genetic transfer of Bacterial Artificial Chromosome (BAC) clones overlapping important regions on the *Cmv1* interval that we report here. The results obtained from this study clearly pinpointed the precise location of *Cmv1*, providing conclusive evidence that *Cmv1* and *Ly49h* are allelic. In addition, these transgenic mice provided an opportunity to investigate several questions regarding the role of Ly49H during MCMV infection such as the threshold of Ly49H$^+$ NK cells for *Cmv1*, the possible genetic heterogeneity of *Cmv1* and the rescue by the transfer of *Ly49h* on different *Cmv1* susceptible backgrounds.

6.2. MATERIALS AND METHODS

6.2.1. Generation of BAC transgenic mice

BAC clones 13J11 and 128D23 spanning the corresponding candidate regions within the *Cmv1* interval were previously described (Depatie et al. 2000). These BAC clones were obtained from RPCI-23 mouse BAC library originated from DNA of C57BL/6 (*Cmv1*^{r} MCMV-resistant) strain (Research Genetics). The BAC DNA was isolated by the alkaline lysis method with gentle handling to prevent the breakage of BAC DNA, linearized by either *Not* I or *Xho* I/*Sal* I digestion and size fractionated on 1% low melting agarose gel using pulsed field gel electrophoresis (PFGE) (6 V/cm, 120 angle, 6 s of switching time for 24 h). The BAC insert DNA was recovered from the gel by β-Agarase I treatment (New England BioLab) without exposure to UV light, as described (Huxley 1998), and purified by dialysis for 4 h against microinjection buffer (10 mM Tris pH 7.5, 0.1 mM EDTA) by floating on a Millipore VSWP 2500 (pore 0.05 um) filter disc. The BAC DNA was microinjected into the pronucleus of fertilized eggs derived from FVB/N at 1.5 ng/μl concentration. Following injection, the eggs were transferred into the oviduct of 0.5-day pseudo-pregnant foster CD-1 female mice. All procedures for generating BAC transgenic mice were performed at the transgenic facility of the Ottawa Hospital Research Institute or McGill University. Transgenic founders were identified via PCR (see below) and subsequently bred to BXD-8, FVB/N and BALB/c mice by serial back-crossing.

6.2.2. Genomic DNA analysis

To identify the presence of BAC DNA, tail DNA was extracted as described in the general materials and methods (chapter 2). Aliquots of 2 μl of this solution were used for PCR reactions. A first round of PCR screening was done using the following set of primers: 5'-GCCGCTAATACGACTCACTATAGGG-3' and 5'-GAAGATTTGCAGGCCAGGAG-3' (amplified a 163 bp fragment from the T7-end of BAC 13J11 vector-insert junction) and 5'-CCGTCGACATTTAGGTGACAC-3' and 5' CTGAGTTCAATTCCTAATTC-3' (amplified a 187 bp fragment from the SP6-end of BAC 13J11 vector-insert junction); 5'-GCCGCTAATACGACTCACTATAGGG-3' and 5'TCCTGCAGAAATCAGAGTTCTGTT-3' (amplified a 74 bp fragment from the T7-end of BAC 128D23 vector-insert junction) and 5'-CCGTCGACATTTAGGT GACAC-3' and 5'-GGGGACTGAAAGATAGAAGTG-3' (amplified a 222 bp fragment from the SP6-end of BAC 128D23 vector-insert junction). To confirm the integrity of BAC DNA in the transgenic mice, several internal polymorphic markers were tested by PCR with previously reported primer pairs (Lee et al. 2001a; Lee et al. 2001b). In addition, *Ly49e* and *D6Ott115* primer pairs (Depatie et al. 1999) were used to determine the *Cmv1* genotype of mice bred into the BALB/c background. To determine the copy number of transgene on founder animal, 20 ng of tail DNA was semi-quantitatively analyzed by PCR at three different cycles and their intensity compared with corresponding sample from homozygous C57BL/6 mice. Oligos specific for *Ly49h* intron1 were used to amplify the C57BL/6-originated copy and the sequence of the oligos were, *D6Ott151*-F: 5'-GTGCTACCACTGAAAACCATTG-3' and *D6Ott151*-R: 5'-CTGTCTCTTGAGTCACCTGCAC-3'.

6.2.3. Detection of transgene expression by PCR analysis

Transgene expression was detected by semi-quantitative RT-PCR. Briefly, total splenic RNA was extracted using Trizol reagent (Life Technologies), and 2 μg of total RNA was reverse transcribed using oligo-dT primers and Superscript II reverse transcriptase according to the manufacturer's protocols (Life Technologies). Gene specific oligonucleotides for the PCR step were, *EST335500*-F: 5'-GGCCGTGGTCGCAGTGAACT-3' and *EST335500*-R: 5'-GCTGGCTTCTCTGGCCACCT-3'; *Ly49d*-F: 5'-GGCTCAAGGAGACACGGAAG-3' and *Ly49d*-R: 5'-TCCCAAAATCTTCCAGATTG-3'; *Ly49h*-F: 5'-AGCCTCTTAGGGGATACAGAC-3' and *Ly49h*-R: 5'-TGTCAAGATAGATAGGAGAGG-3'; *Ly49i*-F: 5'-GATGAATGAGCCGGAGGTC-3' and *Ly49i*-R: 5'-TTTCACTGTTCCATCTGTCCT-3'; *Gapdh*-F: 5'-ACCACAGTCCATGCCATCAC-3' and *Gapdh*-R: 5'-TCCACCACCCTGTTGCTGTA-3'.

6.2.4. Production of anti-Ly49H antibodies

Polyclonal antibodies against Ly49H were generated in rabbits using a bacterial Glutathione S Transferase (GST)-fusion protein encompassing amino acids residues 1 to 44 which corresponds to the entire cytoplasmic domain of Ly49H. The anti-Ly49H serum was adsorbed on GST-sepharose 4B beads and anti-Ly49H antibodies were immunoaffinity purified on GST/Ly49H fusion protein coupled to sepharose beads as described (Beattie et al. 1997).

6.2.5. Flow cytometric analysis

To isolate splenic leukocytes, spleen was ground between the rough surfaces of glass slides and treated with Red Blood Cell Lysing Buffer (Sigma-Aldrich) to remove red blood cells. For isolation of leukocytes from liver, the organ was homogenized with a tissue grinder and the homogenate was passed through fine metal screens to remove large debris and cell clumps. Then, 25% of the filtered hepatic cells were layered over Lympholyte-M (Cedarlane) and centrifuged for 20 min at 1500g at room temperature to remove red blood cells. After centrifugation, a lymphocyte layer at the interface was collected using a Pasteur pipette. NK cells were enriched from spleen and liver leukocytes by magnetic positive separation using anti-DX5 microbeads following the manufacturer's protocol (MACS®; Miltenyi Biotec). The freshly purified $DX5^+$ NK cells were stained for surface expression of DX5 using PE-conjugated murine anti-DX5 (Pharmingen). After washing, the cells were fixed, permeabilized and internally stained for the cytoplasmic tails of Ly49H receptor using the antibody described above. FITC-conjugated anti-rabbit IgG (Pharmingen) was used to stain polyclonal anti-Ly49H that was previously bound. Positively stained cell populations were analyzed by flow cytometry on an Epics analyzer (Coulter).

6.2.6. Statistical analysis

Significance of the differences observed for viral titers or the size of $Ly49H^+$ NK cell population in reference to control non-transgenic groups or between transgenic lines was assessed using the two-tailed Student's *t* test.

6.3. RESULTS

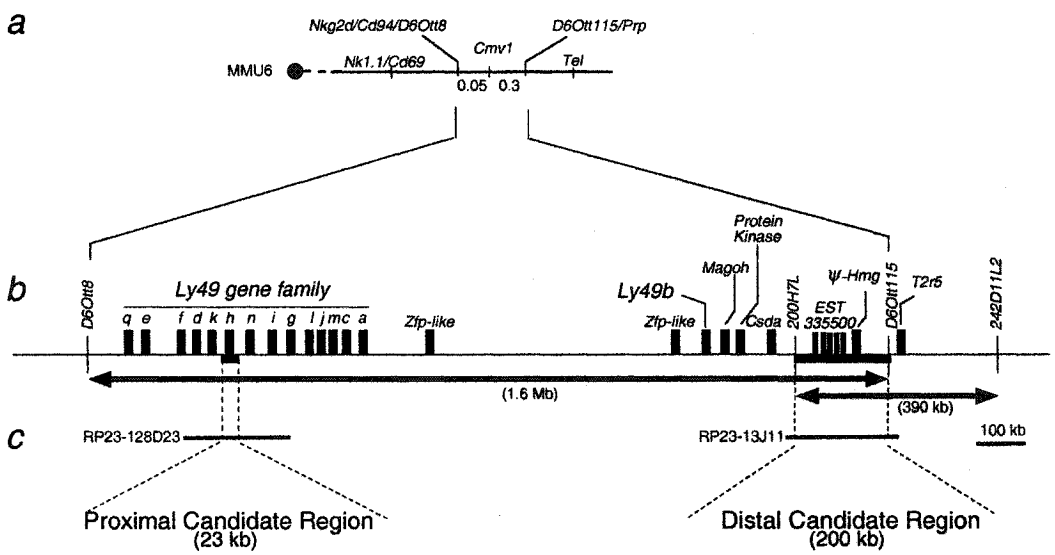
6.3.1. Characterization of BAC clones used in transgenesis

In the absence of an *in vitro* system for *Cmv1*, *in vivo* complementation with BAC clones expressed in transgenic mice was used as a functional assay for the *Cmv1* gene. It was reasoned that introduction of the dominant $Cmv1^r$ (C57BL/6) allele onto a recessive $Cmv1^s$ background should produce F_1 progeny uniformly resistant to MCMV infection. High resolution genetic and physical mapping of the *Cmv1* interval identified a minimal genetic interval that is flanked by the markers *D6Ott8* and *D6Ott115* and which corresponds to a physical distance of approximately 1.6 Mb of genomic DNA (Figure 6.1a and 6.1b) (Depatie et al. 1999; Depatie et al. 2000). Within this large interval, two regions were of particular interest for complementation analysis: a distal candidate region of about 200 kb that corresponds to the common domain for *Cmv1* as defined from two independent mapping efforts (Brown et al. 1999; Depatie et al. 2000), and a proximal candidate region of about 23 kb encompassing *Ly49h* whose absence is associated with susceptibility to cytomegalovirus in BXD-8 mice (Figure 6.1c) (Brown et al. 2001a; Lee et al. 2001a).

In the process of cloning *Cmv1*, we have previously completed a physical map in the *Cmv1* interval using a series of 73 overlapping BAC clones and screened the BAC clones to provide genomic segments fully encompassing each candidate region (Depatie et al. 2000). Based on these considerations, BAC clones 13J11 and 128D23 were identified for complementation analysis (Figure 6.1c). The BAC 13J11 spans the common *Cmv1* interval (200 kb) as shown by the presence of marker *200H7L*, a YAC end defining the proximal boundary of *Cmv1* in Brown et al's map (Brown et al. 1999), as well as *D6Ott115*, a marker defining the distal end of the *Cmv1* interval in Depatie et

al's map (Depatie et al. 2000). Genomic DNA sequence analysis (data not shown) indicated the presence of at least 3 different transcription units in BAC 13J11, including a pseudogene for one of the chromatin proteins of the High Mobility Group (Gariboldi et al. 1995) and the gene for the taste receptor TR2 (Adler et al. 2000) localized distal to *D6Ott115*. The third gene, which presents 5 copies, corresponds to *EST335500* (GenBank Accession No W18806) (see results of chapter 3), a novel spleen specific gene whose expression is absent in the susceptible BALB/c strain (see below). The BAC clone 128D23 is located in the middle of *Ly49* gene cluster where it overlaps the 23 kb deletion present in the BXD-8 strain. This BAC clone contains five *Ly49* genes, three functional genes (*Ly49d, Ly49h and Ly49i*) and two pseudogenes (*Ly49k and Ly49n*) as deduced from its available genomic DNA sequence (GenBank Accession No.: AC090127).

Figure 6.1. Genomic targets for transgenic analysis of the *Cmv1* locus (a) Composite genetic linkage map of mouse chromosome 6 in the vicinity of *Cmv1*. The order and distances of the loci were determined by pedigree analysis. The centromere is represented by a black circle. Recombination frequencies in centimorgans (cM) are shown below the chromosome. *Nk1.1*, *Cd69*, *Cd94* and *Nkg2d* code for C-lectin type receptors. *Prp*: proline-rich protein, *Tel*: translocation-ets-leukemia (Depatie et al. 1997). (b) Blow-up of the minimal genetic interval between *D6Ott8* and *D6Ott115* and the transcriptional map showing candidate genes for *Cmv1*. Arrows indicate the minimal *Cmv1* interval as defined by Depatie et al. 2000 and Brown et al. 1999 (c). Localization of BAC clones used for transgenesis.


a
Nkg2d/Cd94/D6Ott8
D6Ott115/Prp
Cmv1
MMU6
Nk1.1/Cd69
Tel
0.05 0.3
b
Protein Kinase
Magoh
Ψ-Hmg
T2r6
D6Ott8
Ly49 gene family
q e f d k h n i g l j m c a
Zfp-like
Ly49b
Zfp-like
Csda
200H7L
EST 335500
D6Ott115
242D11L2
(1.6 Mb)
(390 kb)
c
RP23-128D23
RP23-13J11
100 kb
Proximal Candidate Region
(23 kb)
Distal Candidate Region
(200 kb)

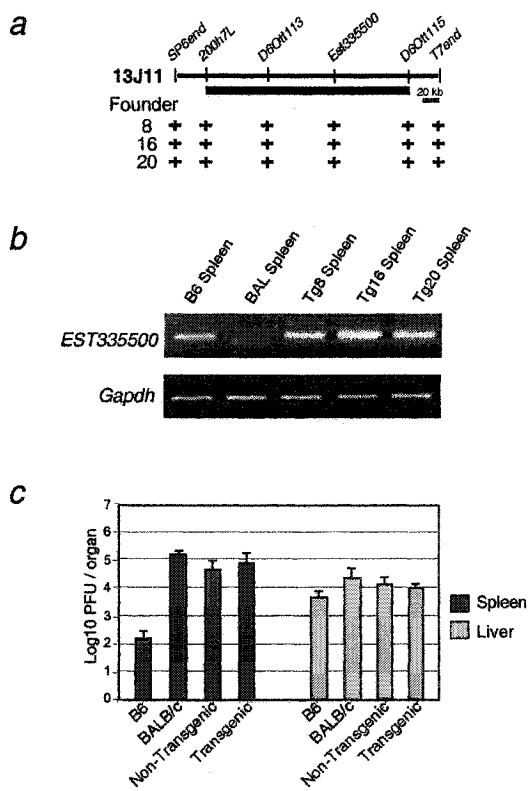
6.3.2. Production and analysis of BAC transgenic mouse lines for clone 13J11

Transgenic mice were generated by injection of purified BAC insert DNA into the male pronucleus of FVB/N zygotes. Transgenic mice were identified by PCR amplification of the BAC vector-insert junctions ends and by the presence of internal polymorphic markers as indicated in Figure 6.2a. In summary, 3 out of 20 founder animals were positive for the transgenic markers and carried an intact 210 kb transgenic construct (Figure 6.2a). The F1 progeny of founder animals with BALB/c were analyzed by the PCR approach, indicating transmission of the transgene to about 50% of their progeny as expected for nonmosaic germ-line transmission.

