Cmv5, a Genetic Modulator of Host Resistance to CMV Infection

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Abstract

Human cytomegalovirus (HCMV) is one of the most ubiquitous viruses in the human population ranging from 40% seroprevalence in developed countries to 100% in developing countries. Although infected individuals are usually asymptomatic, CMV infection may lead to severe deceases and can even be life-threatening to newborns and immunocompromised hosts. The introduction of the mouse cytomegalovirus (MCMV)-mouse model to study viral pathogenesis has aided in the understanding of multiple levels of host-virus interactions, highlighting the role of host genetics. Recently, the Vidal lab has found that the susceptible 129S1 strain of mice modulates documented interactions known to lead to viral clearance in resistant strains (such as C57BL/6) and stays susceptible even when genetic factors necessary to clear the virus were recapitulated in the strain. The aim of this thesis is to characterize this susceptibility and understand which cell types are implicated.

The susceptibility was genetically localized via a QTL analysis to chromosome 9 between 40-92 Mb in 129S1, a region dubbed *Cmv5*. Congenic lines harbouring the *Cmv5* locus in a C57BL/6 background displayed maximal susceptibility between 36 and 48 hours p.i, where Natural Killer (NK) cells, (the main cell type implicated in MCMV clearance) seem to have deficient functional capacities such as granzyme and IFNγ production. This time point correlates directly with deficiencies observed in type I IFN secretion (cytokines necessary to activate NK cells in the context of infection), which dendritic cells (DCs) are known to be responsible for at 36 hours p.i. Interestingly however, the congenic line seemed to have fully functional NK cells upon *in vitro* characterization. Thus it seems as though DCs are the main cell type affected directly by *Cmv5*, who's deficiency indirectly affects NK cell's activation and ability to clear the virus in the context of infection.

All in all, this study has identified a novel genetic defect in the 129S1 strain that directly affects DCs and leads to an inefficient response by NK cells allowing susceptibility to MCMV 36 to 48 hours p.i. This study will also pave the way for future follow up studies that will identify genes and genetic factors responsible for modulating MCMV resistance and lead to a new understanding of host-virus interactions in both mice and humans.

Abrégé

Le cytomégalovirus humain (HCMV) est un virus très répandu chez l'homme, oú la séroprévalence est évaluée à 40% dans les pays développés et jusqu'à 100% dans les pays en voie de développement. Même si l'infection par ce virus est souvent bégnine, elle peut être également responsable de maladies sévères notamment chez les nouveaux nés et les personnes immunodéprimées. L'utilisation du cytomégalovirus murin (MCMV) pour l'étude de la pathogénèse virale a permis d'améliorer notre compréhension des interactions virus-hôte à de multiples niveaux et notamment au niveau du rôle des facteurs génétiques de l'hôte dans le contrôle de l'infection par ce virus. Le groupe du Dr. Vidal a récemment montré que, contrairement à la souche murine C57BL/6, la souche 129S1 est susceptible à l'infection par MCMV même si celle-ci possède tous les déterminants génétiques connus pour être essentiels dans le contrôle de l'infection virale. L'objectif de ma thèse est d'identifier les mécanismes qui sont à l'origine de cette susceptibilité et de déterminer quels sont les types cellulaires impliqués.

Grâce à l'utilisation d'analyses génétiques dites QTL, la susceptibilité à l'infection par MCMV a pu être localisée dans une région 129S1 comprise entre les positions 40 et 92 Mb du chromosome 9 et appelée Cmv5. Des souris congéniques C57BL/6 portant ce locus Cmv5 ont été montrées comme étant très susceptibles à 36 et 48 heures après infection, période pendant laquelle les cellules «Natural Killer»(ou NK sont connues pour être les principaux acteurs de l'immunité anti-MCMV) semblent être déficientes pour certaines de leurs fonctions, comme la production de granzyme et d'IFN-gamma. Ces temps d'infection corrèlent parfaitement avec un défaut de sécrétion d'IFN de type I (cytokines nécessaires pour activer les NKs) par les cellules dendritiques (DCs) 36 heures après infection. En revanche et de façon intéressante, nos études in vitro réalisées à partir des souris congéniques n'ont montré aucune déficiente fonctionnelle au niveau des cellules NK. Ainsi, il semblerait que les DCs soient les principales cellules affectées directement par la région Cmv5, et que cette déficience est ensuite indirectement délétère pour l'activation des cellules NK et leur capacité à contrôler l'infection virale.

En résumé, cette étude a permis l'identification d'une nouvelle déficience génétique associée à la souche 129S1. Celle-ci affecte directement les DCs et,

de ce fait, entraine un dysfonctionnement au niveau des cellules NK. L'ensemble de ces observations constitue des éléments de réponses permettant d'expliquer la susceptibilité MCMV observée 36 et 48 heures après infection. Cette étude devrait également permettre d'émettre de nouvelles hypothèses dans la recherche de facteurs génétiques impliqués dans la susceptibilité/résistance à l'infection par MCMV et d'améliorer notre compréhension des interactions virus-hôte aussi bien chez la souris que chez l'homme.

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Chapter 1

Introduction

1.1 General Introduction

The evolutionary interaction between pathogens and hosts is one that has been developing for millions of years and produced the robust immune responses observed in contemporary organisms. The immune systems of hosts have evolved to be able to detect pathogenic molecules and control infections, where by organisms have even developed specialized molecules, cells and tissues in order to effectively fight of infections. Simultaneously, over time, pathogens have also adapted to evade these immune responses.

If the evolution of the immune system were closely observed, it would be apparent that there are two main arms of immunity; the innate and the adaptive immune response. The innate branch is a nonspecific approach developed by organisms as a primary barrier to infection. Relatively the innate immune responses are clearly more ancient than the developed and specific adaptive immune response. However although older, a well-timed innate im-

mune response could completely clear an infection or provide necessary and vital inputs to the adaptive immune system so as to allow it to function with maximum efficacy. Thus both arms of immunity work together with a common goal: clearing infection and protection of the host.

Murine Cytomegalovirus (MCMV), a model of human Cytomegalovirus (HCMV) has been an exemplary model to study the innate immune response. The reason for this is the long coevolution the virus and host have experienced together, which resulted in highly evolved immune responses in the host and the development of immune evasion techniques by the virus. It has been found that Natural Killer (NK) cells are the main players in the clearance of this virus, and use mostly their pattern-recognition receptors (PRRs) in order to detect virally infected molecules. Although MCMV's interaction with NK cells is relatively well understood, the underlying mechanisms of viral immune evasion and the full picture of NK cells' immune responses have not been fully deciphered. In fact, the study of MCMV pathogenesis is a relatively large field, and as of late has been understood in the context of host genetic aspects that are responsible for modulating anti-viral responses. This thesis explores one such genetic aspect that has been found to modulate a known interaction between NK cells and MCMV infected cells.

1.2 Cytomegalovirus

Cytomegalovirs (CMV) is one of the most ubiquitous viruses in the human population reaching up to 100% seroprevalence in developing countries and 45% in developed countries [1]. There are many species of CMV, each of which is typically host specific (though multiple species can infect the same host) and is capable of being transmitted horizontally as well as vertically. Although under normal circumstances, the consequences of HCMV infection are minimal, it can pose serious risk to newborns and immunocompromised individuals such as HIV patients or transplant recipients [2, 3, 4].

