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The Host Resistance Locus emvl/ly49h Regulates Global Gene Expression in Spleen DX5+ NK Cells in Response to Murine Cytomegalovirus Infection

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The host resistance locus *Cmv1/Ly49h* regulates global gene expression in spleen DX5^{+} NK cells in response to murine cytomegalovirus infection.

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ABSTRACT

In mice, the *Cmv1/Ly49h* locus expressed on Natural Killer (NK) cells determines innate resistance to Murine Cytomegalovirus Virus (MCMV). NK cells provide the first line of defence against infections and tumors through cytokine production or direct cytotoxicity. Acquisition of MCMV resistance in transgenic mice expressing Ly49H, hereafter FVB-Tg (*Ly49h*), demonstrated the critical role of Ly49H in clearance of the infection, and provided an ideal model to characterize the role of NK cells in host defence.

The scoring of viral titers in the visceral organs of Ly49H transgenic (FVB-Tg (*Ly49h*)) mice and their MCMV-susceptible counterparts, FVB, indicated a tissue specific effect of Ly49H independent of genetic background in spleen, lung, kidney and thymus. In the liver, the presence of Ly49H was associated with increased numbers of inflammatory foci, suggesting that Ly49H may facilitate localization of NK cells to the vicinity of infected cells.

To identify genes critical to the initial control of virus replication, comparative gene expression analysis of explanted spleen NK cells from FVB-Tg (*Ly49h*) and FVB mice was carried at 36 hours post-infection. This allows for the onset of Ly49H related mechanisms of host resistance. In contrast to whole spleen samples, RT-PCR from purified NK cells from either mouse strain did not detect MCMV gene expression, indicating that NK cells are not productively infected. Out of 16,000 genes analyzed by microarray, 35 showed greater than 2.5-fold expression difference between resistant and susceptible mice. Genes involved in NK cell proliferation, cytotoxicity and in cell-

mediated immunity, such as the early T lymphocyte activation-1 gene (Eta-1 or osteopontin), showed enhanced expression in NK cells from resistant mice.

On the other hand, NK cells from susceptible mice showed increased expression of pro-inflammatory cytokines such as IFN-γ, MIP2, and TNF-associated receptors, indicating that antiviral cytokines are not sufficient to control viral replication in the absence of *Ly49h*, and that direct killing of virus-infected cells by NK cells expressing Ly49H is required for successful clearance of MCMV.

To characterize the response of NK cells during MCMV clearance further, gene expression patterns were studied in FVB-Tg (*Ly49h*) during the course of infection. Out of 22690 genes analyzed, the expression of 225 genes was significantly changed at 3 days post-infection, when the effect of *Cmv1/Ly49h* is strongest and the viral load is highest. At later time points, the number of genes affected and the level of gene expression gradually returned to normal, in parallel with a decrease in viral titer, indicating that altered expression patterns co-varied with viral burden. More than 50% of the genes upregulated were involved in cell proliferation, metabolism, and transcription, while about 15% were involved in NK cell cytotoxic function, indicating that NK cell blastogenesis and direct killing of infected cells are crucial for MCMV-resistance.

Altogether, our results indicate that the differential pattern of expression between resistant and susceptible mice depends on the presence or absence of *Ly49h* as well as on signals emanating from productively infected cells, such as macrophages and dendritic cells. Genes differentially expressed on MCMV-susceptible NK cells may serve as useful biomarkers for HCMV susceptibility in risk populations, graft recipients in particular.

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CHAPTER 1

INTRODUCTION

Worldwide, 70% to 90% of the population is infected with cytomegalovirus (CMV). CMV-associated diseases become life threatening in immunocompromised individuals such as AIDS patients, graft recipients and neonates (Rawlinson, 1999). Primary CMV infection in the immunocompetent individuals rarely causes serious illness. However uncommonly it can produce a mononucleosis syndrome and myocarditis, pneumonitis and aseptic meningitis are rare but reported complications (Gandhi and Khanna, 2004). In mice, there is clear evidence indicating the presence of a genetic factor contributing to MCMV infection (Scalzo et al., 1990). The identification, and characterization, of host genetic factors controlling natural resistance to viral infection represents an important area of research since it provides the molecular strategies of intervention in the infection.

The murine cytomegalovirus (MCMV) model has provided a useful system to investigate human cytomegalovirus infection since both infections share many similarities in terms of viral structure and cellular immune responses (Britt, 1996).

In mice, natural resistance mediated by NK cells, to MCMV infection is controlled by a dominant chromosome 6 locus, *Cmv1*. *Ly49h* has been identified as *Cmv1* by a positional cloning approach, and transgenic mice generated expressing Ly49H to demonstrate the critical role of Ly49H, an activation Natural Killer (NK) cell receptor, in MCMV resistance (Lee et al., 2001; Lee et al., 2003). Even though the results clearly indicated that *Ly49h* is necessary and sufficient to confer resistance, the precise mechanism as to how the Ly49H NK receptor regulates NK cell immune response during MCMV infection has not been demonstrated in detail. Therefore, understanding gene expression in NK cells ($DX5^+$) would provide useful information to elucidate immune responses mediated by Ly49H during MCMV infection.

My project was to characterize the Ly49H-mediated immune response in NK cells by analyzing global gene expression in purified NK cells using microarray technology on the transgenic mice expressing Ly49H. This chapter provides a general background of CMV, its mouse model of infection and *Cmv1*. In addition, it introduces the microarray technology primarily used for gene expression analysis in the thesis.

1.1. Cytomegalovirus (CMV)

1.1.1. Definition

Cytomegalovirus is a member of the family *herpesviridae,* which also includes herpes simplex virus (HSV) type 1 and 2, varicella-zoster virus, Epstein-Barr virus, and human herpesviruses types 6 and 7 (Roizman B.and P.E.Pellett., 2003). Membership of the family of *herpesviridae* is characterized by the architecture of the virion consisting of a linear double stranded DNA genome, an isosadeltahedral capsid containing the DNA genome, an amorphous asymmetric tegument surrounding the capsid, and an outer envelope carrying viral glycoproteins (Roizman B.and P.E.Pellett., 2003). Herpesviruses are also characterized by their biologic properties, such as their requirement of a large array of enzymes for viral replication, the synthesis of viral DNA and capsid assembly in the nucleus, production of infectious progeny virus accompanied by the destruction of the infected cell and the ability to remain latent (Pass R.F., 2001). This family can be divided into three subgroups; alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae according to biological properties such as their host ranges. CMV belongs to the betaherpesvirinae subfamily based on its restricted host range, long

reproductive cycle and slow growth in culture. The naming of cytomegalovirus reflects that it tends to enlarge the infected cells

1.1.2. Structure (Figure 1.1)

A CMV virion contains a 230 kbp linear double stranded DNA with high GC content. The human CMV genome is made up of unique long and short sequences that are separated by short inverted repeats. In particular, CMV has the largest genome among herpesviruses and represents the highest coding capacity of approximately 200 open reading frames (ORF) in the family (Chee et al., 1990). Approximately 25% of open reading frames (ORFs) function in DNA replication and metabolism. Interestingly, it has been noted that roughly 16% of the CMV ORFs strongly resemble those of the Herpes Simplex-1 virus, the Epstein-Barr virus, and the varicella zoster virus (Soderberg-Naucler and Nelson, 2000).

In CMV, the capsid, or the protein shell surrounding the nucleic acid, has an icosohedral structure that is 100-130 nm in diameter. The capsid consists of a major capsid protein and a minor capsid protein. The minor capsid protein anchors the DNA, while the major capsid protein provides the structural framework of the capsid. The nucleocapsid, which is the capsid plus the genome, takes on an angular shape.

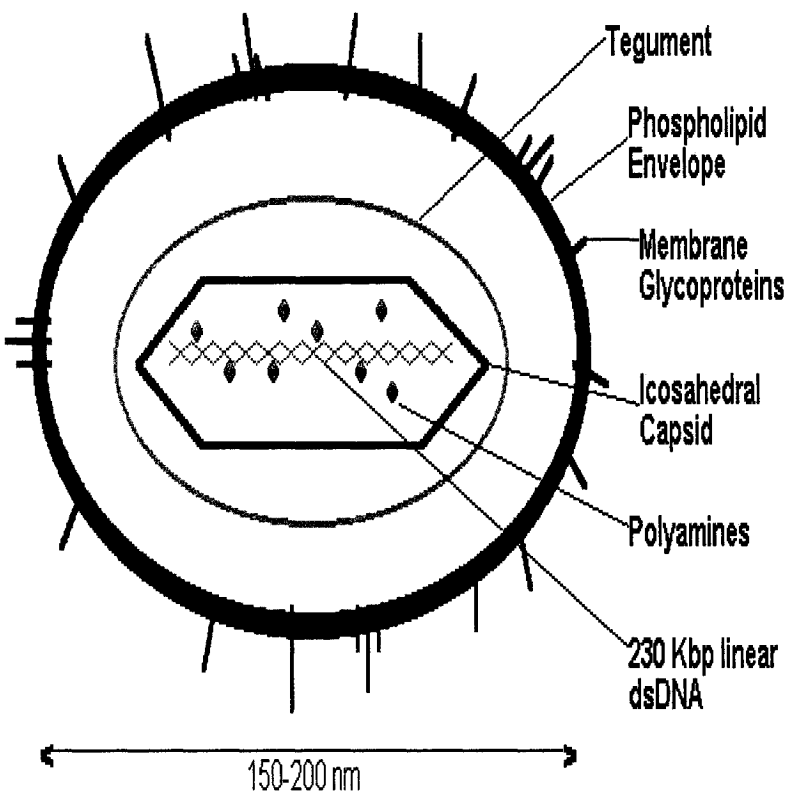
The CMV tegument, also known as the matrix, is composed of globular material that unevenly surrounds the nucleocapsid. It has been estimated that at least twenty proteins make up the tegument and that the vast majority of these proteins are phosphorylated. The tegument is sometimes distributed asymmetrically and its thickness may vary depending on the location of the virion within the infected cell. The function

of most tegument proteins remains uncharacterized, although several transcriptional transactivators have been localized to the tegument (Mocarski, 2003).

The envelope of the human CMV virion carries two prominent herpesvirus-conserved glycoprotein complexes. One is composed of covalently linked, proteolyticaly processed dimers of glycoprotein B (gB) encoded by UL55 and the other is composed of the products of the UL75, UL115, and UL74 genes, gH, gL, and gO, respectively. These glycoprotein complexes seem to play critical roles in the entry into the host cell, which may be common to all herpesviruses.

Viral replication occurs within the nucleus of the host cell and viral envelope forms as assembled nucleocapsids bud from the inner surface of the nuclear membrane or from cytoplasmic membranes. The complex envelopment of CMV appears to involve successive coats derived from the nuclear membrane and from the endoplasmic reticulum or cytoplasmic vesicles (Mocarski, 2003).

Figure 1.1 Structure of HCMV. Schematic cartoon of virion structure of human cytomegalovirus is shown. (This figure was adapted from http://www.virology.net/Big_Virology/ BVDNAherpes.html).


Tegument
Phospholipid Envelope
Membrane Glycoproteins
Icosahedral Capsid
Polyamines
230 Kbp linear dsDNA
150-200 nm

1.1.3. CMV Infection in humans

HCMV rarely causes clinical disease in immunocompetent individuals, although it is responsible for a mononucleosis-like illness upon primary infection, caused by Epstein–Barr virus (EBV) (Gandhi and Khanna, 2004). CMV-associated diseases become life threatening in immunocompromised individuals such as AIDS patients, graft recipients, and in neonates, showing high infectivity and mortality (Soderberg-Naucler and Nelson, 1999; Reusser, 1998). Cytomegalovirus (CMV) is also the most common congenital infection in humans. Congenital CMV infection occurs in approximately 0.5 to 1 percent of all newborns in North America and can result in significant neurological sequelae (Gaytant et al., 2002). Almost all individuals (70% to 90%) have been exposed to HCMV by adulthood and they have developed protective immunity, but a lifelong latent and asymptomatic infection is established with periodic reactivation and viral shedding at mucosal sites such as salivary glands. Cytomegalovirus (CMV) reactivation in immune compromised individuals can cause severe morbidity and mortality. For example, HCMV mediated diseases including retinitis; colitis and encephalitis were found in AIDS patients and have been associated with decreased survival after diagnosis of HIV infection (Pass R.F., 2001). Moreover, the incidence of HCMV infection in the first 100 days following allogeneic bone marrow transplantation (BMT) ranges from 30 – 70%. In the past, HCMV was the leading infectious cause of death in BMT recipients, with mortality rates of untreated HCMV pneumonia of 30 – 60%. Significant HCMV morbidity is also frequently associated with solid organ (kidney, liver, heart) transplantation (Farmer et al., 2001).

The diagnostic hallmarks of active CMV infection include cytomegaly with both nuclear and cytoplasmic enlargement and characteristic intranuclear and intracytoplasmic inclusion bodies. During productive HCMV infection, the virus life cycle is regulated by sequential expression of the immediate early, early, and late genes, ultimately causing a cytopathic effect (Mocarski, 2003). Typical viral cytopathic effects can be seen in a variety of cells, including alveolar pneumocytes, endothelial cells, fibroblast, and exocrine and endocrine glandular epithelial cells. After penetration of susceptible cells by CMV, cellular function does not seem to be very significantly impaired, and the cell may retain its viability despite continual shedding of the virus for prolonged periods. Latent infection can persist for years in the presence of normal immune responses. CMV replicates in the epithelial cells of the respiratory tract, salivary glands, and kidney; in the kidney the tubular cells shed the virus for prolonged periods (Landolfo et al., 2003). Spread via the bloodstream is accomplished by a cell-associated viremia, the virus being associated with both lymphocytes and polymorphonuclear leukocytes. Cell susceptibility to human CMV is strikingly affected by age. Infections in utero result in devastating destruction of the central nervous system, whereas encephalitis from postnatal CMV infection is exceedingly rare (Pass R.F., 2001).

Even though successful control of HCMV infection requires a complex response from most immune cells, it is important to note that NK cells play a primary role in the innate defence against HCMV. The most direct evidence originates from a follow-up study of a patient with a complete lack of NK cells as well as no spontaneous or IL-2-inducible NK cell cytotoxic function. This patient presented extreme sensitivity to

varicella-zoster virus, herpes simplex I and HCMV infections, despite normal antibody and T cell functions (Biron et al., 1989).

Treatment of CMV infection is difficult because there are few options. No vaccine is available and live-virus vaccines have not obtained official approval. In this respect, understanding the early host resistance immune responses against CMV infection at the molecular level could provide important information to develop therapeutic strategies for prevention and treatment of CMV infection.

1.2. Mouse model of CMV infection

Experimental mouse models with MCMV infection have provided an excellent model to study CMV infection because they show many similarities with HCMV in terms of viral structure and cellular immune responses (Cooke and Hill, 2001). More importantly, infecting mice with MCMV mimics virus-induced disease in humans by presenting a large spectrum of clinical manifestations including pneumonitis, hepatitis, and retinitis (Shanley, 1984; Trgovcich et al., 2000; Hayashi et al., 1995). These models presented strong correlations between the severity of pathology and viral titres in target organs.

Furthermore, the critical role of NK cells in defence against MCMV has been found to be relevant to that in humans. Shellam, Bancroft and co-workers (Bancroft et al., 1981) established that increased MCMV susceptibility in *beige* mutant mice was associated with a deficiency in lysosomal activity in NK cells (Shellam et al., 1981). Subsequently, these observations were confirmed in models using selective depletion of NK cells or adoptive transfer experiments. For example, in vivo depletion of NK cells

with antibodies which recognize surface molecules preferentially expressed on NK cells resulted in increased susceptibility to infection with MCMV (Scalzo et al., 1992).

In addition, different strains of inbred mice exhibit striking differences in their level of susceptibility to MCMV infection, indicating genetic components contributing to immune response to MCMV infection (Scalzo et al., 1990). These distinct patterns of susceptibility reveal genetic differences between strains that make the mouse an excellent model to examine the heterogeneous responses seen in humans and facilitate a genetic approach to the mapping of susceptibility traits.

1.3. Immune response to MCMV infection

The immune responses during MCMV infection are complex and mediated by most immune cells belonging to both innate and adaptive immunity. For innate immunity to MCMV infection, NK cells, dendritic cells (DCs), macrophages, and cytokines collaboratively act as the first line of antivirus defence. In addition to the direct antiviral effect before the adaptive immune response is developed, the innate immune response modulates the subset of adaptive immune responses for the most optimal defence. The adaptive immune responses by T and B-lymphocytes help remove the virus at the late phase of infection, thus resulting in a protective effect from the virus-associated pathogenesis.

1.3.1. Innate immune response to MCMV infection

1.3.1.1. Cytokines and chemokines

Cytokines and chemokines are soluble protein factors produced by cells to act in the cells bearing corresponding cytokine receptors. Cytokines and chemokines are usually considered components of innate immunity; however, they are also involved in shaping adaptive immunity at later stages of infection. MCMV infection induces the production of proinflammatory cytokines, such as interleukin12 (IL-12) and type 1 interferon (IFN-α/β), that stimulate NK cell production of other antiviral cytokines. They also induce NK cell proliferation, expression of activation markers and enhance NK cell cytotoxicity (Dokun et al., 2001).

IFN-α and IFN-β exhibit similar biological activities and are induced in many cell types as a consequence of viral infection. Recent papers have indicated that these cytokines are produced by a specific subset of dendritic cells, plasmacytoid dendritic cells (pDC), during MCMV infection (Dalod et al., 2003). Shortly after viral infection, IFN-α/β can induce the antiviral status in both infected cells and neighbouring cells, limiting the spread of further infection. The antiviral effect activated by IFN-α/β results from binding to its heterodimeric receptor and the subsequent activation of the signal transducers and activators of transcription 1 (STAT1) and 2 (STAT2) (Horvath, 2004). The activated and phosphorylated STAT1-STAT2 heterodimers translocate to the nucleus and induce the activation of many antiviral genes including 2-5(A) synthetase, protein kinase R (PKR), dsRNA specific adenosine deaminase (ADAR) and Mx genes. Among these, induced 2-5(A) synthetase, for example, produces 2'-5' oligoadenylates to activate a latent RNaseL, which in turn destroys viral and cellular RNA to inhibit protein synthesis and viral replication. Besides PKR can phosphorylate the translation initiation factor eIF-2α, which also inhibits protein synthesis and, consequently, viral replication.

IFN-α/β also plays an important role in the activation of NK cell responses, such as NK blastogenesis and activation of NK cytolytic function, which provides protective immunity during the early phases of MCMV infection. In later stages IFN-α/β triggers specific $CD8^+$ T cells produce IFN-γ (Welsh, Jr., 1978). Mice depleted of IFN-α/β with neutralizing antibodies or deleted of their receptor by homologous recombination (IFN-α/βR -/- mice) presented a poor NK cell response against MCMV infection(Orange and Biron, 1996) and succumbed shortly after infection (Salazar-Mather et al., 2000).

The role of NK cells during MCMV infection is partially mediated by IFN-γ production. For example, IFN-γ receptor knockout mice present higher viral titers in the liver, suggesting that NK cell-produced IFN-γ is responsible for control of MCMV replication in the liver (Tay and Welsh, 1997). This IFN-γ might exert its direct antiviral effects by inducing inducible nitric oxide synthase (iNOS) in cells such as macrophages, Kupper cells, and hepatocytes. IFN-γ can also be produced by cells of adaptive immunity. For instance, the neutralization of IFN-γ in vivo abolishes the antiviral activity of the $CD4^+$ T cells in the salivary glands, suggesting that $CD4^+$ T cells mediate their antiviral effect by releasing IFN-γ. Furthermore, IFN-γ is required for the antiviral activity of CTLs in long term CD4 depleted mice (Carter and Murphy, 1999).

TNF-α is a major mediator of inflammation and immunity as well as apoptosis, exerting pleiotropic activities (Varfolomeev and Ashkenazi, 2004). TNF-α is an important cytokine mediating antiviral function and helps clear virus infected cells during the early stages of MCMV infection. However, inappropriate continued production of TNF-α has been implicated in the pathogenesis of a wide spectrum of

diseases, including sepsis, autoimmune diseases, and infection. Particularly TNF-α can induce liver damage eventually resulting in death of mice (Orange et al., 1997).

The chemokine macrophage inflammatory protein-1alpha (MIP-1α), a member of the β (or C-C) chemokine family, plays an important role in inflammations induced by certain viral infections. MIP-1α is critical for natural killer (NK) cell recruitment during inflammation, as well as the delivery of IFN-γ to mediate downstream protective responses against MCMV infections in the liver. Production of MIP-1α in virus infected liver cells elicits recruitment of activated NK cells to form distinct inflammatory foci around infected cells and this trafficking is regulated through Type I IFNs. Mice deficient in MIP-1α failed to recruit inflammatory cells and resulted in increased susceptibility to MCMV infection, demonstrating the importance of liver inflammation response in MCMV resistance (Salazar-Mather et al., 1998).

1.3.1.2. Macrophages

Macrophages are important phagocytes during virus infection. They can opsonise and phagocytise infected cells through complements and through Fc receptors. It is important to note that macrophages appear to have dual, even paradoxical, roles during MCMV infection: one as reservoirs for MCMV replication and another as effector cells for eliminating the virus. The infection of macrophages by MCMV plays an important role in the pathogenesis of CMV disease during both acute and latent infection. In both these stages, IFN-γ has an important role (Hamano et al., 1998). During the acute phase of MCMV infection, macrophages are the sites of viral replication in a number of organs, including the spleen and peripheral blood. Moreover,

macrophages aid in the dissemination of MCMV to secondary sites of infection and are one of the cellular sites of latency for MCMV. In spite of their deleterious role in MCMV infection, it is interesting to note that depletion of splenic macrophages significantly enhanced MCMV replication in spleen, indicating that tissue macrophages serve a net protective role and may function as "filters" in protecting other highly permissive cell types from MCMV infection (Hanson et al., 1999). The filtered virus seems to be efficiently cleared by professional antiviral immune responses elicited by macrophages.

Moreover in immune responses, macrophages together with dendritic cells, also act as antigen presenting cells (APC) enabling the adaptive immune response. For example, macrophages activated by IFN-γ can increase MHC expression and antigen processing, resulting in increased antigen presentation to T cells and initiating the coordination of an adaptive immune response. The activation of macrophages is an early event in the immune response, and macrophages are a link between the innate and adaptive arms of the immune system. More importantly, activated macrophages produce nitric oxide (NO) and the NO-mediated antiviral activity of macrophages plays a protective role during MCMV infection (Noda et al., 2001).

1.3.1.3. Dendritic cells (DCs)

The innate recognition of pathogens is important in the production of cytokines and adaptive immune responses and DCs have recently been identified as playing a central role in this process. The innate stimulation of DCs can trigger their differentiation into professional antigen presenting cells (APCs) capable of priming and sustaining the expansion of T cells. DCs are abundant in the skin, mucosa and in

lymphoid organs, and immature DCs take up and process antigens at these sites. Once activated, DCs migrate into lymphoid organs where they initiate a primary immune response. Migration coincides with a reduced antigen processing capacity and an increased cell surface expression of MHC and costimulatory molecules. Activated DCs are essential for the subsequent activation of both naïve and activated T cells (Andrews et al., 2003).

Dendritic cells can be divided into three subgroups based on their expression of surface markers; myeloid DCs, lymphoid DCs, and plasmacytoid DCs (pDC) (Dalod et al., 2003; Liu et al., 2001). The mechanism by which DCs sense viral infections using Toll like receptors (TLRs) has recently been identified. Toll like receptors (TLRs) have emerged as key components of the innate immune system that detect microbial infection and trigger antimicrobial host defence responses. The targets of pathogen associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) and the TLR family is the best-characterized class of PRRs. On binding, they activate signalling cascades leading to the synthesis of proinflammatory molecules. Whereas TLRs 1, 2, 4, 5, and 6 seem to specialize in the recognition of mainly bacterial products, TLRs 3, 7, 8, and 9 specialize in viral detection (Iwasaki and Medzhitov, 2004). In particular, a recent paper demonstrated that pDCs can sense MCMV infection in a Toll like receptor (TLR) 9 dependent manner and thereby produce IFN-α/β and IL12 to establish an antiviral immune response (Krug et al., 2004). Moreover, mice having a defect in their TLR9 and TLR3 signal pathways produced by ENU (N-ethyl-N-nitrosourea) showed an increased susceptibility to MCMV, supporting the importance of TLRs in defence against MCMV infections (Tabeta et al., 2004).

1.3.1.4. Natural Killer (NK) cells

Natural killer cells play critical roles in innate immunity against viruses and other intracellular pathogens by perforin-mediated direct killing and cytokine production. Since my thesis is intended to characterize the central role of NK cells in MCMV infection, NK cells-will be described extensively in a subsequent section.

1.3.2. Adaptive immunity

The adaptive immune response is comprised of a diverse repertoire of effector lymphocytes, such as T and B cells, which provide a highly specific response to pathogens through clonal selection, affinity maturation of their receptors by somatic recombination, and memory of the pathogens encountered.

1.3.2.1. T cells

Although innate immune responses play an important role during early MCMV infection, T lymphocytes are required for the termination of the productive infection and establishment of latency. MCMV infection elicits a strong CD4+ and CD8+ T cell responses, which are necessary for viral clearance from the salivary gland and peripheral organs. The protective capacity of T cells against MCMV infection was determined by an adoptive transfer of MCMV-primed T cells into syngeneic sublethally γ-irradiated hosts (Krmpotic et al., 2003). The adaptive cellular immune response by CTLs is sufficient to clear infection from most tissues and is sufficient for survival. MCMV infection induces a strong Th1 response, characterized by the production of IFN-γ, which helps regulate acute, chronic, and latent viral infection (Wu et al., 2001).

1.3.2.2. B cells

Even though MCMV presents many immunogenic glycoproteins and antibodies which can be easily detected in sera from infected mice, studies on B cell deficient mice demonstrated that the antibodies are not essential for the resolution of the primary infection (Jonjic et al., 1994). These mice clear the virus and establish viral latency with clearance kinetics indistinguishable from normal mice. The reactivation from latency is the only stage at which the absence of antibodies affects the phenotype of MCMV infection, since depletion of cellular immune control in B cell deficient mice resulted in higher viral titers compared with seropositive control animals (Jonjic et al., 1994).