To demonstrate proper expression of genes encoded in BAC 13J11, I took advantage of the fact that only the C57BL/6 allele of *EST335500* is expressed in the mouse spleen. As shown in Figure 6.2b, high levels of *EST335500* RT-PCR product were obtained in spleen of the three transgenic lines as well as from C57BL/6 RNA. No product was observed in BALB/c samples. A control RT-PCR with *Gapdh* primers demonstrates similar results for all samples, indicating that the RNA used as starting material was consistent in quality and quantity in all the animals and that genes in the construct were expressed in transgenic animals (Figure 6.2b).

To examine the effect of BAC 13J11 in resistance to MCMV, transgenic founders were back-crossed with BALB/c, a susceptible strain used for mapping of *Cmv1* (Brown et al. 1999; Depatie et al. 1999). After the acquisition of the context of homozygous $Cmv1^{sBALB}$ haplotype in the *Cmv1* interval, transgenic and non-transgenic siblings were infected with MCMV and viral titers in the spleen were measured 3 days post-infection.

Figure 6.2. Characterization of BAC clone 13J11 transgenic lines (a) Physical map of BAC clone 13J11 with the localization of markers used for identification of transgenic lines by PCR. The black bar indicates the physical domain common to two independent mapping efforts for *Cmv1* (Brown et al. 1999; Depatie et al. 1999) . The (+) indicates a positive result in STS content analysis of transgenic founder animals. (b) RT-PCR for *EST335500* and *Gapdh* control RNA from spleens of wild-type strains and transgenic lines mice. (c) Replication of MCMV in the spleen and liver of inbred and transgenic lines. Viral titers in the organs of 5 mice per group were determined by plaque assay 3 days after infection with 5×10^3 PFU of MCMV.


a
SP6end
200h7L
D6Rt113
Est335500
D6Rt115
T7end
13J11
20 kb
Founder
8
16
20
b
B6 Spleen
BAL Spleen
Tg8 Spleen
Tg16 Spleen
Tg20 Spleen
EST335500
Gapdh
c
Log10 PFU / organ
0
1
2
3
4
5
6
7
B6
BALB/c
Non-Transgenic
Transgenic
B6
BALB/c
Non-Transgenic
Transgenic
Spleen
Liver

As shown in Figure 6.2c, no protective effect was observed in transgenic mice, which presented a viral load identical to BALB/c (*Cmv1*^{s}) and 10^2-10^3 times higher than the resistant C57BL/6 mice. Taken together, these results indicate that neither *EST335500* nor any other genes in the 210 kb region covered by 13J11 confer resistance to cytomegalovirus infection, excluding the presence of MCMV-resistance genes within this genomic domain.

6.3.3. Production and analysis of BAC transgenic mouse lines for clone 128D23

The BAC 128D23 transgenic mice were produced by microinjection of FVB/N (*Cmv1*^{s}) zygotes, and founder animals were analyzed by PCR with several markers as shown in Figure 6.3a to identify transgenic mice. A summary of the BAC transgenic lines and their characteristics is presented in Table 7.1. Out of forty progeny, six founders were shown to present an intact 128D23 insert, while two of them missed one of two BAC ends (Figure 6.3a). Interestingly, founder 043 showed discontinuous STS content, indicating possible rearrangement between *Ly49f* and *Ly49n*. Transgene copy number in founder animals was determined using semi-quantitative PCR with *Ly49h*-specific primers. The range of transgene copy numbers was 1 copy in line Tg915, 2 copies in line Tg831 and 3-4 copies in line Tg832 (Figure 6.3b). Interestingly, copy number correlated well with expression of functional genes present in BAC 128D23 as shown by RT-PCR analysis using *Ly49d*, *Ly49h* and *Ly49i* specific primer pairs (Figure 6.3c). To note, primer pairs were also allele specific for C57BL/6-originated transcripts since no product was detected from non-transgenic FVB/N control RNA.

Figure 6.3. Characterization of BAC clone 128D23 transgenic lines (a) Physical map of BAC clone 128D23 with the localization of markers used for identification of transgenic lines by PCR. The localization of genes present in the BAC clone is also indicated. The (+) indicates a positive result in STS content analysis of transgenic founder animals. Restriction sites of *Xho* I and *Sal* I are shown. (b) Semi-quantitative PCR to determine transgene copy-number on founders. *Ly49h* specific primers were used to amplify 1-7 copies of BAC DNA. Results were compared with those obtained from transgenic lines in 26 cycles of amplification. (c) RT-PCR for genes contained in BAC clone 128D23 and *Gapdh* control RNA from spleens of wild-type mice and transgenic lines. *Ly49h*, *Ly49d* and *Ly49i* oligonucleotide primer pairs were designed to amplify specifically the C57BL/6 alleles (see material and methods).

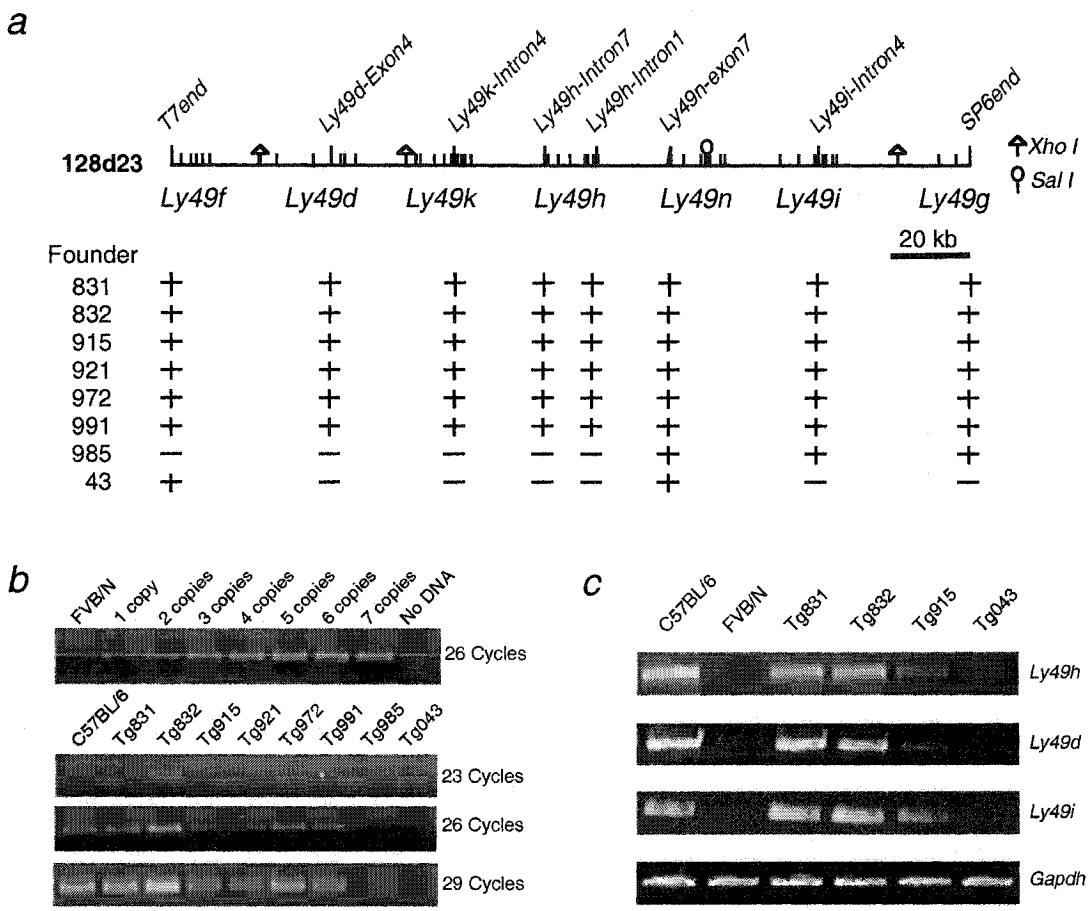

a
T7end
Ly49d-Exon4
Ly49k-Intron4
Ly49h-Intron7
Ly49h-Intron1
Ly49n-exon7
Ly49i-Intron4
SP6end
128d23
Xho I
Sal I
Ly49f
Ly49d
Ly49k
Ly49h
Ly49n
Ly49i
Ly49g
20 kb
Founder
831 + + + + + + + +
832 + + + + + + + +
915 + + + + + + + +
921 + + + + + + + +
972 + + + + + + + +
991 + + + + + + + +
985 − − − − − + + +
43 + − − − − + − −
b
FVB/N
1 copy
2 copies
3 copies
4 copies
5 copies
6 copies
7 copies
No DNA
26 Cycles
C57BL/6
Tg831
Tg832
Tg915
Tg921
Tg972
Tg991
Tg985
Tg043
23 Cycles
26 Cycles
29 Cycles
c
C57BL/6
FVB/N
Tg831
Tg832
Tg915
Tg043
Ly49h
Ly49d
Ly49i
Gapdh

Table 6.1. Summary of genetic and phenotypic analysis of transgenic mice expressing Ly49H

Transgenic line	Integration of BAC DNA	*Ly49h* copy No.	*Ly49h* RNA Expression	Protein expression of Ly49H[a]	MFI of Ly49H$^+$ cells[b]	Viral titer in spleen[c]
212 kb full BAC 128D23						
Tg832	Intact	3-4	+++	45 ± 3 %	5.5 ± 0.4	2.1 ± 0.4
Tg915	Intact	1	+	28 ± 2 %	3.6 ± 0.3	3.1 ± 0.4
Tg985	Partial	0	No	6 ± 1 %	N/D	4.8 ± 0.3
Tg043	Partial	0	No	3 ± 1 %	N/D	4.7 ± 0.4
79 kb *Sal*I-*Xho*I fragment of BAC 128D23						
Tg796	Intact	N/D	No	N/D	N/D	4.5 ± 0.2
Tg814	Intact	N/D	++	35 ± 2 %	4.6 ± 0.3	2.3 ± 0.2

Data analyzed on four mice in each group on FVB/N background, except for Tg814, of which three mice were analyzed. Similar data were obtained for transgenic mice in the context of the BXD-8 or BALB/c.

[a]Indicates the percentage of Ly49H$^+$ population among DX5$^+$ cells in the spleen (mean ± SD).

[b]Indicates median of fluorescence intensity of Ly49H$^+$ cells (mean ± SD).

[c]Indicates $\log_{10}$ PFU/organ (mean ± SD). For controls, viral titers for resistant C57BL/6 and susceptible FVB/N were 2.0 ± 0.3 and 4.5 ± 0.2, respectively.

To monitor expression of Ly49H in different mouse strains, a polyclonal antibody against the cytoplasmic tail of the receptor was produced. This anti-serum stained 52% of DX5$^+$ NK cells isolated from C57BL/6 mouse spleen, consistent with previous results using an antibody directed against the extracellular domain of Ly49H (Smith et al. 2000) (Figure 6.4a). No signal was observed in either FVB/N, BALB/c or BXD-8 indicating that this anti-Ly49H polyclonal antibody does not cross-react with other members of the Ly49 family. In particular, it was shown that, with the exception of *Ly49h*, the BXD-8 strain expresses the full C57BL/6 *Ly49* repertoire, strongly supporting the specificity of the antibody (Lee et al. 2001a).

Interestingly, the RNA expression of *Ly49h* strongly correlated with protein expression as determined by the size of Ly49H$^+$ NK cell population and the level of its expression on single cell. The size of the Ly49H$^+$ NK cell population varied in transgenic lines with 45% and 28% of Ly49H$^+$ NK cells in lines Tg832 and Tg915,

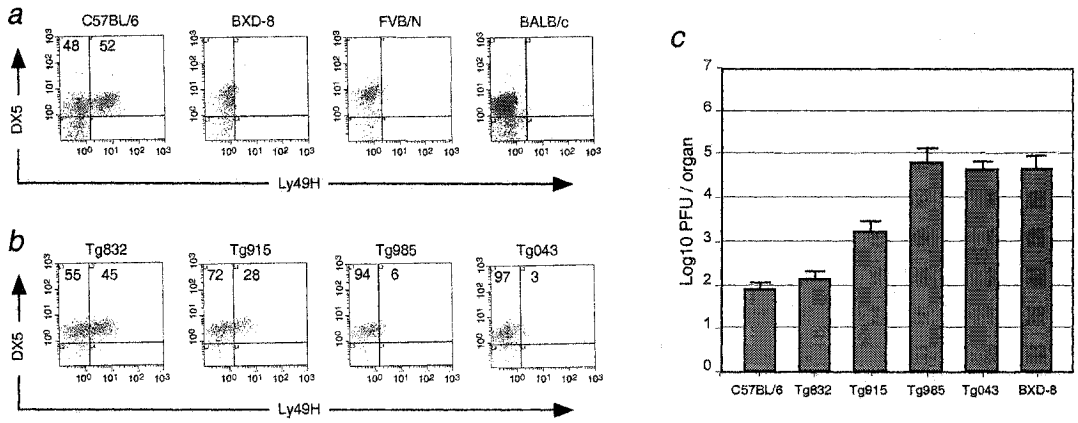
respectively ($P < 0.005$) (Figure 6.4b). Moreover, the median of fluorescence intensity (MFI) of the Ly49H$^+$ NK cells was significantly different with a MFI of 5.5 ± 0.4 for line Tg832 and MFI of 3.6 ± 0.3 for line Tg915 ($P < 0.005$). These differences correlated with RNA expression of *Ly49h* and may depend on either the number of *Ly49h* copy or the site of integration of the constructs. As expected, no significant expression was observed in lines Tg985 and Tg043, which lack *Ly49h* coding sequences.