CMV is a genus of virus that belongs to the Herpesvirales order. It is known to be one of the larger viruses, and has a typical viron structure that is enveloped. In its capsid, each viral particle has a single copy of its double stranded DNA genome. This genome is comprised of approximately 230 megabase pairs (Mbs), predicting to code at least 170 open reading frames (ORFs) [5, 6]. Recently, unpublished work on the MCMV transcriptome conducted in Stipan Jonjic's lab hints that there maybe additional overlapping coding sequences between known open reading frames which would produce much more than just 170 viral gene products.

The virus itself is able to infect multiple niches of cell types ranging from endothelial, epithelial and myeloid cells to muscles to neurons. This falls hand in hand with the fact that CMV infection could cause a whole array of symptoms and may be responsible for various manifestations of disease [7]. In

order to study the dynamics of these diseases, the MCMV-mouse model was established and provided pivotal progress in the understanding of the virus and its manifestations. In a laboratory setting, 2 main strains of MCMV have been used to infect mice: the Smith strain and the Smith derived K181 strain[8]. These strains can be prepared via *in vivo* serial passages in mice to produce a salivary gland derived virus or can be cloned in bacterial artificial chromosomes (BAC) and propagated by transfection in mouse cell lines *in vitro*.

Molecularly, the typical course of infection starts via viral envelope attachment to the host membrane and entry of the nucleocapsid into the cytoplasm. Microtubules then transport this nucleocapsid from the cytoplasm to the nucleus where the DNA is processed and along with host proteins, viral proteins start the initiation of transcription of immediate-early (IE) genes that are eventually responsible for establishing infection and further transcription of early genes encoding for DNA replication [9]. Right after DNA replication, late genes (L) start to be transcribed and are responsible for viral structural proteins and genome packaging proteins. By this point, the virus is capable of lysing the cells they have infected to release viral particles and restart the cycle.

On a more macro level, the infection occurs in stages. Upon intravenous (i.v) or intraperitoneal (i.p) infection in mice, MCMV disseminates through blood and lymphatic routes and infects bone-marrow myeloid cells that seed the infection in visceral organs such as spleen, liver, lungs and salivary glands

as soon as 8 hours post infection (p.i) [10, 11]. Infected stromal cells at this point produce cytokines necessary to activate DCs 36 hours p.i. This causes DCs to produce further cytokines that activate NK cells, the main cell type responsible for controlling CMV replication (the early host response at 8 and 36 hours p.i is further discussed in section 1.4). Once NK cells are activated, they continue to control the virus, however they gain maximum efficiency at approximately 3-4 days p.i due to the up regulation of NK specific receptors that allow for better recognition of infected cells and further potent activation of NK cells (further discussed in section 1.3.3). The spleen and liver are the typical organs used to characterize viral replication in a an experimental setting of acute infection, and it is observed that replication peaks at around day 3-4 and starts to be controlled until no viral particles can be detected by day 10 presumably due to the efficient NK cell function after 3 days of infection. Although no viral particles are detected by traditional plaque assay in these organs on this day, replication continues in the salivary glands for 4-8 weeks.

CMV has also evolved the ability to remain latent in cells; there are no infectious viral particles in the host, but copies of the viral genome linger in cells and could potentially reactivate and cause infection if the host becomes immunosuppressed. After replication ceases and no MCMV viral particles are found by plaque assay in the liver and spleen by day 10 and in the salivary glands by 8 weeks, the viral genome can still be detected in many organs such as the spleen, liver, lungs and heart [12]. Interestingly, it has been found that

macrophages are the major reservoir of latent virus [13].

The MCMV-mouse infection model is an excellent model to study host and CMV viral interaction for multiple reasons. MCMV is a natural pathogen of mice in the wild and its infection seems to trigger analogous responses to HCMV infection in humans [14]. Moreover, the response to MCMV is seems to be dictated based on many genetic host and viral factors, as well as their interactions. Given the range of inbred mouse strains present, the mouse model provides an excellent platform to study the range of genetic determinants that control resistance to CMV.

1.3 NK Cells

1.3.1 General

NK cells are granular lymphocytes that have earned their name through the initial observation of their ability to spontaneously kill Moloney leukemia vius infected cells and produce interferon gamma (IFNγ) without being stimulated in any way [15, 16]. It is now well known that they are also crucial for tumour surveillance, regulation of the adaptive immune response and the control of infection induced by a multitude of virus, CMV being a specifically well studied example [36].

In mice, functional NK cells are characterized by the lack of the cluster of differentiation (CD) 3 receptor (a receptor mostly found on T cells) and the presence of the $\alpha 2\beta 1$ integrin duplex composed of CD49b and CD29. NK cells can also be characterized by the expression of the Natural Killer cell p46 related protein (NKp46) receptor on their surface. In addition, certain strains of mice express the NK1.1 receptor on all NK cells and a minor subpoppulation of T cells (which unlike NK cells express CD3). In humans, NK cells are characterized by CD56 surface expression as opposed to $\alpha 2\beta 1$ integrin (and CD3 is also not expressed) [17].

Various unique subpopulations of NK cells that have the characteristic surface markers but differ in their organ localization have been described. For instance, gut associated mucosal lymphatic tissues have NK cells specialized in the production of IL 22 which is known to contribute significantly to

mucosal immunity [18]. Another subpopulation that has been documented are NK cells resident to the salivary glands (one of the main sites of MCMV latency). These cells were found to be hypo-responsive in terms of inducing their effector functions relative to splenic NK cells, explaining why MCMV preferentially replicates in the salivary gland long after other organs have cleared the virus [19]. Uterine NK cells in the endometrium have also been documented and believed to be involved principally in a regulatory role in tissue homeostasis and remodelling [20]. Splenic and liver NK cells are also very well documented in the context of controlling infections, although they do have different functional profiles which has recently been attributed to which lineage they originate from [21]. Therefore depending on the origin of the NK cell and the organ they develop in, they will mature to have different profiles.

The involvement of NK cells in controlling infection has been documented to take part against a whole range of pathogens including bacteria, parasites and viruses [14]. In humans it is known that NK cell genetic deficiencies can lead to HCMV and other herpesvirus susceptibility [22]. In the context of MCMV, they are a crucial cell population that are mostly responsible for the clearance of the virus. It has been well documented that absence or lack of function in part of NK cells increases susceptibility to MCMV infection [23, 24, 25]. Studies show that if mice have been treated antibodies to deplete NK cells prior to MCMV infection, they exhibit up to a 1000 fold increase in viral titers. Moreover, while control mice clear the virus from

the spleen fully by day 7, NK depleted mice exhibited moderate viral titers up till day 9. Follow up studies have also shown that adoptive transfers of functional/healthy NK cells will rescue NK deficient mice from MCMV replication and disease symptoms [26, 27].

The reasons for NK cell dysfunction and inability to clear viral infections are vast, however they can all be summed up in three broad categories: NK cell development and maturity, NK cell recognition of infection and NK cell effector/killing functions. These three categories need to be understood in order to allow for careful dissection of NK cell dysfunction in any context.

1.3.2 NK Cell Development and Maturity

There are at least four NK cell lineages described in mice: conventional (cNK), thymic, liver and uterine NK cells [21]. Here we will focus on cNK cells, whose progenitors originate in the bone marrow. They are known to populate the spleen, a major organ implicated in viral resistance, and are well characterized for their role in immune defence. The development of cNK cells typically occurs in stages that are necessary in order to prepare enough competent NK cells to ellicit an efficient immune response. If there is a block in development, the cells tend to be hyporesponsive and unable to properly clear infection.