1.4. MCMV immune evasion

A virus capable of establishing persistent infection must be able to avoid the host immune response. Both MCMV and HCMV contain many genes that are dispensable for productive replication and have evolved to subvert the immune system by multiple mechanisms. The most prominent one of these evasive mechanisms is to inhibit antigen presentation with MHC class I molecule and, thereby, to escape $CD8^+$ cytotoxic T lymphocyte recognition of infected cells. For example, MCMV presents three early expressed genes, m04, m06, and m152 (Kleijnen et al., 1997). The m04 gene product gp34 binds to MHC class I molecules in the endoplasmic reticulum (ER) and is then expressed on the cell surface. In addition to its obvious role in escape from CTL responses, its surface expression suggests that m04 may present non-functional MHC class I molecules to decoy natural killer cells in order to cope also with innate immunity.

In addition, gp48 encoded by m06 appears to be particularly efficient in down-regulating cell surface MHC class-I expression by targeting MHC class-I molecules in the ER and rerouting them for lysosomal degradation. Finally, m152, which was first identified as an evasion gene for CMV, mediates retention of peptide-loaded MHC-class-I ß2-microglobulin complexes in the ER and the ER-Golgi intermediate compartment. Similarly, immune evasion genes of HCMV interfere with human MHC (HLA) class I molecule presentation, including US2, US3, US6 and US11. US3 promotes retention of MHC class I in the ER whereas US2 and US11 mediate degradation of MHC class I heavy chain. Interestingly, the genes subverting antigen presentation identified in MCMV and HCMV do not present any sequence similarities, indicating that these evasive strategies evolved after the evolutionary diversification of human and mouse (Pass R.F., 2001).

In addition, CMV contains a number of homologs of host cellular gene products, suggesting that these genes are implicated in viral pathogenesis. A sequence comparison of the complete MCMV and HCMV genomes with published human and mouse genomes has indicated that both CMVs include gene products similar to host genes for MHC class I molecules, T cell receptor deltas, and CC chemokines (Rawlinson et al., 1996). It is important to note that viral homologs of MHC class I molecules can interfere with NK cell surveillance. Since NK cells have the ability to lyse virus infected cells by the recognition of impaired MHC class I expression and, thereby, compensate the disability of CTL in the recognition of reduced MHC class I expression, viral MHC class I decoys could inhibit NK cell mediated killing of virally infected cells. For example, experiments with mutant MCMV have demonstrated that the deletion of the MHC class I homolog gene, m144, resulted in reduced virus replication, suggesting that the m144

gene product serves as a "decoy MHC" molecule and inhibits NK lysis by binding to unknown inhibitory receptors on the NK cell (Farrell et al., 1997). Additionally, the murine cytomegalovirus CC chemokine homolog MCK-2 (m131-129) has been identified as an important determinant of dissemination during primary infection by recruiting permissive leukocytes into the infection site (Fleming et al., 1999).

1.5. NK cells

1.5.1. Development

NK cells are large, granular lymphocytes that constitute an important component of the innate immune system and provide an early barrier against viral infection and tumor development. They are bone marrow–derived lymphocytes, distinct from T and B cells since their development does not require gene rearrangement for their receptors. An intact bone marrow microenvironment is prerequisite for the complete phenotypic and functional maturation of NK cells. For example, Interleukin 15 (IL15), produced by stromal cells in the bone marrow, is critical for NK development and differentiation. In mice, both immature and fully mature NK cells leave the bone marrow and travel to lymphoid compartments around the body, including the spleen, lymph nodes, lungs, and liver. NK cells constitute only a small population of cells (about 2.5% of splenic leukocytes) and localize to the red pulp of the spleen and the sinusoidal region of the liver. The half life of mature NK cells in the periphery appears to be about seven to ten days based on the data from the survival of adoptively transferred NK cells (Yokoyama et al., 2004).

1.5.2. NK surface markers

Asialo ganglio-N-tetraosylceramide (AGM-1), NK1.1, and DX5 are the surface markers most frequently used to identify murine NK cells. These molecules are expressed on NK cells isolated from a variety of compartments including the spleen and liver. NK1.1 expression is limited to NK cells in a few inbred mouse strains, such as C57BL-related strains, which restricts its application in most other inbred mice. However, both AGM-1 and DX5 (mouse CD49b antigen) are detectable on NK cells in all tested strains (Arase et al., 2001). In contrast to the AGM-1 expression found in most granulocytes, DX5 is expressed at high levels in the vast majority of mouse NK cells and at lower levels in NKT cells ($CD3^+CD49b^+$), providing a useful reagent to recognize NK cells in mice not expressing NK 1.1.

1.5.3. Function in MCMV infection

The functions of NK cells are regulated by a balance of signals transmitted by receptors on NK cell surfaces. First, NK cells are activated by many cytokines such as IL2, IL12, IL15, IL18 and IFN α/β, which promote their IFN-γ production and cytotoxic activity against virus infected cells (Biron et al., 1999). In addition, NK cells also respond to signals transmitted by NK specific receptors, which are often divided into inhibitory and activation receptors. Initially, the surveillance of virus infected cells by NK cells was explained by the missing-self hypothesis (Karre et al., 1986). According to that idea, every NK cell presents at least one inhibitory receptor that recognizes a self-MHC class I molecule on healthy cells and generates inhibitory signal, thereby protecting them from NK killing. However, virus infected cells that have downregulated

MHC class I expression become susceptible to NK killing due to the absence of the inhibitory signal.

More recently, evidence supporting the importance of activation receptors in NK activity against virus-infected cells has been described. For example, NKG2D recognizes several antigens that are induced on virus infected cells during HCMV and MCMV infections. Furthermore, the identification of Ly49H, the other NK activation receptor, which recognizes a MCMV viral antigen on infected cells and transmits activation signals, has demonstrated the high impact of activation receptors upon the roles of NK cells during virus infections (Lanier, 2005).

As aforementioned, NK cells play important roles during MCMV infection by using a number of effector mechanisms, including direct cytotoxicity and release of cytokines. In humans, the critical role of NK cells in CMV infection was well illustrated by the case of the first female patient described as specifically lacking NK cells. Despite having normal antibody and memory T cell functions, this patient suffered life-threatening HCMV, chicken pox and severe HSV infections (Biron et al., 1989). Studies of human NK responses to HCMV *in vitro* found that they were mediated by large granular lymphocytes and were independent of interferon. In vitro studies have shown that fibroblasts infected with certain HCMV strains but not others can be susceptible to NK cell–mediated lysis (Borysiewicz et al., 1985).

In mice, many studies demonstrate that NK cells function in resistance to MCMV infection. Beige mice, which are genetically deficient in functional NK cells, have increased susceptibility to MCMV. In other mice, depletion of NK cells, using a specific antibody that recognizes an antigen preferentially expressed on NK cells, led to

more serious infections after MCMV infection (Shellam et al., 1981). In the spleen, cytolysis of infected cells by NK cells plays a significant role in limiting MCMV viral replication. Cytotoxicity in NK cells is carried out by granule exocytosis of membrane pore forming molecules (perforin) and granzymes. Mice having disrupted perforin and granzyme A/B genes showed high susceptibilities to the early stages of MCMV infection (Riera et al., 2000). In particular, the identification of the specific interaction between the Ly49H NK activation receptor and MCMV glycoprotein m157 proved the NK's role in the direct killing of MCMV infected cells (Arase et al., 2002). The detailed function of Ly49H NK cells in MCMV infection will be introduced in the following section.

In the liver, in addition to the direct killing function, IFN-γ-mediated antiviral functions of NK cells seem to be more important in the control of MCMV replication than spleen. Blocking NK cell produced IFN-γ significantly enhanced viral replication and virus-induced pathologies in the livers of MCMV infected mice (Tay and Welsh, 1997).

Besides the direct role during MCMV infection, NK cells also have an immunomodulatory function regulating the function of other immune cells. For example, NK cells are able to maintain CD8α+DC populations during the early infection stage of MCMV. During later stage of MCMV infection reciprocal interaction between NK cells and $CD8\alpha^{+}$DCs continues; at this stage, IL-12 and IL-18, derived from $CD8\alpha^{+}$DCs can keep on activating NK cells (Andrews et al., 2003). An additional immunoregulatory role of NK cells can be observed in their production of IFN-γ, which helps to drive the development of a Th1 polarized T cell response (Boehm et al., 1997).

1.6. *Cmv1* locus and *Ly49* receptor

Cmv1 is a genetic locus that controls early MCMV replication in the spleen, bone marrow, and thymus. *Cmv1* presents two alleles, either CMV resistant allele (*Cmv1*^{r}) or CMV susceptible allele (*Cmv1*^{s}). Early genetic analysis indicated that *Cmv1* was a single autosomal dominant gene (Scalzo et al., 1990). Experiments of in vivo depletion of NK cells and bone marrow chimeras demonstrated that the effect of *Cmv1* is mediated by NK cells (Scalzo et al., 1992). Initial mapping of the *Cmv1* locus using recombinant inbred strains localized *Cmv1* to the NK cell gene complex (NKC) on distal chromosome 6 (Yokoyama and Plougastel, 2003). In mice, the NKC is a 2 Mb genomic region that contains genes encoding for a variety of genes important for NK cell function. Most NKC-encoded genes are cell surface receptors of the C-type lectin superfamily predominantly expressed in NK cells, including *Nkg2* and *Ly49* gene families. Interestingly, many phenotypic traits associated with innate immune functions or susceptibility to infections and diseases have been localized to the NKC, supporting the importance of the region for innate immunity and NK cell function. The identities of several loci have been identified. For example, *Ly49d* was identified as the *Chok* locus and more recently *Nkg2d* has been implicated to be important in diabetes (Melanitou et al., 1998).

The *Ly49* gene family encodes disulfide-linked homodimeric type II receptors, which are expressed in overlapping subsets of the total NK cell population and some T cell subpopulations. So far, most Ly49 receptors specifically bind to classical MHC class I molecules. While most Ly49 family members encode inhibitory Ly49 receptors,

which contain an ITIM motif in the cytoplasmic region and deliver inhibitory signals via the SHP-1 or SHP-2 tyrosine phosphatase, some receptors, including Ly49D and Ly49H, can generate activation signals for NK cells due to the lack of an ITIM motif in their cytoplasmic domain. Instead, activating Ly49 receptors, Ly49D and Ly49H, possess a positively charged amino acid, arginine, in their transmembrane domains. Arginine can associate with the DAP12 adaptor molecule that has negatively charged amino acids in the transmembrane region (Smith et al., 1998). DAP12 possesses an ITAM motif in the cytoplasmic region and delivers activation signals through the recruitment of Syk and ZAP70 tyrosine kinases.

Three independent groups identified that *Cmv1* encodes Ly49H based on both genetic and functional analyse (Lee et al., 2001), demonstrating that Ly49H is responsible for MCMV resistance. Two groups took advantage of inbred strain BXD8 mice that derived from $Cmv1^r$ C57BL/6 and $Cmv1^s$ DBA/2 parents. This strain is particularly interesting since it is highly susceptible to MCMV infection despite having a C57BL/6 haplotype at *Cmv1*. Extensive investigation of the susceptibility in the BXD8 mice using genetic linkage analysis, in vivo genetic complementation and genomic DNA analysis demonstrated that a deletion of 23 kb encompassing *Ly49h* in BXD8 mice is responsible for their susceptible phenotypes. This was further corroborated by in vivo depletions of *Ly49h* positive NK cells using an antibody specific to Ly49H that renders C57BL/6 mice susceptible.

Ly49H is exclusively expressed in approximately half of the NK cell population in resistant C57BL/6 mice. The activation of Ly49H by monoclonal antibodies resulted in calcium mobilization, cytokine production and the killing of target cells, suggesting that stimulation of $Ly49H^+$ NK cells by an MCMV infection-related ligand is critical to

the NK function in MCMV resistance (Daniels et al., 2001). Using a Ly49H/DAP12 transfected T hybridoma reporter system, a mouse cytomegalovirus (MCMV) protein, m157, glycoinositol phospholipids (GPI)-linked protein structurally related to non classical MHC, was identified to be the MCMV infection-related ligand binding to Ly49H (Arase et al., 2002).

1.7. Transgenic mice expressing Ly49H

To formally demonstrate the role of Ly49H in the resistance to MCMV infection, transgenic mice expressing Ly49H were generated by introducing BAC clones 128D23 into the FVB susceptible background (Lee et al., 2003). The BAC clone 128D23 is located in *Ly49* gene cluster where it overlaps the 23-kb deletion identified in the BXD-8 strain (Lee et al., 2001). 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/EMBL/DDBJ accession no. AC090127).

Based on RNA and protein expressions of the *Ly49h* transgene among founder animals, one line Tg832 was identified to show Ly49H expressions similar to that of C57BL/6 among many founders, showing about 45% of Ly49H expression on NK cells by FACS analysis. This line also presented splenic viral titers comparable to those of resistant C57BL/6 (10^2 PFU/spleen). Initial characterization of the transgenic mice indicated that identical immune responses in the context of Ly49H to wild type mice could be reproducible in the transgenic mice. First, a similar decrease in virus titer was observed in B6 and transgenic mice, indicating that virus replication is controlled by

Ly49H between days 1 and 2 in both mice. Second, specific amplification of Ly49H NK cells identified in B6 mice during MCMV infection was also observed in the transgenic mice. At 7 days post infection, almost 70% of NK cells are Ly49H positive compared with 44% in uninfected mice. Even though other two functional genes, *Ly49d* and *Ly49i*, were introduced into transgenic mice together with *Ly49h*, these genes have been excluded for any role in resistance to MCMV infection by previous functional analysis. Since *Ly49h* is the only identified gene responsible on MCMV resistance, these results demonstrated that the transgenic mice can serve as useful models to investigate the role of Ly49H in NK mediated resistance to MCMV infection (Lee et al., 2003; Salazar-Mather et al., 1998).

1.8. Microarray technology and Data analysis

1.8.1. Microarray technology

With the completion of the full genomic sequence of many organisms, microarray technology provides a powerful approach to study global gene expression patterns. Traditional methods for analyzing gene expression, such as Northern blot analysis, differential RNA display, and RT-PCR, can only analyze one, or a few, genes at a time, offering limited insights into the complex nature of cellular processes. In contrast, DNA microarray is a much more powerful tool in studying genome wide gene expression, providing an opportunity to obtain the expressions of numerous genes with a single experiment.

There are two basic types of array technology (Figure 1.2): spotted microarrays, in which pre-synthesized single-strand or double-strand DNAs are bound, or printed,

onto glass slides and high-density oligonucleotide arrays, in which sets of oligomers are synthesized in situ on glass wafers using a photolithographic manufacturing process. Although both types of arrays can produce comprehensive expression profiles, there are some fundamental differences between the two approaches (Harrington et al., 2000).

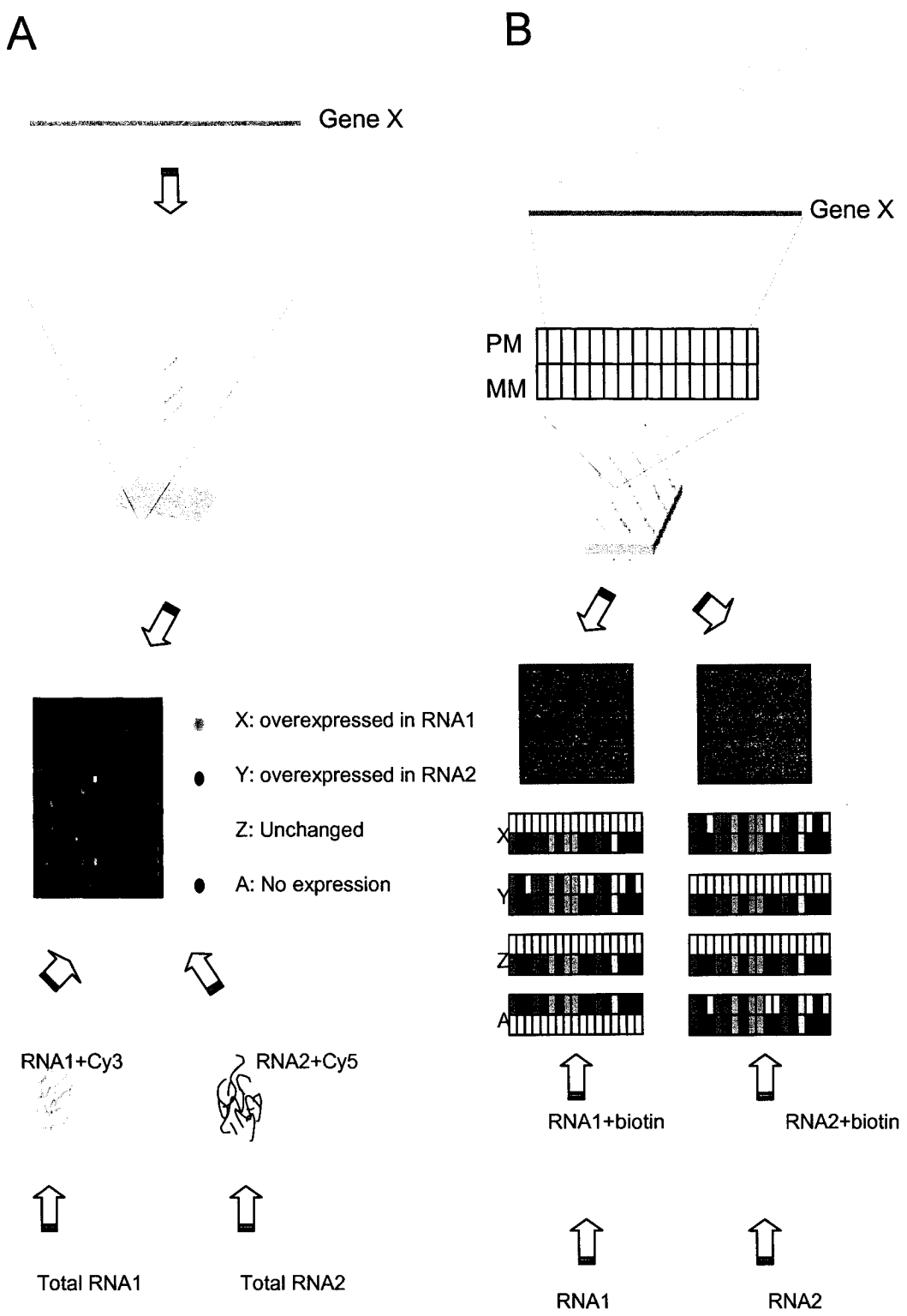
On spotted arrays, single DNA fragments that are greater than several hundred base pairs in length are amplified by PCR and printed at specified sites on glass slides using high precision arraying robots. RNAs from control and experimental samples are reverse transcribed to cDNA and labelled by incorporating fluorescently tagged nucleotides. Two different fluorophores (generally Cy3- and Cy5-dUTP) are used to label cDNA and are hybridized to the array. Relative amounts of a particular gene transcript in the two samples are determined by measuring the signal intensities detected for both fluorophores and calculating signal ratios. Spotted microarrays can be produced in-house or accessed through commercial service providers.

On high density oligonucleotide arrays (Affymetrix, Inc, Santa Clara, CA) a gene is represented by 15–20 different 25-mer oligonucleotides that are unique and sequence-specific. The use of mismatch (MM) control oligonucleotides that are identical to their original sequences (perfect match (PM)) except for a single base difference makes specific detection for a corresponding gene. Cross-hybridization of this MM control is considered as a background and is subtracted from the PM signal. In the high density oligonucleotide arrays sample mRNA is reverse transcribed to oligo-dT-primed cDNA, converted to biotinylated cRNA, and detected by staining with a fluorescent dye coupled to streptavidin. Each sample is hybridized to a separate array. Differences in mRNA levels between samples are determined by comparison of any two hybridization signal

intensities produced on separate arrays of the same array type (http://www.affymetrix.com/technology/ge_analysis/index.affx).

Figure 1.2 Schematic overview of array preparation and expression assay for spotted cDNA microarrays and high-density oligonucleotide microarrays (modified from reference 1616). A. Spotted cDNA microarrays Array preparation: Amplified DNAs by PCR are printed on glass using high-precision arraying robots. Target preparation: RNA from two different samples is used to synthesize single-stranded cDNA in the presence of nucleotides labelled with two different fluorescent dyes (Cy3 and Cy5-dUTP). Both samples are mixed and hybridized to the array surface resulting in competitive binding of differentially labelled cDNAs to the corresponding array DNAs. Scanning of the array with two different wavelengths provides relative signal intensities for two dyes and ratios of mRNA abundance for the genes represented on the array.

B. High-density oligonucleotide microarrays Array preparation: Array is produced by in situ synthesis (using photolithography) of 15–20 short oligonucleotides chosen from the mRNA reference sequence of each gene. Target preparation: RNA from different samples is reverse transcribed to double-stranded cDNA, in vitro transcribed to biotin labelled cRNA and detected by fluorescent dye conjugated with stretavidin. Signal intensities of probe array element sets on different arrays are used to calculate relative mRNA abundance for the genes represented on the array. X, Y, Z, and A indicate that genes which are upregulated, downregulated, no change, and no expression in sample RNA1 compare to RNA2.


A
B
Gene X
Gene X
PM
MM
X: overexpressed in RNA1
Y: overexpressed in RNA2
Z: Unchanged
A: No expression
X
Y
Z
A
RNA1+Cy3
RNA2+Cy5
RNA1+biotin
RNA2+biotin
Total RNA1
Total RNA2
RNA1
RNA2

1.8.2. Normalization of data

Microarray experiments generate a substantial amount of experimental variation, making it difficult to identify specific biological interests. In particular, systematic variations between experimental conditions (technical variations) can bear no relation to the biological differences between experiments. The normalization of data can help to minimize these technical differences between chips and reveal more clearly biological differences between samples.

The most common approach to normalize microarray data is total intensity normalization (Hackl et al., 2004). This is based on the simple assumption that equal quantities of RNA for each sample were used for starting material. For examples, if the sizes of the RNA molecules are comparable, the number of RNA molecules should also be the same in each sample. Therefore, the same number of labelled molecules from each sample should hybridize to the arrays and, if all other conditions were equal, the total hybridization intensities summed over all probes in the arrays should be the same for each sample.

1.8.3. Data analysis

It has been known that the variability of microarray results can be significant, especially for genes with low expression levels, and replication is needed to gain confidence in the data. Replication has been shown to reduce markedly the number of potential false positive results. However, it is very difficult to perform multiple replicates because of high costs or limitations of the sample amount. Scientists have had

to supplement microarray data with a variety of other analytical method in order to use its findings optimally.

A simple older method for finding interesting genes in experiments is to compare expression profiles of two or more samples for differentially expressed genes by their fold changes. Scientists can decide fold change depending on their experimental model (typically 2 to 4 fold). However, in order to detect important genes that may have small, but reproducible, changes in expression, scientists need statistically designed experiments and data analyses that produce not only estimates of relative expression, but also error bars for those estimates. Error bars provide a basis to decide which features in the data likely represent interesting biology and which are likely to have arisen by chance. The calculation of a t-statistic (such as student t-test and ANOVA) is the most applied method. Following this, the combination of the analysis of fold change and statistical analysis was found to offer a more restricted list of the important genes involved in an experimental model (Quackenbush, 2002). In other analysis, clustering analysis has been used to group together genes with similar patterns. This provides interesting or novel genes that are controlled by the same transcriptional factors or are in the same biological pathway, since there is a tendency of redundant representation of genes clustered together and we know that genes of similar function cluster together (Eisen et al., 1998). Normally, the lists of genes or the gene clusters gene are studied by analysis of individual genes. However, this makes it difficult to put them into a meaningful biological framework. Some methods have been resolved this problem successfully; Mapping of gene expression data onto sequences, finding a common potential regulator, or integrating genes into pathway information. Scientists have also used the ontology made available by the Gene Ontology (GO) consortium, which

organizes information for molecular function, biological processes and cellular components for a number of different organisms in order to construct a functional categorization of gene expression data. Several tools, such as DAVID, FatiGO, GOminer, and Mappfinder are available to perform an interpretation of the list of differentially expressed genes by GO automatically (Osier et al., 2004). Because of the complexity of the data sets generated by microarray experiments, the use of data-analysis software is essential. Several data-analysis tools have been developed by commercial suppliers (such as GeneSpring from Silicon Genetics, and Stratagene), and others are available from many public sources (such as http://genome-www4.stanford.edu/MicroArray/SMD/ restech.html).

1.8.4. Validation of microarray data

The biggest consideration in using DNA microarrays is the fact that the regulation of mRNA levels is only one aspect of biological control. Protein levels are also controlled at several post-transcriptional levels, and protein activity is controlled by post-translational modification. It is also possible that certain DNA elements on the array simply fail to detect the right transcript species, as a result of cross-hybridization or secondary structure, which leads them to be classified as unchanged genes. Therefore, results from a microarray experiment should be properly validated by alternative methods such as northern hybridization, RNase protection or RT-PCR, western blot, Flow Cytometry analysis (FACs), RNAi, or gene knockout experiments.

1.9. Osteopontin

As the name indicates, *Opn* was originally identified to encode a protein that is produced by osteoblasts and abundant in bone (Reinholt et al., 1990). OPN is expressed on many different cell types such as osteoclast, osteoblasts, kidney and nerve cells. OPN is also expressed on activated immune cells such as T cells, NK cells, and macrophages (Denhardt and Guo, 1993).

In bone, it facilitates the attachment of osteoclasts to the bone matrix via an interaction with cell surface molecules, such as integrin and CD44 (Weber et al., 1996). Accordingly, mice deficient in the *Opn* gene have a subtle abnormal bone phenotype, with delayed and impaired bone resorption. However, recent papers have indicated that the expression of *Opn* is not restricted to the bone but extends to the immune system.

In the immune system, *Opn* plays a critical role in the induction of the Th1 immune response. OPN is a phosphorylated glycoprotein secreted by activated macrophages, T lymphocytes, NK, and NKT cells. This cytokine has been reported to mediate important cellular matrix, and cell-cell interactions. In particular, the roles of OPN in chemotaxis, leading to the migration of macrophages and dendritic cells to the sites of inflammation have been demonstrated (Weber et al., 2002). The activation of T lymphocytes results in an increase in OPN transcription, hence its alternative designation as *Eta-1* (Weber et al., 1996). Importantly, $OPN^{-/-}$ mice have shown increased susceptibility to Herpes Simplex virus-type I and *Listeria monocytogenes* infection (Liaw et al., 1998).

1.10. Statement of objectives

The main objective of my masters project was to initiate the functional dissection of the role of Ly49H in NK mediated antiviral immunity against MCMV by taking advantages of MCMV-resistant mice, FVB-Tg (*Ly49h*), and their MCMV-susceptible counterpart, FVB mice.

The specific aims of this thesis were:

1) The analysis of phenotypic differences of FVB-Tg (*Ly49h*) compared to wild type, FVB, and C57BL/6 mice in order to characterize this transgenic mouse model and role of *Ly49h* in viral replication and the formation of inflammatory foci.