6.3.4. Ly49H confers resistance to MCMV infection

The phenotypic consequences of BAC128d23 expression in resistance to infection with MCMV was evaluated in the progeny of a cross between transgenic animals and the susceptible mouse strain BXD-8. This strain expresses the full $Ly49^{C57BL/6}$ repertoire with the exception of *Ly49h* (Lee et al. 2001a). As shown in Figure 6.4c, transgenic expression of the 212 kb BAC 128D23 clone conferred resistance to MCMV infection in BXD-8 mice. Among the four BAC 128D23 lines included in our analysis, line Tg832 is of particular interest because it presented splenic viral titers comparable to those of resistant C57BL/6 mice (10^2 PFU/spleen), whereas line Tg915 presented an intermediate phenotype (10^3-10^4 PFU/spleen) (Figure 6.4c). As expected, lines Tg985 and Tg043 lacking *Ly49h* allowed uncontrolled viral replication at the same level as those of susceptible strains (10^4-10^5 PFU/spleen).

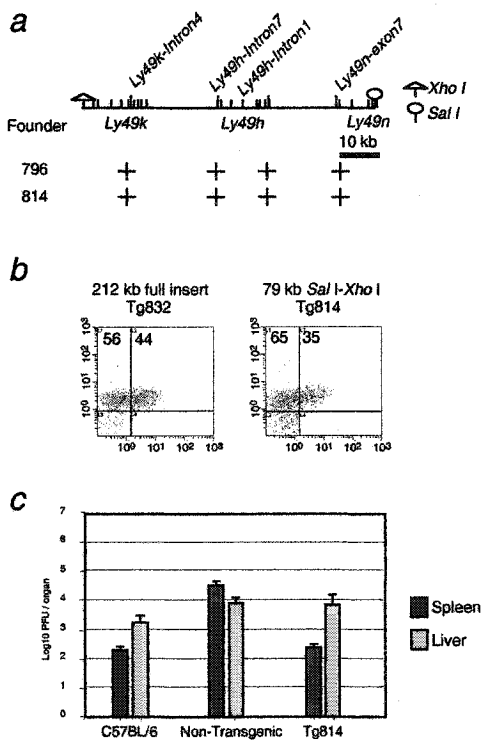
Figure 6.4. Acquisition of MCMV-resistance in BAC clone 128D23 transgenic lines

(a) Enriched NK cell preparations from spleens of wild-type strains were stained with the mAb DX5 and the rabbit polyclonal antibody against the cytoplasmic domain of the Ly49H peptide reported here. The numbers in the density plots indicate the percentage of $DX5^+$ lymphocytes either $Ly49H^-$ or $Ly49H^+$. (b) Enriched NK cell preparations from spleens of BAC 128D23 transgenic lines were stained with the monoclonal antibody DX5 and the rabbit polyclonal antibody against the cytoplasmic domain of the Ly49H. The numbers in the density plots indicate the percentage of $DX5^+$ lymphocytes either $Ly49H^-$ or $Ly49H^+$. (c) Replication of MCMV in the spleen of wild-type and transgenic lines. Viral titers in the spleen of 5 mice per group were determined by plaque assay 3 days after infection with 5×10^3 PFU of MCMV. Statistically significant differences were observed between transgenic (Tg832, Tg915) and nontransgenic mice at *P* values of <0.0005.


a
C57BL/6
BXD-8
FVB/N
BALB/c
48
52
DX5
Ly49H
b
Tg832
Tg915
Tg985
Tg043
55
45
72
28
94
6
97
3
DX5
Ly49H
c
Log10 PFU / organ
0
1
2
3
4
5
6
7
C57BL/6
Tg832
Tg915
Tg985
Tg043
BXD-8

To reduce the rescuing interval further, the same functional assay was used to test a 79 kb *Xho* I and *Sal* I fragment of BAC128D23 encompassing a single functional gene, *Ly49h*. Out of two transgenic lines carrying the complete construct (Figure 6.5a), only one, line Tg814, was shown to express Ly49H in about 35% of $DX5^+$ NK cells (Figure 6.5b). Phenotypic analysis of line Tg814 demonstrated that transgenic animals acquired MCMV-resistance upon introduction of the 79 kb transgene (Figure 6.5c). It is important to note, however, that at this early time-point the effect of *Cmv1* (or the transgene) is observed primarily in the spleen (see below) (Scalzo et al. 1990). The proper expression of Ly49H in line Tg814 indicates that *Cmv1* gene and its requisite regulatory sequences are contained within the genomic construct. These results showed that MCMV susceptibility can be reversed by proper expression of a single functional gene, *Ly49h*, thereby providing the formal demonstration of allelism between *Ly49h* and *Cmv1*. In addition, partial recovery observed in line Tg915 shows a gene dosage effect indicating a threshold value in providing resistance above 28% of $Ly49H^+$ cells.

Figure 6.5. A 79 kb genomic construct containing Ly49H confers resistance to MCMV infection (a) Physical map of a 79 kb fragment with the localization of markers used for identification of transgenic lines by PCR. The localization of genes present in the BAC clone is also indicated. Restriction sites of *Xho* I and *Sal* I used to generate the 79 kb fragment were shown. The (+) indicates a positive result in STS content analysis of transgenic founder animals. (b) Enriched NK cell preparations from spleens of lines Tg832 and Tg814 were stained with the mAb DX5 and the rabbit polyclonal antibody against the cytoplasmic domain of the Ly49H. The numbers in the density plots indicate the percentage of $DX5^+$ lymphocytes either $Ly49H^-$ or $Ly49H^+$. (c) Replication of MCMV in the spleen and liver of wild-type and transgenic lines. Viral titers in the spleen of 5 mice per group were determined by plaque assay 3 days after infection with 5×10^3 PFU of MCMV.


a
Ly49k-Intron4
Ly49h-Intron7
Ly49h-Intron1
Ly49n-exon7
Xho I
Sal I
Founder
Ly49k
Ly49h
Ly49n
10 kb
796
814
b
212 kb full insert
Tg832
79 kb Sal I-Xho I
Tg814
56
44
65
35
c
Log10 PFU / organ
Spleen
Liver
C57BL/6
Non-Transgenic
Tg814

6.3.5. *Ly49h* confers resistance to MCMV infection independently of the genetic background

With the exception of the 23 kb deletion encompassing *Ly49h*, the susceptible BXD-8 and the resistant C57BL/6 strains are identical at the *Cmv1* locus (Lee et al. 2001a). In contrast, classical inbred strains present two independent susceptible haplotypes. In particular, susceptible BALB/c and A/J (used for genetic mapping) and FVB/N (used for transgenesis) present unrelated haplotypes for all loci analyzed in the vicinity of *Cmv1* (Lee et al. 2001b). These results indicated two independent origins for the MCMV-susceptibility $Cmv1^s$ allele and suggested the possibility of genetic heterogeneity at *Cmv1*, such as mutations in alternate *Ly49h* members in different inbred strains. In addition, a BALB/c modifier gene of MCMV resistance has been proposed to be present in the distal candidate region (Brown et al. 1999). Moreover, the susceptibility to MCMV has been shown to be controlled by various cytokines and chemokines such as IFN-α, β, IL-12, TNF and macrophage inflammatory protein 1α (MIP-1α) (Dalod et al. 2002; Orange and Biron 1996a; Salazar-Mather et al. 1998) whose expression is very diverse in different genetic backgrounds (Charles et al. 1999; He et al. 1995). Thus the rescue by the transfer of *Ly49h* might be influenced by the variable level of expression of these molecules.

To evaluate a possible effect of the genetic background in MCMV susceptibility, the response of transgenic mice to MCMV infection was tested in the context of a homozygous $Cmv1^{sBALB}$ and $Cmv1^{sFVB}$ background. Five animals from lines Tg832 and Tg915 were analyzed as described above. As shown in Figure 6.6a and 6.6b both lines supported similar levels of MCMV replication to those obtained in the BXD-8

background, supporting a critical role for Ly49H in MCMV resistance independent of the genetic background.

6.3.6. Regulation of trans-Ly49H expression in transgenic mice

The identification of Ly49H as the critical resistance factor in host response to MCMV predicted that cells expressing Ly49H should display evidence of specific activation during MCMV infection in vivo. Soon after MCMV infection, there is a general activation of NK cells to produce IFN-γ and proliferate in the spleen (Dokun et al. 2001a). At later time points, there is a specific expansion of $Ly49H^+$ cells in C57BL/6 mice (Dokun et al. 2001a), while expression of MIP-1α promotes recruitment of NK cells and formation of inflammatory foci in the infected liver (Salazar-Mather et al. 1998).

To investigate whether control sequences for clonal expansion are present in the transgenic construct, Ly49H expression was followed by FACS analysis of $DX5^+$ NK cells isolated from spleen and liver over a one-week period following infection. The kinetics of the $Ly49H^+$ NK cell expansion was identical for wild-type C57BL/6 mice and the line Tg832 (Figure 6.7a). The percentage of splenic and liver NK cells expressing Ly49H gradually increased during infection (Figure 6.7a). By day 7, the proportion of NK cells in spleen and liver expressing Ly49H was about 70%, compared to only 50% in spleens of mock-infected mice. Importantly, the increased percentage of $Ly49H^+$ cells in the liver was accompanied by an enhanced control of viral titers in these organs, supporting a direct role for this receptor in MCMV-resistance in the liver (Figure 6.7b). Taken together, these results indicate a proliferation of $Ly49H^+$ NK cells during

infection and demonstrate that regulation of the *Ly49h* transgene is indistinguishable from that of its endogenous counterpart in C57BL/6 mice, possibly due to specific activation through the Ly49H receptor itself.

Figure 6.6. Ly49H confers resistance to MCMV infection independently of the genetic background Replication of MCMV in wild-type and transgenic Tg832 and Tg915 mice in the context of homozygous FVB/N ($Cmv1^{sFVB}$) (a) and BALB/c ($Cmv1^{sBALB}$) (b) was shown. Haplotypes of *Ly49* family for $Cmv1^{r}$ (C57BL/6), $Cmv1^{sFVB}$ and $Cmv1^{sBALB}$ are shown; black boxes, C57BL/6 alleles; hatched boxes, FVB alleles; gray boxes, BALB/c alleles; white boxes indicate the absence of alleles. Viral titers in the spleen and liver of 5 mice per group were determined by plaque assay 3 days after infection with 5×10^3 PFU of MCMV.

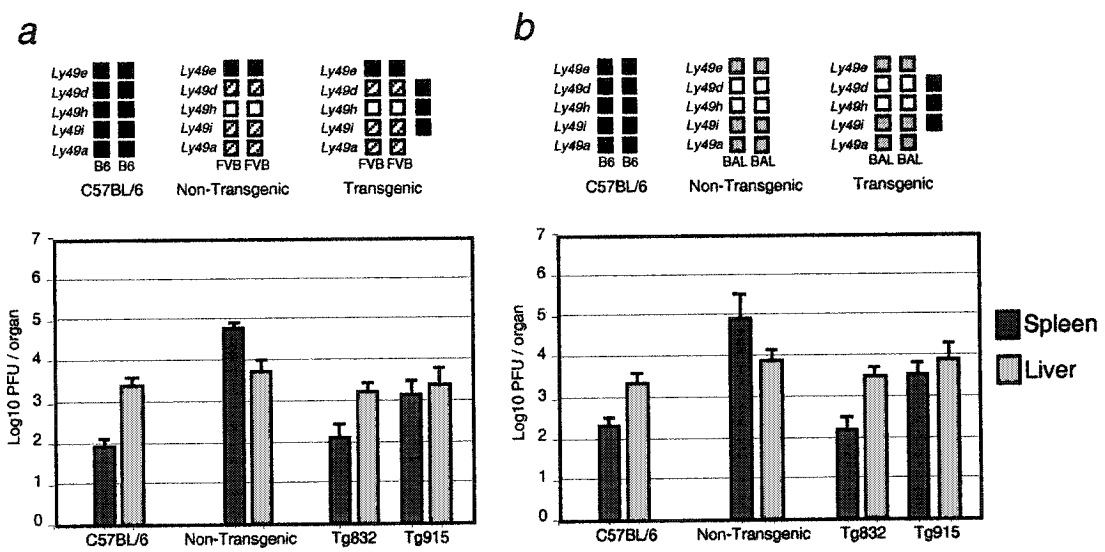
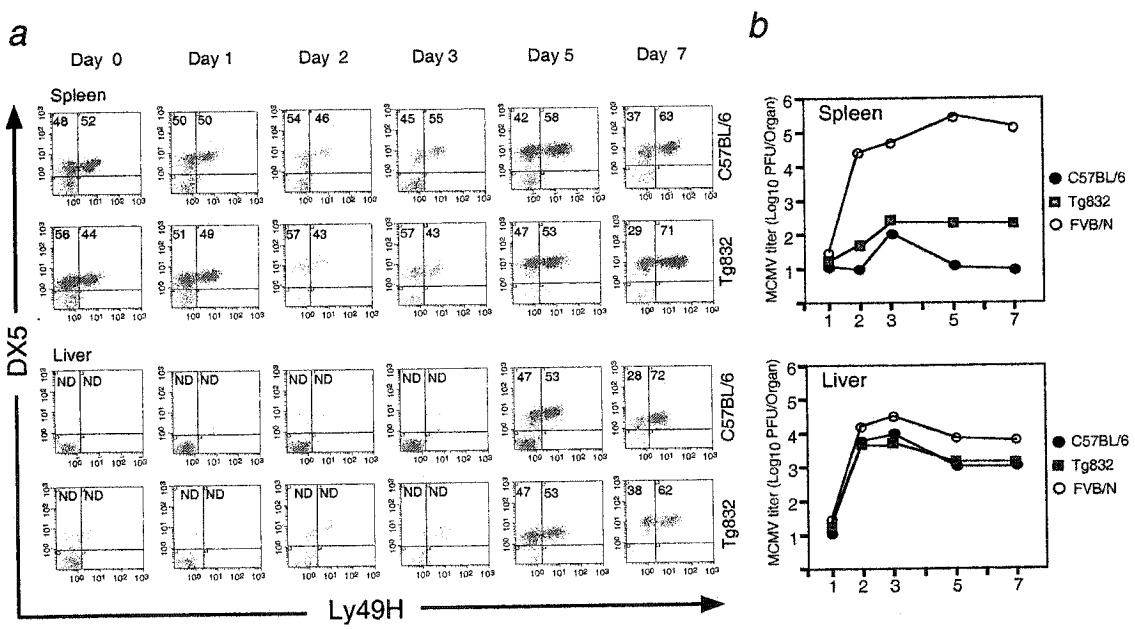

a
Ly49e
Ly49d
Ly49h
Ly49i
Ly49a
B6 B6
C57BL/6
FVB FVB
Non-Transgenic
FVB FVB
Transgenic
Log10 PFU / organ
0
1
2
3
4
5
6
7
C57BL/6
Non-Transgenic
Tg832
Tg915
b
BAL BAL
Spleen
Liver