Developed NK cells are typically differentiated from precursor cells by changes in surface receptor expression, and although the stages of development in terms of receptor changes have been well documented, the transcription factors critical to this process are beginning to be understood. Specifically, the development of NK cells requires certain transcription factors [28] of which NFIL3 (or E4BP4) has been dubbed an NK cell specific factor [29]. In addition, splenic cNK cells require IL 15 and its cognate receptor IL15R for efficient development as mice deficent in either of these receptos/ligand pair are devoid of splenic cNK cells [30].

In mice there are generally 5 stages of development, however the amount of stages differs based on who is describing the process since arbitrary checkpoints are defined for simplicity in a continuously progressing process. The first stage, where the cells are still identified as NK precursors in the bone

marrow is characterized by CD122 and NKG2D surface expression, and the absence of NK1.1. The second stage is hallmarked by the acquisition of NK1.1, TRAIL ligand and CD94, followed by the third stage where they start loosing TRAIL expression and acquire CD11b and the Ly49 receptors (which will be further discussed in section 1.3.3). Only after this third stage are the cells considered to be "mature" NK cells, capable of efficient cytokine secretion and effector functions. The fourth stage is characterized by the surface expression of CD27, CD49b and CD43 and cell proliferation. This stage usually occurs while NK cells are still in the bone marrow, but soon after the acquisition of CD49b the they leave the bone marrow and migrate to the spleen. Once in the periphery, NK cell development moves on to the fifth stage, which can be further subdivided into four sub-stages correlating to how "mature" an NK cells is. In the order of least to most mature, they are $CD11b^{-}CD27^{-}$, $CD11b^{-}CD27^{+}$, $CD11b^{+}CD27^{+}$ and finally $CD11b^{+}CD27^{-}$ [31, 32]. It has been observed that $CD11b^{+}CD27^{+}$ NK cells have the most potent effector functions and low thresholds of activation, however $CD11b^{+}CD27^{-}$ are the main NK cell sub-population responsible for non-self rejection [33, 34, 35].

Genetic defects in genes involved in maturation of NK cells have been documented in both the human and mouse context [36, 37]. In both cases a gene mutation causes a halt in NK cell maturity either directly, or indirectly by affecting a cell type heavily involved in NK maturity, eventually leading to detrimental phenotypes upon infections known to be controlled by NK

cells. Thus in recent years, genetic approaches have been heavily utilized in elucidating the root source of defects in NK cell maturity [38, 39, 40].

1.3.3 NK Cell Recognition

The ability of NK cells to distinguish self from non-self is the prominent ability and enables these cells to effectively fight off infection while preventing autoimmunity. In fact, one of the first characterizations of NK cells was their ability to reject radiolabelled tumour cells that do not express the Major Histocompatibility Complex I (MHC-I), while not being able to kill the same cells expressing MHC-I [41]. Interestingly, many viral infections are capable of down regulating MHC I expression on the surface of infection cells, which NK cells then recognize as an aberrant, non-self cell. Moreover, cytosolic viral peptides degraded via the proteosome are also loaded onto MHC I receptors generally, and thus an infected cell having viral proteins in its cytosol will express degraded viral proteins on their surface MHC I molecules, which some specific NK cell receptors are capable of recognizing [42].

In mice, the receptors that detect MHC-I on NK cells belong to the C-type lectin-like family, specifically the Ly49 family of receptors clustered in the Natural Killer gene Complex (NKC) on chromosome 6. The receptors' expression have been well documented in many strains of inbred and wild mice and were found to be highly variable in gene copy numbers and polymorphic [43, 44, 45, 46]. Moreover, Ly49 receptors present inhibitory and activating receptors that are stochastically expressed in overlapping NK cell subsets. In humans, NK receptors are the Killer Ig-like Receptors (KIR) and are found on chromosome 19q12.4. KIR receptors have multiple alleles and were found to belong to one of 2 major haplotypes; Haplotype A which con-

tains mostly inhibitory receptors and Haplotype B which is a combination of both activating and inhibitory receptors. These sets of receptors in mice and humans are a great example of convergent evolution because the receptors are not phylogenetically related however they bind the same ligands and exhibit the same downstream signalling pathways.

The immunological role of the Ly49 receptors has been extensively studied and found to be particularly involved in NK response to MCMV. Activating Ly49 receptors and their ligand interactions have been relatively well characterized in mice. In general, the activating Ly49 receptors have a lower affinity to MHC I molecules than inhibitory Ly49 receptors. In the context of infection however, they bind to MHC I molecules complexed with a viral peptide which leads to a downstream signalling in the NK cells that potentiates NK cell effector functions. Activating Ly49 receptors do not necessarily need to have a MHC I associated ligand; it has been documented early in MCMV studies that non-MHC I linked ligands such as viral proteins expressed on the surface of target cells can activate NK cells [47].

Although different activating Ly49 receptors have a very wide range of ligands and react differently to different H2 haplotypes, they generally have the same downstream signalling. These receptors contain a positively charged arginine (sometimes lysine) residue in their intracellular domain, allowing them to interact with negatively charged moieties on adaptors such as DAP10 and DAP12 that contain Immunoreceptor Tyrosine-based Activating Motifs (ITAM). Upon receptor ligation, adaptor proteins are recruited to the intra-

cellular tail of the receptor and are phosphorylated by Src family kinases. This interaction eventually leads to downstream signal amplification that ultimately leads the NK cell to exhibit proliferation, cytotoxic and killing functions (discussed in section 1.3.4). Other, non-Ly49 activating receptors such as NK1.1 and NKp46 follow the same general downstream pathways but utilize adaptor proteins other than DAP12. NK1.1 uses CD3ζ and NKp46 uses FcϵRI-γ which both also contain ITAM motifs [48].

Inhibitory Ly49 receptors comprise most of the documented Ly49s. Their main ligand seems to be MHC I, and they generally have high affinity for the molecule, however different Ly49s show variable affinities to different haplotypes of H2 expressed in MHC I. Also, much like activating receptors, inhibitory receptors also follow a common downstream signalling pathway. Inhibitory receptors contain Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIM) in their intracellular domains, which upon receptor ligation, triggers the phosphorylation of the tyrosine moiety by Src family kinases. This provides a docking site for the recruitment of cytoplasmic SH2-domain-containing protein tyrosine phosphatase 1 or 2 (SHP1 or SHP 2) that eventually leads to downstream signal amplification, causing a dampening of activating signals and a general hypo-responsivness of NK cells [48]. In general, inhibitory signals dominate over activating signals [49].

Therefore Ly49 receptors have a wide array of ligands that trigger them providing a complex web of interaction between activating and inhibitory signals and depending on the balance of these signals, the final output of NK

cells will be dictated. Two relevant activating Ly49 receptors that relevant to the basis of this thesis are Ly49H that interacts with a non-H2 linked ligand on target cells and Ly49P that interacts with a viral particle / MHC-I complex expressed on the surface of infected target cells. Both are discussed in further detail below.