2) The comparative global gene expression analysis of splenic $DX5^+$ cells from FVB-Tg (*Ly49h*) and FVB mice at 1.5d MCMV infection, in order to identify genes critical in the early control of virus replication.

3) The analysis of global gene expression patterns in splenic $DX5^+$ cells of FVB-Tg (*Ly49h*) at 0d, 3d, 5d and 8d MCMV infection to investigate the roles of NK cells during MCMV infection.

4) The investigation of the role of *Opn* genes in MCMV resistance using *Opn* knockout mice.

CHAPTER TWO

MATERIALS AND METHODS

2.1. VIRUS

The Smith strain of MCMV, originally purchased from the American Type Culture Collection (ATCC, Rockville, MD), was used in all the experiments. Following this purchase, we prepared stock virus. 19-21 day old weaning BALB/c mice (10 female BALB/c mice) were infected with 5×10^3 Plaque Forming Units (PFU) of MCMV. After 17 to 21 days of infection, the mice were sacrificed and their salivary glands were removed and pooled in a 50 ml tube containing 20 ml of Dulbecco Minimal Essential Medium (DMEM; Gibco/BRL). The harvested salivary glands were washed once with DMEM, added in an equal volume of DMEM containing 20% heat-inactivated Fetal Bovine Serum (FBS) (Gibco/BRL) and 2 × Pen/Strep antibiotics (7 ml for 10 mice). This mixture was homogenized for 10 sec at speed 15 using a Polytron homogenizer (Model: PT-MR 2100, Switzerland). Centrifugation was performed at 3000 rpm for 15 min at 4°C and a supernatant was aliquoted, and stored as master stock in liquid nitrogen. In order to obtain the working stock (of higher virulence), the same procedure was repeated and the final solution was stored in 200 µl aliquot as working stock in liquid nitrogen. For virus titration, one vial from the working stock was thawed rapidly in a 37°C water bath and serially diluted 10-fold in DMEM containing 2% of FBS. PFUs of these diluted viruses were determined in triplicate by standard plaque assay as described below.

2.2. MICE AND INFECTION

C57BL/6, FVB/N and 129/J were purchased from either The Jackson (Bar Harbor, ME) or Charles River (Wilmington, MA) Laboratories. *Ly49h* transgenic mice

were obtained using previously described methods (Lee et al., 2003). Osteopontin knockout mice were provided by Dr. Hakim (Neurological Center, Ottawa) (Rittling et al., 1998). Four $Opn^{-/-}$ mice and wild type mice were obtained from The Ottawa Heart Institute and transferred to the animal care facility at the Faculty of Medicine, University of Ottawa. 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. For infection, 6-9 week old mice were intraperitoneally injected with a volume of 0.2 ml in PBS containing 5×10^3 PFU of working stock MCMV. All comparison groups of mice were age-and sex-matched.

2.3. MOUSE EMBRYONIC FIBROBLAST (MEF) PREPARATION

Female BALB/c mice were sacrificed at 14 to 16 days of pregnancy, and embryos were placed on sterile 80 mm Petri dishes containing Phosphate Buffered Saline (PBS). Organs such as the liver, heart and blood were removed to the best of possibility, and remaining embryo particles were finely minced to about 3 mm diameter. After washing with PBS three times, chopped embryos were trypsinized with 0.25% Trypsin-EDTA (Gibco-BRL) at 4°C overnight. The next day, excessive trypsin was removed on ice carefully, and embryos containing residual trypsin were incubated at 37°C for 20-30 min. Warm DMEM (1 ml for 100 mg) containing 10% FBS, 1 × Pen/Strep and 25 mM Hepes was added and embryos were gently dispersed by pipetting up and down. Cells were centrifuged for 10 min at 500 g and resuspended in 2 ml DMEM containing 10% FBS per embryo. Cell concentration was determined and cells

were seeded at 1×10^5/ ml in DMEM on 14 cm tissue culture dishes. When cells were semi-confluent in 2 to 5 days, the cells were removed by trypsinization, centrifuged, and resuspended in DMEM containing 20% FBS and 10% Dimethyl Sulfoxide (DMSO) at a concentration of 1 to 2×10^6 cells/ml. Aliquots were stored in liquid nitrogen or at -80°C.

2.4. MCMV PLAQUE ASSAY

Two mice per group in each experiment were used for MCMV plaque assays after three days of MCMV infection to study the effect of Ly49H in different organs after MCMV infection. Three different experiments were used for data analysis. The average of six mice was used for representative results. Organs (spleen, liver, lung, heart, kidney, bone marrow, thymus, and salivary gland) were aseptically harvested, weighed, and then individually homogenized in ice cold DMEM containing 2% FBS, using a Polytron homogenizer. After clarification, by centrifugation at 2,000 g for 20 min at 4°C, supernatants were serially diluted for plaque assay. In microarray experiments, three to five mice were used for plaque assays in each experiment. Spleens were aseptically harvested and a 1/5 portion was taken from each spleen for plaque assay. The average of log PFUs was used for representative results. For the Osteopontin experiments, whole spleens and livers were used for plaque assays. The results represent the average of log PFUs from 3 to 5 mice per each group.

Frozen MEF cells were thawed and rinsed with DMEM medium supplemented with 1 × Pen/Strep and 10% heat inactivated FBS (10% FBS). Cells were grown for 3 days until they reached confluence. At 1 day before the plaque assay, MEF cells were

seeded in a 24 well tissue culture plate at 1.6 x 10^5 cells/ml in DMEM (10% FBS). After incubation for 24 h at 37 °C in a 5% CO_2 incubator, the monolayers were washed once with DMEM containing 2% FBS, and infected with 0.2 ml of serial dilutions of organ homogenates (1/2, 1/10, and 1/100). The virus was allowed to adsorb for 60 min. at 37 °C in a 5% CO_2 incubator, and then DMEM containing 1% low-melting agarose (Gibco-BRL) and 10% FBS were overlaid onto the plates. Plaques were developed for three days. After three days, cells were fixed with 10% formalin for 10 min and MCMV plaques were revealed by staining with 1% methylene blue in 70% ethanol.

2.5. TAIL DNA EXTRACTION AND GENOTYPING

Tail DNA was extracted using the modified alkaline lysis method (Truett et al., 2000). Briefly, a 2-mm tail biopsy was incubated in 600 ml 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 s and neutralized by adding 50 ml of 1 M Tris (pH 7.0). Aliquots of 2 µl of this solution were used for PCR reactions. SV151 was used to detect the transgene *Ly49h* in FVB mice. The sequences were SV151-F, 5′-gtg cta cca ctg aaa acc att g-3′, SV151-R, 5′- ctg tct ctt gag tca cct gca c-3′.

For Osteopontin knockout mice, genotyping was performed using three primer pairs to determine the genotypes of the *Opn* and NKC region. For *Opn* genotypes, a wild type allele was analyzed with the primers, Eta1-F, 5′- acc att cgg atg agt ctg atg-3′ and Eta1-R, 5′- cct gct taa ccc tca cta aca-3′, and a knockout allele with the primers, Eta1-F, 5′- acc att cgg atg agt ctg atg-3′ and PGK-1-F, 5′-agc ggg aag gga ctg gct gct a-3′. For

NKC genotyping, DNA was analyzed with the primers, SV143-F, 5′- gct tac ata cat aca att gc-3′ and SV143-R, 5′- gtc aat aaa cag tat cct ca-3′.

2.6. HISTOLOGY

Spleen, liver, kidney, lung, thymus, and heart samples were harvested at 3 days of MCMV infection with 5×10^3 PFU from 2 mice of C57BL/6, FVB, and FVB-Tg (*Ly49h*). A small piece of each sample (1/10 of whole organ) was fixed in 10% neutral buffered formalin. Fixed tissues were sent to the lab of Dr Douglas Frank at the University of Ottawa. They paraffin embedded tissues and stained them with haematoxylin and eosin (H&E). Briefly, paraffin-embedded tissue slides were deparaffinised and rehydrated in the following order, toluene (VWR#CAT07353), 100% ethanol, and deionized water. These slides were stained with filtered haematoxylin first for 5 min, and washed with water. Slides were dipped in acid ethanol (1 ml concentrated HCl + 400 ml 70% ethanol) for a few seconds and washed again. After staining with eosin for 5 min, slides were dipped in 100% ethanol, xylene until they cleared, and cover slipped. H&E stained slides were analyzed microscopically. The nuclei of the cells stain bluish and the cytoplasms of the cells stain pink. Inflammatory foci, defined as discrete clusters containing between 6 and 60 nucleated cells, were identified to observe histological differences in mice. They were also quantified under microscopy at a magnification of 100, as clusters of cells in $8 \times 1\text{mm}^2$ per slide. Slide pictures were taken at magnifications of 40 and 100.

2.7. $DX5^+$ CELL ISOLATION

To isolate splenic mononuclear cells, the spleen was ground between rough surfaces of glass slides and lysed with Red Blood Cell Lysing Buffer (Sigma-Aldrich) to remove red blood cells individually. 3 to 5 spleens were pooled in one tube to increase NK yield. NK cells were enriched from splenic leukocytes by magnetic positive separation using anti-DX5 microbeads following the manufacturer's protocol (MACS®; Miltenyi Biotec). Cell suspensions were centrifuged at 300g for 10 min and cell pellets were resuspended in 90 µl of buffer (PBS, 0.5% BSA, and 2 mM EDTA) per 10^7 cells. 10 µl of CD49b (DX5) microbeads per 10^7 cells were added into cell suspension and it was incubated for 15 min at 4 °C. Cells were washed, centrifuged again, and resuspended up to 10^8 cells in 500 µl of buffer. LS MACS columns were placed in the magnetic field of MACS separator. Columns were prepared by rinsing with 3 ml of buffer and cell suspensions were applied onto the column. Only the labelled cells were attached onto the column. Columns were removed from separator and immediately flushed out with 5 ml of buffer to obtain labelled $DX5^+$ cells. 2×10^6 cells were used for FACS analysis to see purity of cells and 70 to 80 % of $DX5^+$ cells were obtained.

2.8. FLOW CYTOMETRIC ANALYSIS (FACS)

DX5 and Ly49H expressions were analyzed in every experiment in this thesis to see their proper expression in mice. The freshly purified $DX5^+$ NK cells were stained for surface expression of DX5 using PE-conjugated murine anti-DX5 (Pharmingen). Cells were washed, fixed, and permeabilized for 25 min at room temperature in the presence of 4% paraformadehyde and 0.1% saponin in PBS buffer. After two washes in the presence of 0.1% saponin, the cells were stained with the antibody for the cytoplasmic

tails of Ly49H, for 30min at room temperature. FITC-conjugated anti-rabbit IgG (Pharmingen) were used as a secondary antibody for the detection of polyclonal anti-Ly49H that was previously bound. For Osteopontin knockout mice flow cytometric analysis, Six F2 mice were used. Single-cell suspensions were prepared by liberase (Roche Applied Science, Indianapolis, IN) treatment into spleens to increase yield of dendritic cells (Martin et al., 2000).1 $\times 10^6$ of splenocytes were preincubated on ice for 30 min with Fc blocker (Pharmingen, San Diego, CA) to reduce non-specific staining. Lymphoid DCs were detected by staining with FITC conjugated -anti-Cd11c and PE conjugated-anti-CD8α (Pharmingen, San Diego, CA). Cell staining was analyzed by flow cytometry on an Epics analyzer (Coulter, Hialeah, FL).

2.9. MICROARRAY ANALYSIS

Total RNA was prepared from freshly isolated $DX5^+$ cells using Trizol reagent and quality was assessed on the 1.2% formaldehyde agarose gel electrophoresis. 10 ug of total RNA for MG U74A chips and 200 ng of total RNA for MOE430A chips were used for the experiments. The labelling, hybridization and reading in microarray experiment was performed with in the collaboration with the Ottawa Genome Center. RNA quality was assessed using Agilent Bioanalyser 2100. Oligonucleotide array hybridization and scanning were performed according to Affymetrix® (Santa Clara, California) protocols in the Ontario Genomics Innovation Centre at Ottawa Cancer Center. In brief, for U75A chips, 10 μg of total RNA from the splenic $DX5^+$ cells of FVB-Tg (*Ly49h*) and FVB at 1.5 days of MCMV infection were used for first-strand cDNA synthesis with T7-linked oligo-dT primers. 200ng of total RNA form the splenic $DX5^+$ cells of FVB-Tg (*Ly49h*) at 0, 3, 5, and 8 days of MCMV infection for MOE430A

chips, were used for first-strand cDNA synthesis with T7-linked oligo-dT primers. For MOE430A chips, Eukaryotic Small Sample Target Labelling method was processed. 50 ng of total RNA was used for two cycles of cDNA synthesis. In vitro transcription (IVT) reactions were performed in batches to generate biotinylated cRNA targets, which were chemically fragmented at 95°C for 35 minutes. Fragmented biotinylated cRNA (10 μg) was hybridized at 45°C for 16 hours to Affymetrix® high density oligonucleotide array mouse MG U74A and MOE430A chips, which contains 15000 and 22000 probe sets representing well-substantiated mouse genes. The arrays were washed and stained with streptavidin- phycoerythrin (SA-PE, final concentration of 10 μg/ ml). Signal amplification was achieved using a biotinylated anti-streptavidin antibody. The array was scanned according to the manufacturer's instructions (Affymetrix Genechip® Technical Manual, 2001). Scanned images were inspected for the presence of obvious defects (artefacts or scratches) on the array. To minimize discrepancies due to variables such as sample preparation, hybridization conditions, staining, or array lot, the raw expression data was scaled to a target intensity of 1500 using Affymetrix® Microarray Suite 5.0 software. The average difference for each probe set is an average of the differences between the "perfect match" (PM) and control "mismatch" (MM) probe intensities and is directly related to the level of expression of the transcript. A comparative analysis of the expression profiles of samples was carried out on GeneSpring™ software version 6.0 (Silicongenetics, Redwood, California). The text file data generated from The Ontario Genomics Innovation Centre were imported into GeneSpring and data was normalised in two ways: "per chip normalisation" and "per gene normalisation". For "per chip normalisation", all expression data on a chip is normalised to the 50th percentile of all values on that chip. For "per gene normalisation"

the data for a given gene is normalised to the median expression level of that gene across all samples. GeneSpring filtered a gene list MAS call flags (present or absent). If the gene was expressed as present at least once in each duplicate chip, it was retained in a gene list. Gene analysis by fold change (more than 2.5 fold change) was performed at normalized intensity between samples. Only genes expressing such a difference in duplicate or triplicate experiments were retained. The statistical comparison of expression between FVB-Tg (*Ly49h*) and FVB within each time point was done by applying a one way ANOVA test ($P< 0.05$) to the genes that had passed a 2.5-fold change filter, flag filter (present call in 1 of 2 samples) or individual group of genes generated by Venn diagram in time course experiments. Genes were regrouped by their molecular functions or cellular processes, using the annotation from Silicongenetics (Redwood, California) and http://david.niaid.nih.gov/david/beta/index.htm. A gene tree was made by clustering the genes that had been grouped by their annotation according to a standard correlation of their gene expression patterns.

2.10. RNA PREPARATION AND SEMI QUANTITATIVE PCR

Total spleen and $DX5^+$ cell RNA were prepared using the TRIzol reagent (Invitrogen) following the manufacturer's recommendations. RNA quality was confirmed using 1.2% formaldehyde agarose gel. The first-strand cDNA synthesis from extracted RNA was generated in a final volume of 50 µl using an MMLV Reverse Transcription System (Invitrogen). To perform PCR, specific primers were designed for the genes using sequences obtained from GenBank (Table 2.1). Semi-quantitative PCR

was performed normally with 22-30 cycles. Every 2-3 cycles, 5 μl of PCR products were taken for gel electrophoresis. *Gapdh* PCR was used for internal control.

Table 2.1 Primer used for semi quantitative PCR

Gene name	Accession No	Primer name	Sequence	T^a	size
m157 (MCMV)	AY228667	sinia-m157-F	act tcg gtc gcg ttc atg tat g	58	227bp
		sinia-m157-R	gga ttc gac gtt ata tgt gat g		
Osteopontin	X13986	Eta1-F	acc att cgg atg agt ctg atg	60	650bp
		Eta1-R	cct gct taa ccc tca cta aca		
Cd7	D31958	CD7-F	aga aga acc tga cca tca cc	57	409bp
		CD7-R	tgt aag ctc tgc tga aac aa		
AA657044	AA657044	AA657044-F	tga ggt tac tgg gag tga tg	57	256bp
		AA657044-R	cta ttg ggg tgt ttc tct tt		
CD160	AF060982	By55-F	tcc ttg gaa ctg ttc ccc tga	63	~500bp
		By55-R	cct gca atg tgg cag gca aac		
IFN alpha	x01974	IFNalpha-F	atg gct ag(ag) ctc tgt gct ttc ct	57	500bp
		IFNalpha-R	agg gct ctc cag a(ct)t tct gct ctg		
IFN beta	NM_010510	IFNbeta-F	cat caa cta taa gca gct cca	57	350bp
		IFNbeta-R	ttc aag tgg aga gca gtt gag		
IFN gamma	NM_008337	IFN-F	acg cta cac act gca tct tg	58	530bp
		IFN-R	ttc aaa tag tgc tgg cag aa		
MIP2 (Cxcl2)	X53798	MIP2-F	gaa caa agg caa ggc taa ctg a	58	203bp
		MIP2-R	aac ata aca aca tct ggg caa t		
Saa3	NM_011315	Saa3-F	ctg cta aag tca tca gcg atg c	58	~100bp
		Saa3-R	gcc cca ctc att ggc aaa c		
CD14	X13987	CD14-F	gga agc cag aga aca cca tcg	62	413bp
		CD14-R	gca ggg ctc cga ata gaa tcc		
TNF-alpha	NM_013693	TNF-a-F	ctt gtc tac tcc cag gtt ctc ttc	62	317bp
		TNF-a-R	aac acc cat tcc ctt cac aga gca		
Gapdh	XM_111622	GAPDH F	acc aca gtc cat gcc atc ac	58	500bp
		GAPDH R	tcc acc acc ctg ttg ctg ta		
DX5 (Itga2)	NM_008396	CD49b-F	ttc ccc tca tga taa tga aac c	55	103bp
		CD49b-R	gca gtc ata gcc aac agc aa		
Ly49h	NM_010650	Ly49H-1-F	agc ctc tta ggg gat aca gac	60	1kb
		Ly49H-R	tgt caa gat aga tag gag agg		

2.11. REAL TIME PCR USING SYBR GREEN

One-tube RT-PCR was performed using QuantiTect™ SYBR® Green RT-PCR (Qiagen) at 50°C for 30 min, 95°C for 15 min, and 45 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s. Primer sequences for *Opn* are; QTOPN-2-F, 5'-ctt tca ctc caa

tcg tcc cta c-3', QTOPN-2-R, 5'- gaa tgc tca agt ctg tgt gtt t-3' and for *Gapdh*; QTGAPDH2-F, 5'- cat ggc ctt ccg tgt tcc ta-3', QTGAPDH2-R, 5'- gcg gca cgt cag atc ca-3'. The expression of osteopontin (*Opn*) was determined in relation to *Gapdh* on an ABI Prism 7000 (Applied Biosystems). The quantification of the amplified product was done on a cycle-by-cycle basis via the acquisition of a fluorescent signal generated by binding of the fluorophore SYBRGreen (Qiagen) to double-stranded DNA. The difference in quantities of *Opn* mRNA from splenic $DX5^+$ cells between FVB and FVB-Tg (*Ly49h*) was calculated using a relative standard curve method based on user bulletin #2 of Applied Biosystems. Briefly, standard curves were prepared for both *Opn* and endogenous control *Gapdh*. For each experiment sample (FVB and FVB-Tg (*Ly49h*)), the amount of *Opn* and *Gapdh* was determined by the appropriate standard curve. Then, the *Opn* amount was divided by the *Gapdh* amount to obtain a normalized target value. The normalized *Opn* value was divided by *Opn* from FVB (calibrator) value to generate the relative expression levels. Following amplification, melting curves were acquired on the SYBR channel using a ramping rate of 1°C/60s between 75 and 99°C to see the specific amplification (one product) of each gene.

2.12. STATISTICAL ANALYSIS

The significance of the differences observed from viral titers in different organs, at day 3 MCMV infection among mice was assessed using ANOVA (Analysis Of Variance between groups) test. For microarray analysis, the statistical comparison of gene expression between FVB-Tg (*Ly49h*) and FVB within each time point was done by

applying a one way ANOVA test ($P<0.05$) according to the manual of GeneSpring software.

CHAPTER THREE

RESULTS

3.1. Phenotypic difference of MCMV resistance mice, FVB-Tg (*Ly49h*), compared to susceptible mice (FVB).

3.1.1. Determination of viral titers in 8 visceral organs after 3 days of MCMV infection

Since most lymphocytes are susceptible to CMV infection, CMV can replicate in almost all organs and cause many types of viral pathogenesis including hepatitis, myocarditis, pneumonia and retinitis (Stoddart et al., 1994). In an experimental model of MCMV, the dissemination of MCMV from sites of initial replication in the peritoneal cavity to various organs occurs via the blood stream. In resistant mice, MCMV is rapidly cleared from the visceral organs and later from the salivary glands.

During the acute phase of MCMV infection, NK cells have been reported to be critical for innate regulation in an organ-specific manner. NK cells regulate MCMV infection differently in the spleen and liver, which indicates that a different host defense mechanism maybe responsible for the control of MCMV replication in organs. For example, according to a study by Tay and Welsh (Tay and Welsh, 1997), NK cells primarily utilize perforin-mediated cytotoxicity to lyse virus infected cells in the spleen, but control infection in the liver with IFN-γ. The mouse locus *Cmv1* has been known to control early viral replication in the spleen; however, it has only a minor effect in the liver, suggesting the involvement of perforin in its function. Recent studies have identified an activating NK receptor, Ly49H, as *Cmv1* and support the critical role of a NK cell receptor in the perforin mediated control of MCMV infection in the spleen.

Transgenic mice (FVB-Tg (*Ly49h*)) with the susceptible FVB background have

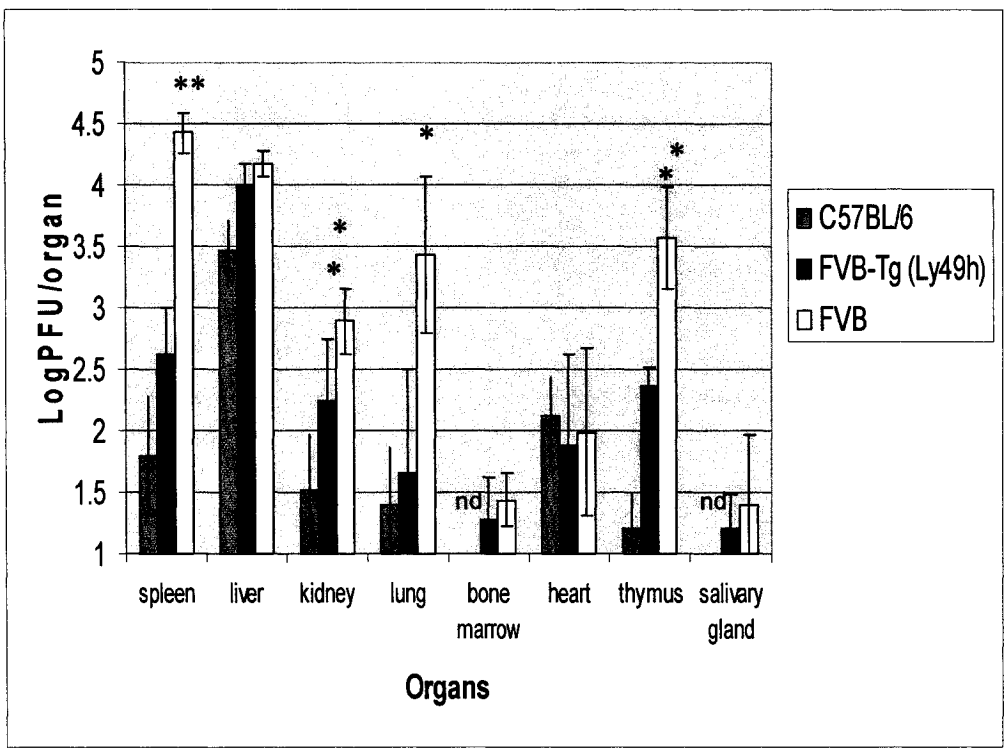
been generated (Lee et al., 2003) and have demonstrated the critical role of Ly49H in the clearance of MCMV infection in the spleen. However, the effect of Ly49H was not significant in controlling MCMV in the liver, consistent with the characteristics of *Cmv1* locus. To investigate the organ specificity of *Cmv1/Ly49h* in the control of MCMV, I tried to investigate the effect of *Cmv1/Ly49h* in most visceral organs of transgenic mice at day 3 post infection of 5×10^3 PFU MCMV in comparison with both resistant C57BL/6 (B6) and susceptible FVB mice (Figure 3.1).

As shown in the Figure 3.1, the virus titers in the organs of both B6 and FVB-Tg (*Ly49h*) mice (N=6 in each group), which express Ly49H, are significantly lower than those in their counterpart FVB mice. This result indicates that *Ly49h* controls MCMV replication significantly in most tested organs, including spleen (most affected), kidney, lung, and thymus. This result also indicated that the tissue specific effect of Ly49H in the spleen, lung, kidney and thymus can be observed independently of the genetic background because similar resistances were observed in both B6 and FVB mice with Ly49H expression.

A slightly more susceptible phenotype was observed in the spleens, kidneys, thymuses and lungs of FVB-Tg (*Ly49h*) mice compared with B6 mice. This can be explained by lower expression of Ly49H in the FVB-Tg (*Ly49h*). Previous investigation of the FVB-Tg (*Ly49h*) mice demonstrated weaker Ly49H expression in transgenic mice compared to B6 mice (44% versus 52% of $DX5^+$ cells based on FACS result) (Lee et al., 2003). In contrast, the *Ly49h* effect in the liver, bone marrow, and salivary gland at this stage is minor, as shown by the similar viral load among B6, FVB-Tg (*Ly49h*), and FVB. Interestingly, the viral replication in hearts seems not to be controlled by *Ly49h,* because the organ showed the similar viral load irrespective of the presence of *Ly49h* at day 3

MCMV post infection. Taken together, these results indicate that different host defense mechanisms are responsible for the control of MCMV replication in different organs, and Ly49H has a tissue specific effect independent of genetic background in spleen, lung, kidney, and thymus at 3 days of MCMV infection.