Figure 6.7. Kinetics of trans-Ly49H expression (a) Ly49H expression was followed during a one-week period in the transgenic line Tg832 and C57BL/6 by staining with the mAb DX5 and a rabbit polyclonal antibody against the cytoplasmic domain of the Ly49H. The numbers in the density plots indicate the percentage of $DX5^+$ lymphocytes either $Ly49H^-$ or $Ly49H^+$. Enriched NK cell preparations were obtained from spleen and liver tissues after 0, 1, 2, 3, 5 and 7 days post infection. The proportion of NK cells in liver until 3 day post infection was not determined owing to the insignificant number of $DX5^+$ cells on samples. (b) A fraction of the organ homogenates used in (A) were used to determine the course of MCMV infection at the indicated time-points in transgenic Tg832 mice and wild-type C57BL/6 (*Cmv1*^{r}) and FVB/N (*Cmv1*^{s}) controls. Viral titers were determined by plaque following infection with 5×10^3 PFU of MCMV.


a
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
Spleen
Liver
C57BL/6
Tg832
DX5
Ly49H
48 52
50 50
54 46
45 55
42 58
37 63
56 44
51 49
57 43
57 43
47 53
29 71
ND ND
47 53
28 72
38 62
b
Spleen
Liver
MCMV titer (Log10 PFU/Organ)
C57BL/6
Tg832
FVB/N

6.4. DISCUSSION

Ly49h was identified as a strong candidate for *Cmv1* based on the following observations: a) it mapped within the minimal genetic and physical intervals delineating the *Cmv1* region (Depatie et al. 2000); b) a 23 kb deletion encompassing *Ly49h* in the context of a C57BL/6 (*Cmv1*^{r}) is associated with susceptibility (Lee et al. 2001a); c) expression of *Ly49h* is restricted to *Cmv1*^{r} mice (Brown et al. 2001a; Lee et al. 2001a); d) *Ly49h* encodes for a C-type lectin activating receptor specifically expressed on NK cells (Smith et al. 2000); e) depletion of Ly49H$^+$ NK cell populations abrogates MCMV-resistance (Brown et al. 2001a; Daniels et al. 2001); f) Ly49H recognizes an MCMV-encoded molecule, m157, a GPI-membrane bound protein that is expressed early during infection (Arase et al. 2002; Smith et al. 2002).

While these data provide compelling evidence of the role of *Ly49h* in MCMV infection, important questions remain to be clarified concerning the association between *Ly49h* and *Cmv1*: a) an independent genetic mapping effort identified a region distal to the *Ly49* cluster as associated with the MCMV-susceptibility phenotype (Brown et al. 1999), this region –referred to as the distal candidate region- was also detected by haplotype analysis in genealogically and phylogenetically unrelated inbred strains of mice (Lee et al. 2001b); b) the minimal *Cmv1* physical interval was shown to contain other genes that were potential candidates for an infection resistance locus. For example *Csda*, that encodes a putative repressor for human GM-CSF, a key molecule for macrophage maturation (Coles et al. 1996), or the spleen specific gene, *EST335500,* described here, are both localized within the distal candidate region; c) functional analysis with monoclonal antibodies could be misleading because the possibility of cross-reactivity with a yet to be identified member of the *Ly49* family; d) the presence of

two independent origins for the *Cmv1*^{s} alleles, *Cmv1*^{sFVB} or *Cmv1*^{sBALB}, suggested that MCMV-susceptibility could be associated with either independent mutations on the same gene or mutations in tightly linked genes within the *Cmv1* interval (Lee et al. 2001b).

To address these questions, I carried out experiments in which I transferred cloned copies of two important regions within the minimal *Cmv1* interval onto a susceptible (*Cmv1*^{sFVB} or *Cmv1*^{sBALB}) genetic background and analyzed the effects of this transfer on resistance to infection. In the present study, it was shown that transfer of DNA constructs that overlap the distal candidate region did not affect resistance, whereas transfer of genomic constructs overlapping *Ly49h* caused gain-of-function on an otherwise susceptible background. Moreover, using a 79 kb genomic fragment containing no functional gene other than *Ly49h*, the phenotypic rescue obtained in these experiments was complete, unambiguous and quantitative. Combined with the previous positional cloning studies and the antibody depletion experiments, the transgenic transfer of *Ly49h* presented here unequivocally establishes that *Ly49h* and *Cmv1* are allelic.

These results did not detect either a gain-of-function following genetic transfer of the distal construct BAC 13J11 or a loss-of-function in *Ly49h*-transgenic animals in the BALB/c context. A possible effect of genes distal to the *Ly49* cluster was previously suggested by genetic analysis using crosses between the MCMV-resistant C57BL/6 and the MCMV-susceptible BALB/c or A/J mouse strains (Brown et al. 1999). The gene *EST335500* presented here was of particular interest because: it is flanked by markers defining the distal region involved in MCMV-resistance; it is specifically expressed in the spleen, which is a main site of control of MCVM replication; and its transcript is present in tissues of *Cmv1*^{r} C57BL/6 mice. In contrast, it is absent in those of *Cmv1*^{s}

BALB/c or A/J mice, indicating a possible role in MCMV-resistance. However, lack of complementation with 13J11 transgenic mice indicates that polymorphisms in this genomic domain, including those identified in *EST335500*, do not affect the MCMV-response, as measured by splenic or liver viral titers. However, it cannot be ruled out at this time the possibility of a MCMV-strain specific effect as we have used the original Smith strain of MCMV (Selgrade et al. 1981), while others have used the strain K181 (Brown et al. 1999; Forbes et al. 1997; Scalzo et al. 1999). K181 is considered to be a more virulent strain, since at a similar infectious dose it produces more plaques and cytopathic effects than the Smith strain, which might allow the detection of more subtle effects of genomic regions distinct from *Ly49h* in an anti-viral response (Hudson et al. 1988). The transgenic models reported here will allow us to test this possibility.

The use of genomic DNA in transgenesis has the advantage of allowing the function and regulation of genes to be studied in conditions approaching their natural context. Indeed, this is the first study reporting successful production of mice that are transgenic for an activating NK receptor, whereas several transgenic mice expressing inhibitory *Ly49s* were previously generated (Hanke and Raulet 2001; Liu et al. 2000; Lowin-Kropf and Held 2000; Oberg et al. 2000). *Ly49* gene expression is known to be regulated in an isoform-specific fashion, by a process that restricts the number of *Ly49* genes expressed per single NK cell and generates clonal variability (Kubota et al. 1999; Raulet et al. 2001; Smith et al. 2000). Furthermore, Ly49H protein is expressed on the surface of 56% of $NK1.1^{+}CD3^{-}$ splenic NK cells (Smith et al. 2000), the cell population known to be engaged by *Cmv1*. Using a polyclonal antibody specific for the cytoplasmic tail of Ly49H we observed the appearance of the Ly49H protein in transgenic NK cells and restoration of resistance to infection, suggesting that the

regulatory sequences for proper expression of the gene are contained within the genomic fragment used for transgenesis. Moreover, trans-Ly49H expression seems to be NK cell specific since no expression was observed in other lymphocyte lineages, such as T or B cells, in flow cytometric analysis of total splenocytes. So far, little is known about cis- and trans-elements that regulate activating Ly49 receptors. A comparative analysis of the promoter regions of mouse *Ly49* genes indicated the presence of two consensus binding sites for the T-cell factor 1 (TCF-1), a member of the high mobility group protein family (Cadigan and Nusse 1997), immediately upstream of a predicted TATA box (Wilhelm et al. 2001). Using TCF-1 -/- mice, Held and co-workers demonstrated that TCF-1 was essential for acquisition but not maintenance of expression of Ly49 receptors, such as Ly49A and Ly49D, as the size of the Ly49A and D populations were greatly reduced in the knockout mice in contrast to the level of receptor expression in individual cells (Held et al. 1999). The results indicate that sequences within the 79 kb genomic construct are sufficient to direct proper acquisition and maintenance of Ly49H expression by a mechanism distinct to that involving TCF-1. This mechanism, however, would also act at the transcriptional level, since Ly49H protein levels, determined by both the size of $Ly49H^+$ population and MFI per individual cell, parallels *Ly49h* RNA expression levels in the various transgenic lines produced here.

The observation that the transgenic line Tg915 presented a $Ly49H^+$ cell population of reduced size and splenic viral titers intermediate to those present in $Cmv1^r$ or $Cmv1^s$ mouse strains revealed a previously unforeseen gene dosage effect for Ly49H function and defined a threshold level of $Ly49H^+$ NK cells (28%) necessary for full resistance to MCMV infection. This result indicates that line Tg915 carries a hypomorphic *Cmv1* allele, which is an allele with reduced expression compared to

Cmv1^{r}. This leads one to predict that transgenic overexpression of *Cmv1/Ly49h* in more than 50% of NK cells should have the hypermorphic effect of increased resistance -that is lower splenic viral titers and shorter span of the acute infectious phase- in comparison with C57BL/6. However, I have not observed higher levels of *Ly49h* transcript in any of the transgenic animals obtained, suggesting that upper levels of transcript and/or protein expression are tightly regulated. This might reflect the fact that expression of *Ly49* genes is developmentally controlled (Raulet et al. 2001). It is thought that NK cells acquire Ly49 receptors until they generate sufficient inhibitory signals to balance their activation signals (Raulet et al. 2001; Lowin-Kropf et al. 2002). Thus, overexpression of activating receptors could be deleterious for mouse development by preventing a process that is crucial to ensure the induction of NK cell self-tolerance.

Complementation was observed in two independent genetic backgrounds carrying either the susceptibility allele *Cmv1*^{sBALB} or *Cvm1*^{sFVB}. I have shown that a group of susceptible strains including FVB/N and 129 carry a similar haplotype at *Cmv1* that is related to that of C57BL/6, and different from susceptible strains such as BALB/c that do not share alleles at any of the 13 loci studied (Lee et al. 2001b). This is particularly noteworthy at the level of the sequence and functional diversity for genes of the *Ly49* family.

In a recent report, Arase et al. established that Ly49H was being activated by a second viral-encoded class I homologue known as m157 (Arase et al. 2002). Interestingly, m157 also engages the inhibitory receptor Ly49I from 129 mice but it does not bind to NK cells from BALB/c mice (Arase et al. 2002). While the presence of Ly49I in FVB/N mice remains to be confirmed, initial cloning experiments (data not shown) and haplotype analysis (Lee et al. 2001b) indicate that FVB/N and 129 share a

similar *Ly49* repertoire. Thus, results presented here indicate that engagement of Ly49H is sufficient to override inhibitory signals prompted by either m144 or m157.

Important next questions are whether Ly49H is a component of the innate immune system that is 'hard-wired' to detect MCMV (Smith et al. 2002) or whether it is less restricted in that it recognizes other yet to be identified ligands. In addition to the *Cmv1* gene, the murine NKC region has been linked to loci contributing to susceptibility to cutaneous leishmaniasis (Beebe et al. 1997), ectromelia virus (Delano and Brownstein 1995) and herpes simplex I virus (Pereira et al. 2001). These susceptibility traits all involve NK-cell-mediated immunity, and the C57BL/6 mouse presents the active, "resistant" allele. Transgenic mice reported here will be instrumental to test the proposal that such functions are strictly dependent on activating members of the *Ly49* family and will support a direct role of these molecules in recognition of foreign antigens.

CHAPTER SEVEN

GENERAL SUMMARY AND DISCUSSION

7.1. GENERAL SUMMARY

In mice, natural resistance to infection with cytomegalovirus is controlled by a dominant chromosome 6 locus, *Cmv1* (Scalzo et al. 1990). *Cmv1* is expressed in target organs such as the spleen, bone marrow and thymus where it determines natural killer cell activity against virally infected targets (Welsh et al. 1990; Scalzo et al. 1992). Initial mapping of *Cmv1* indicated that it is located on the distal part of chromosome 6 linked to the NK cell gene complex (NKC) (Brown et al. 1997a). Based upon the function and NK-specific expression, all members of the NKC were considered potential candidates for *Cmv1*. Because of the genetic complexity of the NKC and the absence of a reliable *in vitro* assay to assess susceptibility/resistance to MCMV, a positional cloning approach based on meiotic mapping and the definition of a minimal genetic interval for the localization of *Cmv1* seemed the most rational approach to identify the locus. Previous efforts of our laboratory have successfully generated a comprehensive genetic and physical map in the *Cmv1* interval. As a result, *Cmv1* has been localized to a 0.35-cM genetic interval, closely linked to the *Ly49* gene family (Depatie et al. 1999). A high-resolution physical map of the *Cmv1* genetic interval based on long-range restriction mapping by PFGE and the assembly of a contig of YAC and BAC clones translated the genetic interval into 1.6 Mb of genomic DNA (Depatie et al. 2000).

Following the earlier stage of the approach, the next steps taken to finally clone *Cmv1* have been described in this thesis. The thesis reported (1) the generation of a transcription map in the *Cmv1* interval (Chapter 3), (2) the haplotype analysis of the *Cmv1* region in a panel of 17 inbred mice (Chapter 4), (3) the study of the susceptibility of the recombinant inbred strain BXD-8, suggesting that *Ly49h* and *Cmv1* are allelic (Chapter 5) and (4) *In vivo* complementation analysis by BAC transgenic mice

expressing Ly49H to formally demonstrate the allelism between *Ly49h* and *Cmv1* (Chapter 6).