Ly49H The discovery of Ly49H activating receptor harboured in the *Cmv1* genetic locus in the NKC was pivotal in the understanding of NK mediated resistance to MCMV. In relation to other activating receptors, the ly49H response is one of the most potent NK cell-activating signal and its importance became apparent after multiple studies, which showed that any alteration in *Cmv1* through genetic manipulations led to significant susceptibility to MCMV [50, 51, 52, 53]. The specific Ly49H resistance starts at around 3 days p.i with MCMV and induces a potent activation in NK cell subsets that express Ly49H that eventually leads to viral clearance.

The natural ligand of Ly49H is m157, a MCMV encoded MHC-I homologue [54, 55]. Once m157 has bound to Ly49H, the downstream signaling happens as it would for most activating receptors, and specifically through the ITAM containing DAP12 adaptor protein as previously described (Figure 1.1). Since MCMV infection naturally downregulates MHC-I in infected cells, it has been hypothesized that the reason that MCMV has evolved to code for an MHC-I homologue is to evade NK cells. The fact that Ly49H expressing NK cells have evolved to detect this viral homologue exemplifies

the deep evolutionary relationship between MCMV and its host.

Ly49P Unlike Ly49H, Ly49P does not recognize m157, but a complex including at least a classical MHC-I complexed with the viral protein m04. This interaction however only activates NK cells when the H2 MHC-I gene is H2-D^k. For this reason, Ly49P was first characterized in the MA/My strain that naturally harbors the $H2^k$ haplotype [56]. The necessity for these two conditions was also confirmed via multiple crosses that combined different NKCs and H2 haplotypes. BALB/c mice that have Ly49P in their NKC but the $H2^d$ MHC-I haplotype have an inactive Ly49P and are susceptible to MCMV, but the phenotype can be rescued by genetically introducing the $H2^k$ haplotype to the BALB/c background [57]. Interestingly, the presence of $H2^k$ is necessary for Ly49P activation but this activation can be overridden by the presence of $H2^b$ or $H2^q$. Crosses that created mice that have a heterozygous H2 haplotype (such as $H2^k$ and $H2^q$) nullified the activating interaction between Ly49P and the m04 presenting $H2^k$ MHC-I molecule [49], and leads to relative MCMV susceptibility in these mice.

The downstream signalling of Ly49P happens in a very similar manner to Ly49H and other receptors of the same family (Figure 1.1), ultimately leading to cell-mediated cytotoxicity and cytokine secretion.

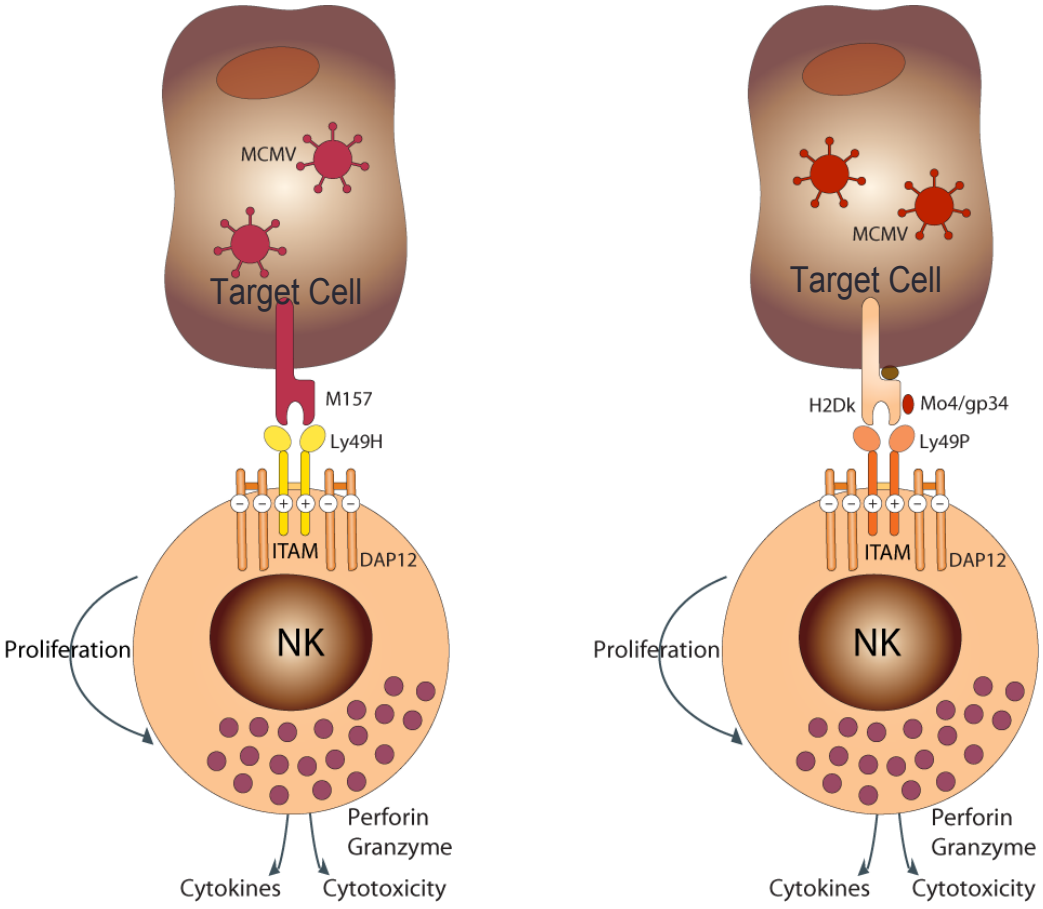

Figure 1.1 – A brief schematic of the mechanism of recognition of Ly49H and Ly49P. On the left, an NK cell expressing Ly49H on its surface is triggered by the viral protein m157 on the surface of infected cells. This causes a potent activation of NK cells which induces proliferation and the effector functions of NK cells. To the right, and NK cell expressing Ly49P recognizes the viral protein m04 that is mounted of the MHC-I molecule (specifically $H2^k$ haplotype). This causes similar activation as Ly49H and triggers the same responses. (Figure adapted from [36])

1.3.4 NK Cell Activation and Effector Functions

NK cells need to be activated before efficiently producing their effector functions. This activation can happen as per the previously discussed activating receptors, however they also require other stimulants in order to deliver a potent immune response. The presence of pro-inflammatory cytokines such as type I IFN and interleukins (IL) 12, 2, 15, 18 and 21 are necessary in order to potentiate the effector functions of NK cells [58].

The activation and subsequent effector functions of NK cells can also be modulated by their education/ licensing as they mature in the periphery. It is generally thought that specific inhibitory receptor triggering on NK cells potentiate the stimulation of the NK cell if subsequently activated. Indeed, it has been found that the higher the binding affinity of inhibitory NK receptors to their ligand, the greater greater levels of IFNγ produced when the NK cell is stimulated [59]. Hence licensing has been defined as a mechanism by which only the NK cells that can recognize MHC I are rendered competent. For this reason humans and mice that naturally lack MHC I (an inhibitory ligand) have hypo-responsive NK cells when assayed *in vitro* [60, 61, 62]. There is still much work necessary to fully understand the impact of NK cell education/ licensing on activation in the context of MCMV infection however, since mice lacking MHC I still display efficiently activated NK cells and clear MCMV infections as well as mice expressing MHC I [63].