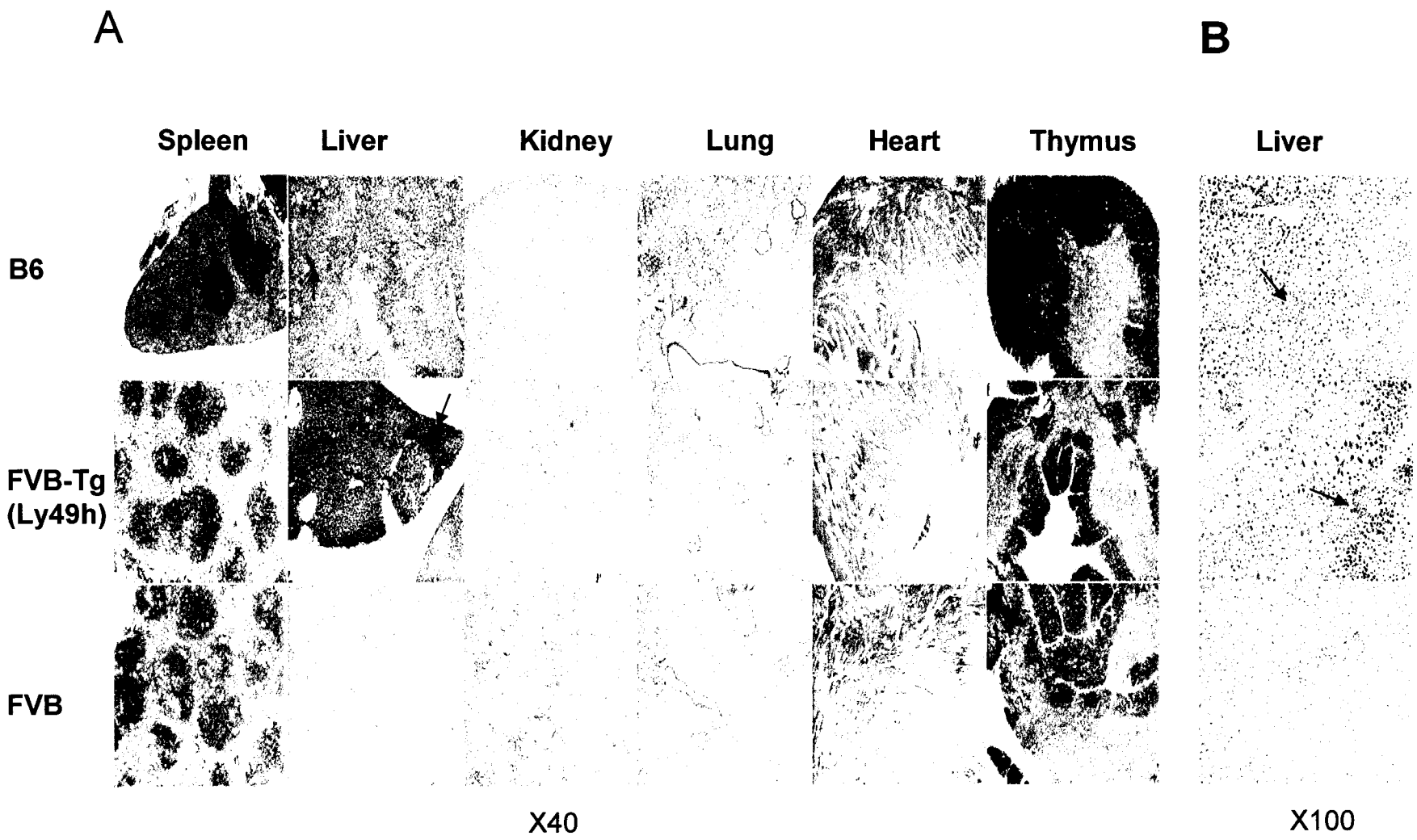
Figure 3.1 Phenotype assessments from visceral organs at 3 days post infection of MCMV. The virus titers from three independent experiments were integrated and present together. In total, six mice per group were used. Plaque assays were performed on eight visceral organs (spleen, liver, lung, kidney, heart, thymus, bone marrow and salivary glands) harvested after 3 days of MCMV infection. **: $p<0.001$, *: $p<0.05$. nd indicates samples where no plaques were detected.


5
4.5
4
3.5
3
2.5
2
1.5
1
LogPFU/organ
**
*
*
*
*
nd
nd
spleen
liver
kidney
lung
bone marrow
heart
thymus
salivary gland
Organs
C57BL/6
FVB-Tg (Ly49h)
FVB

3.1.2. Increased formation of inflammatory foci in the liver in FVB-Ly49H$^+$ mice

As an alternative parameter for phenotype against MCMV infection, I investigated the histological differences in organs to determine the effect of the presence of *Ly49h* at 3 days post-infection. For the histological analysis by H&E staining, the organs that had been used for the plaque assay were once again used. Searching only for inflammatory foci, we found that the significant difference between the 3 types of mouse tissue was the large number of inflammatory foci in the livers of mice expressing Ly49H. In these mice, the response to MCMV infection results in the formation of inflammatory foci characterized as discrete clusters of small, nucleated cells throughout the liver. Biron and colleagues have shown that inflammatory foci colocalize with viral antigens, and that 80% of the cells in the foci are NK cells (Orange et al., 1997). At 3 days of MCMV infection, the inflammatory foci in B6 and FVB-Tg (*Ly49h*) were clearly visible, and greater in number than in FVB mice in H&E staining (Figure 3.2). To quantify the MCMV-induced inflammatory changes, the total numbers of inflammatory foci per section were counted in 8 × 1mm^2 areas of liver sections. Whereas there were very few (2 to 5) inflammatory foci observed in FVB, 25 to 40 inflammatory foci were observed in FVB-Tg (*Ly49h*) and B6 mice. The determination of the total number of inflammatory foci in B6, FVB-Tg (*Ly49h*), and FVB indicate that the formation of inflammatory foci is dependent on the expression of Ly49H. Nevertheless, this analysis should be performed with more mice to demonstrate statistical significance, since I analyzed only 2 mice per group at this stage.

Figure 3.2 H and E staining of visceral organs after 3 days of MCMV infection
The same mice (two mice per group) used for plaque assay in figure 3.1 were evaluated for histological differences in various organs. Tissues were fixed in 10% formaldehyde and paraffin embedded. Inflammatory foci in the liver are clearly found and numerous in resistant mice whereas FVB show very few foci. Black arrows indicate representative inflammatory foci in the liver. Histological sections are shown at a magnification of A) ×40 and B) ×100.


A
B
Spleen
Liver
Kidney
Lung
Heart
Thymus
Liver
B6
FVB-Tg
(Ly49h)
FVB
X40
X100

3.2. Comparative analysis of gene expression of NK cells using Microarray technology

Because of the great advantages in analyzing the expression of thousands of genes at once, DNA microarray has been used to identify important genes that are differentially expressed in response to viral infections (Bolt et al., 2002). In an effort to better characterize the functionalities of NK cells and *Ly49h* in the response to MCMV infection, I conducted a comprehensive gene expression analysis using microarray technology with enriched NK cells (70-75% $DX5^{+}$) from Ly49H transgenic mice and their non-transgenic counterparts upon MCMV infection. Transgenic mice expressing Ly49H in a susceptible genetic background (FVB) provide a useful model for comparative microarray analysis, and provide an opportunity to study Ly49H function within an identical genetic background. A microarray experiment requires replicates to ensure the significance of differential gene expression. Moreover, in this model, in which NK gene expression is analyzed, the RNA amount from the NK cell population (nearly 3-5% of total splenic leukocytes) was very limited. Therefore, NK cells were purified from a pool of mice (5 to 10 mice per experiment) to prepare total RNA, and analyzed gene expression from duplicate chips per sample.

Since I purified NK cells from mice of FVB genetic background, of which NK1.1 expression is absent, DX5, another pan-NK marker present on NK cells in nearly all strains, was used to isolate NK cells from these animals.

3.2.1. Comparative gene expression analysis of splenic NK cells between FVB-Tg (*Ly49h*) and FVB mice at uninfected stage

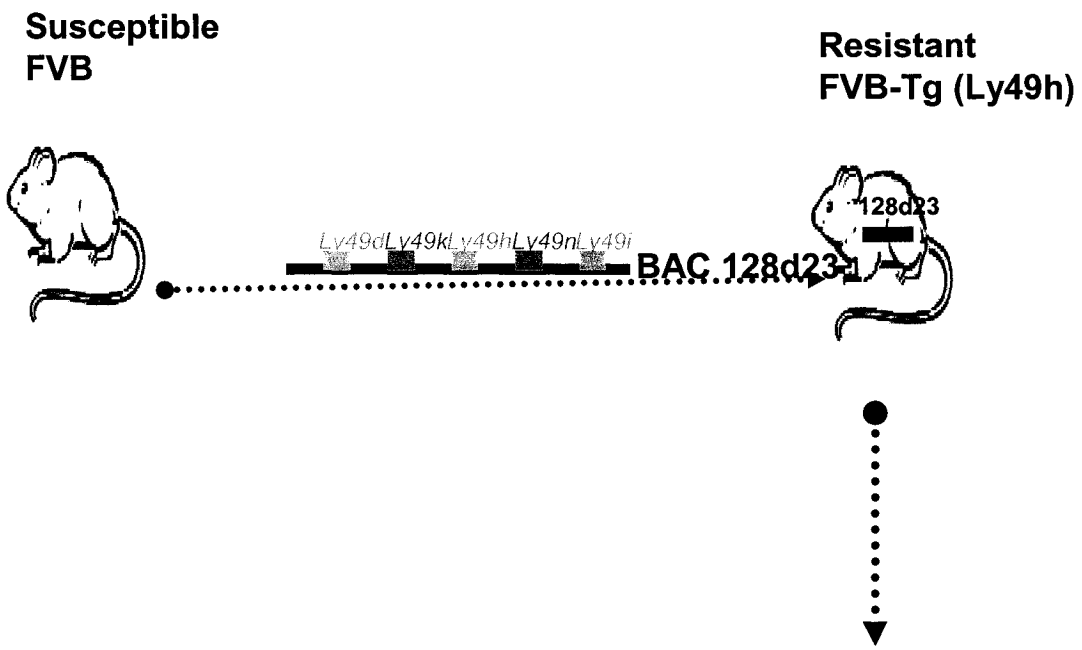
As stated in the Introduction, FVB-Tg (*Ly49h*) mice were generated by introducing 128d23 BAC DNA into FVB mice. This BAC DNA includes three functional *Ly49* genes, two activating receptors (*Ly49d* and *Ly49h*) and one inhibitory receptor (*Ly49i*), and two non functional Ly49 receptors (*Ly49n* and *Ly49k*). Before using these mice to analyze gene expression during MCMV infection, it was important to evaluate the gene expression of these mice without infection because transgenesis can cause many unexpected changes in the mRNA expression of unrelated genes due to the random incorporation of BAC DNA into the mouse genome. In addition, the appropriate expression of transgenes in the BAC construct should be confirmed.

A comparative analysis of the microarray results of the splenic NK cells obtained from uninfected mice of FVB-Tg (*Ly49h*) and FVB mice generated 8 genes, which changed their gene expression (Figure 3.3). As a consequence of 128d23 BAC introduction into FVB, *Ly49h* and *Ly49c/i* were highly expressed in FVB-Tg (*Ly49h*) by an 84 and 9.5 fold difference, respectively. The expression of *Ly49d* could not be assessed in this experiment since it was not included on the MOE430A chip, while *Ly49n*, and *Ly49j* are pseudogenes. Nevertheless, the expression of *Ly49d* in transgenic mice has previously been confirmed in RT-PCR analysis (Lee et al., 2003). Six genes were downregulated in FVB-Tg (*Ly49h*) mice: NADH dehydrogenase 1 alpha subcomplex 7 (*Ndufa7*), cyclin dependant kinase inhibitor 1B (*Cdkn1b*), metastasis associated 1 (*Mta1*), ribosomal protein S10 (*Rps10*), Metadherin (*Mtdh*), and one EST. None of these genes was identified to show any related function in NK cells according to a Pubmed search. However, there is strong evidence that these genes might be closely

related to each other. Metahedrin is a cell surface protein of breast tumors and is known to inhibit experimental lung metastasis (Brown and Ruoslahti, 2004). Metastasis associated 1 (Mta1) has been identified as a subunit of NURD (nucleosome remodelling and histone deacetylation), which contains not only ATP-dependent nucleosome disruption activity, but also histone deacetylase activity, which usually associates with transcriptional repression. It interacts with MAT1 (Menage A Trois-1), cyclin dependent kinase-activating kinase (CAK) complex ring finger factor, and has been suggested to be involved in the inhibition of CAK-induced transactivation function of ER by recruiting HDAC (Bowen et al., 2004). In addition *Rps12* (ribosomal protein S12) has been known to be cotranscribed with the gene NADH dehydrogenase alpha subcomplex3 in wheat and maize (Gualberto et al., 1988), suggesting that *Ndufa7* and *Rps10* might be related. They are both localized on chromosome 17.

Overall, very few genes were identified for their modulation in gene expression, upon the BAC integration into the mouse chromosome. These data showed that transgenic mice showed acquisition of a proper expression of transgenes, in particular *Ly49h*, without major perturbation in the expression of other genes, supporting the applicability of transgenic mice for microarray analysis.

Figure 3.3 Genes differentially expressed on NK cells from FVB and FVB-Tg (*Ly49h*) mice. Three functional genes (*Ly49d, h, i*) and two non functional genes (*Ly49f, n*) from C57BL/6 genes were introduced into a pronuclei egg of FVB. Duplicate microarray experiments were performed using transgenic FVB-Tg (*Ly49h*) and FVB wild type mice. Five mice per group were used in one experiment. Genes present on both experiments were applied for one way ANOVA test ($p<0.05$) using GeneSpring 6.2 and genes which were differentially expressed are listed. The fold changes between FVB and FVB-Tg (*Ly49h*) are indicated in red and blue, and chromosomal location is also indicated. A: Absent, P: Present on microarray data.



Probe Name	Gene	Gene bank #	Common name	FVB	FVB-Tg (Ly49h)	Fold change	p<0.05	Chromosome
Genes upregulated in FVB-Tg (Ly49h)								
1425417_x_at	Ly49h	U12889	killer cell lectin-like receptor, subfamily A, member 8	A	P	84	0.05	6
1425436_x_at	Ly49c/i	U49865	killer cell lectin-like receptor, subfamily A, member 3	P	P	9.5	0.00	6
Genes downregulated in FVB-Tg (Ly49h)								
1436567_a_at	Ndufa7	C88880	NADH dehydrogenase 1 alpha subcomplex, 7	P	A	3.8	0.02	17
1457285_at		BB354728	RIKEN cDNA 5730493B19	P	P,A	3.3	0.04	?
1434045_at	Cdkn1b	BB354528	cyclin-dependent kinase inhibitor 1B	P	P,A	3.3	0.03	6
1438723_a_at	Rps10	BI900577	ribosomal protein S10	P	P	3	0.05	17
1417295_at	Mta1	NM_054081	metastasis associated 1	P	P	3	0.01	12
1434882_at	Mtdh	AV083741	Metadherin	P	P,A	2.8	0.05	15

3.2.2. Comparative gene analysis of splenic NK cells from FVB-Tg (*Ly49h*) and FVB mice at early MCMV infection (at 1.5 days of MCMV infection)

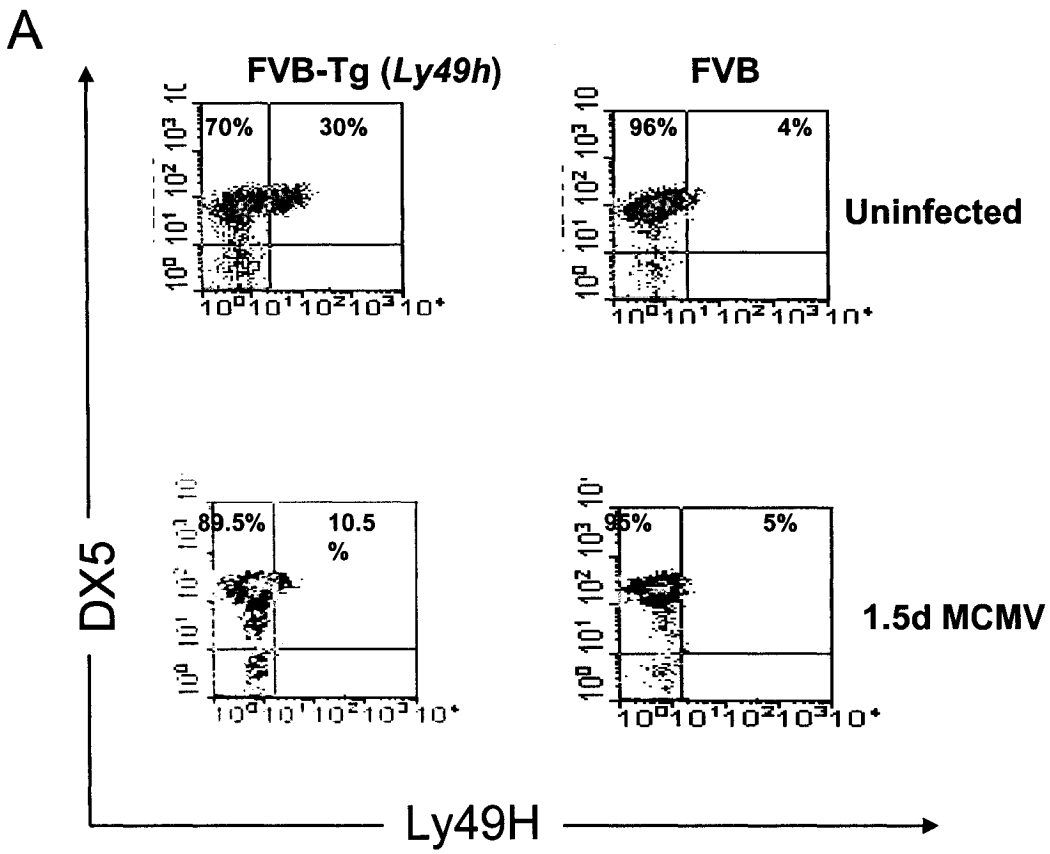
Cmv1/Ly49h controls MCMV infection at early stages in the spleen before adaptive immune responses are fully activated. To understand the roles of *Ly49h* in the early control of MCMV, I decided to compare NK gene expressions between FVB-Tg (*Ly49h*) transgenic mice and their wild type counterparts, FVB mice, by microarray analysis. Within 36 hours of experimental infection, a time point that is relevant to the activation of both innate and acquired host immune mechanisms, a two-fold difference of Log10 PFU was observed between the MCMV-resistant FVB-Tg (*Ly49h*) and the MCMV-susceptible FVB/N strain (Figure 3.4). This indicates that Ly49H-related mechanisms of host resistance are operative at this very early time point. In addition, after day 3, severe cellular destruction was observed in the spleens of susceptible mice, which prevented us from acquiring good quality RNA for microarray experiments.

To conduct the microarray experiments, NK cells were enriched by purifying $DX5^+$ cells from total splenocytes. A portion of purified cells was stained with antibodies for DX5 and Ly49H to evaluate NK purity and Ly49H expression. 70 to 75% of $DX5^+$ cells were obtained from total splenocytes and used for the preparation of RNA for microarray analysis. Compared to uninfected mice, $Ly49H^+$ subsets were decreased at 1.5 days of MCMV infection in FVB-Tg (*Ly49h*) mice, resulting in 11% of $Ly49H^+$ cells among total $DX5^+$ cells. At this time point, the active Mip-1α -mediated migration of splenic NK cells to the liver has been reported (Salazar-Mather et al., 1998). Possibly, this decrease of $Ly49H^+$ subset among $DX5^+$ NK cells may be due to the preferential

migration of Ly49H$^+$ NK cells since these cells can be activated through the recognition of MCMV-infected cells.

The comparison of normalized intensities on chips identified that 35 out of 15000 genes were differentially expressed more than 2.5 fold between FVB-Tg (*Ly49h*) and FVB (Table 3.1). The clustering of these genes by their functions and patterns of normalized intensity using GeneSpring 6.2 indicated four major groups (Figure 3.5): 1; the *Ly49* gene family. In line with the observation in uninfected samples, *Ly49h*, *Ly49d*, and *Ly49i* genes were all upregulated in FVB-Tg (*Ly49h*) following MCMV infection; 2) genes expressed on NK and T cells; 3) genes related to transcription, ubiquitination, and development, and 4) genes expressed during inflammation.

Figure 3.4 Expression of Ly49H correlates with control of MCMV viral titer at 1.5 days post-infection. A. Enriched NK cell preparations from spleens of FVB-Tg (*Ly49h*) and FVB at 0 and 1.5 days post MCMV infection 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 $Ly49H^+$. B. Replication of MCMV in the spleen of FVB-Tg (*Ly49h*) and FVB mice. Viral titers in the spleen of 5 mice per group were determined by plaque assay after 1.5 days infection with 5×10^3 PFU of MCMV.


A
FVB-Tg (Ly49h)
FVB
70%
30%
96%
4%
Uninfected
89.5%
10.5
%
95%
5%
1.5d MCMV
DX5
Ly49H

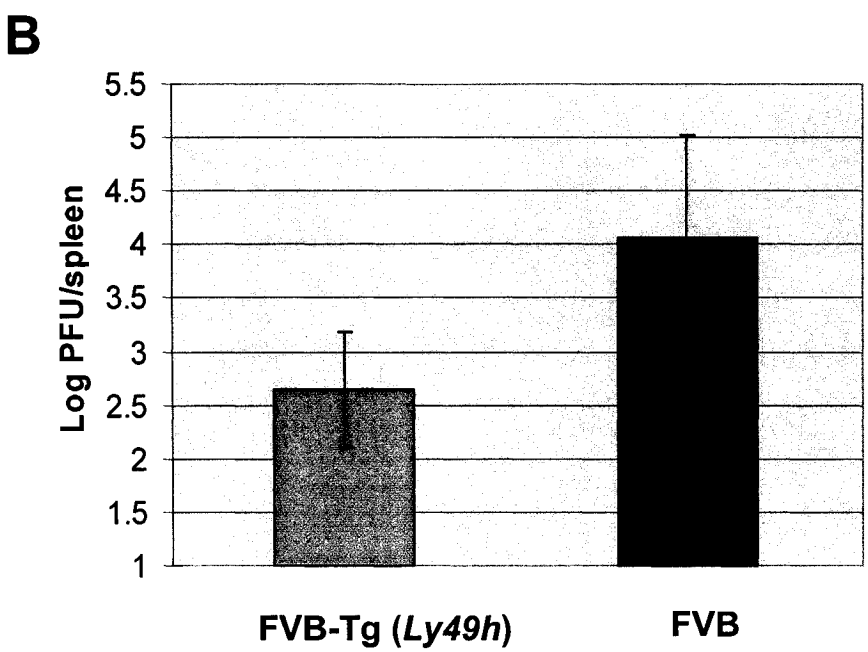
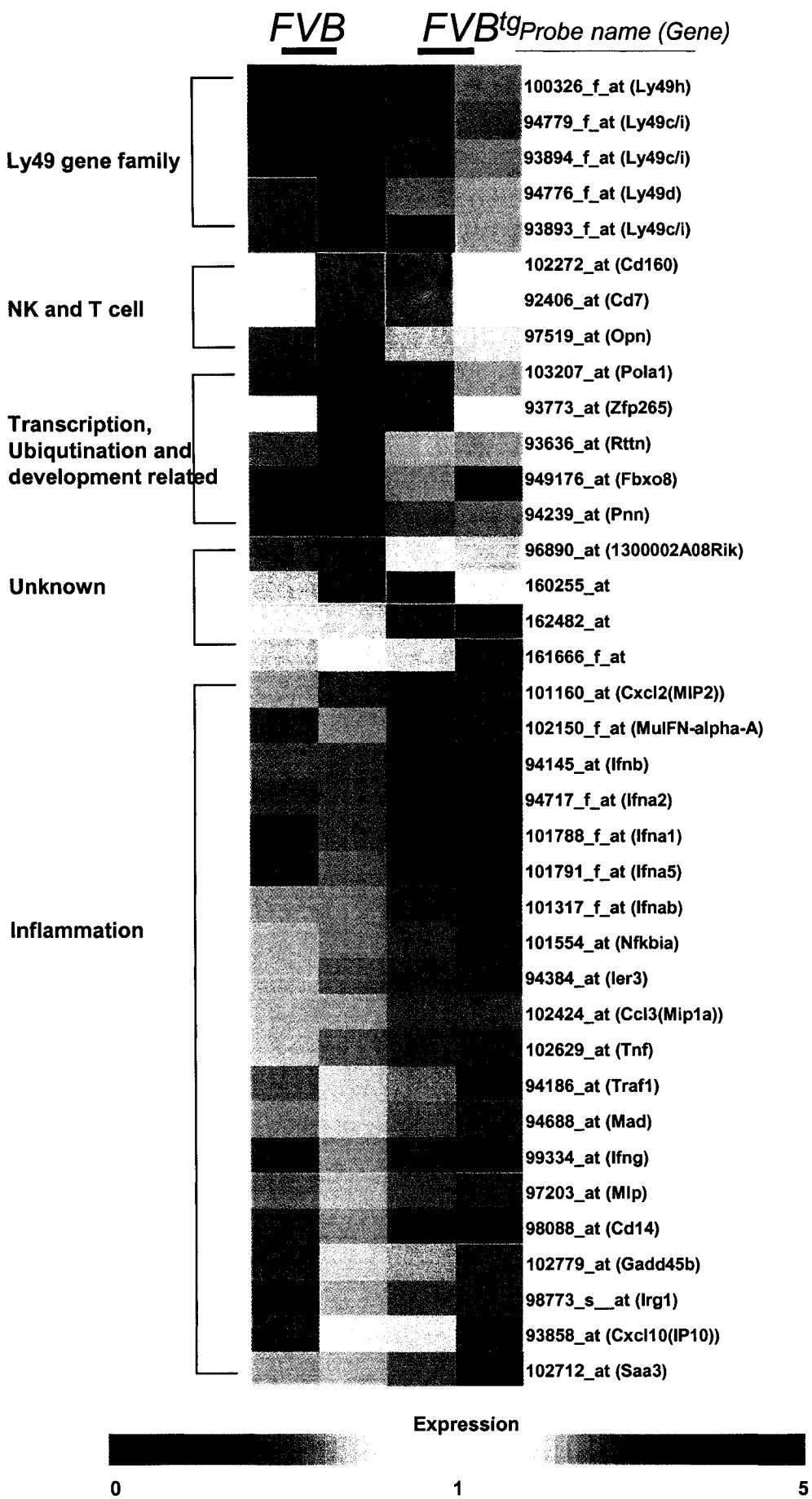

B
Log PFU/spleen
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
FVB-Tg (Ly49h)
FVB