The generation of a transcription map is a prerequisite step in the positional cloning approach. Taking advantage of BAC contig fully representing the *Cmv1* interval, I generated a transcription map for the *Cmv1* region, which represents at least 19 genes comprising a complete *Ly49* gene cluster and other new candidate genes. Recently, the whole *Ly49* gene cluster of C57BL/6 strain has been fully sequenced and the result confirmed our localization of the *Ly49* gene family (Depatie et al. 1999; Wilhelm et al. 2002). I investigated several genes (*Ly49b*, *Csda* and *EST335500*) for their candidacy using RT-PCR and mutation analysis. Although I showed that most candidates are highly expressed in spleen, intense allelic differences for transcripts analysed in the *Cmv1* interval precluded assessment of their candidacy based upon mutation analysis. In addition, the complex structural organization of the *Ly49* gene family further complicated the analysis of the *Cmv1* domain as many of the *Ly49* genes have different copy numbers and genomic organization in inbred strains (Takei et al. 1997; Anderson et al. 2001), making it difficult to distinguish allelic variants of the same or of distinct *Ly49* gene copies.

As an alternative for fine mapping, I applied haplotype analysis of a set of loci linked to *Cmv1* to a panel of 17 inbred strains, demonstrating the presence of 3 major classes of independent haplotypes at this region. A class present in the CMV-resistant strain C57BL/6J and its close relative C57BL/10J showed the same haplotype. The second class, present in six strains including FVB, carried a susceptibility allele named $Cmv1^{sFVB}$ related to that of $Cmv1^{r}$ strains. The third class, present in strains including BALB/cJ, A/J, DBA/2J which are known to carry the susceptibility allele, $Cmv1^{s}$, by

genetic mapping (re-named *Cmv1*^{sBALB}), carried a unique haplotype (Lee et al. 2001b). Close inspection of the *Cmv1*^{sFVB} haplotypes indicated allele sharing from proximal markers, ruling out *Ly49e*, *Ly49f* and *Ly49d* as *Cmv1* candidates. However, the region in disequilibrium with susceptibility extended distally to *Ly49d*. These results, together with the localization of *Cmv1* as determined by the two previously described genetic maps, (Brown et al. 1999; Depatie et al. 2000) made it difficult to rule out a possible contribution of several linked loci to the susceptibility/resistance phenotype.

Studies using the recombinant inbred strain BXD-8 were instrumental to pinpoint the genetic defect associated with MCMV susceptibility. The BXD-8 line was of particular interest since it was highly susceptible to MCMV infection but harbored a C57BL/6 haplotype at *Cmv1*. Previous linkage analysis using (BXD-8/Ty × C57BL/6)F2 populations together with genetic complementation experiments with congenic strains at *Cmv1*, indicated that the CMV susceptibility trait of BXD-8 was localized to the minimal *Cmv1* interval (Lee et al. 2001a). Using STS content analysis and pulse-field gel electrophoresis, I identified that a 23 kb deletion in BXD-8 mice, encompassing *Ly49h*, was associated with the susceptibility. By analysing the expression patterns of the entire *Ly49* gene family, I revealed that absence of the Ly49H activating receptor correlated with MCMV susceptibility, pointing to allelism between *Ly49h* and *Cmv1* (Lee et al. 2001a). This was further corroborated by treatment of MCMV-resistant C57BL/6 mice with an anti-Ly49H antibody which resulted in the acquisition of a susceptible phenotype (Brown et al. 2001a; Daniels et al. 2001).

Lastly, to formally demonstrate the role of *Ly49h* in host resistance to viral infection, I introduced the *Ly49h* gene into a susceptible background using BAC transgenesis. The transfer of the *Ly49h* gene successfully conferred resistance to MCMV

in genetically susceptible mice. Investigation of *Ly49h* gene expression by RT-PCR and FACS analysis demonstrated a strong correlation between the level of *Ly49h* expression and the control of viral replication, providing definitive proof of *Ly49h/Cmv1* identity (Lee et al, 2003). Furthermore I investigated the rescuing effect of *Ly49h* in the context of a $Cmv1^{sBALB}$ and $Cmv1^{sFVB}$ background to evaluate a possible effect of the genetic background in MCMV susceptibility. The genetic transfer of *Ly49h* to these two backgrounds rescued their susceptible phenotypes, indicating that a critical role for Ly49H in MCMV resistance is independent of the genetic background.

7.2. ROLES OF Ly49H IN MCMV RESISTANCE

NK cells contribute to antiviral immunity by two distinct mechanisms, their cytotoxicity and cytokine release (Biron et al. 1999). Cytotoxicity would require direct contact between NK cells and virus-infected cells, as well as the presence of positive signaling from the target to effector cells for release of cytolytic molecules such as perforin and granzymes. For example, IFN-α/β has been known as a strong inducer of natural cytotoxicity of NK cells (Orange and Biron 1996a). Natural cytotoxicity defines the capacity of NK cells to spontaneously kill targets without prior exposure or stimulation. Although its functional roles were clearly demonstrated by NK-mediated tumor killing, a prominent role for NK-mediated lysis of virus infected cell has not been established.

NK cells produce cytokines with antiviral functions, including IFN-γ, during MCMV infection (Figure 7.1). In contrast to the limited evidence supporting a role of NK cell cytotoxicity, the importance of NK cell-produced IFN-γ in antiviral defense has been clearly established. Such IFN-γ production during viral infection is a consequence

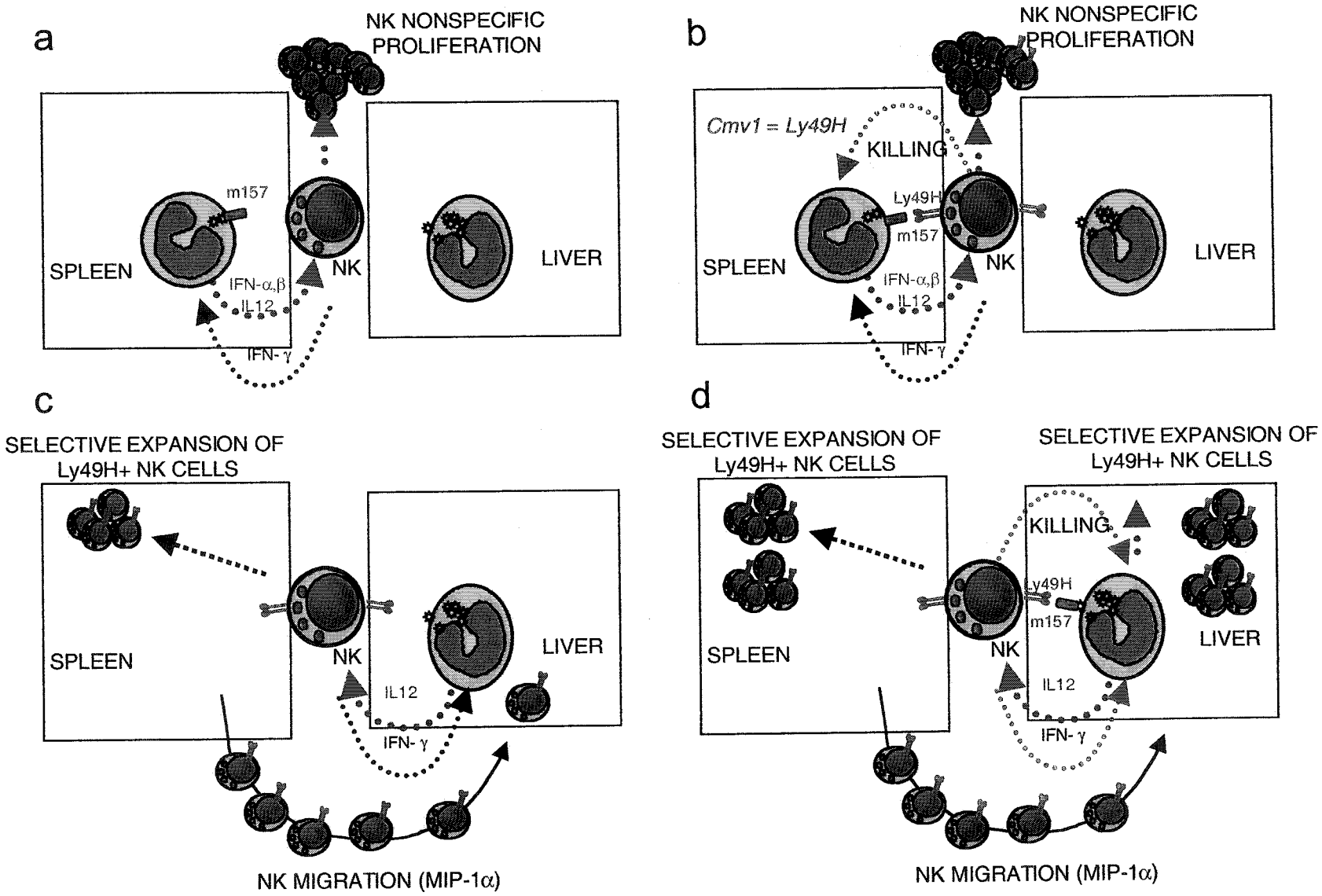
of virus induced IL-12 (Orange and Biron 1996a). During MCMV infection, the NK cell IFN-γ response is systemic for the first 2 days post infection (Pien et al. 2000; Dokun et al. 2001a). IFN-γ is critical for protection against death following challenge of mice with high dose of MCMV, where NK cell IFN-γ production contributes to inhibition of early MCMV replication, particularly in the liver (Dokun et al. 2001a). The effect may result from several mechanisms. Secreted IFN-γ induces many antiviral mediators such as PKR, Mx and OAS, which in turn induce apoptosis in virus infected cells and antiviral defenses dependent upon inducible nitric oxide synthase (iNOS) (Tay and Welsh 1997); IFN-γ also promotes antigen presentation by stimulating macrophages and dendritic cells (DC) (Moretta 2002), demonstrating its dual functions in innate and adaptive immunity. Given the potential for high systemic levels, NK cell-produced IFN-γ might act distally. However, even under the conditions of high systemic IFN-γ production during MCMV infection, peak antiviral functions in the liver require proximity of NK cells, which is mediated by a chemokine, MIP-1α (Salazar-Mather et al. 1998). In mice deficient in MIP-1α, NK cell accumulation in liver during MCMV infection is markedly reduced, resulting in the increased virus replication in the liver.

If Ly49H has been adapted to be a critical host factor conferring resistance against MCMV, its role might be mediated by these two major mechanisms, cytotoxicity and cytokine release. Clearly engagement of Ly49H by monoclonal antibodies results in redirected lysis of target cells and IFN-γ production (Gosselin et al. 1999), supporting increased cytotoxicity and IFN-γ production as possible mechanisms of resistance to MCMV infection. The activating of the Ly49H receptor transmits signals through an adapter molecule called DAP12 which associates through a non-covalent interaction

(Gosselin et al. 1999). The intracellular domain of DAP12 contains an immunoreceptor tyrosine-based activation motif (ITAM) which becomes phosphorylated upon ligand binding and recruits the cytoplasmic protein tyrosine kinases Syk and ZAP70 (Hatada et al. 1995; McVicar et al. 1998). So far, major responses of NK cells to Ly49H signaling have been demonstrated: 1) killing of virus infected cells, 2) production of IFN-γ 3) specific expansion of Ly49H+ NK cells (clonal expansion) 4) NK cell migration to liver (Figure 7.1). Although the significance of each effect differs in organs such as spleen and liver, these events seem to occur in an orchestrated manner.

Cmv1/Ly49h has been shown to control MCMV replication in the spleen and the mechanism is mediated in an IFN-γ independent and perforin dependent manner (Tay and Welsh 1997). Interestingly, control of virus replication in the liver is mediated in an inverse manner, and is IFN-γ dependent and perforin independent, consistent with the observation that *Cmv1* has only a minor effect in the liver at early time-points after infection (Scalzo et al. 1990).

Figure 7.1. Major cellular events mediated by Ly49H signaling in a time-course of MCMV infection. (a) 1-2 days post infection in the absence of Ly49H NK receptor. Nonspecific activation of NK cells such as proliferation and IFN-γ production occur in the spleen. The NK cytotoxicity might be enhanced by IFN-α/β produced by infected cells, however the response is not sufficient to specifically kill virus infected cells. (b) 1-2 days post infection in the context of Ly49H. In the spleen, NK cells bearing the Ly49H NK receptor specifically recognize and kill MCMV infected cells. In addition, nonspecific activation of NK cells such as proliferation and IFN-γ production, without regard to Ly49H, occur in the spleen. *Cmv1/Ly49H* has only a minor effect in liver at early time-points. (c) 3-4 days post infection. Viral load in spleen becomes negligible in resistant mice. At this time point, Ly49H+ NK cells are specifically amplified and migrate from spleen to liver mediated by the chemokine MIP-1α. (d) 6-8 days post infection. Specific expansion of Ly49H+ NK cells continues in thr spleen and the liver. Ly49H NK cells might control viral replication in the liver through either direct killing or IFN-γ production.


a
NK NONSPECIFIC PROLIFERATION
m157
SPLEEN
NK
LIVER
IFN-α,β
IL12
IFN- γ
b
NK NONSPECIFIC PROLIFERATION
Cmv1 = Ly49H
KILLING
Ly49H
m157
SPLEEN
NK
LIVER
IFN-α,β
IL12
IFN- γ
c
SELECTIVE EXPANSION OF Ly49H+ NK CELLS
SPLEEN
NK
LIVER
IL12
IFN- γ
NK MIGRATION (MIP-1α)
d
SELECTIVE EXPANSION OF Ly49H+ NK CELLS
SELECTIVE EXPANSION OF Ly49H+ NK CELLS
KILLING
Ly49H
m157
SPLEEN
NK
LIVER
IL12
IFN- γ
NK MIGRATION (MIP-1α)

Clearly, transgenic mice expressing Ly49H showed 10^2-10^3 times lower splenic virus titers than their susceptible counterparts (chapter 6), indicating that direct killing of virus infected cells is mediated by the Ly49H NK receptor (Figure 7.1b). This was further supported by recent papers showing the direct Ly49H recognition of the MCMV molecules m157 on virus infected cells (Arase et al. 2002; Smith et al. 2002). It seems that natural cytotoxicity driven by IFN-α/β is not sufficient to kill MCMV infected cells since nontransgenic mice show high levels of IFN-α/β expression, indicating the absolute requirement of a signal mediated by Ly49H/DAP12 for efficient killing (Figure 7.1a; Kim et al. unpublished data). The importance of Ly49H/DAP12 in MCMV resistance has also been supported by the generation of KARAP/DAP12 mutant mice bearing a nonfunctional ITAM. In these mice, a considerable increase in MCMV titers were observed in the spleen (30-40-fold) and in the liver (2-5-fold), showing a crucial role for a particular activating signaling pathway through DAP12 in the NK cell-mediated resistance to infection (Sjolin et al. 2002).