The activation of NK cells generally causes an up regulation of killer cell lectin-like receptor (KLRG1) and CD69 expression on the cell surface which is

a general marker for NK cell activation [64, 65]. Once fully activated, NK cells display their effector functions in a wide array of ways. They secrete many cytokines and chemokines such as Tumor Necrosis Factor alpha (TNF- α), IL-3, Granulocyte-macrophage colony stimulating factor (GM-CSF), chemokine (c-c motif) ligand 3 (CCL3), CCL4, CCL5, but most importantly IFNγ. These aid in the recruitment and activation of various other cells that are necessary in order to clear an infection [66]. NK cells can also elicit direct effector functions to fight pathogens; target killing can occur via cell surface receptors such as TNF-related apoptosis-inducing ligand (TRAIL) [67], but more commonly occurs via the release of cytotoxic granules such as perforin or granzyme. Perforin is a membrane disrupting protein and granzyme is a serine protease that eventually leads to target cell apoptosis [68].

1.4 Role of DCs in Activation of NK cell Anti-MCMV Function

It is important to note that the primary sensors of MCMV are epithelial and myeloid innate immune cells such as dendritic cells (DCs). DC activation occurs mainly via pathogen detection by Toll-Like Receptors (TLRs) and pro-inflammatory cytokines such as TNF-α and IL-1β [69]. They are one of the few cells specialized in antigen presentation which serves to activate the innate immune system early on during infection, but in the context of MCMV infection, they are indispensable for NK cell activation.

DCs can be subdivided into 2 main groups; plasmocytoid DCs (pDCs) that are of lymphoid origin and conventional DCs (cDCs) of myeloid origin. pDCs are of significant importance in MCMV infection as they promote early activation of NK cells. Upon infection mature pDCs detect viral CpG via their TLR9/MyD88 pathway, which causes them to be the main producers of IL-12 and type I IFN; cytokines that are necessary for the non-specific activation of all NK cells. This response usually happens at around 36 hours p.i in mice and is known as the second wave of type 1 IFN secretion [70].

Studies have also documented activation of NK cells occurring prior to 36 hours p.i with MCMV, which happens during the first wave of IFN type I secretion that occurs at around 8 hours p.i [11]. Splenic stromal cells located in the marginal zone are among the first cell types to get infected and start to express the Lymphotoxin β receptor (LTβR) on their surface and secrete

the chemokine C-X-C motif chemokine 13 (CXCL13). CXCL13 attracts B cells and induces them to produce Lymphotoxin $\alpha\beta$ (LT$\alpha\beta$) on their surface that triggers the LTβR on the surface of stromal cells, leading to a potent production of IFN type I via the NF-κb pathway [71]. The surge in IFN type I is the first known time point of NK cell activation occurring at 12 hours p.i and is characterized by increased NK IFNγ production.

These 2 waves of IFN type I are necessary to initially control the early replication of MCMV in an NK cell dependent manner [11]. This control however is due to a nonspecific NK cell activation, meaning it does not trigger any specific NK cell populations [71] such as Ly49H or Ly49P expressing NK cells; the first wave occurs around 8 hours p.i and the second around 36 but specific Ly49 triggering does not occur before approximately 3 to 4 days p.i, making these 2 first waves of IFN type I secretion essential for the control of MCMV infection in an NK dependent manner [11].

1.5 The Genetic Approach to Deciphering Host Susceptibility to Infection

The recent advances in the field of genomics have led to the optimization of techniques that have been instrumental in the identification of many human diseases, including but not limited to cancer, diabetes and Chron's disease [72, 73, 74]. In humans, most genetic based diseases are a result of complex interactions between many genes (polygenic interaction) and the environment. Each of these genes contribute to varying extents to the overall phenotype and have a varying penetrance throughout the population. For these reasons, the discovery of complex trait disease alleles cannot be determined as it is done with monogenic traits [75], and was a daunting task before the recent advances in genomics. A popular statistical model that has developed to detect defective alleles that result in disease is the quantitative trait loci (QTL) approach.

The QTL approach utilizes the movement of alleles within family members of a pedigree in order to correlate genomic regions to a continuously distributed phenotype expressed in affected individuals. In order to know the alleles in the individuals, polymorhic markers such as single nucleotide polymorphisms (SNPs) and simple sequence length polymorphism (SSLP) are utilized and the individuals in question are genotyped across their genome. The general accepted method used to correlate the linkage of a marker in a genomic region to a phenotype is the logarithm of odds (LOD), where

the greater the LOD score, the higher the likelihood that the specific genomic region is contributing to the phenotype. The genomic region that has been found to significantly contribute to the phenotype is called a QTL. The usefulness of mouse genetic analysis and specifically QTL analysis has been undoubtedly proven and aided in the discovery of multiple diseases related NK cell related deficiencies [36].

Mice are an ideal genetic model for these tests because it is feasible to obtain large families relatively quickly, but more importantly because they are very close to humans genetically making them a very relevant context to study human diseases [76]. QTL analysis in mice is generally carried out by crossing two inbred strains of mice, both having a consistent phenotype within the strain but a significantly different one across the strains. The F1 generation is usually intercorssed to produce a large F2 generation having a normal distribution of alleles from both strains (an assumption of QTL analysis). The F2 is subsequently phenotyped and genotyped and statistical techniques evaluate the probability that a specific marker is linked with the phenotype [77, 78]. Genome-wide thresholds of significance are then assessed via multiple permutations of the original data.

In order to confirm a QTL, the standard approach is to create congenic strains. These are strains of mice that have only one genetic region usually bearing a QTL from a donor strain introduced into the background of a recipient (usually a resistant strain). These mice are achieved through first crossing two strains, followed by successive backcrossing to a pure breed of

the desired background. This allows the isolation of the QTL independently of the recipient background to determine the extent at which it contributes to the primary phenotype.

The main disadvantage of a QTL analysis is that the resolution is limited by the recombinations that take place during the process of meiosis, which generally causes the resolution of QTLs harbouring disease allele to be in the order of megabase pairs. If the purpose of the study is to identify the specific genes within a QTL that contribute to disease, it would not be feasible to study the hundreds of genes within a QTL region in order to determine candidacy. A more reasonable approach would be the production of sub-congenic lines, which are lines of mice that have a smaller region of the QTL and are generated by additional backcrossing and intercrossing. The use of multiple sub-congenic lines allows the observation of sections of the QTL separately in order to discover which regions truly contribute to the disease, in turn reducing the amount of genes that could be candidates. The production of sub-congenic strains also allows to determine if one or more regions, or their interactions within the QTL contribute to the phenotype.

1.6 Rationale, Hypothesis and Objectives

1.6.1 Rationale

Recent unpublished work in Silvia Vidal's laboratory has shown that the genetic background of mice modulates the function of Ly49P. For instance, the mouse strain 129S1 is susceptible to MCMV; the virus seems to be able to replicate more efficiently in this strain and reach higher titers relative to other resistant strains. These mice also share a common Ly49 haplotype with the naturally resistant MA/My mice (i.e. they express Ly49P) but possess the $H2^b$ haplotype and thus their NK cells cannot be activated via Ly49P. However, unlike other strains, the combination of Ly49P and $H2^k$ in the progeny from an F2 cross between 129S1 and BALB.K (who do not express Ly49P but have the $H2^k$ haplotype) failed to provide protection against MCMV (Figure 1.2). These findings suggest that a genetic modulator of MCMV-resistance may be present in 129S1 mice.