Table 3.1 Genes differentially expressed on NK cells from FVB and FVB-Tg (*Ly49h*) mice at 1.5 days post-infection. Genes whose expression is differentially expressed more than 2.5 fold in the splenic $DX5^+$ cells between FVB and FVB-Tg (*Ly49h*) at day 1.5 post MCMV infection. Genes were selected by fold change and then grouped by their function based on Pubmed search. P: present, A: Absent

Probe Name	Gene	Gene bank #	Common name	FVB	FVB^{tg}	Fold change
Genes are upregulated in FVB^{tg}						
Ly49 gene family						
100326_f_at	Ly49h	U12889	killer cell lectin-like receptor, subfamily A, member 8	P	P	10.3
93893_f_at	Ly49c/i	U56404	killer cell lectin-like receptor subfamily A, member 3	P	P	6.9
93894_f_at	Ly49c/i	U49866	killer cell lectin-like receptor, subfamily A, member 3	P	P	7.4
94776_f_at	Ly49d	U10090	killer cell lectin-like receptor, subfamily A, member 4	P,A	P	5.2
94779_f_at	Ly49c/i	U34891	killer cell lectin-like receptor, subfamily A, member 3	A	P	24.3
NK and T cell						
102272_at	Cd160	AF060982	CD160 antigen	P	P	2.6
92406_at	Cd7	D31956	CD7 antigen	P	P	2.6
97519_at	Opn	X13986	Osteopontin	A	P	13.5
Transcription, Ubiqutination and Development related						
103207_at	Pola1	D13543	polymerase (DNA directed), alpha 1	P,A	P	5.4
93636_at	Rttn	AA146482	rotatin	A	M,P	4.3
93773_f_at	Zfp265	AI835041	zinc finger protein 265	P,A	P	2.8
94239_at	Pnn	Y08701	pinin	A	P	8.5
94917_at	Fbxo8	AI844932	F-box only protein 8	A	P	15.2
Unknown						
96890_at	1300002A08Rik	AW060971	RIKEN cDNA 1300002A08 gene	P,A	P	2.9
160255_at				P,A	P	3.2
Genes are upregulated in FVB						
Inflammation						
101160_at	Cxcl2 (MIP2)	X53798	chemokine (C-X-C motif) ligand 2	P	A	5.3
101317_f_at	Ifnab	L38698	Mus musculus interferon alpha-B (IFN-alpha-B) gene	P	A	4.0
101554_at	Nfkbia	U57524	Mus musculus I kappa B alpha gene	P	P	3.0
101788_f_at	Ifna1	X01974	Mouse gene for interferon alpha 1 (MuIFN-alpha 1).	P	A	16.6
101791_f_at	Ifna5	X01971	Mouse gene for interferon alpha 5 (Mu IFN-alpha 5).	P	A	6.4
102150_f_at	MuIFN-alpha-A	M28587	Mouse alpha leukocyte interferon gene	P	A	6.9
102424_at	Ccl3 (Mip-1α)	J04491	chemokine (C-C motif) ligand 3	P	P	2.5
102629_at	Tnf	D84196	Mus musculus TNFA gene for tumor necrosis factor alpha	P	A	3.2
102712_at	Saa3	X03505	serum amyloid A (SAA) 3	P	M,A	12.3
102779_at	Gadd45b	X54149	growth arrest and DNA-damage-inducible 45 beta	P	P	2.9
93858_at	Cxcl10 (IP10)	M33266	chemokine (C-X-C motif) ligand 10	P	P	2.7
94145_at	Ifnb	K00020	interferon beta, fibroblast	P	A	16.0
94186_at	Traf1	L35302	Tnf receptor-associated factor 1	P	P	3.0
94384_at	Ier3	X67644	immediate early response 3	P	P	3.1
94688_at	Mad	X83106	Max dimerization protein	P	P	2.9
94717_f_at	Ifna2	K01238	Mouse gene for interferon alpha 2 (Mu IFN-alpha 2).	P	A	16.3
97203_at	Mlp	X61399	MARCKS-like protein	P	P	3.0
98088_at	Cd14	X13333	CD14 antigen	P	P	4.7
98773_s_at	Irg1	AI323667	immunoresponsive gene 1	P	P	3.9
99334_at	Ifng	K00083	interferon gamma	P	P	5.3
Unknown						
162482_at				P	A	4.1
161666_f_at				P	P	2.6

Figure 3.5 Functional clustered view of genes listed in table 1. The candidate genes in Table 3.1 were clustered by their expression patterns using GeneSpring 6.2 and reorganized by their functions. The original microarray data for candidate genes was integrated into GeneSpring 6.2 software. Individual signal intensities were normalized to their median value. A clustering view was made for each functional group of Table 3.1 based on its gene expression patterns. All views were put together in a large clustering view.


FVB
FVBtg
Probe name (Gene)
Ly49 gene family
NK and T cell
Transcription, Ubiqutination and development related
Unknown
Inflammation
100326_f_at (Ly49h)
94779_f_at (Ly49c/i)
93894_f_at (Ly49c/i)
94776_f_at (Ly49d)
93893_f_at (Ly49c/i)
102272_at (Cd160)
92406_at (Cd7)
97519_at (Opn)
103207_at (Pola1)
93773_at (Zfp265)
93636_at (Rttn)
949176_at (Fbxo8)
94239_at (Pnn)
96890_at (1300002A08Rik)
160255_at
162482_at
161666_f_at
101160_at (Cxcl2(MIP2))
102150_f_at (MuIFN-alpha-A)
94145_at (Ifnb)
94717_f_at (Ifna2)
101788_f_at (Ifna1)
101791_f_at (Ifna5)
101317_f_at (Ifnab)
101554_at (Nfkbia)
94384_at (Ier3)
102424_at (Ccl3(Mip1a))
102629_at (Tnf)
94186_at (Traf1)
94688_at (Mad)
99334_at (Ifng)
97203_at (Mip)
98088_at (Cd14)
102779_at (Gadd45b)
98773_s__at (Irg1)
93858_at (Cxcl10(IP10))
102712_at (Saa3)
Expression
0
1
5

To validate the gene expression of the 35 candidate genes, I performed semi-quantitative RT-PCR with gene-specific oligos. *Gapdh* and *DX5* were used as internal controls for equal amounts of RNA and the purity of NK isolation, respectively. In addition, the expression of these candidates at 1.5 days post infection was compared to that in naïve mice of both FVB and FVB-Tg (*Ly49h*) mice. 11 genes were confirmed for their differential expressions by semi-quantitative RT-PCR (Figure 3.6). Among candidates of upregulated genes in resistant FVB-Tg (*Ly49h*) mice, greater gene expressions of *Ly49h*, *Opn*, *Cd7*, *Cd160*, and *AA657044* were confirmed. The gene expression of *Ly49h* was only present in resistant FVB-Tg (*Ly49h*) mice and absent in susceptible mice, as expected. In addition, the number of Ly49H+ NK cells was decreased at 1.5 days infection, consistent with the FACS results (Figure 3.4). It has been proposed that the loss of spleen NK cells is a result of migration to other organs such as liver. Although the gene expressions of *Ly49i* and *Ly49d* were not assessed in this experiment , a previous report by Lee et *al* had shown increased expression of these genes in FVB-Tg (*Ly49h*) mice (Lee et al., 2003).

Two different gene expression patterns were observed for the genes expressed higher in FVB-Tg (*Ly49h*) mice. The gene expressions of *Cd7*, *Cd160*, and *AA657044* were reduced upon MCMV infection in both FVB-Tg (*Ly49h*) and FVB mice. In contrast, the gene expression of *Opn* was completely absent in naïve mice, and became highly induced at 1.5 days infection, indicating that the *Opn* may play a role during MCMV infection, and that its role is further strengthened in the presence of Ly49H. The gene expression analysis of other candidates in resistant mice such as *Pnn, Pola I, and*

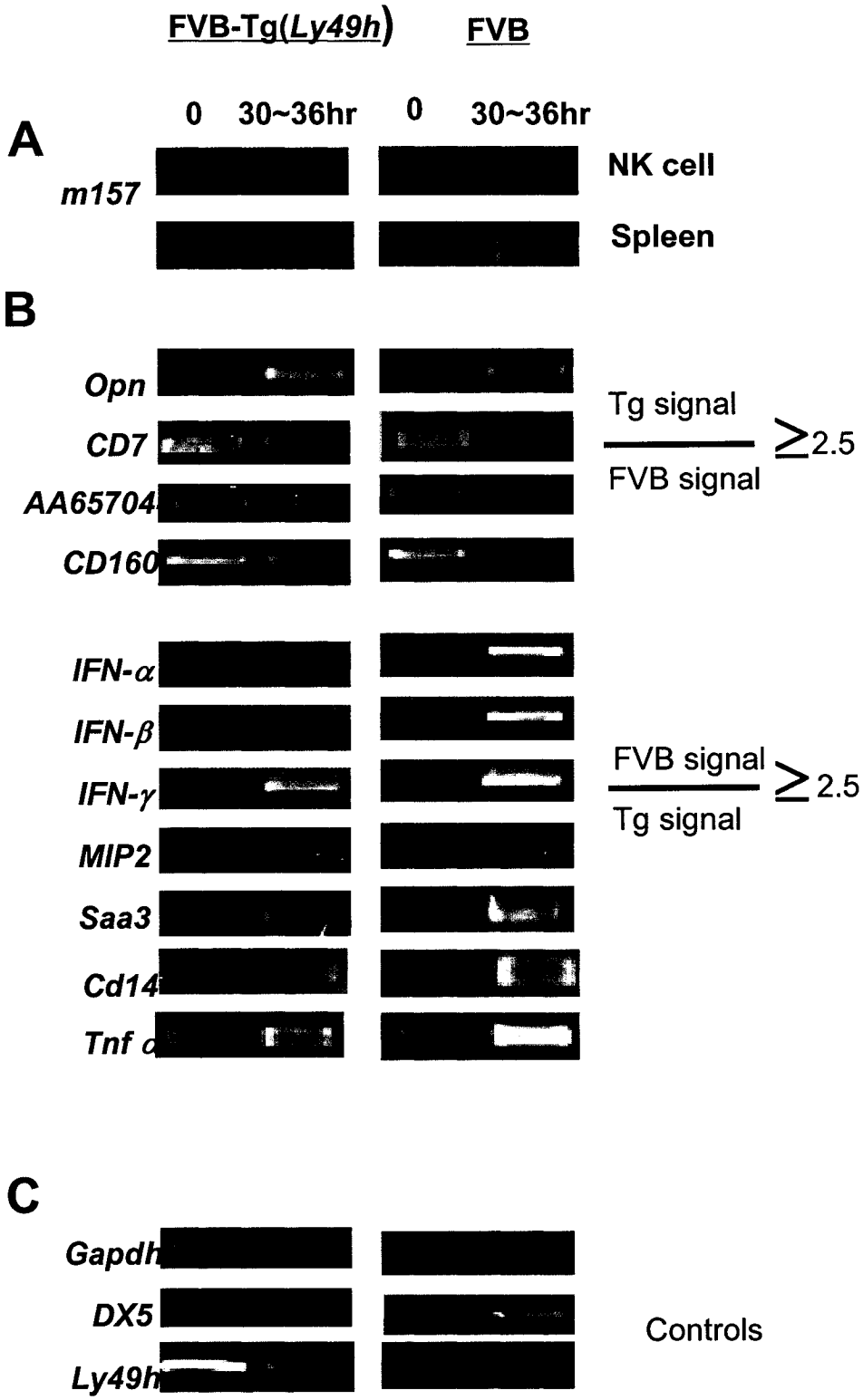
Fbxo8 failed to detect any difference between resistant and susceptible mice. *Rttn*, a gene related in embryogenesis (Faisst et al., 2002), and *Zfp65*, a gene probably related to posttranscriptional mechanisms (Adams et al., 2000), was not subjected to RT-PCR analysis due to poor current information about cDNA sequences.

Among genes more expressed in susceptible mice, the gene expressions of most proinflammatory cytokines such as *Ifn-α*, *Ifn-β*, *Ifn-γ*, *Mip2*, *Saa3*, *Cd14*, and *Tnf-α* were confirmed by semi-quantitative PCR. All these genes were highly induced upon MCMV infection and were more highly expressed in susceptible mice.

3.2.3 Comparative expression of MCMV ORF, m157

To determine the presence of MCMV in NK cells, we monitored the expression of the transcript m157, an immediate early-expressed glycoprotein gene. As shown in RT-PCR for m157 (Figure 3.6), there was no transcript in NK cells, in spite of active transcripts in total spleen cells. These results suggest that $DX5^+$ NK cells are not productively infected with MCMV.

Figure 3.6 Confirmation of differential NK cell gene expression in FVB and FVB-Tg (*Ly49h*) at 1.5 days post infection by semi-quantitative PCR. To detect MCMV, primers for an early expressed MCMV gene, m157, were used. The numbers of PCR cycles for each candidate gene were optimized for visualization of differential expressions. *Gapdh and Cd49b* were used for a control of RNA amount used in RT-PCR and the NK cell purity, respectively.


FVB-Tg(Ly49h)
FVB
0
30~36hr
0
30~36hr
A
m157
NK cell
Spleen
B
Opn
CD7
AA65704
CD160
Tg signal / FVB signal ≥2.5
IFN-α
IFN-β
IFN-γ
MIP2
Saa3
Cd14
Tnf α
FVB signal / Tg signal ≥2.5
C
Gapdh
DX5
Ly49h
Controls

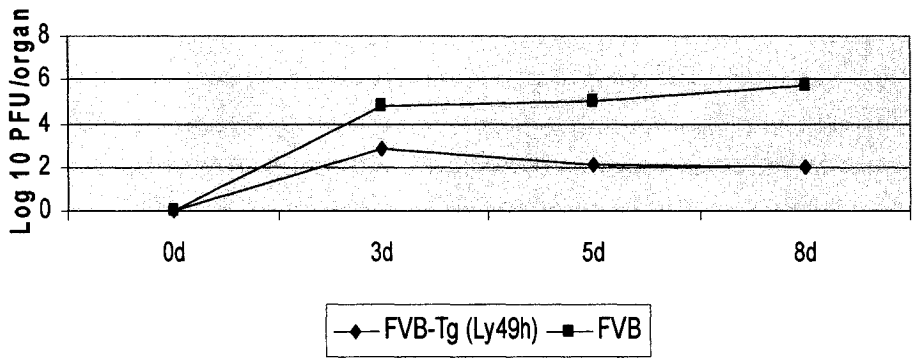
3.3. Global gene expression analysis in NK cells during MCMV infection using microarray

3.3.1. Global gene expression analysis for the splenic NK cells of FVB-Tg (*Ly49h*) after 0, 3, 5, and 8 days of MCMV infection

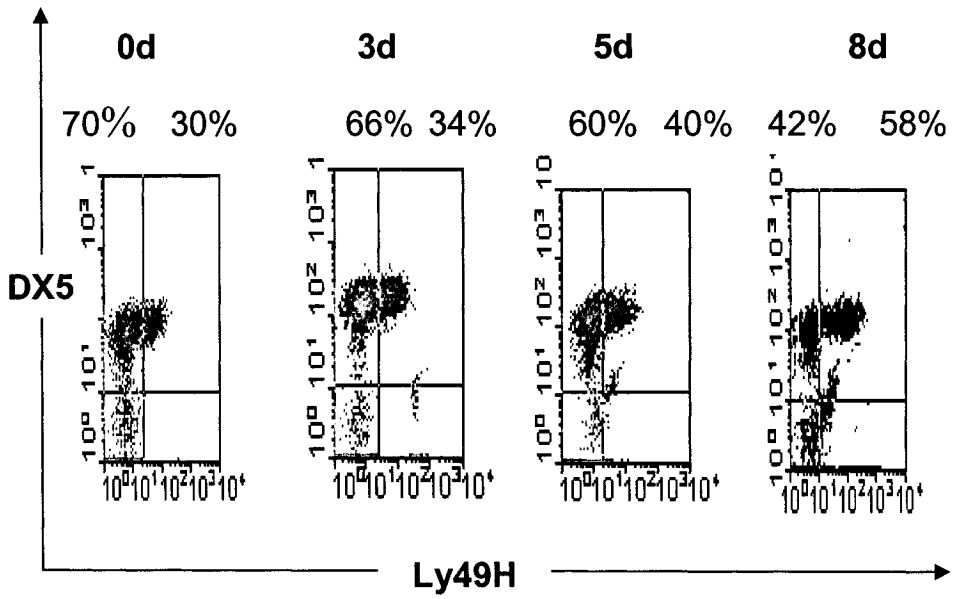
I analyzed global gene expression profiles in NK cells isolated from the spleens of FVB-Tg (*Ly49h*) mice during the course of MCMV infection using microarray. The purity of NK cells after magnetic separation was 70 to 75% based on the staining with mAb DX5 through the entire time course. Viral replication in the spleens of FVB-Tg (*Ly49h*) mice, at these time points transiently increased at day 3 and decreased afterward, while that in the FVB spleens continuously increased afterward (Figure 3.7 A). The proportion of $Ly49H^+$ NK cells increased steadily during the course of infection from 30% at day 0, to approximately 60% at 8 days post infection (Figure 3.7 B), demonstrating that the specific expansion of $Ly49H^+$ cells could be reproduced in our transgenic mice. Moreover, microarray data indicated that the expression of *Ly49h* is increased by more than 3-fold at day 8 in duplicate microarray experiments, indicating that Ly49H NK cell expansion is also accompanied by transcriptional upregulation of the gene (Figure 3.7 C).

Figure 3.7 Expression of both Ly49H mRNA and Ly49H protein in NK cells correlates with the control of MCMV in the spleen during the course of infection. A. Viral titer in the spleen of wild type and transgenic mice, which was used for microarray experiment Virus titer reached maximum at 3 days infection and decreased afterward in transgenic mice expressing Ly49H whereas viral titer in spleen of FVB continuously increase. B. $Ly49H^{+}$ cells were expanded with time course. Enriched NK cell preparations from spleen of transgenic mice were stained with the DX5 and anti-Ly49H polyclonal antibody. The numbers in the density plots indicate the percentage of $DX5^{+}$ cells either $Ly49H^{+}$ or $Ly49H^{-}$. C. Normalized intensities of *Ly49h* expression in microarray during infection were shown.

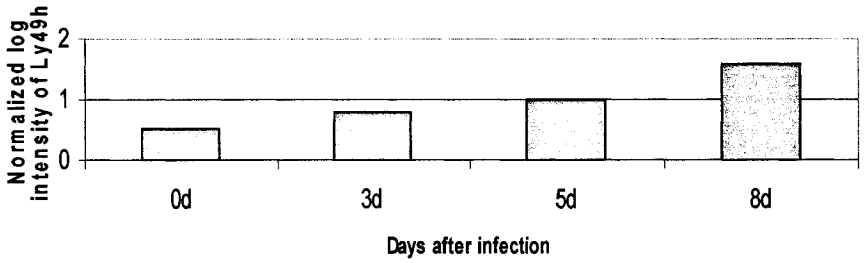
A


Log 10 PFU/organ
8
6
4
2
0
0d
3d
5d
8d
FVB-Tg (Ly49h)
FVB

B


0d
3d
5d
8d
70% 30%
66% 34%
60% 40%
42% 58%
DX5
Ly49H

C


Normalized log intensity of Ly49h
2
1
0
0d
3d
5d
8d
Days after infection

A comparative analysis was performed for microarray results at each time point using Genespring 6.2. To give significance and minimize the number of candidate genes, two parameters were chosen from GeneSpring software. First, I selected candidate genes by their fold changes (more than 2.5 fold compared to 0D at each time point). Second, these selected genes by their fold changes were subjected to a one–way analysis of variance (ANOVA) test ($p<0.05$), which allows us to determine if one given factor, a different time point in this case, has a significant effect on gene expression behaviour. Out of 22690 genes on MOE430A chip, 213 genes were selected as differentially expressed genes at any time point compared to that of naïve mice.

The number of genes differentially expressed at each time point is illustrated in Figure 3.8 as a Venn Graphic showing that majority genes are turned on at 3 days of MCMV infection (193 genes), a time point showing peak virus titer. However, the number of these differentially expressed genes was decreased at later time points, showing 66 genes at 5, and 30 genes at 8 days of MCMV infection. This pattern was similar to that of virus load during these time points and suggests that most of these genes are induced to control virus replication in the spleen.

As we can see in the Venn graphic, a group of genes were differentially expressed only at a specific time point, while some were differentially expressed at two overlapped time points. 137 genes were differentially expressed at day 3, 10 at day 5 and only 7 at day 8. 36 genes were differentially expressed at days 3 & 5 and 3 genes at days 3 & 8 and days 5 & 8. 17 genes were differentially expressed at all three time points. Since each gene was chosen by its fold changes, some genes were more than 2.5 fold upregulated or downregulated upon infection. Using this Venn Graphic and their up

and downregulated patterns at each time point, 11 distinct patterns of gene expression were identified and gene lists were generated for each pattern with its expression profiles (Figure 3.9). Currently, extensive studies on these gene lists in each pattern are still in progress in the Vidal lab. Since the analysis of 213 genes in 11 patterns is very challenging, I have investigated several important genes and, as discussed below, shown data of this initial analysis.

Among *Ly49* genes, the gene expression of *Ly49g* (*Klra7*) was continuously increased along the time course of MCMV infection, consistent to previous reports. Others in the Vidal lab have published data which shows an upregulation in the cell surface density of Ly49G2, a splice variant of Ly49G (an inhibitory receptor), at days 3 and 4 (Depatie et al., 1999). *Tay et al* also showed proportional increases in $Ly49A^+$ and $Ly49G2^+$ cells, but decreases in $Ly49C^+$ and $Ly49D^+$ cells within the splenic $NK1.1^+$ population at 3 days post-MCMV infection (Tay et al., 1999). Whereas *Ly49g* is implicated in tumor killing, the functional role of Ly49G in MCMV infection is still elusive.

NK cytotoxicity utilizes the granule exocytosis pathway, which consists of the pore-forming protein perforin (*Pfp*) and a family of serine proteases known as granzymes (*Gzms*) to kill infected cells by inducing apoptosis. The gene expressions of perforin and granzyme A were greatly modulated at day 3, and decreased later. The gene expressions of granzyme B were also modulated at day 3and 5 and decreased at day 8, while those of granzyme K were modulated later at day 8 of MCMV infection. The upregulated gene expression of cytotoxic granules was consistent with the known increased NK cell cytotoxic activity at day 3 of MCMV infection, and direct killing is actively occurring at that time point. This data also suggests that the Granzyme subfamily may have a

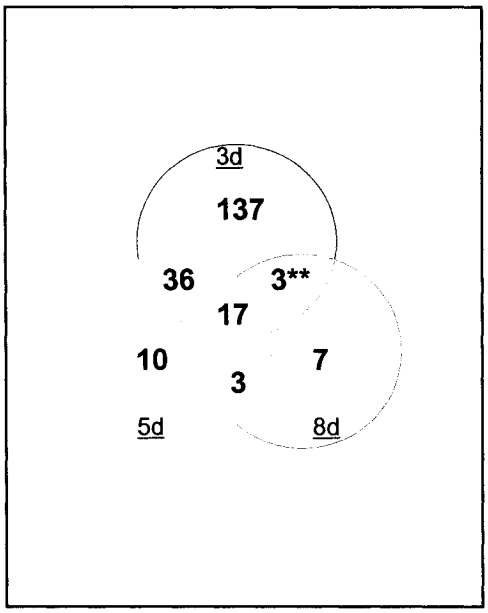
different role in MCMV infection at different time points. For example, Granzymes A and B upregulated early are important for the clearance of virus-infected cells, while Granzyme K has different unknown roles, since its expression was increased at day 8 of MCMV infection, at a time point at which viral replication is almost resolved. Another possibility is that these observations may also reflect a difference in the protein stabilities of the different Granzymes.

Another interesting observation is the gene expression of two important cytokines, which elicit contrary roles in immune responses. During the time course of MCMV infection, Interleukin (IL-10) and Interleukin18 (IL-18) showed completely opposite patterns of gene expression. For example, IL-10 was greatly increased at day 3, while IL-18 was decreased at this time point. Previous studies on the roles of these two cytokines in MCMV infection have shown that transient increased expression of IL-10 and IFN-γ was observed in lung tissue at day 3 of MCMV infection (Wu et al., 2001), and IL-18 has been implicated in the expansion of $Ly49H^+$ NK cells during MCMV infection. IL-10 has been known to block IFN-γ production by IL-12 or IL-18 via NK-1R (Weinstock et al., 2003). IL-10 is an inhibitor of activated macrophages and dendritic cells and, as such, regulates innate immunity and cell-mediated immunity. Moreover, IL-10 inhibits the production of IL-12, co-stimulator molecules, and MHC-II molecules, all of which are needed for cell-mediated immunity. IL-10 is produced by macrophages mainly, and Th-2 cells. IL-18, mainly produced by macrophages, stimulates the production of IFN-γ by NK cells and T-lymphocytes and thus induces cell-mediated immunity. A recent study showed that the activating Ly49D NK cell receptor can

potently synergize during co-stimulation with IL-12 and IL-18 for selective production of IFN-γ (Ortaldo and Young, 2005).

Figure 3.8 Genes differentially expressed in spleen NK cells during the course of MCMV infection. Duplicated microarray experiments were performed on Affymetrix MOE430A chips. Splenic $DX5^+$ cells were purified from FVB-Tg (*Ly49h*) at 0, 3, 5, and 8 days post infection. Three to five mice were used for each microarray experiment. All the data analyses were performed using GeneSpring 6.2 software. A) Venn graphic representing the number of genes selected for gene modulation more than 2.5 fold compared to day 0 during time course infection and are passed one way ANOVA test using GeneSpring ($p<0.05$). Gene expression fold changes were compared day 3 versus day 0, day 5 versus day 0, and day 8 versus day0. B) Downregulated and upregulated gene numbers after infection at each time point. The number of genes illustrated in Figure A were separated into upregulated and downregulated groups, and compared to day 0 at each different time point.