Hence, how does the interaction between Ly49H/DAP12 and m157 generate the activation signal to release perforin and other cytotoxic granules such as granzyme A/B? The most extensively studied activating NK receptor with respect to its cytotoxic signaling is CD16. Similar to the signaling of Ly49H/DAP12, signaling via CD16 activates nuclear factor of activated T cells (NFAT) and results in the production of cytokines, including IFN-γ and chemokines, suggesting that their signal transduction upon ligand binding seem to be similar. Accumulating evidence concerning the CD16 activation has indicated that the signaling cascade that induces NK cytotoxicity is quite similar to TCR-induced signal transduction in T cells. Phosphorylation of the ITAMs is

probably mediated by a src family kinase, thereby facilitating recruitment of Syk and ZAP70. Downstream events linking the phosphorylation of Syk to the release of perforin include the phosphorylation of numerous signaling molecules such as SLP-76, phospholipase C, Vav family guanine nucleotide exchange factor and PI3-kinase. In particular, studies using inhibitors that block kinase activation to extrapolate upstream and downstream events demonstrated phosphoinositode 3-kinase (PI3K) as a central mediator, presenting a detailed picture of a signaling cascade: Syk – PI3K – Pac – Rho – Erk2 – perforin release (Jiang et al. 2000). In this case, blocking either Syk kinase or PI3K prevents the activation of the Erk2 cascade, thereby abolishing the release of perforin and the cytotoxic activity of NK cells.

What is the physiological significance of Ly49H+ NK cells in the liver? It has been known that the *Cmv1* locus does not significantly contribute to control of viral replication in liver (Scalzo et al. 1990), while IFN-γ is a crucial mediator for control of viral replication in liver (Tay and Welsh 1997). At first glance, IFN-γ production is not a feature of Ly49H+ NK cells as IFN-γ is expressed by NK cells irrespective of their surface expression of Ly49H (Dokun et al. 2001a). Instead, there is NK cell proliferation and IFN-γ production within 2 days post infection, without regard to Ly49H (Figure 7.1b). At this stage, IFN-γ can be detected in the serum, showing systemic IFN-γ production. The viral-induced NK cell proliferation is not virus specific as it also follows other virus infections such as vaccinia virus (Dokun et al. 2001a). This non-specific proliferation is thought to be mediated by proliferative cytokines such as IL-2 or IL-15 (French and Yokoyama 2003). However, levels of IFN-γ fall below detection in spleen or serum at 4 days post infection (Pien et al. 2000). In contrast to the early

response, at this time point, there is a specific activation of Ly49H+ NK cells resulting in clonal expansion and migration to the liver (Dokun et al. 2001a) (Figure 7.1c). This response is MCMV-specific, is not seen in vaccinia virus infection, and is mediated by an Ly49H signal since it can be blocked by anti-Ly49H mAb. I have demonstrated that the specific NK cell proliferation occurs independently of the genetic background, further supporting the role of Ly49H in this response (Lee et al. 2003). By 7 days post infection, in the spleen, the proportion of NK cells expressing Ly49H is almost 70%, compared with 44% in the spleen of uninfected mice (chapter 6). Interestingly, at this time point, there is also an enrichment of Ly49H+ NK cells in the liver explained by the NK cell migration from spleen to liver, which follows a transient disappearance of splenic NK cells at 2 days post infection (Dokun et al. 2001a; Lee et al. 2003) (Figure 7.1d). It is important to note that this expansion has little additional effect on the control of viral replication in spleen since viral loads become negligible in the spleen by day 3 after infection in resistant mice (Dokun et al. 2001a; Lee et al. 2003).

Thus, it is conceivable that the specific activation of Ly49H+ NK cells may be adapted for controlling virus replication in liver. In contrast to the early stages of MCMV infection, when nearly equivalent amounts of viral replication are observed in the livers of resistant and susceptible strains, I observed a reduced viral titers in the liver concomitant with an increased percentage of Ly49H+ NK cells in the organ, supporting a direct role for this receptor in MCMV resistance in the liver (Chapter 6).

Therefore, I would like to propose that Ly49H+ NK cells have a dual protective role in spleen and liver. 1. Direct cytotoxicity: Ly49H+ NK cells are able to directly recognize and kill viral infected cells in the spleen at an early time points of MCMV infection. 2. Immunomodulatory role: Expanded Ly49H+ NK cells in liver at later

stages of MCMV infection can secrete IFN-γ locally, which is a critical mediator in the control of MCMV replication. Indeed, a more prolonged presence of IFN-γ has been detected in liver compared to spleen and serum, supporting this notion (Pien et al. 2000). The precise mechanism of IFN-γ in the restriction of viral replication still remains to be determined, however secreted IFN-γ might activate expression of several effector genes including iNOS. Alternatively, Ly49H+ NK cells may directly kill virus infected cells in the liver in a similar manner to that in spleen (Figure 7.1d). Altogether the specific proliferation of Ly49H+ NK cells may be important in the control of viral replication in the liver; in contrast "selective expansion" contributes little to immediate NK cell function in the spleen.

7.3. THE LIGAND OF THE Ly49H NK RECEPTOR

The identification of the Ly49H activating receptor as the protein responsible for the MCMV organ specific resistance/susceptibility phenotype prompted the search for the ligand of the Ly49H NK activation receptor. Since all Ly49 receptors characterized to date, both inhibitory and activating, have been shown to bind to classical MHC class I molecule (Anderson et al. 2001), it was predicted that Ly49H would also bind to a MHC class I molecule or a similar molecule, thereby transmitting activation signals to NK cells to kill virus infected cells. Two possibilities existed for the origin of the ligand of Ly49H: 1) the infected cell itself produces a MHC class I molecule or a MHC class I like molecule or; 2) MCMV encodes a MHC class I like molecule that is recognized by Ly49H-expressing NK cells. Recently, two groups independently reported that Ly49H directly binds to a viral protein containing an MHC class I fold encoded by MCMV ORF

m157 (Arase et al. 2002; Smith et al. 2002). These investigators used Ly49H/DAP12-transfected T hybridomas carrying an NFAT inducible reporter as a readout for Ly49H activation. Using deletion mutations of MCMV (Arase et al. 2002) or a bioinformatics approach (Smith et al. 2002), both groups found that m157-infected fibroblasts, but not uninfected fibroblasts, elicited an Ly49H-dependent response. Furthermore Ly49H activation was maintained when infected fibroblasts from β2-microglobulin-deficient or β2-microglobulin/TAP double deficient mice were used (Arase et al. 2002). This latter result suggests that classical MHC class I molecules are not involved in Ly49H activation since class I heavy chain expression is dependent upon complex formation with peptide and β2-microglobulin. m157 may have been previously overlooked as a class I homologue since it is not related to class I at the primary sequence level. Interestingly, molecular modeling has recently revealed that m157 has a class I-like structure (Arase et al. 2002; Smith et al. 2002).

The recognition of the m157 protein by the activating Ly49H receptor provides the first example of direct recognition of a virally encoded molecule by a member of the Ly49 family. Ly49D is the only other activating member of the Ly49 family for which ligands have been identified, this being the classical MHC class I molecule H2-D^d and a hamster classical MHC I molecule (Nakamura et al. 1999; Furukawa et al. 2002). Thus it would seem that Ly49H represents a component of the innate immune system that is 'hard-wired' to detect MCMV infection. Direct recognition of infection by the innate immune system is generally associated with activation of the Toll-like receptor family of proteins or the complement system (Janeway and Medzhitov 2002). To date, the only other example of 'direct' recognition of viral infection by NK cells is recognition of the

hemagglutinin of influenza virus and the hemagglutinin-neuraminidase of Sendai virus by the activating molecules NKp44 (Arnon et al. 2001) and NKp46 (Mandelboim et al. 2001). However, the significance of this interaction *in vivo* during influenza virus infection is difficult to assess as this recognition is dependent on ubiquitously expressed sialic acid residues. Certainly there are many examples of NK recognition of virally-infected cells via the downregulation of MHC class I molecules (Farrell et al. 1997), and there are activating receptors (NKG2D) that recognize ligands that are up-regulated on cells that are 'stressed' via a variety of mechanisms, including viral infection (Bauer et al. 1999; Cerwenka et al. 2000). But these latter receptors detect 'danger signals' on a 'global sense' whereas Ly49H, it would seem, is a highly specific receptor of the innate immune response. Indeed, Ly49H was activated in the presence of MCMV but not the closely related mouse γ-herpesvirus 68 (Arase et al. 2002). An obvious question is whether Ly49H is really MCMV-specific or whether it is less restricted and recognizes other yet to be identified ligands. Or was MCMV sufficiently ubiquitous in the ancestral mouse population to drive the evolution of an MCMV-specific innate immune receptor?

7.4. MHC CLASS I, MHC CLASS I-LIKE AND MCMV

Host and pathogen are evolutionarily competing against each other. Hosts are evolving to avoid deleterious infections by pathogens whereas pathogens are trying to evolve to overcome resistance that hosts may have evolved. The competition (arms race) between mice and murine cytomegalovirus (MCMV) is well observed in the context of the MHC class I molecule. MHC class I plays a central role in combatting viral infection by presenting viral antigen to CD8+ cytotoxic T cells. In particular, CD8+ cytotoxic T cells are the major mediator of immune responses against MCMV infection. For this

reason, MHC class I molecules might be a prime target for immune evasion by MCMV. To avoid activation and recognition by the adaptive immune system, MCMV has evolved mechanisms to downregulate MHC class I molecules on infected cells. At least three MCMV genes (m04, m06 and m152) have been identified that interact with MHC class I in infected cells, thereby affecting the expression of MHC class I.

Class I downregulation renders the infected cell susceptible to recognition by NK cells expressing Ly49 inhibitory receptors, in accordance with the 'missing self' hypothesis of Karre et al (Karre et al. 1986). (Figure 7.2) Recent identification of at least 12 MHC-like ORFs in the MCMV genome suggests that many different gene products may be available to able MCMV to escape this NK cell-mediated killing. The roles of several ORFs containing a putative MHC-like fold have been investigated. m144 was first investigated for its possible role in immune evasion because it showed nucleotide sequence similarity with murine MHC class I. Other ORFs encoding molecules with potential MHC-like folds were identified by structural homology analysis even though they show little similarity to MHC class I at the sequence level (Smith et al. 2002). Indeed, m144 is the first MCMV encoded class I homologue that was identified to confer resistance to NK cell mediated attack in susceptible BALB/c mice (Farrell et al. 1997). *In vivo* experiments have shown that deletion of the m144 gene results in decreased viral titers in MCMV susceptible BALB/c mice. Thus, m144 is likely the ligand for an inhibitory NK receptor; however, the identity of this receptor is currently unknown.

More recently, the m152 gene, which, as mentioned earlier, downregulates MHC class I expression and protects infected cells from CD8+ T cell surveillance (Krmpotic et al. 1999), was also identified as containing an MHC-fold in its protein sequence and to

contribute to an additional mechanism ro evade from NK cell mediated killing in susceptible BALB/c mice (Krmpotic et al. 2002) (Figure 7.2c). MCMV mutants that do not expess m152 are highly susceptible to early control by NK cells in vivo, which is reversed by antibody depletion of NK cells (Figure 7.2d). In BALB/c mice, NKG2D ligands on fibroblasts are specifically downregulated by m152, thereby inhibiting the activation of NK cells. The ligand of NKG2D upon MCMV infection was first thought to be H60, but has been recently confirmed as retinoic acid early inducible 1 (RAE-1) protein (Lodoen et al. 2003). During MCMV infection, the transcription of all five known RAE-1 genes was induced, however their expression was specifically downregulated by m152. The expression of H60 was not affected by m152 expression. It is important to note that m152, which contains a MHC fold, inhibits the presentation of both a genuine MHC class I molecule and RAE-1 (another MHC like molecule) on cell surface, suggesting the structural significance of the MHC like motif for the critical interaction each other.

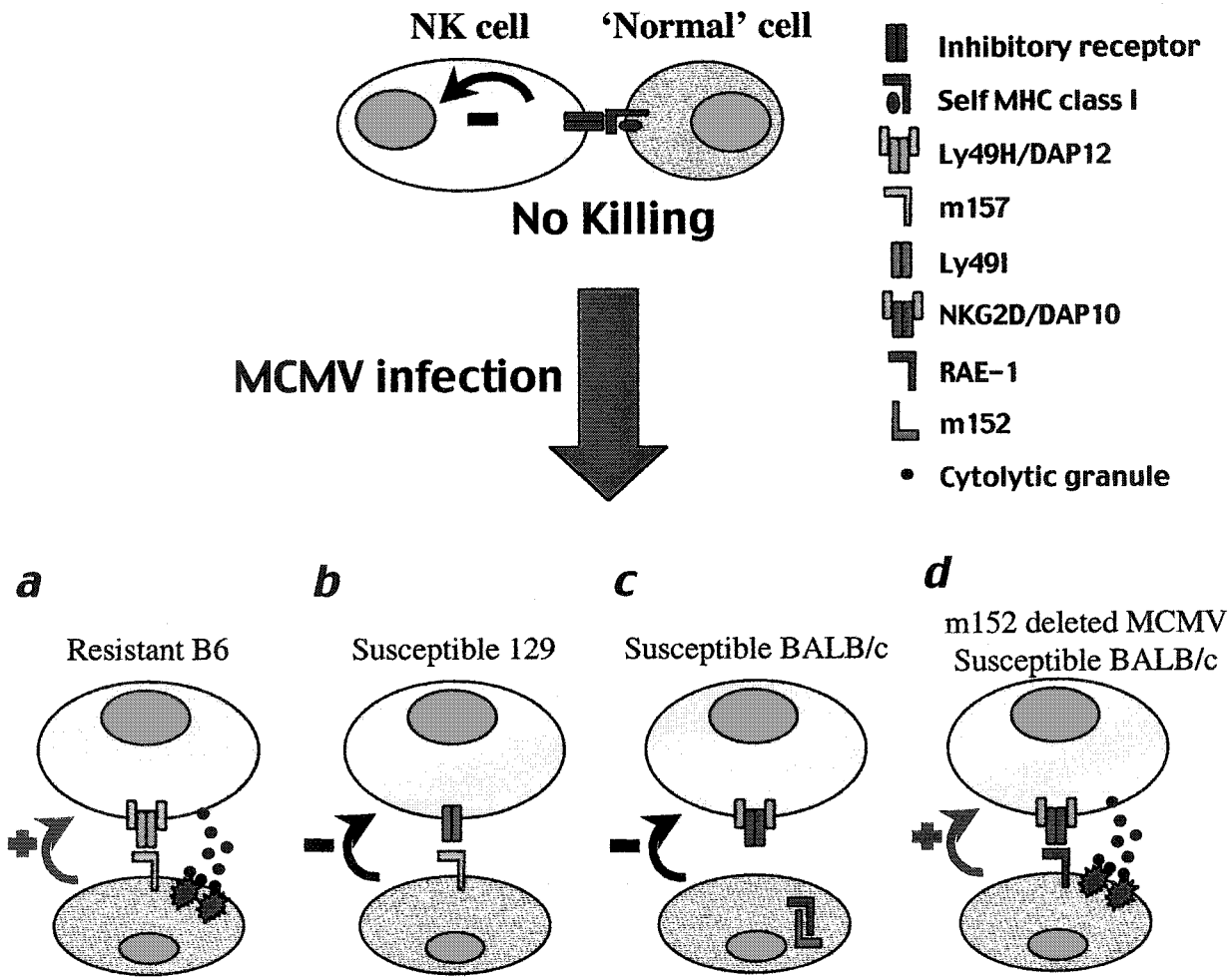
m157, another MCMV ORF containing an MHC fold, was first identified as a ligand of activation receptor Ly49H (Figure 7.2a). The expression of a virally-encoded ligand for an activating Ly49 receptor initially seems counterintuitive, however further studies showed that m157 also engages the inhibitory receptor Ly49I in the MCMV-susceptible 129/J strain (Arase et al. 2002) (Figure 7.2b), suggesting that m157 may have evolved as a mechanism to escape NK cell killing by targeting inhibitory receptors in susceptible mice. These results suggest that Ly49H may have recently evolved as a countermeasure against the virally-encoded m157 class I homologue, providing an overriding, activating signal to the NK cell, and promoting the elimination of infected cells and the arrest of virus spread. Some data support this notion: 1) the *Cmv1*^{r}