Ly49H potency also seems to be modulated in the 129S1 background (whose NKC does not have Ly49H naturally). Previous results also conducted in Silvia Vidal's laboratory have shown that an F1 cross between C57BL/6 and 129S1 to introduce Ly49H to the 129S1 background has a intermediate phenotype, which is surprising as Ly49H is inherited in a dominant fashion in the C57BL/6 background. Another finding that hints at a genetic modulator to MCMV resistance in the 129S1 background.

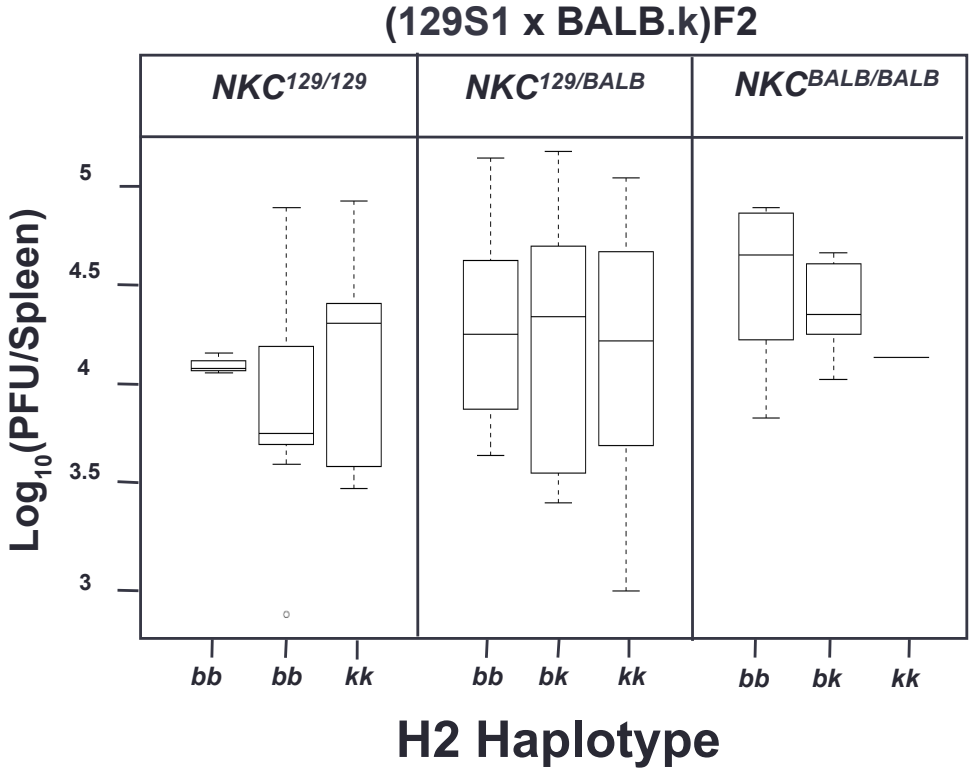

Figure 1.2 – The effect of different combinations of MHC-I and NKC haplotypes was assessed in the 129S1 background in order to rescue the effect of Ly49P. The graph shows viral titers 3 days p.i with MCMV. The $H2^k$ MHC-I haplotype was introduced to the 129S1 background via breeding to BALB.k mice, however this did not confer the relative resistance expected from a functional Ly49P receptor. In fact, no significant differences were observed between any of the groups (Desrosiers and Vidal unpublished data).

1.6.2 Hypothesis

It has been well documented that there are functional interactions and complex cross-talk between NK cells, dendritic cells, macrophages, B cells and even stromal cells in the spleen are required for NK cells to be fully activated [11, 79], leading ultimately to an efficient host immune response and viral clearance. Although this modulator may be found primarily on NK cells, it could also act through a secondary mechanism on NK cells through one of these cells. Therefore it is hypothesized that a unique QTL in the genetic background of the 129S1 strain controls NK cell antiviral function either directly at the level of the NK cell, or indirectly via another cell type necessary for inducing efficient NK cell function.

1.6.3 Objectives

1. Confinement of the genetic region(s) responsible for the susceptibility on the 129S1 background is the primary goal. To achieve this, a F2 generation must be utilized for a QTL statistical analysis in order to determine the genetic loci responsible for the modulation of MCMV susceptibility.

2. Creation of congenic line(s) bearing the susceptibly locus (discovered in objective 1) will be done in order to assess the effects of the genetic regions that modulate MCMV resistance independently from the rest of the 129S1 background.

3. Reassessment of the MCMV susceptibility phenotype in the congenics is the next necessary step in order to confirm the that the susceptibility locus alone is enough to recapitulate the phenotype. If the phenotype is recapitulated, the congenic strain NK cells will be further characterized at the level of activation, recognition and functional capacity. This will be crucial in identifying the nature of the genetic defect and if it manifests directly at the level of NK cells.

4. Characterizations of the kinetics of infection will be conducted in order to determine the optimal time range where the susceptibility manifests. These experiments should provide preliminary data regarding the cell type the QTL affects; whether it be at the level of NK cells (i.e. it is an intrinsic NK cell defect) or on another cell type.

5. Utilizing published exome sequences will allow the interrogation of SNPs that are differential between C57BL/6 and 129S1 and cause coding variants known to affect protein translation. This will pave the way for future studies that aim to identify the specific genes in the genetic locus identified that are involved in the susceptibility.

Chapter 2

Materials and Methods

2.1 Mice

C57BL/6 and 129S1 were purchased from Jackson Laboratories and housed in the Goodman Cancer Center Animal Facility. F2 mice used for QTL mapping were created by intercrossing both pure breed strains for 2 generations. To create the congenic line, an F4 generation was created in order to maximize recombinations which was then followed by 2 generations of backcrossing to pure breed C57BL/6 mice. Subsequently, a large number of F2N2 males (n=90) were primarily genotyped using SSLP markers on chromosome 9, where 10 mice were found to be heterozygous in the region between 40-92 Mb. These mice were sent for a high density SNP scan at the University of Toronto where the rest of their genome was genotyped using 873 differential SNP markers. One best male having the QTL and the least amount of 129S1 contamination was identified and a panel of SSLP markers was created in order to genotype the offspring for further genetic clean up (Fig-

ure A.1). By the F4N4 generation, promising males and females were found with minimal contamination form the 129S1 background and intercrossed to produce the full congenic mice having a pure C57BL/6 backgrounds and the 129S1-CHR9/40-92 Mb or C57BL/6 $Cmv5^{129}$).

Three subcongenic lines were created using an intercross between F4N2 mice that displayed interesting recombinations and subsequently backcrossing it to a pure breed for 5 generations. The mice were then genotyped in order to determine homozygous subcongenics having a pure C57BL/6 background and 129S1 CHR9:70-92; 76-92 and 85-92 Mb. A table of markers used to genotype chromosome 9 in sub-congenics and congenics is provided in the appendix (Table A.2).

2.2 Virus and Infection

Stock MCMV was prepared by passaging the virus (Smith strain ATCC) twice in weanling (3 weeks) BALB/c mice. After every passage, salivary glands were extracted and homogenized as previously described [80]. Viral titers of the stock virus were evaluated in vitro by standard plaque assays on a confluent BALB/c mouse embryonic fibroblasts (MEF) monolayer, as previously described [81].