A


3d
137
36
3**
17
10
7
3
5d
8d

B

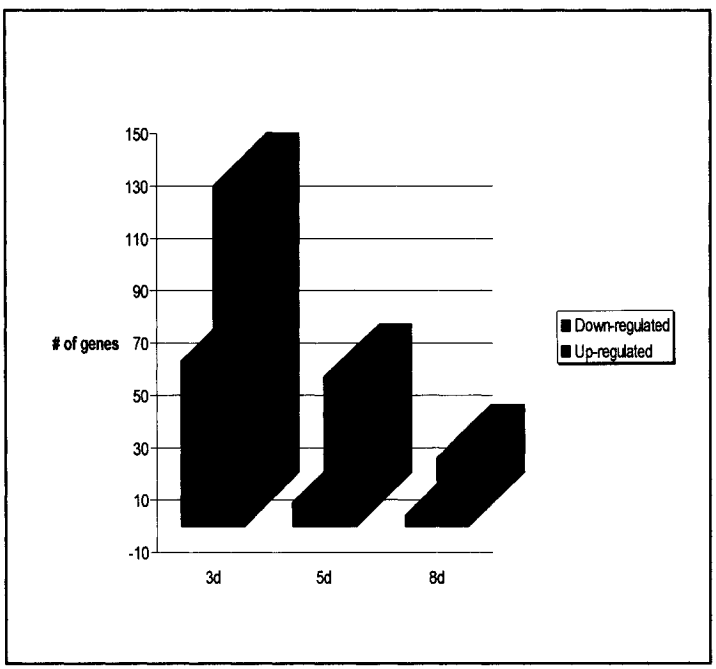
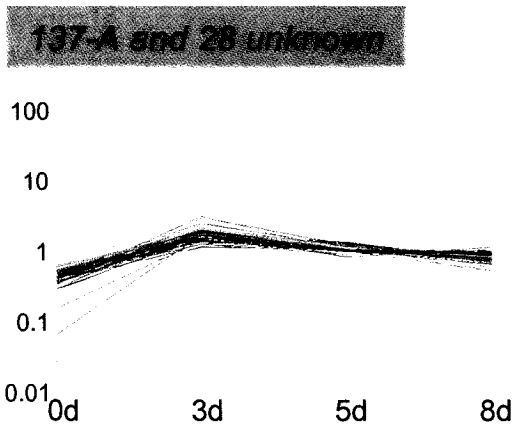
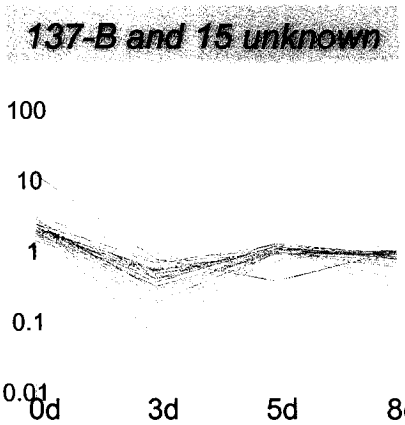

150
130
110
90
# of genes 70
50
30
10
-10
3d
5d
8d
Down-regulated
Up-regulated

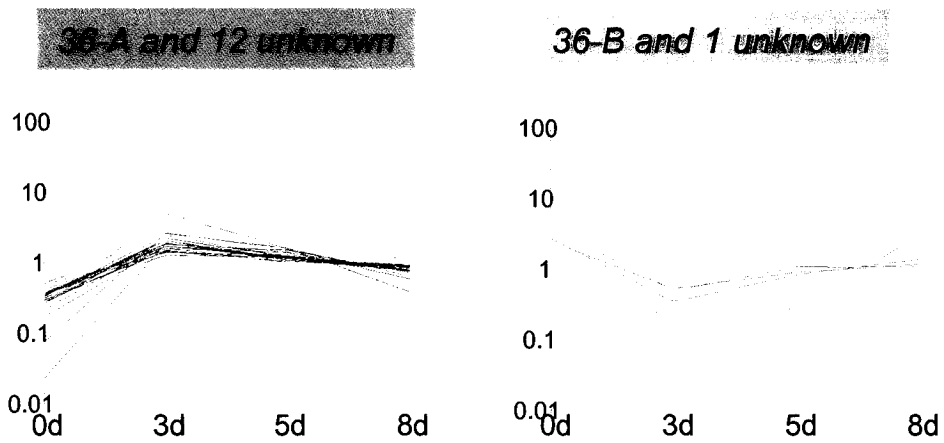
Figure 3.9 213 genes shows different patterns of expression during MCMV infection. 11 distinct pattern of expression were generated using GeneSpring 6.2 and their gene lists were indicated in each table. The gene expression at 3, 5, and 8 d were separately compared to 0d. 137-A and 28 unknown: Genes whose expressions were highly upregulated at 3d only. 137-B and 15 unknown: Genes whose expressions were highly downregulated at 3d only. 36-A and 12 unknown: Genes whose expressions were highly upregulated at 3d and 5d. 36-B and 1 unknown: Genes whose expressions were highly downregulated at 3d and 5d. 17-A and 5 unknown: Genes whose expressions were highly upregulated at 3d, 5d, and 8d. 17-B: Genes whose expressions were highly downregulated at 3d, 5d, and 8d. 10-A: Genes whose expressions were highly upregulated at 5d only. 10-B and 2 unknown: Genes whose expressions were highly downregulated at 5d only. 3-A and 1 unknown: Genes whose expressions were highly upregulated at 5d and 8d. There were no highly downregulated genes at 5d and 8d. 7-A and 2 unknown: Genes whose expressions were highly upregulated at 8d only. There were no highly downregulated genes at 8d only. 3**-A and 1 unknown: Genes whose expressions were highly upregulated at 3d and 8d. There were no highly downregulated genes at 3d and 8d.



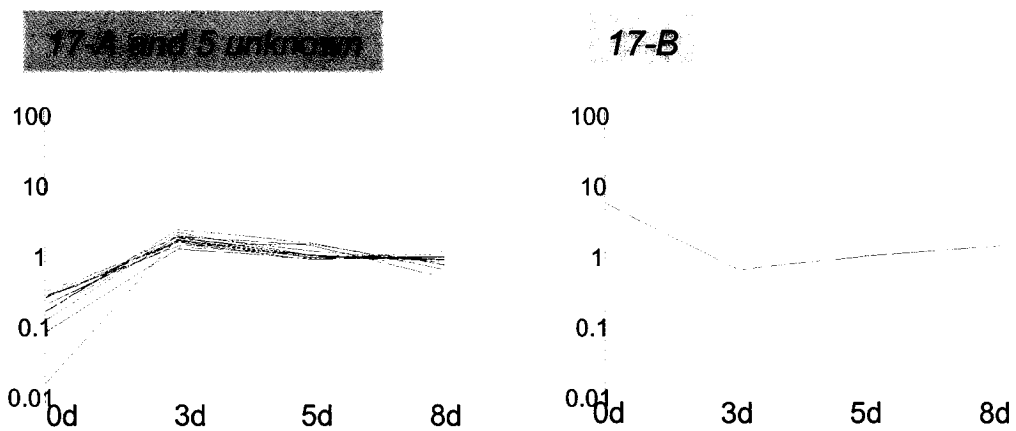
Probe Name	Gene	Gene bank #	Common name
1424161_at	Ddx27	BC011321	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27
1450330_at	Il10	NM_010548	interleukin 10
1421828_at	Kpna3	BM213828	karyopherin (importin) alpha 3
1429117_at	Tradd	BB749262	TNFRSF1A-associated via death domain
1449060_at	Kif2c	NM_134471	kinesin family member 2C
1448266_at	Edf1	AB030185	endothelial differentiation-related factor 1
1448899_s_at	Rad51ap1	BC003738	RAD51 associated protein 1
1433893_s_at	Spag5	BM208112	sperm associated antigen 5
1424942_a_at	Myc	BC006728	myelocytomatosis oncogene
1453851_a_at	Gadd45g	AK007410	growth arrest and DNA-damage-inducible 45 gamma
1451745_a_at	Znhit1	BC026751	zinc finger, HIT domain containing 1
1416834_x_at	Ndufb2	NM_026612	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2
1417057_a_at	Ppid	BC011499	peptidylprolyl isomerase D (cyclophilin D)
1423423_at	Grp58	BF319868	glucose regulated protein
1420878_a_at	Ywhab	NM_018753	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide
1452314_at	Kif11	BB827235	kinesin family member 11
1423520_at	Lmnb1	AA270173	lamin B1
1451064_a_at	Psat1	BC004827	phosphoserine aminotransferase 1
1424511_at	Stk6	U80932	serine/threonine kinase 6
1421083_x_at	Banf1	NM_011793	barrier to autointegration factor 1
1448505_at	C1d	NM_020558	nuclear DNA binding protein
1427094_at	Pole2	AF036898	polymerase (DNA directed), epsilon 2 (p59 subunit)
1421081_a_at	Banf1	NM_011793	barrier to autointegration factor 1
1423969_at	Nup37	BC011102	nucleoporin 37
1450694_at	Fkbp2	NM_008020	FK506 binding protein 2
1452040_a_at	Cdca3	BI081061	cell division cycle associated 3
1416641_at	Lig1	NM_010715	ligase I, DNA, ATP-dependent
1424156_at	Rbl1	U27177	retinoblastoma-like 1 (p107)
1417523_at	Plek	AF181829	pleckstrin
1416258_at	Tk1	NM_009387	thymidine kinase 1
1417216_at	Pim2	NM_138606	proviral integration site 2
1435429_x_at	Rps27l	AV111399	ribosomal protein S27-like
1417125_at	Ahcy	NM_016661	S-adenosylhomocysteine hydrolase
1416309_at	Nusap1	BC009096	nucleolar and spindle associated protein 1
1450920_at	Ccnb2	AK013312	cyclin B2
1460469_at	Tnfrsf9	BM250782	tumor necrosis factor receptor superfamily, member 9
1422483_a_at	Cycs	NM_007808	cytochrome c, somatic
1460168_at	Slbp	NM_009193	stem-loop binding protein
1423775_s_at	Prc1	BC005475	protein regulator of cytokinesis 1
1423589_at	Arpc4	BG145444	actin related protein 2/3 complex, subunit 4
1434380_at		BM241271	Diabetic nephropathy-related gene 1 mRNA, partial sequence
1451080_at	Usp1	BC018179	ubiquitin specific protease 1
1416746_at	H2afx	NM_010436	H2A histone family, member X
1449839_at	Casp3	BG070529	caspase 3, apoptosis related cysteine protease
1448632_at	Psmb10	NM_013640	proteasome (prosome, macropain) subunit, beta type 10
1426789_s_at	Ssrp1	BC024835	structure specific recognition protein 1
1422601_at	Serpinb9	NM_009256	serine (or cysteine) proteinase inhibitor, clade B, member 9
1435122_x_at	Dnmt1	BB165431	DNA methyltransferase (cytosine-5) 1
1416902_a_at	Cox5b	NM_009942	cytochrome c oxidase, subunit Vb
1419480_at	Sell	M36005	selectin, lymphocyte
1452659_at	Dek	AK007546	DEK oncogene (DNA binding)
1451862_a_at	Prf1	M23182	perforin 1 (pore forming protein)
1454716_x_at	Cox5b	AA960638	cytochrome c oxidase, subunit Vb
1419573_a_at	Lgals1	NM_008495	lectin, galactose binding, soluble 1
1417898_a_at	Gzma	NM_010370	granzyme A



Probe Name	Gene	Gene bank #	Common name
1420282_s_at	Isp2	D19363	implantation serine protease 2
1419018_at	Psx1	NM_008955	placenta specific homeobox 1
1426204_a_at	Oprl	AF043276	opioid receptor-like
1421118_a_at	Gpr56	NM_018882	G protein-coupled receptor 56
1417623_at	Slc12a2	BG069505	solute carrier family 12, member 2
1449109_at	Socs2	NM_007706	suppressor of cytokine signaling 2
1448688_at	Podxl	AF290209	podocalyxin-like
1455332_x_at	Fcgr2b	BM224327	Fc receptor, IgG, low affinity IIb
1425639_at	Centa2	BC027165	centaurin, alpha 2
1420464_s_at	Pira6	NM_011093	paired-Ig-like receptor A6
1416318_at	Serpinb1a	AF426024	serine (or cysteine) proteinase inhibitor, clade B, member 1a
1434768_at	Cln2	AI266910	ceroid-lipofuscinosis, neuronal 2
1415973_at	Marcks	AW546141	myristoylated alanine rich protein kinase C substrate
1418493_a_at	Snca	NM_009221	synuclein, alpha
1425546_a_at	Trf	AF440692	transferrin
1423586_at	Axl	AA500897	AXL receptor tyrosine kinase
1419476_at	Adamdec1	NM_021475	ADAM-like, decysin 1
1417492_at	Ctsb	M14222	cathepsin B
1419905_s_at	Hpgd	AV026552	hydroxyprostaglandin dehydrogenase 15 (NAD)
1422122_at	Fcer2a	M99371	Fc receptor, IgE, low affinity II, alpha polypeptide
1450570_a_at	Cd19	NM_009844	CD19 antigen
1422645_at	Hfe	AJ306425	hemochromatosis
1417268_at	Cd14	NM_009841	CD14 antigen
1422869_at	Mertk	NM_008587	c-mer proto-oncogene tyrosine kinase
1416930_at	Ly6d	NM_010742	lymphocyte antigen 6 complex, locus D
1425346_at	Zfp318	BB168536	zinc finger protein 318
1450430_at	Mrc1	NM_008625	mannose receptor, C type 1
1417932_at	Il18	NM_008360	interleukin 18
1449164_at	Cd68	BC021637	CD68 antigen
1416985_at	Ptpns1	AB018194	protein tyrosine phosphatase, non-receptor type substrate 1
1448182_a_at	Cd24a	NM_009846	CD24a antigen
1417640_at	Cd79b	NM_008339	CD79B antigen
1452463_x_at	Igk-V8	BG966217	immunoglobulin kappa chain variable 8 (V8)
1418243_at	Fcna	NM_007995	ficolin A
1449401_at	C1qg	NM_007574	complement component 1, q subcomponent, gamma polypeptide
1423226_at	Ms4a1	BB236617	membrane-spanning 4-domains, subfamily A, member 1
1417061_at	Slc40a1	AF226613	solute carrier family 40 (iron-regulated transporter), member 1
1452431_s_at	H2-Aa	AF119253	histocompatibility 2, class II antigen A, alpha
1417184_s_at	Hbb-y	BC027434	hemoglobin Y, beta-like embryonic chain

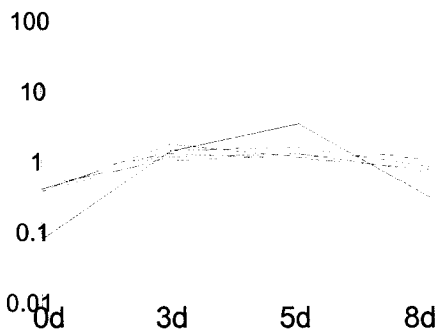


Probe Name	Gene	Gene bank #	Common name
1452458_s_at	Ppil5	BC022648	peptidylprolyl isomerase (cyclophilin) like 5
1418293_at	Ifit2	NM_008332	interferon-induced protein with tetratricopeptide repeats 2
1416076_at	Ccnb1	NM_007629	Ccnb1-rs13
1419641_at	Purb	BM220305	purine rich element binding protein B
1422993_s_at	Refbp2	NM_019484	RNA and export factor binding protein 2
1416939_at	Pyp	NM_026438	pyrophosphatase
1426349_s_at	Tmpo	AA153892	thymopoietin
1439040_at	Cenpe	BG068387	centromere protein E
1415810_at	Uhrf1	BB702754	ubiquitin-like, containing PHD and RING finger domains, 1
1428315_at	Ebna1bp2	AK007491	EBNA1 binding protein 2
1418191_at	Usp18	NM_011909	ubiquitin specific protease 18
1429295_s_at	Trip13	AK010336	thyroid hormone receptor interactor 13
1419169_at	Mapk6	BC024684	mitogen-activated protein kinase 6
1417244_a_at	Irf7	NM_016850	interferon regulatory factor 7
1423135_at	Thy1	AV028402	thymus cell antigen 1, theta
1449009_at	Tgtp	NM_011579	T-cell specific GTPase
1417141_at	Igtp	NM_018738	interferon gamma induced GTPase
1417082_at	Anp32b	NM_130889	acidic nuclear phosphoprotein 32 family, member B
1425084_at	Ian3	BC026200	immune associated nucleotide 3
1452014_a_at	Igf1	AF440694	insulin-like growth factor 1
1422041_at	Pilrb	NM_133209	paired immunoglobin-like type 2 receptor beta
1415947_at	Creg	BC027426	cellular repressor of E1A-stimulated genes
1417714_x_at	Hba-a1	NM_008218	hemoglobin alpha, adult chain 1



Probe Name	Gene	Gene bank #	Common name
1416558_at	Melk	NM_010790	maternal embryonic leucine zipper kinase
1421546_a_at	Racgap1	NM_012025	Rac GTPase-activating protein 1
1448226_at	Rrm2	NM_009104	ribonucleotide reductase M2
1427161_at	Lek1	BE848253	leucine, glutamic acid, lysine family 1 protein
1425815_a_at	Hmmr	BC021427	hyaluronan mediated motility receptor (RHAMM)
1415878_at	Rrm1	BB758819	ribonucleotide reductase M1
1439377_x_at	Elovl1	BB041150	elongation of very long chain fatty acids -like 1
1454694_a_at	Top2a	BM211413	topoisomerase (DNA) II alpha
1426817_at	Mki67	X82786	antigen identified by monoclonal antibody Ki 67
1417185_at	Ly6a	BC002070	lymphocyte antigen 6 complex, locus A
1419060_at	Gzmb	NM_013542	granzyme B
1448780_at	Slc12a2	BG069505	solute carrier family 12, member 2

10-A



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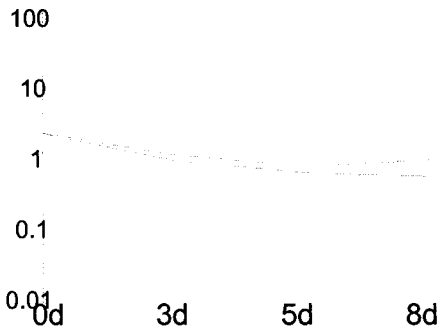
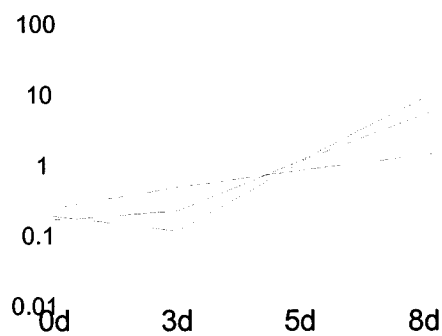


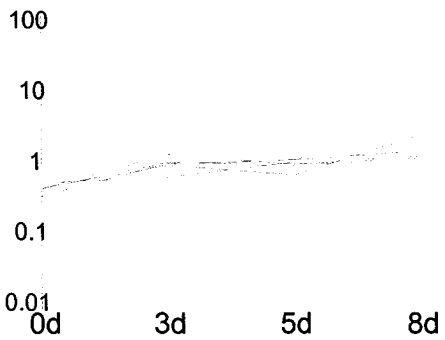
Probe Name	Gene	Gene bank #	Common name
1417314_at	H2-Bf	NM_008198	histocompatibility 2, complement component factor B
1449322_at	Ptp4a1	BC003761	protein tyrosine phosphatase 4a1
1426534_a_at	Arfgap3	BG067878	ADP-ribosylation factor GTPase activating protein 3
1416605_at	Nola2	BC024944	nucleolar protein family A, member 2
1421855_at	Fgl2	BF136544	fibrinogen-like protein 2
1425005_at	Klrc1	AF106008	killer cell lectin-like receptor subfamily C, member 1
1425142_a_at	Hnrpd	BC011172	heterogeneous nuclear ribonucleoprotein D
1448330_at	Gstm1	NM_010358	glutathione S-transferase, mu 1

3-A and 1 unknown



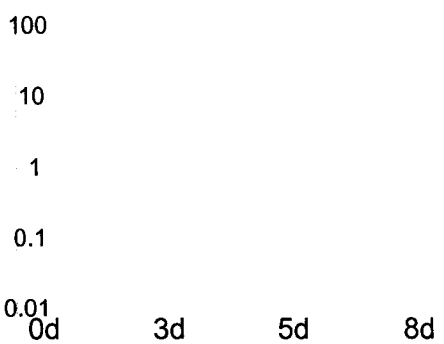
Probe Name	Gene	Gene bank #	Common name
1426171_x_at	Klra7	U10095	killer cell lectin-like receptor, subfamily A, member 7
1451632_a_at	Igh-1a	BC018535	immunoglobulin heavy chain 1a (serum IgG2a)

7-A and 2 unknown



Probe Name	Gene	Gene bank #	Common name
1422280_at	Gzmk	AB032200	granzyme K
1436930_x_at	Hmbs	BB000512	hydroxymethylbilane synthase
1454943_a_at	Paxip1	BM935413	PAX interacting (with transcription-activation domain) protein 1
1416433_at	Rpa2	BC004578	replication protein A2
1452661_at	Tfrc	AK011596	transferrin receptor

3 -A and 1unknown



Probe Name	Gene	Gene bank #	Common name
1449077_at	Eraf	NM_133245	erythroid associated factor
1416034_at	Cd24a	NM_009846	CD24a antigen

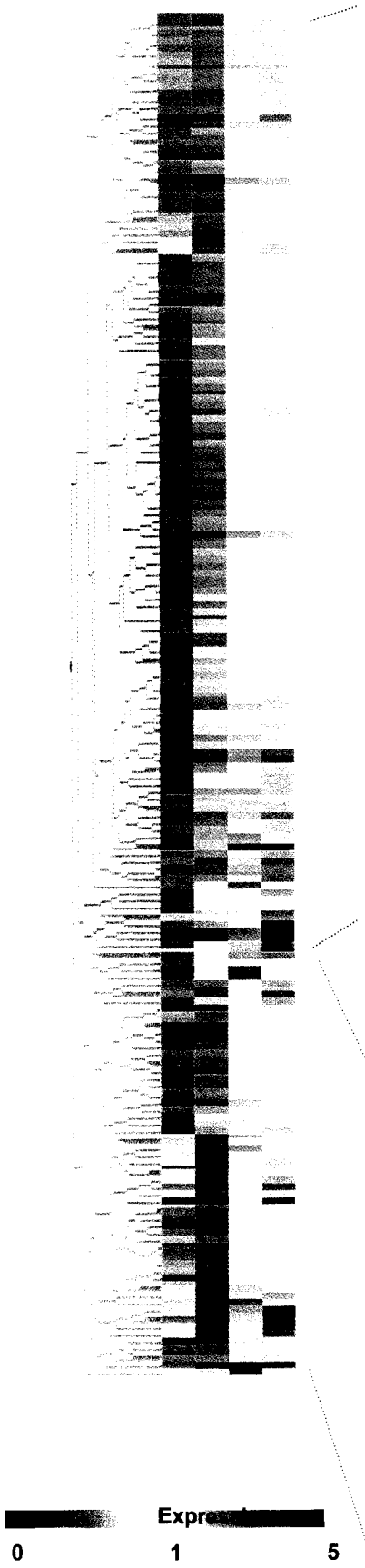
There was also an increase in the gene expression of Interferon Regulatory Factor 7 (IRF7) at days 3 and 5 post-MCMV infection. IRF7 is an interferon inducible transcription factor required for the induction of delayed early interferon alpha genes and the onset of a potent antiviral state. IRF7 is regulated in two ways. It is synthesized in response to IFN and it is activated by phosphorylation in virus infected cells (Marie et al., 1998). The critical role of IRF7 in the induction of type 1-interferon genes upon virus infected cells is well established. IFN-β and multiple IFN-α genes belong to the type 1 interferon family, and are normally activated upon virus infection. IRF3 (interferon regulatory factor 3), which is constitutively expressed in many cell types, can induce IFN-α4 and IFN-β in virus infected cells. Positive feedback of the initial interferon results in IRF production and induces the other members of the IFN-α family by phosphorylation of IRF7 (Marie et al., 1998; Sato et al.,). However, in this time course experiment, I failed to observe any interferon gene expression at any time point. During MCMV infection, Type I IFN production is transient by showing a sharp peak at day 1.5 and resides at day 2 post infection. Therefore, IRF7 at day 3 and 5 post infection might be involved in the transcriptional activation of other genes.

As an alternative approach to interpret microarray data, I used hierarchal tree and gene ontology tools to analyze 213 candidate genes in context of their functions. The Hierarchal tree was created by the standard correlation of 213 genes based on their signal intensities (Figure 3.9) using GeneSpring 6.2. Most of the gene expressions were modulated after infection and seemed to return to the basal level at Day 8. They can be subdivided into two groups: induced and reduced after MCMV infection. Using FatiGO

tools available on their website (http://fatigo.bioinfo.cnio.es), the genes of these two groups were interpreted by their gene ontologies and gene ontology terms representing more than 10% of total genes (213 genes) were presented in Figure 3.10. Among the genes induced after infection, the majority of genes (more than 80%) were related to cell proliferation (37%), DNA (17%) or protein (31%) metabolism, and transcription (15%). On the other hand, genes related to transport (24%), defense mechanisms (22%), and protein metabolism (16%), were downregulated. Since NK cells are activated and do undergo blastogenesis, during MCMV infection, and an increased yield of NK cells was observed in NK isolation at 3d and 5d, this result suggests that these upregulated genes belonging to groups of cell proliferation or DNA metabolism are involved in NK blastogenesis and activation. Nevertheless, approximately 10% of upregulated genes were also related to cell death. Selective death of NK cells in the liver after MCMV infection is observed along with the maturation and expansion of NK cells (Robbins et al., 2004). Moreover, activating NK cells are known to undergo apoptosis through CD2 stimulation or by granzyme leakage (Ida et al., 2003). Therefore, it is possible that the majority of NK cells from the peripheral system are proliferating for the MCMV clearance and some activated NK cells undergo apoptosis.

Although further extensive studies for these gene lists have to be done, these gene lists lay the foundation to dissect the specific NK cell pathways activated in response to infection. Moreover, my data will be useful to identify critical pathways for the transcriptional regulation of genes induced upon virus infection by analyzing upstream sequences of coexpressing genes.

Figure 3.10 Clustering view of NK cell genes differentially expressed during the course of MCMV infection. 213 genes, which illustrated in Figure 3.9 A were clustered by their standard correlation of normalized log intensity using GeneSpring 6.2. Based on their expression pattern compared to day 0, genes were divided into two categories, which were upregulated and downregulated after infection. Total genes in each category were interpreted with their Gene Ontology using FatiGO web GO tool (http://fatigo.bioinfo.cnio.es). Since many genes are involved in many different functions, a gene can be found in multiple GO terms. GO terms with more than 10% of total genes were presented in this figure.