resistance allele is relatively uncommon in a panel of 17 inbred mice, as shown in our haplotype analysis (Chapter 4). 2) The expression of *Ly49h* is also very rare in inbred and wild-driven inbred mice (Girard and Vidal, unpublished data). These data suggest that Ly49H activation receptor is very uncommon in the general mouse population. The unique existence of Ly49H in C57BL-related strains suggests that Ly49H evolved recently and that there has been no significant pressure on the m157 gene to be deleted or mutated.

The roles of other putative MCMV MHC class I like molecules still remains to be determined; however, they might be associated with immune evasion by binding to as yet unidentified inhibitory receptors. Interestingly, BALB/c mice are also susceptible to MCMV and yet they lack alleles for both Ly49H and Ly49I. Therefore perhaps the other class I homologue, m144, plays an inhibitory role on NK cell function in this strain.

Lastly, it is interesting to note that MHC molecules can sometimes confer susceptibility to virus infection independently of their role in the immune response by serving as receptors for virus entry. For example, transfection of macrophages with sequences encoding MHC molecules such as $H2K^d$, D^d or K^b renders these cells, which are major reservoirs for MCMV, sensitive to MCMV infection (Price 1994).

Figure 7.2. Consequences of MCMV infection on inbred mice depending on different innate immune recognition mediated by NK cell receptors. Normal expression of self MHC class I in the uninfected cell protects the cell from NK cell mediated lysis because MHC class I is recognized by class I-specific inhibitory NK receptors. The outcome upon MCMV infection is determined by which NK receptor plays a role in early infection. (a) Downregulation of self MHC class I and expression of MCMV encoded m157 on the virus infected cell facilitate Ly49H/DAP12-mediated NK cell activation in resistant C57BL/6 mice, resulting in the killing of the virus infected cell. (b) The same MCMV encoded m157 is recognized by the inhibitory Ly49I receptor in susceptible 129 mice, preventing NK cell activation. (c) Susceptible BALB/c mice lacking Ly49H and Ly49I are further compromised by MCMV encoded m152, which downregulates the expression of the cellular ligand for activation of the NKG2D receptor, RAE-1 (Lodoen et al. 2003), preventing NKG2D-mediated activation. (d) BALB/c cells infected by an MCMV mutant lacking m152 express RAE-1, which permits BALB/c NK cells to be activated. In the figure, "-" indicates overall NK cell inhibition and "+" indicates NK cell activation.


NK cell
'Normal' cell
No Killing
MCMV infection
Inhibitory receptor
Self MHC class I
Ly49H/DAP12
m157
Ly49I
NKG2D/DAP10
RAE-1
m152
Cytolytic granule
a
Resistant B6
b
Susceptible 129
c
Susceptible BALB/c
d
m152 deleted MCMV
Susceptible BALB/c

7.5. ROLE OF LY49H IN NK CELL INTERACTIONS WITH OTHER INNATE IMMUNE CELLS

Recent data showed that activation of Ly49H mediates direct killing of viral infected cells and IFN-γ secretion by NK cells (Dokun et al. 2001a; Daniels et al. 2000). However, these NK cell functions have been shown to be controlled by other factors in sophisticated regulatory pathways (Biron 2001a). Both IL12 and IL18 released from infected cells play critical roles in the production of systemic or splenic IFN-γ by NK cells during MCMV infection because mice genetically deficient in either IL-18 or IL-12p35 exhibited up to 95% reduction in IFN-γ responses (Pien et al. 2000). Surprisingly, IFN-γ responses were preserved in the livers of IL-18-deficient, but not in IL-12p35-deficient mice. Under conditions where mice lacking IL-12p35 exhibited 100% mortality, those lacking IL-18 survived, suggesting that preservation of local, hepatic IFN-γ production is critical for host defense against MCMV infection. The type I IFNs (IFN-α/β) also have major roles in innate immune responses to viral infections as potent antiviral cytokines that are rapidly upregulated in infected cells (Biron 2001a). During the course of MCMV infection, IFN-α/β enhance NK cell cytotoxicity through signal transducer and activator of transcription (STAT) 1 signalling, but they do not stimulate the release of IFN-γ (Cousens et al. 1999; Nguyen et al. 2000). By contrast, IL12 stimulates the production of IFN-γ through STAT4. In the absence of STAT1, IL12 stimulated IFN-γ production is increased markedly, which suggests that IFN-α/β signalling through STAT1 negatively regulates the IL12/STAT4-dependent production of IFN-γ, possibly preventing the indiscriminate release of cytokines by NK cells (Biron

2001a). Taken together, these studies indicate that two major antiviral functions of NK cells, cytotoxicity and IFN-γ production, are influenced by cytokines secreted by other innate components such as macrophages and dendritic cells.

It was first thought that specific expansion of Ly49H+ NK cells can be achieved in the context of the Ly49H receptor itself and probably through its ligand binding. However, the mechanism of specific proliferation of Ly49H+ NK cells seems to be more complex than initially thought. Recent studies have shown that reciprocal interaction between NK cells and DCs (Andrews et al. 2003) is required to achieve specific expansion of Ly49H+ NK cells during MCMV infection. In particular, it has been shown that CD8α+ DCs are essential for the expansion of Ly49H+ NK cells via a mechanism that is IL12 and IL18 dependent. The critical requirement of IL12 and IL18 was demonstrated by a significant defect of Ly49H+ NK cells expansion in C57BL/6 mice lacking either IL-12, IL-18 or both. Interestingly, an effective Ly49H+ NK mediated early response is required to maintain splenic CD8α+ DC populations within the first 4 days after infection, while splenic CD8α+ DCs are lost in susceptible mice lacking Ly49H. These results indicate a reciprocal relationship between CD8α+ DCs and Ly49H+ NK cells. It remains to elucidate the molecular mechanism by which Ly49H+ NK cells mediate the survival of CD8α+ DCs. IFN-γ , a prime candidate mediator, has been excluded since mice lacking IFN-γ undergo the CD8α+ DC dependent expansion of Ly49H+ NK cells. Similar to NK cells, DCs are considered as sentinels of the immune system. Their primary function is to alert the aquired immune response by presenting antigens to naïve T cells. The functional interaction between DCs and NK cells indicates that DCs also have a regulatory role in innate immunity by

stimulating NK cells. Therefore the reciprocal interaction between NK cells and DCs might provide a coordinated mechanism that is involved not only in the regulation of innate immunity, but also in the modulation of the subsequent adaptive response to MCMV infection.

7.6. Ly49H IN RELATION TO OTHER PHENOTYPIC TRAITS OF THE NKC

A remarkable number of phenotypic traits associated with immune function or susceptibility to disease have been mapped to the NKC (see section 1.3.2). An immediate question resulting from the identification of *Ly49h* is whether *Ly49h* contributes to any of these phenotypic traits. The genes encoding some of these traits have been mapped and are known to be Ly49H-independent. For example, *Chok*, which controls tumor target killing by mouse NK cells, has been shown to correspond to Ly49D (Idris et al. 1999). Also MCMV-susceptible BXD-8 mice (Ly49H-) remain resistant to infection with ectromelia virus (Brownstein et al. 1991) suggesting that *Rmp1*, the resistance to ectromelia virus, is distinct from Ly49H. The herpes simplex virus (HSV) resistant locus, termed *Rhs1*, mapped proximal to the core NKC suggesting it is also distinct from *Ly49h/Cmv1* (Pereira et al. 2001). Although the precise genes confering the *Rhs1* and *Rmp1* effects have not been determined, they might encode activating NK cell receptors similar to *Cmv1*. It is still possible that other phenotypic loci contributing to susceptibility to cutaneous leishmaniasis and insulin-dependent diabetes are associated with the *Ly49h* gene since susceptible phenotypes are found in $Cmv1^s$ strains such as BALB/c or NOD whereas the C57BL/6 (Ly49H+) mice are resistant to these traits. Investigation of the possible roles of *Ly49h* in these phenotypes

would be facilitated by transgenic mice expressing Ly49H and is in progress in our laboratory.

Indeed, it is worth emphasizing at this point that the *Ly49* locus is both polygenic and highly polymorphic. Different strains of mice contain distinct alleles for many of the *Ly49* genes, making it difficult to make predictions regarding the evolution of the locus. More detailed analysis of the genomic organization of the *Ly49* cluster in two different strains of mice (C57BL/6 and 129/sv) (Makrigiannis et al. 2002) shows a plasticity in gene content between haplotypes, similar to that described for the *KIR* gene family (Wilson et al. 2000). For the same reason one needs to be cautious when drawing assumptions regarding the ligand-specificity of Ly49 molecules, particularly when making inter-strain comparisons, since subtle changes of sequence in the extracellular domains may result in profound differences in ligand specificity. MHC class I receptors such as the Ly49 receptors and the functionally analogous human KIR receptors (Long 1999), particularly those of the inhibitory class, are thought to evolve in parallel with the highly polymorphic MHC class I locus (Trowsdale 2001). However, the realization that Ly49H binds to a virally-encoded class I homologue adds a new wrinkle to this evolutionary scheme and suggests that evolutionary pressures for these interesting families of receptors extend beyond the ability to recognize 'missing-self'.

7.7. NK RECEPTORS AND HCMV INFECTION

NK cells are also thought to play a critical role in HCMV infections, suggesting an important role of NK receptors in HCMV infection. Analogous to the emerging role of the activating receptor Ly49H in MCMV infection, the activating receptor NKG2D seems to play a critical role in recognition of infected cells during HCMV infection.

NKG2D can provide a costimulatory function for NK and T cells when coupled to the signalling chain of DAP10, which contains a motif for phosphatidyl inositol 3 kinase (PI3-kinase) (Groh et al. 2001; Wu et al. 1999). More recently, the ability of NKG2D to trigger costimulation in $CD8^+$ T cells and cytotoxicity in NK cells was domonstrated to arise from its differential association with the intracellular signaling molecules DAP10 and DAP12, respectively (Zompi et al. 2003; Billadeau et al. 2003). In activated mouse NK cells, the NKG2D receptor associates with two intracellular adaptors, DAP10 and DAP12, which trigger PI3-kinase and Syk family protein tyrosine kinases, respectively. Interestingly, cytotoxicity, but not cytokine production, can be triggered by NKG2D in activated NK cells lacking either DAP12 or the Syk family members Syk and ZAP70, suggesting that the DAP10-PI3K pathway is sufficient to initiate NKG2D-mediated killing of target cells (Zompi et al, 2003). These results highlight signaling divergence in the effector functions of NKG2D and demonstrate that alternative associations between a receptor and its adaptors may provide mouse NK cells with more choices through which to trigger cytotoxicity. However, the exact contribution of DAP10-PI3K mediated cytotoxocity to the control of CMV infection still remains to be identified.

The ligands for human NKG2D include the polymorphic MHC class I-related molecules MICA/MICB (Bauer et al. 1999) and the ULBP family of MHC class I-related molecules (Cosman et al. 2001). These ligands are not expressed by most normal tissues but are upregulated in many epithelial tumor cells (Groh et al. 1999), in stressed cells (Groh et al. 1996) and in cells infected with human cytomegalovirus (Groh et al. 2001). Interestingly, the HCMV encoded protein, UL16, seems to block the binding of MICB and ULBP 1 and 2 to NKG2D on human NK cells (Cosman et al. 2001), thus providing a potential escape mechanism for the virus.

In addition, like MCMV, HCMV encodes an MHC class I homologue, UL18, which engages an inhibitory receptor (leukocyte immunoglobulin-like receptor-1, LIR-1). Downregulation of endogenous MHC class I molecules with concurrent engagement of inhibitory receptors would allow UL18 to provide relief from both the adaptive and innate immune systems. However, this proposition has become controversial as the cellular receptor for UL18, LIR-1, is mainly expressed in macrophages, not NK cells (Cosman et al. 1997), and later studies found UL18 enhanced NK cell killing (Leong et al. 1998). Maintaining the thread of 'measure-countermeasure' between the infectious agent and the immune system, and considering the prevalence of HCMV in the human population, we expect that there are likely to be HCMV-specific activating receptors present in human leukocytes that function in a manner analogous to Ly49H.