For phenotype determination, infections were conducted in mice aged between 7 and 9 weeks were infected intravenously with 50,000 PFUs of MCMV (or 12,500 PFU of a different virus stock for the QTL mapping

infections). Viral titers of mouse organs (spleen and liver) were evaluated in vitro by standard plaque assays on a confluent BALB/c MEF monolayer, as previously described.

2.3 Flow Cytometry and BrDU incorporation assay

Organs from infected or uninfected mice were collected in 5 ml of RPMI supplemented with 10% FBS and single cell suspensions were obtained by grinding the organs with cell strainers. The cells were treated with red cell lysis buffer (Sigma) or Ack's lysis buffer, washed with PBS and stained for extracellular markers using specific antibodies. For intracellular staining, cells were fixed using BD cytofix/cytoperm kit according to manufactures' protocol. For BrDU incorporation assay, mice were injected with 2mg of 5-Bromo-2'-deoxyuridine (BrDU)(Sigma), and spleens were extracted 3 hours after injection. Extracellular staining was done as described and BrDU staining was done using FITC BrDU Flow kit (BD) according to the manufacturers protocol.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

cDNA was obtained from 2 µg of RNA extracted using Trizol according to the manufacturers instructions, treated with DNaseI and finally reverse transcribed with MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. RNA levels were quantified using the Platinum SYBR Green PCR master mix (Invitrogen), according to the manufacturer's instructions. Duplicate cycle threshold (CT) values were analyzed with the comparative C(T) method and the relative amount of mRNA was obtained by normalizing to the endogenous hypoxanthine-guanine phosphoribosyltransferase (HPRT) reference levels as previously described [82].

2.5 Cell Labeling and *in vivo* Rejection of MHC1$^{-/-}$ Cells

Spleens from congenic $Cmv5^{129S1}$ or their $Cmv5^{C57BL/6}$ littermates and MHC I deficent mice, lacking H2-D^{b} and K^{b} (kindly provided by Hidde L. Ploegh. Cambridge, Massachusetts) were collected in 5 ml of complete RPMI and ground using cell strainers to get single cell suspensions. The cells were treated with Ack's lysis buffer or red blood cell lysis buffer (Sigma) and stained. In particular each of the cell fractions were stained either with CFSE (Invitrogen), or eFluor670-proliferation dye (eBiosciences). 10mM CFSE or

eFluor670-proliferation dye were resuspended each in 1 ml of cRPMI. The cells were re-suspended in 1 ml of complete RPMI, to which 1 ml of particular dye was added. This was followed by the incubation for 5 min at 37°C and three washes with PBS 10% FBS. The cells were then mixed in a 1:1 ratio, (MHC I deficent + $Cmv5^{129S1}$) or (MHC I deficent + $Cmv5^{C57BL/6}$), and a total of 10^6 cells were injected per mouse for 24 hours. Finally the data was analyzed using [(% of MHC I deficent cells in sample / % of MHC I deficent cells in injection mix) / (% of self cells in sample / % of self cells in injection mix)].

For NK cell depletions, mice were injected i.v. with 35 µl of anti-asialo GM1 antibody (Wako) [81]. After 24 hours, mice were infected as described.

2.6 *Ex vivo* stimulations

Spleens from mice were collected individually in 5 ml of complete RPMI and ground using cell strainers from each sample individually. The cells were treated with Ack's lysis buffer and $5*10^6$ cells from each mouse spleen were stimulated individually in 500µl of complete RPMI containing either 10ng/ml IL12 and 50ng/ml IL18; 50ng/ml of IL15; 50 ng/ml of IL15 and 50ng/ml of IL18; 100ng/ml of phorbol 12-myristate 13-acetate (PMA) (a direct protein kinase C activator) and 1mg/ml of Ionomycin (IONO) (a calcium ionophore) or $75*10^4$ RMAs-m157 cells. Cells were then incubated for 5, 24 or 48 hours at 37°C. GolgiStop (BD) was added 4 hours before the end of each incubation,

and FACS staining was done as described in the flow cytometry section.

For the antibody stimulations, cells were treated as above and aliqouted into a 96-well 2HB Immulon plate that has been coated with purified antibodies against either NK1.1; NKp46; Ly49H or a cocktail of all mentioned antibodies. Cells were stimulated in complete RPMI media with protein transport inhibitors (GolgiStop or Golgiplug; BD) for 5 h at 37°C, and then stained.

2.7 Linkage/ QTL Analysis

434 F2 mice (C57BL/6 X 129S1) were genotyped and 114 mice were found to have homozygous Ly49H. These 114 mice were the ones that were used in QTL mapping. Viral titers in the spleen 3 days p.i (quantified via plaque assay) were used as the phenotype to detect linkage. The mice were genotyped with around 250 informative markers using the Illumina platform (http://www.tcag.ca/facilities/microarray.html2) at the The Center for Applied Genomics (Toronto). Further genotyping was conducted manually using PCR in the CHR9 region of interest using SSLP or RFLP in order to get a better resolution. Genome scan analyses were performed using the R/qtl package [83], utilizing the scan one function to determine genome wide LOD scores. Significance was determined using 100,000 permutations (see section 1.5 for details).

2.8 Statistical Analysis

Sample sizes and number of experimental replica are indicated in figure legends. Data is displayed as the mean with error bars showing the standard error of mean. Differences between the two groups were calculated using the Prism statistical and graphing software (graphpad). Pair-wise comparisons between the two groups were conducted using a two- tailed unpaired t-test was used and differences with $P<0.05$ were deemed significant.

Chapter 3

Results

3.1 Mapping of a Genetic Modulator of MCMV Resistance: *Cmv5*

The first step in understanding the nature of the susceptibility to MCMV present in the 129S1 strain would be to delimit the genetic area responsible. In order to accomplish this, a cross between the two pure breed C57BL/6 and 129S1 strains to produce a heterozygous F1 was conducted. This generation was further intercrossed and expanded to produce (C57BL/6 X 129S1) F2 offspring. To control for the effect of Ly49H, the F2 offspring were genotyped using the D6MIT61 SSLP marker which lies in the Ly49h gene on the NKC. A total of 114 mice were found were homozygous for Ly49h which were used for the QTL mapping.

The Ly49h homozygous F2 mice were infected i.p for 3 days with 12,500 PFU of MCMV and then splenic viral titers were determined via plaque assay. DNA extracted from the mice was subsequently genotyped with 240

polymorphic SNP and SSLP markers genome wide. Using the genotypes and phenotypes, a QTL analysis was conducted which produced a significant peak on chromosome 9 (Figure 3.1). The LOD support interval defining our 95% confidence interval was calculated using a 1.5 LOD drop (taking a 1.5 LOD score lower that the significance threshold of 0.05 calculated) and was found to be between positions 60-92 Mb with a maximum LOD score of 6.4 (Figure 3.2). This genetic locus at chromosome 9 from 60-92 Mb was dubbed *Cmv5*.

The peak marker on this mapping was SSLP D9MIT8. When the mice used in the mapping were grouped based on the alleles of this SSLP marker, mice homozygous for the 129S1 alleles at D9MIT8 showed a 1 Log_{10} increase in MCMV viral titer in the spleen relative to their litter mates having homozygous C57BL/6 alleles. Interestingly, the heterozygotes seem to show an intermediate phenotype (Figure 3.3).