Gene Ontology Term	Precentage in upregulated genes
Cell proliferation (Rrm1, Cdca3, Pole2, Mapk6, Prc1, Spag5, Ebna1bp2, Mki67, Lig1, Top2a, Kif11, Rbl1, Ccnb2, Stk6, Uhrf1, H2afx, Rrm2, Ccnb1, Cenpe, Gadd45g, Anp32b, Casp3, Myc, Nusap1)	37%
Protein metabolism (H2-Bf, Mapk6, Refbp2, Stk6, Melk, Gzma, Ccnb1, Usp18, Fkbp2, Ywhab, Ppid, Il10, Spag5, Kpna3, Rps27l, Gzmb, Pim2, Gadd45g, Nola2, Casp3, Psmb10, Igh-1a)	31%
DNA metabolism (Dnmt1, Ahcy, Banf1, Lig1, Top2a, Tk1, Rad51ap1, H2afx, Rrm1, Pole2, Rrm2, Myc)	17%
Transcription (Dnmt1, Hnrpd, Rbl1, Dek, Irf7, Tmpo, C1d, Uhrf1,Myc, Ssrp1)	15%
Cell organization and biogenesis (Ebna1bp2, Hnrpd, H2afx, Cenpe, Kif2c, Kif11, Arpc4, Nusap1, Igh-1a)	14%
Defense response (H2-Bf, Ifit2, Il10, Klra7, Tnfrsf9, Gadd45g, Casp3, Sell, Igh-1a)	14%
Transport (Refbp2, Ywhab, Arfgap3, Kpna3, Uhrf1, Nup37, Igh-1a)	10%
Programmed cell death (Cycs, Gzma, Tradd, Gzmb, Pim2, Gadd45g, Casp3, Myc)	10%

Gene Ontology Term	Precentage in upregulated genes
Transport (Hba-a1, Hbb-y, Fcna, C1qg, Slc12a2, Fcgr2b, Trf, Slc40a1, Ptpns1, Mrc1, Ctsb)	24%
Defense response (H2-Aa, C1qg, Cd14, Il18, Fcgr2b, Ms4a1, Hfe, Fcer2a, Ly6d, Cd79b)	22%
Protein metabolism (Serpinb1a, Mertk, Isp2, Eraf, Adamdec1, Axl, Ctsb)	16%
Cell surface receptor linked signal transduction (Igf1, Fcgr2b, Gpr56, Adamdec1, Cd79b)	11%

3.3.2. The kinetics of gene expression of candidate genes, which were differentially expressed at 1.5 days infection in the presence of Ly49H

The 36hr time point was chosen for the identification of candidate genes that might be important in the early NK immune response against MCMV. This is because phenotypic differences between transgenic mice and their counterparts become (and continue to be) detectable at this time point and, indicating that genes involved in the control of virus replication are being expressed at this time point. The microarray analysis at 1.5 days produced a list of genes modulated in the presence of Ly49H, so it is important to investigate the gene expression of these candidate genes during the time course of infection (Figure 3.11). This analysis will help to understand the possible functions of these genes from initiation to resolution during MCMV infection.

Among the *Ly49* gene family that are upregulated in FVB-Tg (*Ly49h*) mice at 1.5d , *Ly49h* gene expression continuously increased through later time points, consistent with the specific expansion of $Ly49H^+$ NK cells at 8 days of MCMV infection in C57BL/6 mice. This also indicates that the expansion of $Ly49H^+$ NK cells is regulated by transcription. In contrast, the expressions of Ly49c/i do not show any obvious changes, suggesting that the expansion of subsets of NK cells is specific to NK cells expressing Ly49H during MCMV infection. The expression of the other transgene, *Ly49d,* could not be evaluated at this time because a probe for this gene does not exist in the MOE430A chip used in this experiment.

It was particularly interesting to see that most candidate genes upregulated in transgenic mice, such as *Spp1*, *Cd160*, and *Cd7,* are upregulated at 3 days of infection where virus replication is still being controlled by activated NK cells. More importantly,

the expression of these genes decreased at later time points where very low viral titers are observed. This pattern of gene expression was observed in several candidate genes such as *Ccl3*, *Gadd45b*, *Cxcl10*, *Ifn-γ*, and *Mlp,* which were upregulated in susceptible mice. Although it is unclear whether Ly49H directly or indirectly triggered this gene expression, this data indicates that the expression of these genes is controlled by the presence of virus replication, suggesting their function in antiviral immunity.

Among candidate genes showing higher expression in susceptible mice, the expression of *Cd14* was unique, showing a sharp disappearance after MCMV infection. CD14 is a molecule critical for lipopolysaccharide (LPS)-dependent signal transduction, and therefore an important innate immune component in sensing microbial infections. Interestingly, CD14 has been implicated in HCMV infection. HCMV can activate inflammatory cytokine responses through CD14 and TLR2 (Compton et al., 2003), and elevated CD14 levels from serum samples of HCMV infected patients after liver transplantation was observed (Zipfel et al., 2001), suggesting that CD14 is part of the sensing machinery of HCMV infection. Accordingly, in the Ly49h transgenic model, *cd14* downregulation accompanies the decrease in viral load.

Genes expressed in greater numbers in susceptible mice, including most inflammatory cytokines such as, *type 1 IFNs*, *Tnf-α, Traf1*, *Mad*, *Irg1* and *Saa3*, were not present at any later time points. Although, these genes were highly induced at 1.5 days of infection, they became absent later in resistant transgenic mice, indicating that they are expressed in the presence of active MCMV infection.

The expressions of *Spp1*, *Cxcl10*, and *Ifn-γ* showed the most remarkable induction at 3 days of infection. *Spp1*, *Cxcl10*, and *Ifn-γ* are known to be important cytokines, and chemokine inducing type 1 immune responses.

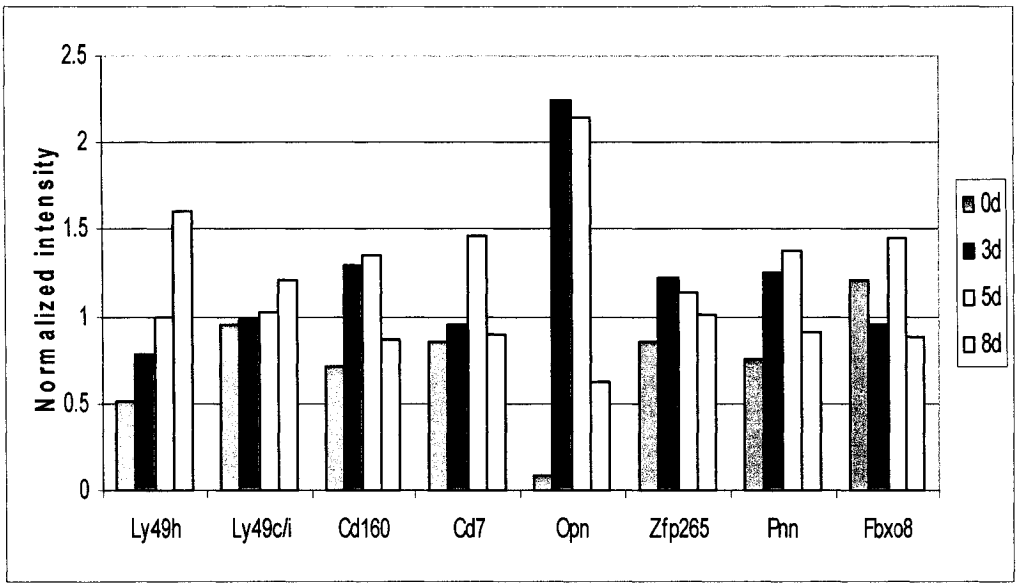
CXCL10, also known as IP10 (interferon-gamma inducible protein 10), encodes a protein with amino acid homology to platelet factor-4 and beta-thromboglobulin (Luster et al., 1985). *Cxcl10* is regulated by *Ifn-γ* and secreted from a variety of cells. Studies in mice deficient in *Cxcl10* showed *Cxcl10* plays a role in the generation and delivery of an effector T-cell response (Dufour et al., 2002). Recent papers have shown the induction of *Cxcl10* in intracerebral and atherosclerosis models of MCMV infection, according with our observation.

IFN-γ is characterized as an antiviral gene, and its function in MCMV infection has been extensively investigated. The secretion of IFN-γ by NK cells has a number of functions that play a role in controlling acute MCMV infection. For examples, it activates macrophages during MCMV infection (Heise and Virgin, 1995), increases the antigen presentation by MHC class 1 (Hengel et al., 1994), inhibits lytic MCMV replication and gene expression (Gribaudo et al., 1993), and releases of IL-12. Via IL-12, the NK-cell produced IFN-γ contributes to the differentiation of T cells into Th1 cells (Mosmann and Sad, 1996). Studies in IFN-γR$^{-/-}$ and IFN-γ$^{-/-}$ mice also support its protective role in MCMV infections because these mice were significantly more susceptible to MCMV infection. IFN-γ is also important in chronic and latent infections. Mice lacking IFN-γ receptors (IFN-γR$^{-/-}$) had difficulty clearing infections and developed chronic inflammation (Presti et al., 1998). Moreover, Loh et al have shown significantly increased viral titers in the spleens and livers of IFN-γ$^{-/-}$ mice compared to

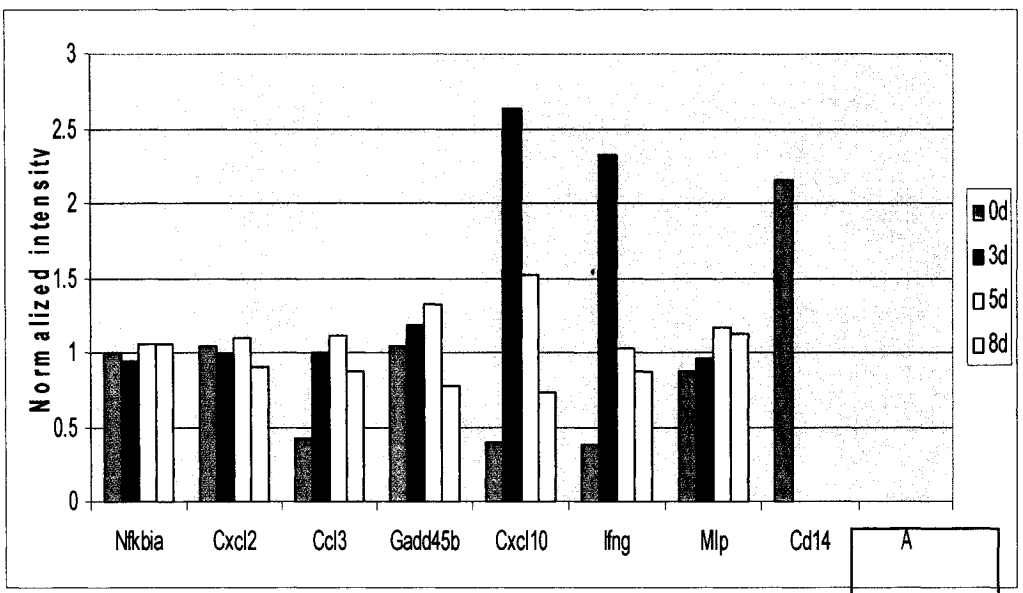
wild type B6 mice after MCMV infections. They also observed increased viral titers after depletion of IFN-γ in both organs in perforin (Pfp) deficient mice, suggesting that IFN-γ has a regulatory function that is independent of Pfp (Loh et al., 2005).

Figure 3.11 Expression patterns during the course of MCMV infection of genes differentially expressed on NK cells from FVB and FVB-Tg (*Ly49h*) at 1.5 days post-infection. The gene expression patterns of candidate genes obtained from comparing NK gene expression between FVB-Tg (*Ly49h*) and FVB at 1.5 days of MCMV infection (Figure 3. 5, 1.5d experiment) were examined in NK cells from FVB-Tg (*Ly49h*) during time course experiments A. The expression patterns of genes, which are upregulated in FVB-Tg (*Ly49h*) in 1.5d experiment. B. The expression pattern of genes which are upregulated in FVB in 1.5d experiment .Genes in the red box were absent in NK cells of FVB-Tg (*Ly49h*) during the time course experiment while their expressions were highly increased in susceptible mice at 1.5d MCMV infection.

A



B



A
Ifna1
Tnf
Saa3
Ifnb
Traf1
Mad
Ifna2
Irg1

3.4. Investigation of the function of the Osteopontin (*Opn*) gene in host resistance to MCMV infection

Among candidate genes upregulated during MCMV infection, the gene showing the most dramatic induction, *Osteopontin* (*Opn*), encodes an arginine-glycine-aspartate (RGD)-containing cytokine that activates macrophages to produce Th1 cytokines such as IFN-γ and IL-12, and downregulates the macrophage expression of Th2 cytokine IL-10. Interestingly, mice deficient in the *Osteopontin* gene, have shown impairment of type 1 immunity to viral and bacterial infections, resulting in increased susceptibility to Herpes Simplex virus and Listeria infection. Nevertheless, the role of *Opn* in MCMV infection has not been tested. Therefore, the dramatic induction of *Opn* expression during MCMV infection and the previous role of *Opn* in herpesvirus infection prompted me to investigate the role of *Opn* in the resistance to MCMV infection.

3.4.1. Real time PCR analysis of *Opn* expression in the splenic NK cells between FVB-Tg (*Ly49h*) and FVB

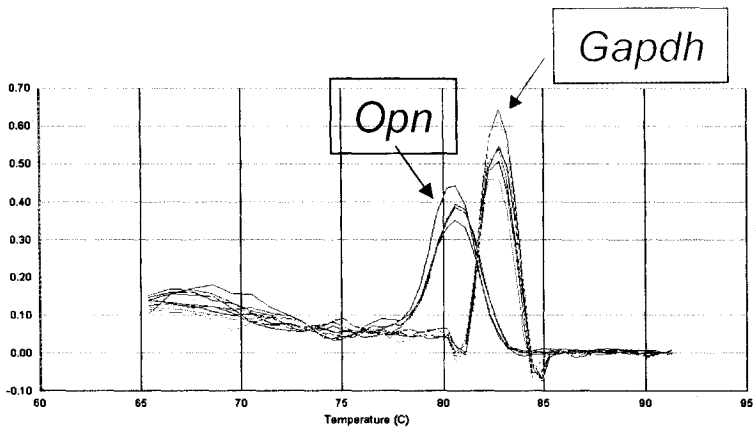
In order to determine the difference in mRNA expression of *Opn* between resistant transgenic mice and their susceptible counterpart mice quantitatively, I performed real time PCR using SYBR green dye. For comparison of *Opn* gene expression in NK cells of FVB-Tg (*Ly49h*) mice to that of FVB mice at early MCMV infection, total RNA was purified from NK cells isolated at 1.5 days post infection. Since SYBR green can integrate any double strand DNA during amplification, it is essential to validate specific amplification of *Gapdh* and *Opn* using melting curve analysis. As shown in Figure 3.12, there was only one PCR product during each PCR,

supporting specific amplification. A relative standard curve method was applied to quantify *Opn* gene expression using *Gapdh* as a control for normalization.

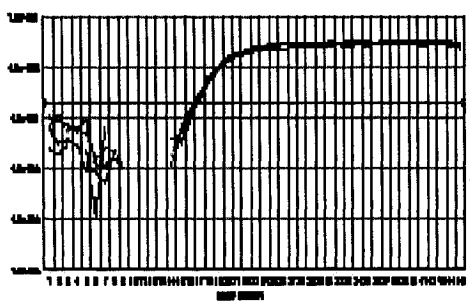
A comparison of normalized signals of *Opn* from each sample indicated that *Opn* is expressed in FVB-Tg (*Ly49h*) mice 16 fold more than in FVB mice, consistent with previous microarray and semi quantitative PCR results. In addition, as shown in the section 3.2, *Opn* gene expression was greatly modulated in the splenic $DX5^+$ cells of FVB-Tg (*Ly49h)* during the course of infection. Taken together, these data suggests that *Opn* is a novel candidate involved in the immune response to MCMV infection.

Figure 3.12 Confirmation by quantitative PCR of differential *Opn* expression in NK cells from FVB and FVB-Tg (*Ly49h*) mice at 1.5 days post-infection. A. Melting curve analysis for *Opn* and *Gapdh* B. Amplification plot for *Gapdh*. C. Amplification for *Opn* between FVB-Tg (*Ly49h*) and FVB D. Relative expression of *Opn* to *Gapdh* in the splenic DX5+ cells at 1.5 days of infection between FVB-Tg (*Ly49h*) and FVB Numbers on Y axis indicate fold difference.

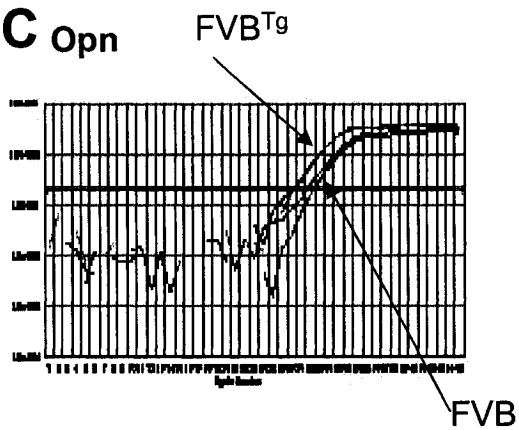
A



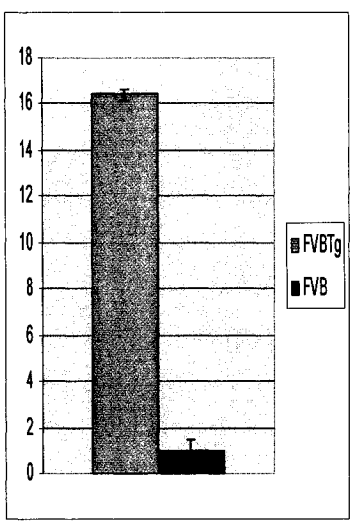
B Gapdh



C Opn



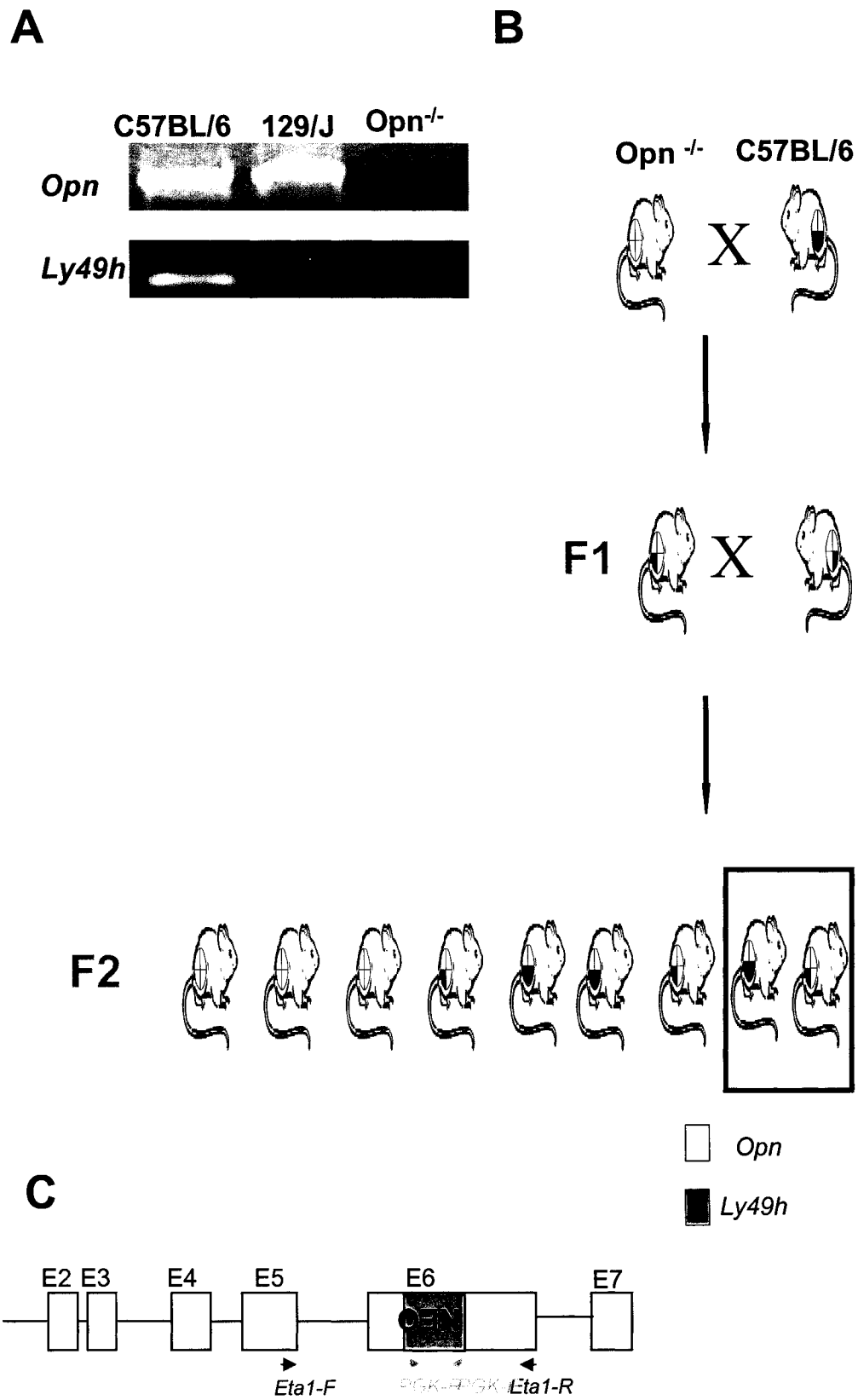
D



3.4.2. Genotyping of *Opn*-deficient mice

Opn-deficient mice were originally generated by targeted gene mutagenesis as described elsewhere (Rittling et al., 1998) and are maintained in a mixed 129/SV × C57BL/6 background. *Opn*-deficient mice do not show any detectable change in development and osteogenesis. Four *Opn*-deficient mice were obtained and transferred to the Animal Care Facility in Roger-Guindon hall at the University of Ottawa for this study. Since these mice had been maintained in a mixed background of MCMV resistant (Biron et al., 1989)(B6) and MCMV susceptible (129) strains and the study was intended to test the function of *Opn* in MCMV resistance upon the presence of *Ly49h*, it was important to examine which strain (B6 or 129) NKC is originated from and, more specifically, whether the *Ly49h* gene is present. Mice were genotyped using two primer pairs, Eta-1 and SV151, which are specific for *Opn* and *Ly49h,* respectively (Figure 3.13). Genotype analysis indicated that these mice contain neither *Opn* nor *Ly49h*. In order to investigate the role of *Opn* in the MCMV resistance in the context of *Ly49h*, one *Opn*-deficient mouse was backcrossed with C57BL/6 mice and the F1 offspring were interbred to generate F2 progeny containing various genetic combinations. 44 F2 mice were obtained and their genotypes were analyzed with SV143, Eta-1 and PGK to monitor the NKC region, *Opn*, and the KO construct respectively. Specifically, genotype analysis with two pairs of oligo, Eta-1 and PGK, was necessary to discriminate heterozygote mice from homozygote mice for the *Opn* gene. As a result of the genotype analysis of 44 mice with these genetic markers, 4 mice of $Opn^{-/-}Ly49h^{+/+}$, and 7 mice of $Opn^{-/-}Ly49h^{+/-}$ which showed the absence of *Opn* and the presence of *Ly49h*, were obtained. Interestingly, there were no mice having $Opn^{-/-}Ly49h^{-/-}$ genotype from this cross.

Figure 3.13 Generation of *Opn*$^{-/-}$*Ly49h*$^{+/+}$ mice. A. Tail DNA of Osteopontin knockout mice obtained from Ottawa Heart Institution and control mice, C57BL/6 and 129/J, were genotyped using specific primers recognizing NKC region (*Ly49h*) and *Osteopontin*. B. A scheme of generation of *Opn*$^{-/-}$*Ly49h*$^{+/+}$ mice. Osteopontin knockout mice obtained from Ottawa Heart Institution were crossed with C57BL6, and their F1 mice were intercrossed to generate multiple genetic combinations of F2 mice. C. The targeted disruption of the *Opn* locus in the paper of *Rittling et al* and position of primer used for genotyping of *Opn* to select Osteopontin knockout mice. The green box labelled *oen* is the neomycin phosphotransferase gene.


A
C57BL/6
129/J
Opn-/-
Opn
Ly49h
B
Opn -/-
C57BL/6
X
F1
X
F2
Opn
Ly49h
C
E2
E3
E4
E5
E6
E7
Eta1-F
Eta1-R

3.4.3. Investigation of the role of *Opn* in MCMV infection

I investigated the possible role of *Opn* in MCMV resistance using *Opn*-deficient mice. The first obvious question was whether *Opn* is involved in the Ly49H mediated killing of MCMV infected cells. To answer the question, I investigated the susceptibility of mice of genotypes, $Opn^{-/-}Ly49h^{+/+}$, $Opn^{+/-}Ly49h^{+/+}$, and $Opn^{+/+}Ly49h^{+/+}$, against MCMV infection of 5×10^3 PFU for 3 days by determining virus titers in the spleen. Virus titers in the liver were also evaluated as controls for appropriate infections. As shown in Figure 3. 14 A, I failed to observe any increased susceptibility in *Opn*-deficient mice compared to their counterparts. In contrast, a highly increased virus titer was observed in mice without Ly49H, which was expected.

In addition, I investigated *Opn*-deficient mice for their viral loads against a high dose of MCMV infection (1×10^4 PFU). Again, the viral titer did not correlate to the genotype of *Opn* gene beyond 8 days of infection (Figure 3.14 B and C). Taken together, these results indicated that the control of MCMV is not influenced by *Opn*.