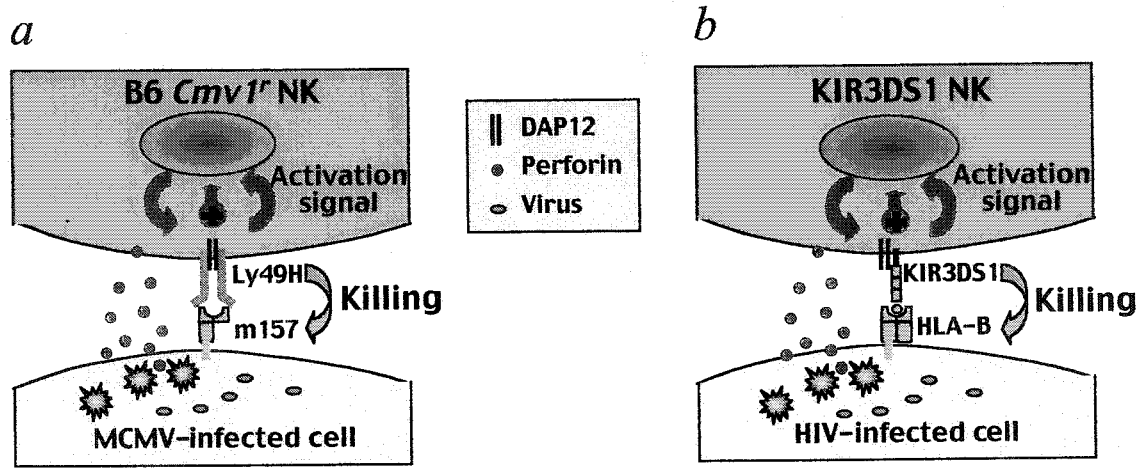
Prime candidates for this type of recognition include members of the KIR family of molecules. As previously mentioned, the human KIR molecules are structurally distinct from the murine Ly49 molecules (KIR contain immunoglobulin type domains versus the C-type lectin domains of the Ly49 molecules) and are encoded by a different locus (KIR are encoded in the LRC rather than the NKC). However, KIRs are thought to represent the functional equivalents of the Ly49 molecules in terms of their ability to recognize downregulation of self MHC molecules during viral infection. Like the murine Ly49 family prior to the discovery of Ly49H, KIR molecules (both inhibitory and activating) are thought to recognize only self MHC class I molecules (Raulet et al. 2001). Therefore, based on the activity of Ly49H, it seems entirely plausible that the KIR molecules, the activating KIR receptors in particular, may have a hard wired capacity to recognize common viral infections through positive recognition of virally-encoded ligands. While the ligands of KIR activating receptors remain to be identified, it

is interesting to note that the presence of the activating KIR3DS1, along with alleles of HLA-B, have an epistatic protective effect on AIDS progression, supporting this possibility (Figure 7.3.) (Martin et al. 2002).

7.8. CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the identification of the activating Ly49H receptor as an important molecule for the activation of NK cells during the early stages of MCMV infection in the mouse constitutes an important step forwards in understanding the function of these enigmatic cells. This finding provides additional evidence that NK cells have a capacity to discriminate between infected and uninfected cells despite the lack of variant receptors that are characteristic of cells of the adaptive immune system. Selective recognition of pathogens is a key step for an effective innate immune response. The major targets of innate immune recognition are pathogen associated molecular patterns (PAMPs). PAMPs represent conserved molecular structures produced only by microbes and essential for their survival. Peptidoglycan and lipopolysaccharide are good examples of bacterial PAMPs. Although recognition of viral double-stranded RNA is an other example of PAMP recognition, viruses pose a particular problem for specific recognition since all the components of the virus are synthesized and assembled in the host cell.

Figure 7.3. Direct recognition of virus infected cells by NK cell receptors. (a) NK cell recognition and killing of MCMV infected cells in mice. The recognition of the m157 protein by the activating Ly49H receptor provides the first example of direct recognition of a virally encoded molecule by a NK cell receptor. Ly49H associates with the adapter molecule DAP12 through a charged arginine residue in its transmembrane domain. DAP12 possesses an ITAM in its cytoplasmic domain that serves as a docking site for Syk and Zap70 protein tyrosine kinases. Upon Ly49H binding to m157, a cascade of tyrosine phophorylation is initiated leading to cellular activation and perforin-mediated killing of the virus-infected cell. (b) Proposed NK cell recognition and killing of HIV infected cells in humans. Human KIR is considered to be a functional homologue of mouse Ly49. An epistatic interaction between KIR3DS1 and HLA-B delays progression to AIDS, suggesting that HLA-B behaves as a ligand for KIR3DS1 [32]. The peptide presented on HLA-B that is responsible for this interaction still remains to be identified. Since KIR3DS1 receptor is also associated with the adaptor molecule DAP12, the intracellular signaling cascade leading to cellular activation and killing of virus-infected cells seems to be similar to that triggered by mouse Ly49H.


a
B6 Cmv1r NK
Activation signal
Ly49H
m157
Killing
MCMV-infected cell
DAP12
Perforin
Virus
b
KIR3DS1 NK
Activation signal
KIR3DS1
HLA-B
Killing
HIV-infected cell

So far, two alternative strategies have been shown to be used by NK cells to detect unhealthy or infected cells: 1) recognition of the absence of self in the case of MHC class I recognition by NK cell inhibitory receptors; 2) recognition of altered self, in the case of stress molecule recognition (ULBPs, H60, RAE). Genetic analysis of naturally occurring variation in the host response has now led to the identification of a gene (*Cmv1/Ly49*h) that is involved in the direct recognition of viral infection by a cellular component (NK) of the innate immune system. Many cytokines and chemokines are activated on MCMV infection. These molecules affect NK cell functions such as trafficking, proliferation and cytotoxicity. Reciprocal interactions between NK cells and other innate immune cells such as DCs, macrophages and NKT cells should be investigated to understand the roles of the Ly49H receptor in the context of *in vivo* MCMV infection.

Clearly, fundamental questions concerning the activating receptor Ly49H remain to be answered. For example, what are the structural determinants of m157 recognition? Does Ly49H recognize cognate ligands from other pathogens? Which are the effector molecules that ultimately couple the Ly49H/DAP12 pathway to gene transcription, and what is the full range of downstream targets following MCMV infection? Infections with multiple pathogens in well-described *in vitro* reporter systems (Arase et al. 2002), together with *in vivo* analysis of the ectopic expression of Ly49H in sensitized backgrounds, such as the diabetes prone NOD mouse, are necessary to clarify the relationship of Ly49H with NKC related phenotypes (Rogner et al. 2001). This, coupled with whole cell expression analysis using micro-array technology will put a wealth of information about the mechanisms of Ly49 action at our disposal.

What is the relevance of these results to humans? A possible role for activating KIR receptors in HCMV susceptibility can be addressed in association studies for KIR alleles in target populations such as neonates, bone marrow transplant patients, or solid organ transplant recipients. Considering the conserved effector functions of NK cells (cytotoxicity and cytokine production), it is likely that the biological significance of NK cells in virus infections of mice and humans is very similar. A better understanding of NK mediated resistance to virus infection in mice could eventually help us to manipulate NK cells in therapeutic strategies for the treatment of infectious diseases in humans.

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CONTRIBUTIONS TO KNOWLEDGE

The research reported in this thesis was carried out by Seung-Hwan Lee, with the following exceptions. The following people have contributed to the experimental work presented in this thesis:

The genotype analysis presented in the thesis was partially performed by John Gita (Chapter 4) and Sonia Girard (Chapter 5) under Seung-Hwan Lee's supervision. Dr. Pierre Lepage, a bioinformatician at the Montreal Genome Center, provided assistance in the analysis of sequences of BAC 13J11 (Chapter 3) and in the generation of phylogenetic tree (Chapter 4). *In vivo* complementation analysis of BXD-8 with congenic lines was performed by Denis Marcina (Introduction of the Chapter 5). BAC transgenesis was carried out at two institutions, Ottawa Hospital Research Institute and McGill University (Chapter 6). Yves de Repentigny and Dr. Rashmi Kothary at Ottawa Hospital Research Institute were responsible for the generation of BAC 13J11 transgenic mice. Janice Penny, Dr. Michael Tremblay and Dr. Philippe Gros at McGill University were responsible for the generation of BAC 128D23 transgenic mice. Dr. Pascal Duplay at the University of Quebec provided a polyclonal antibody against Ly49H (Chapter 6).

Publications

1. Seung-Hwan Lee, Ken Dimock, Douglas A Gray, Nicole Beauchemin, Kathryn V. Holmes, Majid Belouchi, John Realson, Silvia M. Vidal. Maneuvering for advantage: the genetics of mouse susceptibility to virus infection. *Trends in Genetics*. 8, 447-457, 2003.
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3. Seung-Hwan Lee, Ahmed Zafer, Yves de Repentigny, Rashmi Kothary, Michel L. Tremblay, Philippe Gros, Pascale Duplay, John R. Webb and Silvia M. Vidal. Transgenic expression of the activating natural killer receptor Ly49H confers resistance to cytomegalovirus infection in genetically susceptible mice. *The journal of Experimental Medicine* 197, 515-526, 2003.
4. Eduardo Diez, Seung-Hwan Lee, Susan Gauthier, Silvia M. Vidal and Philippe Gros. *Bircle* (*Naip5*) is the Legionella pneumophila resistance locus *Lgn1*. *Nature Genetics* 33, 55-60, 2003.
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Abstracts

1. Seung-Hwan Lee, Ahmed Zafer, Pascale Duplay, John W. Webb and Silvia M. Vidal. Study of the activating natural killer cell receptor Ly49H on BAC Transgenic mice: A new application to investigate host resistance to cytomegalovirus infection. at the Keystone Symposia, Tahoe City, California, March, 2002.

2. Seung-Hwan Lee, Ahmed Zafer, Pascale Duplay, John R. Webb and Silvia M. Vidal. Transgenic expression of the activating natural killer cell receptor Ly49H: A new tool to study host resistance to cytomegalovirus infection. at the 8th annual Ottawa Life Science Conference, Ottawa, Nov. 2001.

3. Seung-Hwan Lee, Yves de Repentigny, Rashmi Kothary, Abdelmajid Belouchi, Philippe Gros and Silvia M. Vidal. Phenotypic assessment of genomic candidate regions for *Cmv1* using transgenic BAC rescue in mice. at the 15th International Mouse Genome Conference, Edinburgh, Scotland, October, 2001.

4. Seung-Hwan Lee, Yves de Repentigny, Rashmi Kothary, Abdelmajid Belouchi, Philippe Gros and Silvia M. Vidal Transgenic BAC rescue in mice identifies the host resistance gene to cytomegalovirus infection, *Cmv1*. at the 10th annual meeting of Canadian Genetics Diseases Network, St-Sauveur, Quebec, April, 2001.

5. Seung-Hwan Lee, Maria Busà, Ahmed Zafer and Silvia M. Vidal. Susceptibility to cytomegalovirus infection is associated with deletion of the natural killer cell lectin-like receptor Ly49H. at the Department Poster Day, Ottawa, April, 2001.

6. Seung-Hwan Lee, Yves de Repentigny, Rashmi Kothary and Silvia M. Vidal. Cytomegalovirus infection: assessment of genomic candidate regions for *Cmv1* using transgenic BAC rescue in mice. at the 7th annual Ottawa Life Science Conference, Ottawa, Nov. 2000.

7. Seung-Hwan Lee, Abdelmajid Belouchi, and Silvia M. Vidal. Human GTX: Genomic Organization, cDNA Cloning, Tissue-specific expression and Chromosomal mapping to 10q26. at the 43rd meeting of CFBS, Ottawa, June 2000.

8. Seung-Hwan Lee, John Gitas and Silvia M. Vidal. Haplotype mapping indicates that the mouse susceptibility allele to cytomegalovirus infection, *Cmvls*, is derived from two independent mutations at the 43rd meeting of CFBS, Ottawa, June 2000.

9. Laneuville, G. Trudel, K.S. Kim, S.H. Lee and H.K. Uhthoff. Identification of six differentially expressed genes in the cartilage of joint with contractures at the 31st Lauretian Conference of Rheumatology, Val David, Quebec, February 2000.

10. Seung-Hwan Lee, Chantal Depatie, Sonia Girard, Philippe Gros and Silvia M. Vidal Physical and transcription map of a 2 -Mb region overlapping the host resistance locus *Cmv1* on distal mouse chromosome 6 at the 6th annual OLSC conference, Ottawa, Nov. 1999.

11. Seung-Hwan Lee, Chantal Depatie Amanda Stafford, Sonia Girard, Philippe Gros and Silvia M. Vidal Carte physique de la region contenant le locus de resistance *Cmv1* sur le chromosome 6 de la souris. at the 67th congress of ACFAS, Ottawa, May 1999.

Oral Presentations

1. Genetic susceptibility to cytomegalovirus infection: the *Cmv1* locus and its role in natural killer cell function at the Host Resistance Lecture Series, McGill University, November, 2003

2. Molecular cloning and characterization of mouse host resistant gene *Cmv1* at the seminar for post-doctoral position, The University of Texas, Southwestern, April, 2003

3. Molecular cloning and characterization of mouse host resistant gene *Cmv1* at the seminar for post-doctoral position, Brown University, March, 2003

4. Molecular cloning and characterization of mouse host resistant gene *Cmv1* at the seminar for post-doctoral position, Massachusetts Institute of Technology, March, 2003

5. Study of the activating natural killer cell receptor Ly49H on BAC Transgenic mice: A new application to investigate host resistance to cytomegalovirus infection at the Keystone Symposia, Tahoe City, California, March, 2002

6. Molecular analysis of mouse host resistant gene *Cmv1* at the Department Seminar at the University of Ottawa, Ottawa, Ontario, January, 2002

7. Transcriptional Map of a 2-Mb region overlapping the Mouse Natural Resistant Locus *Cmv1*. at the Department Seminar at the University of Ottawa, Ottawa, Ontario, December, 2000

Honours

1. CIHR Postdoctoral Fellowship awarded by Canadian Institutes of Health Research: 2004-

2. Graduate Student Scholarship awarded by Keystone Symposia: March 2002

3. 2nd Graduate Student Poster Award awarded by Ottawa Life Sciences Conference: November 2001

4. Graduate Student Scholarship awarded by 15th International Mouse Genome Conference: October 2001

5. BMI travel award by the Department of Microbiology and Immunology, University of Ottawa: October 2001

6. CIHR Doctoral Research Scholarship awarded by Canadian Institutes of Health Research: May 2001- 2004

7. Excellence Scholarship awarded by University of Ottawa: May 1999- 2003

8. SAD (Strategic Areas of Development) Scholarship awarded by University of Ottawa: September 1999- August 2001

9. Ontario Graduate Scholarship awarded by Ontario Ministry of Education and Training: May 1999- April 2001

10. International Scholarship awarded by University of Ottawa: September 1998- August 2000

11. Admission Scholarship awarded by University of Ottawa: September 1998- April 1999