Opn has been known to attract macrophages and dendritic cells to the site of inflammation and mediate efficient type-1 immune responses through the differential regulation of macrophage IL-12 and IL-10 cytokine expression. During MCMV infection, $CD8\alpha^+CD11c^+$ cell population (a lymphoid dendritic cell subset) was identified to play a critical role in the specific expansion of Ly49H. IL12 and IL18 seem to be major mediators in the mechanism because the specific expansion was abolished in mice deficient in IL12, IL18 or both genes. Of interest, $Ly49H^+$ NK cells are necessary to maintain the $CD8\alpha^+CD11c^+$ dendritic cells in the early stage of infection. Since the *Opn*

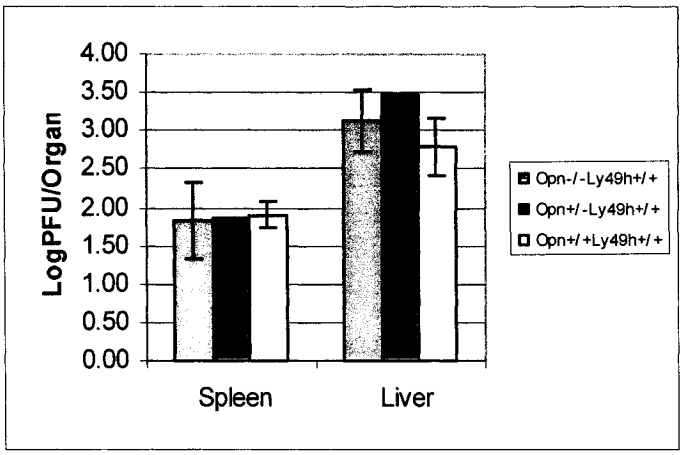
gene activates IL12 production and it is highly induced in the presence of Ly49H signalling, it is conceivable that *Opn* is involved in the preservation of the population. To test this hypothesis, I compared the $CD8\alpha^{+}CD11c^{+}$ cell population using total splenocytes between wild type mice and *Opn*-deficient mice after 8 days of MCMV infection. Five major populations based on the staining with CD11c and CD8α such as $CD8\alpha^{-}CD11c^{hi}$, $CD8\alpha^{-}CD11c^{dim}$, $CD8\alpha^{+}CD11c^{hi}$, $CD8\alpha^{+}CD11c^{dim}$, and $CD8\alpha^{+}CD11c^{-}$ were analyzed for their percentage in total splenocytes. As shown in the Figure 3.15, most populations were comparable among tested mice irrespective of the presence of the *Opn* gene, except for the cell population characterized as $CD8\alpha^{+}CD11c^{dim}$. Even though the exact identity of this population is still not clear, the percentage of the lymphoid dendritic cells is increased in mice deficient in either the *Opn* or *Ly49h* gene, suggesting possible immune disregulations in the absence of a signalling axis from *Ly49h* to *Opn*.

Nevertheless, these experiments were done with very small numbers of mice and further experiments are required for the confirmation of these results. It is also important to note that all the experiments with *Opn*-deficient mice were done using F2 (129 and B6) progeny showing high genetic heterogeneity, which increased the difficulty of analyzing their phenotypes against MCMV infection. For example, the body weights in each group were highly variable and body weight is one parameter determining susceptibility against MCMV infection. To generate *Opn*-deficient mice in pure B6 genetic background, the serial backcross to B6 mice is currently ongoing. The role of *Opn* in resistance to MCMV infection will be re-evaluated once such mice are achieved. Taken together, I failed to observe an increased susceptible phenotype in *Opn*-deficient mice based on virus titers in spleens and mortality, which excludes the direct role of *Opn*

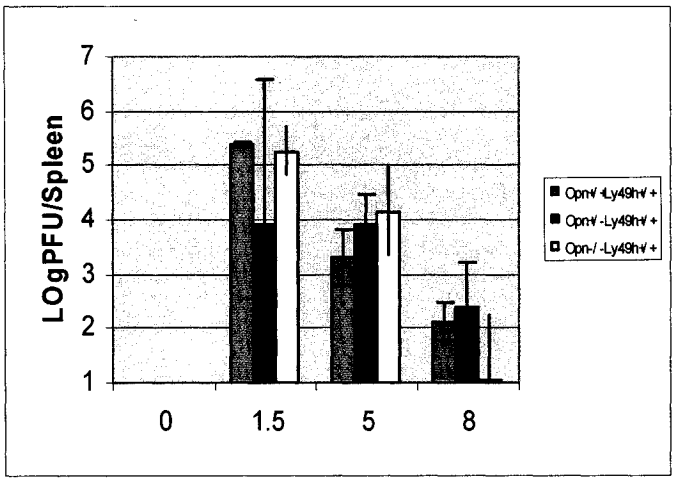
in the resistance against MCMV infection. Nonetheless, *Opn*-deficient mice showed increased numbers of a subset of lymphoid dendritic cells, suggesting the possible role of *Opn* in the immune regulation of other cells during MCMV infection.

Figure 3.14 Susceptibility after MCMV infection in Osteopontin knockout mice. A. Viral titers in the spleen and liver at 3 days of MCMV infection B. Viral titers in the spleen with high doses (1~2 × 10^4 PFU).C. Viral titers in the liver with high doses (1~2 × 10^4 PFU) Viral dose were adjusted based on body weights. D. Blue indicates $Opn^{-/-}$ $Ly49h^{+/+}$, red indicates $Opn^{+/-}Ly49h^{+/+}$, and yellow indicates $Opn^{+/+}Ly49h^{+/+}$.

A


4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
LogPFU/Organ
Spleen
Liver
Opn-/-Ly49h+/+
Opn+/-Ly49h+/+
Opn+/+Ly49h+/+

B


7
6
5
4
3
2
1
LOgPFU/Spleen
0
1.5
5
8
Opn+/+Ly49h/+
Opn+/-Ly49h/+
Opn-/-Ly49h/+

C

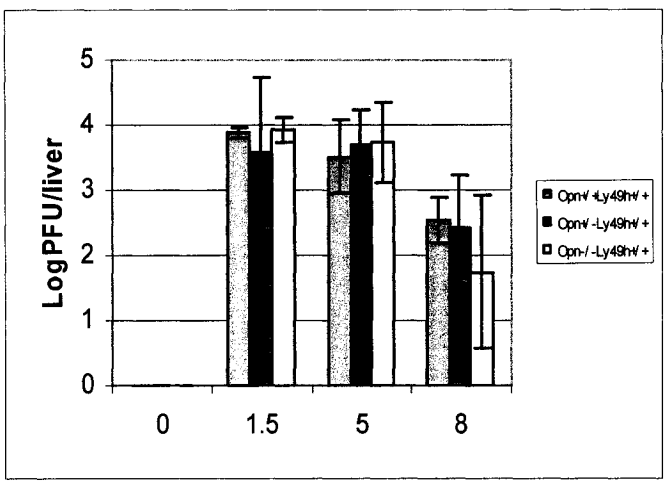
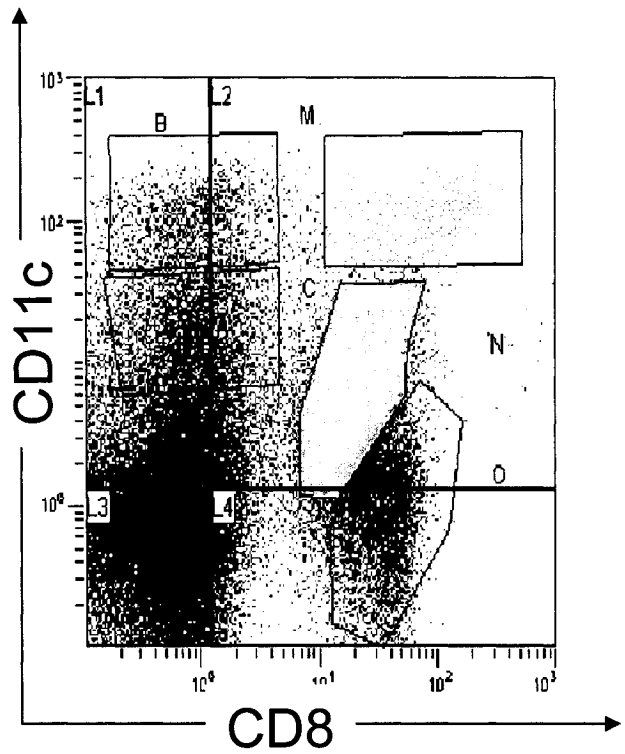

5
4
3
2
1
0
LogPFU/liver
0
1.5
5
8
Opn+/+Ly49h/+
Opn+/-Ly49h/+
Opn-/-Ly49h/+

Figure 3.15 Modulation of specific dendritic cell (DC) population in Osteopontin knockout mice at late MCMV infection Total splenocytes were obtained from mice at 8 days of MCMV infection with liberase treatment to get dendritic cells and stained with mAb against CD11C and CD8. Five different cell populations were analysed (B, C, M, N, and O) and the percentages of cells gated in these areas were calculated.



		% of cell gated in the area				
	Mice ID\Area	B	C	M	N	O
OPN-/- Ly49H+/-	3375	1.31	4.47	0.4	7.76	5.65
	3377	1.75	4.7	0.68	6.92	4.3
OPN+/+ Ly49H+/-	3304	1.32	4.62	0.6	4.4	5.88
	3373	1.6	4.16	0.66	3.6	5.8
OPN+/+ Ly49H+/+	3351	1.92	5.86	0.36	1.96	7.96
OPN+/+ Ly49H-/-	3380	1.7	4.55	0.71	5.59	3.08

CHAPTER SEVEN

GENERAL SUMMARY AND DISCUSSION

7.1. GENERAL SUMMARY

The identification of *Ly49h* as *Cmv1* and the subsequent generation of transgenic mice expressing Ly49H have clearly demonstrated that the NK activation receptor Ly49H is necessary and sufficient to confer natural resistance against MCMV infection. In addition, transgenic mice expressing Ly49H provided an ideal model for identifying the Ly49H-mediated cellular response against MCMV infection, including 1) the early control of viral replication, 2) the specific amplification of the Ly49H+ NK cell population, and 3) the compartmental migration of NK cells from spleen to liver. Despite numerous studies addressing NK cell-mediated immune responses, the precise role of Ly49H *in vivo* was difficult to evaluate because *Ly49h* exists only in mice of C57BL background. In this study, I have taken advantage of MCMV-resistant mice, FVB-Tg (*Ly49h*), and their MCMV-susceptible counterparts, FVB mice, to initiate the functional dissection of the role of Ly49H in the control of viral replication, and the roles of NK cells in the clearance of MCMV virus.

First, to understand this transgenic mouse model better, I compared phenotypes of transgenic mice to those of wild type mice (FVB). The determination of viral titers in 8 visceral organs showed that the introduction of *Ly49h* significantly reduced viral titers in mouse spleens, lungs, kidneys and thymuses at 3 days following MCMV infection, but not in the livers, hearts, bone marrows, and salivary glands, indicating a tissue-specific effect of Ly49H independent of the genetic background. Even though viral titers were similar, the numbers of inflammatory foci in liver, characterized by discrete clusters of nucleated cells, increased in a Ly49H-dependent manner.

Next, I used DNA microarray technology for a comparative gene expression analysis of explanted NK cells from FVB-Tg (*Ly49h*) and FVB mice at 1.5 days post-MCMV infection to identify genes critical to the initial control of virus replication. I determined that NK cells do not produce an m157 transcript that can be detected by RT-PCR amplification in the RNA of NK cells from FVB-Tg (*Ly49h*) and FVB mice. However, the transcript is amplified from total spleen RNA, indicating that NK cells are not productively infected with MCMV. Out of 16,000 genes analyzed, 35 showed greater than 2.5-fold expression differences between resistant and susceptible mice. NK cells from susceptible mice showed an increased expression of pro-inflammatory cytokines such as IFN-γ, MIP2, and TNF associated receptors. In sharp contrast, NK cells from resistant animals showed increased expressions of markers associated with components of cytotoxicity in NK and T cells, cell-mediated immunity, and transcriptional regulation. In addition, *Ly49h, Ly49d, and Ly49i*, which are carried in the transgenic construct, were also upregulated. These results suggest that the differential pattern of expression between resistant and susceptible mice depends upon the presence or absence of *Ly49h,* as well as on signals emanating from productively infected cells, such as macrophages and dendritic cells. Data also indicates that, at this relatively high viral dose, antiviral cytokines are not sufficient to control viral replication in the absence of *Ly49h*, and that the direct killing of virus-infected cells by NK cells expressing Ly49H is crucial for the successful clearance of MCMV.

Then, to investigate the roles of NK cells during MCMV clearance, global gene expression patterns in $DX5^+$ cells, isolated from the spleens of FVB-Tg (*Ly49h*) mice, were investigated at 0, 3, 5, and 8 days MCMV post-infection using microarrays. The percentage of $Ly49H^+$ cells increased with the time course of infection and

corresponding mRNA levels in the microarray also correlated with this protein expression. Out of 22690 genes analyzed, the expression of 213 genes was significantly changed after infection compared to those of uninfected animals. The numbers of genes that are modulated at each time point, and the viral loads of these time points showed a similar pattern; both reached maximum at 3d and eventually decreased at 5d and 8d, suggesting that the expressions of most genes were modulated in the context of viral load. The 213 genes were clustered by their expression patterns, mainly into two groups; upregulated and downregulated during infection. The majority of genes (70%) were upregulated after infection. I also found that major populations of genes are involved in cell proliferation, metabolism, and transcription, which suggests their potential roles in NK blastogenesis and activation.

Lastly, I investigated the role of the *Opn* gene, one of the most intriguing candidate genes identified here, in the control of MCMV infection. In microarray data, *Opn* was highly upregulated in the NK cells of FVB-Tg (*Ly49h*) mice at 1.5d experiment, and this gene expression was confirmed by semi-quantitative and real time PCR. In addition, the gene expression of *Opn* was greatly modulated during the time course of MCMV infection. I also investigated the role of the *Opn* gene in the resistance to MCMV infection by analyzing phenotypic parameters for susceptibility, such as viral loads in spleens and livers and weight loss during infection, using *Opn*-deficient mice. *Opn*-deficient mice failed to show any significant phenotypic differences during MCMV infection when compared with controls. This result indicates that OPN may not be directly involved in the resistance to MCMV infection.

7.2. DISCUSSION

Studies revealing immunity against MCMV infection have demonstrated that many immune barriers, mediated by distinct sets of immune cells, exist to control virus replication (Webb et al., 2002; Beutler et al., 2005). Among these immune cells, NK cells are particularly appropriate players in controlling viral replication during the early stage of virus infection because they can be activated without prior sensitization or priming. For an effective T-cell response upon virus infection, specific T-cell receptors for epitopes of a certain pathogen must undergo a rearrangement of TCR genes and clonal expansion. This response results in a comprehensive, highly specific, adaptive response; however, it requires 5-8 days. Therefore, other immune cells should take care of the invading pathogen before the full activation of adaptive immunity. Unlike T cells, NK cells do not need any gene rearrangement for their receptors. Further, NK receptors are encoded with invariant genes, thereby allowing them to suppress viral invasion in the early days of infection and earn time for the activation of adaptive immune responses (Lanier, 1998).

Roughly, there are two mechanisms by which NK cells perform this function (Biron et al., 1996). The first involves the secretion of various cytokines, such as IFN-γ, TNF-α, and chemokines. The anti-viral cytokines interfere with viral replication, activate other immune cells, or induce the programmed cell death of the infected cells, whereas chemokines recruit immune cells to virus infected cells. The second mechanism employed by NK cells is to destroy virus-infected cells directly. This involves the exocytosis of perforin, accompanied by the release of granzymes and activating caspases that trigger programmed cell death in the infected target cells. Alternatively, a Fas-Fas

ligand (FasL) interaction has also been identified as inducing programmed cell death by activating caspase-dependent pathways in infected cells (Riera et al., 2001); however, its role in the control of MCMV infection is still unclear.

It is noteworthy to mention that the antiviral responses of NK cells are triggered by positive signals mediated by IFN-α/β, IL12 or NK cell- target cell contact through various cytokine receptors and NK receptors (Biron et al., 1999). In particular, IFN-α/β has been known to enhance NK cell cytotoxicity against NK-sensitive target cells through Signal Transducer and Activator of Transcription (STAT) 1 signalling, however the molecular mechanism of the activation is still not clear. Even though IFN-α/β can activate NK cells, increased IFN-α/β observed in susceptible FVB mice failed to control viral replication, which emphasizesing the importance of a recognition system for the efficient killing of virus-infected cells. Therefore, it is clear that Ly49H mediates such recognition during MCMV infection. At the same time, the failure of IFN- to control viral replication indicates that even a higher production of antiviral cytokines and chemokines in the absence of Ly49H failed to compensate for Ly49H-mediated killing, suggesting that each immune barrier has distinct functions for the control of viral replication. For example, whereas IFN-α/β and IFN-γ mediate the inhibition of intracellular viral replication and immunoregulate other immune cells, NK cells use their receptors to recognize and eliminate virus-infected cells intercellularly via cell-cell contact between effector cells and target cells.

The mechanisms by which NK cells counteract viral infections are organ-dependent. The organ specificity of *Cmv1*/Ly49H resistance has been identified by showing that its great effect in the control of MCMV happens in the spleen, but not in

the liver. Considering that Ly49H destroys virus-infected cells in a perforin-dependent and IFN-γ-independent manner (Tay and Welsh, 1997), this organ specificity might validate the hypothesis that NK cells use Perforin and IFN-γ in an organ-dependent manner to regulate acute MCMV infection. However, this hypothesis has been challenged by a recent study. *Loh et al*, have demonstrated that both Perforin and IFN-γ are important to regulate acute MCMV infection in the spleen and liver by analysing virus titers in these two organs in $Pfp^{-/-}$, IFN-$\gamma^{-/-}$ and NK-deficient mice (Loh et al., 2005). Therefore, it is interesting to investigate the effects of Ly49H in the control of MCMV replication among different organs. The determination of MCMV titers at 3days infection indicated that Ly49H mostly controls MCMV replication in spleens but also in lungs, thymuses, and kidneys. Lower viral titers in B6 and transgenic mice expressing Ly49H were used as indicators of the specific effect of Ly49H in these organs. In addition to its minor effect in the liver, Ly49H seemed to have no effect in the control of MCMV replication in the heart. However, *Vliegen et al,* using real time PCR for quantification of the MCMV genome, have shown that there are clear differences in MCMV replication at later time points between resistant B6 and susceptible BALB/c mice (Vliegen et al., 2003). Therefore, a careful investigation of the full window of innate immune responses (up to 6 days post infection) should be required to conclude the organ-specificity of Ly49H in the restriction of MCMV replication. Another possibility is that; since all organs are connected by the blood vasculature, active MCMV replication in any organ will indirectly affect the MCMV titers of neighbouring organs. Moreover, lesser responsiveness to the effect of Ly49H in the liver and heart might be due to their compact parenchyma cells, which prevent NK cells from easily

accessing virus-infected cells. Indeed, analyses of MCMV replication in the liver revealed different kinetics of the control of viral titers compared to the spleen, showing one log lower titer in Ly49H$^+$ resistant mice by day 6 post infection.

It was interesting to identify an increased number of inflammatory foci in the livers of Ly49H$^+$ mice during MCMV infection. A possible interpretation of this data is that Ly49H-m157 interactions between NK cells and MCMV-infected cells may facilitate the localization of NK cells to the infected targets. Alternatively, Ly49H$^+$ NK cells might be more responsive to cytokines secreted by the infected cells, and therefore, be found more frequently in their vicinity. These possibilities could be tested with the same type of assays, using a virus strain deleted at the *m157* gene. It has been previously shown that infection with mutant delm157 reverts the resistance phenotype in C57BL/6 mice into susceptibility because the Ly49H axis cannot be activated (Voigt et al., 2003; French et al., 2004). If direct contact with Ly49H-m157 is necessary to the formation of inflammatory foci, these should be lacking in mice infected with delm157.

The analysis of gene expression profiles using enriched DX5$^+$ cells from FVB-Tg (*Ly49h*) and FVB using microarray technology allowed us to understand NK-specific killing of MCMV-infected cells better during infection in the context of Ly49H. We confirmed the upregulation of genes such as CD160 (BY55), CD7, and *Opn* (*Eta-1 or Spp1*) in FVB-Tg (*Ly49h*) at 36 h post-infection. Since these genes have been implicated in the cytotoxicity of NK and T cells, or cell-mediated immune responses, this supports their candidacy for roles in the control of MCMV infection. CD160 has been known to acts as a coreceptor in TCR signal transduction of CD8$^+$ CD28$^-$ T lymphocytes in humans (Nikolova et al., 2002). In NK cells, it is also expressed as an Ig-like activating NK cell receptor on the majority of circulating non-proliferating CD56^{dim}CD16$^-$ NK

cells, and induces NK cytotoxicity and cytokine production such as IFN-γ, TNF-α, and IL-16 through the engagement of the HLA-C molecule (Barakonyi et al., 2004). The CD7 antigen is a cell surface glycoprotein found on T and NK cells. It is one of the earliest antigens to appear on cells of the T-lymphocyte lineage and the most reliable clinical marker of T-cell acute lymphocytic leukemia (Baker et al., 1990). Studies in CD7-deficient mice demonstrated that CD7 plays important roles in regulating cytokine production and the cytotoxicity of T and NK cells. These mice showed both a reduced sensitivity to LPS-induced shock syndromes and the possible role of CD7 in the maturation of $NK1.1^+$ T cells (Sempowski et al., 1999). The cross-linking of CD7 on NK cells has been reported to induce membrane calcium flux, adhesion to fibronectin, and production of IFN-γ (Rabinowich et al., 1994). Osteopontin (OPN) was originally identified as the protein that is produced by osteoblasts and involved in the anchoring of osteoclasts to the mineral of bone matrix (Reinholt et al., 1990). Recent numerous studies showed that *Opn* is also important in the functioning of immune cells, including T lymphocytes and NK cells (Gravallese, 2003). The importance of *Opn* in immune responses is emphasized by the fact that *Opn* mediates Th1 responses by inducing IL-12 production in microbial infections (O'Regan et al., 2000). Mice deficient in *Opn* have greatly increased susceptibility to Herpes simplex virus-type I and *Listeria monocytogenes* infection (Ashkar et al., 2000), indicating the critical role of *Opn* in host resistance to infections. However, these results have been challenged recently by Abel et al (Abel et al., 2005) who could not demonstrate any difference between wild-type and *Opn*-deficient mice in the response against *Listeria monocytogenes*. Similarly, our results using Opn knockout mice did not show any direct role of *Opn* in the control of

MCMV titers. However, we cannot exclude at this point that the induction of *Opn*, a Th1 type cytokine, may provide some immunoregulatory role to NK cells at this, or later, time points in the infection. Further studies will be required, first monitoring specific protein expression during infection, and detailed phenotypic analysis in knock-out models in a homogeneous background.

Since cytokines are important in antiviral responses, it was surprising to discern the higher gene expressions of many proinflammatory cytokines in the NK cells of susceptible FVB mice. The critical role of cytokines has been appreciated by observations that mice deficient in either cytokines or cytokine-related genes such as IFNs and Mip-1α showed increased susceptibility to, and mortality from, MCMV infection (Salazar-Mather et al., 2002). At 1.5 days MCMV infection, NK cells from susceptible mice showed increased expressions of inflammatory cytokines such as members of the type I IFNs, IFN-γ, MIP2, TNF-α, and TNF associated-receptors. Even though they are certainly antiviral cytokines, the high virus replication observed during the active expression of these cytokines suggests that antiviral cytokines are not sufficient to control viral replication in the absence of *Ly49h*.

Furthermore, it is conceivable that such overexpression of these cytokines in FVB mice might have deleterious effects by generating immunopathological effects such as tissue damage or vascular collapse. I have observed that the spleens of susceptible mice underwent severe necrosis (characterized as stiff spleens and very low yield of viable cells) at 3 days of MCMV infection, suggesting that proinflammatory cytokines play a harmful role in tissue pathology (Nian et al., 2004). The TNF-α/ TNF-α receptor superfamily is interesting since it has been identified as the major mediator

responsible for tissue destruction (Hehlgans and Pfeffer, 2005; Dempsey et al., 2003). Although TNF-α is an important proinflammatory cytokine in anti-viral immunity when its production is tightly regulated, uncontrolled persistent TNF-α production can exacerbate illness by inducing tissue damage. It is tempting to suggest a follow-up of the cytokine levels in the sera of risk patients for HCMV infection, to determine if similar correlates can be drawn with human viral susceptibility.

In the next paragraphs, however, I would like to pose on several concerns about applying microarray data to understand the biological system. Even though microarray technology is now commonly used for global gene expression, many discrepancies have been observed between gene expression and protein abundances in many studies (Lian et al., 2001). Since the level and activity of protein can be modulated at the post-transcriptional and post-translational levels, it is necessary to validate microarray data by protein analysis or functional assay. The other important concern in our microarray experiment relates to the antibody used for NK purification, DX5. $DX5^+$ cells are comprised of NK cells and NKT cells. Approximately 2% and 0.5% of NK and NKT cells, respectively, are present in naïve splenic leukocytes. Even though NKT cells are clearly stained by the DX5 antibody, the staining intensity is a half log lower than that of NK cells, and the weak expression of CD49b (the antigen of DX5 antibody) might reduce the contamination of NKT cells. Indeed, I did not observe NKT cells expressing lower DX5 staining when confirming the purity of $DX5^+$ cells. The last concern is on the subject of the purity of the $DX5^+$ cells used for the microarray experiment. Although total RNA was obtained from enriched $DX5^+$ cells (70 to 80%), there remained 20 to 30 % non-DX5 cells, suggesting that some modulated genes may be expressed in $DX5^-$

cells. For example, Plasmacytoid DCs (PDCs), characterized as $CD8\alpha^{+}CD11c^{dim}Ly6G/C^{+}$ $CD11b^{-}$ (after infection), are known to be major producers of IFN-α/β and IL-12 in response to MCMV infection (Dalod et al.,). Therefore, increased IFN-α/β expression in susceptible FVB mice at day 1.5 might come from $DX5^{-}$, in particular pDC cells. However, the expressions of IL-12, another cytokine highly expressed by pDC cells, were absent in all microarray experiments, possibly alleviating the concern of pDC contamination in the IFN-α/β production. $DX5^{+}$ cells seemed to not be a major producer of TNF-α during MCMV infection in vivo (Lee and Biron, Unpublished data), even though NK cells have been reported to express TNF-α transcripts upon the cross-linking of Ly49D activating receptors in vitro (Ortaldo and Young, 2003).

Nevertheless, I observed expression of genes that are unlikely to be expressed in NK cells, suggesting the presence of transcripts from the contamination of other cells. For example, the expression of genes such as Hemoglobins (*Hba-a1 and Hbb-y*) has not been reported at the level of NK cells so far. Taken together, a careful validation of these gene expressions on NK cells has to be performed in order to make conclusions about their role in NK cells, in response to MCMV infection.

Overall, in this thesis, I used transgenic mice expressing Ly49H to characterize functionalities of NK cells during MCMV infection. In spite of concerns mentioned above, a comparative analysis of whole genome expression studies of $DX5^{+}$ cell populations could be helpful in identifying previously unrecognized genes and pathways of defence mechanisms in MCMV infection. Despite clear structural differences between activation-NK receptors in mice and humans, for example, C-type lectin Ly49

receptors in mice *versus* killer cell immunoglobulin (Ig)-like (KIR) receptors in humans, it has been proposed that these signal through identical intracellular pathways to activate (or turn off) essentially the same genes (Colucci et al., 2002). Therefore, based on the proven importance of NK cells in HCMV infection, and the similarities in NK receptors in mice and humans, our data could provide valuable information to investigate HCMV infection in immunocompromised patients such as neonates, graft recipients, and HIV patients.

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