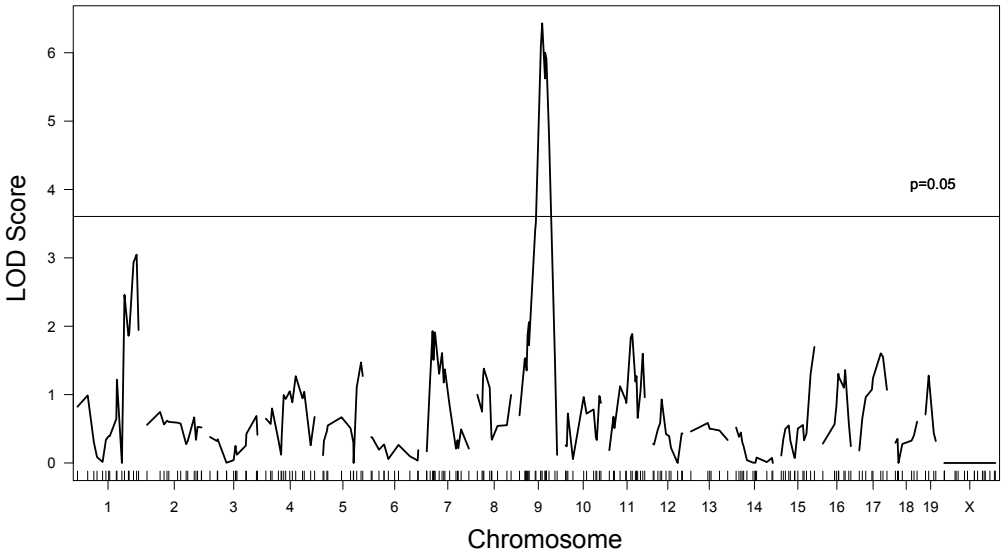

Figure 3.1 – The QTL analysis output of n=114 (C57BL/6 X 129S1) F2 mice genotyped using 240 markers genome wide. QTL output for the entire genome shows a clear significant peak on chromosome 9.

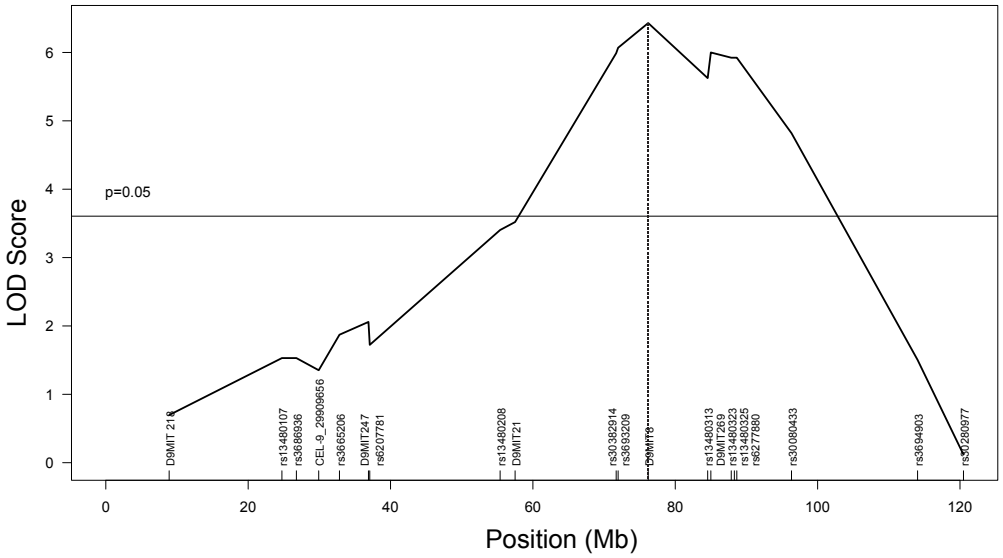

Figure 3.2 – QTL output for only chromosome 9. Labels on the X-axis specify the marker used in the QTL analysis. A closer look at the peak shows a significant LOD support interval (1.5 LOD drop) between approximately positions 60 and 92 Mb(*Cmv5*), with the maximum LOD score being 6.4 at the peak marker D9MIT8.

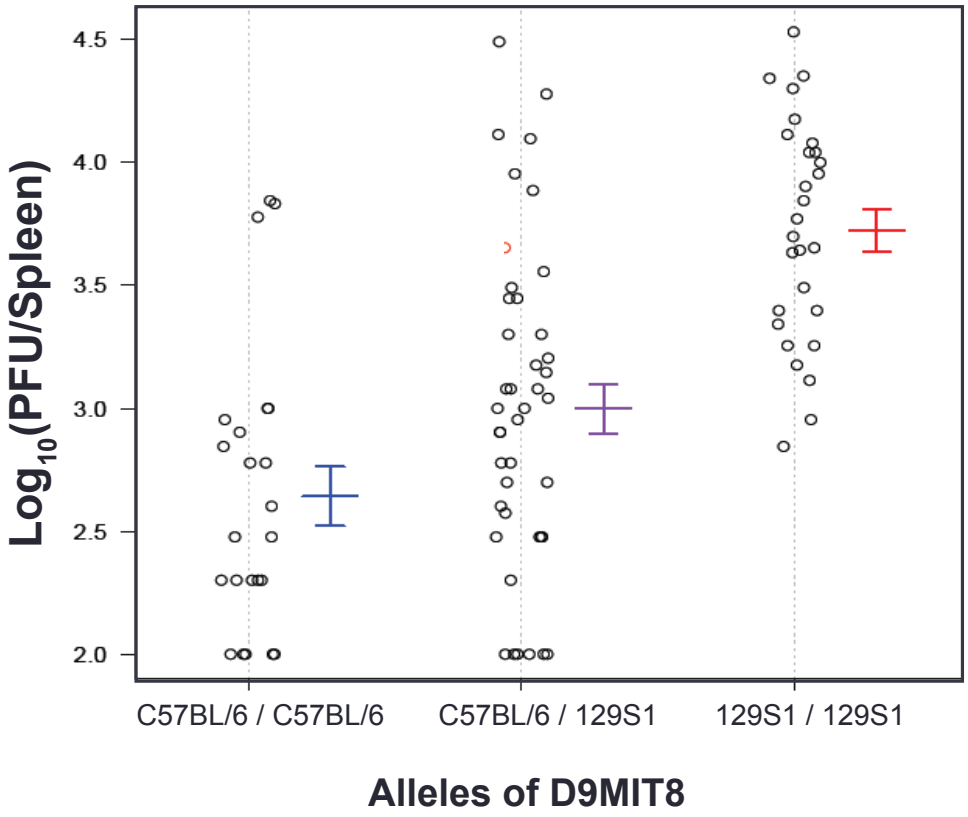

Figure 3.3 – (C57BL/6 X 129S1) F2 MCMV viral titers 3 days p.i (quantified by plaque assay) grouped by D9MIT8 alleles. F2 mice bearing homozygous 129S1 alleles show a 1 Log_{10} increase in viral titer relative to their litter mates bearing C57BL/6 D9MIT8 alleles. The heterozygous seem to display an intermediate phenotype.

3.2 Breeding and Confirmation of MCMV Susceptibility in Congenic Mice

In order to further characterize the contribution of *Cmv5* to MCMV susceptibility, the QTL would have to be isolated from the 129S1 MCMV susceptible background onto a C57BL/6 MCMV resistant background. The congenic line was started by creating an F4 generation, to maximize recombinations in the region of *Cmv5* region which allowed for the eventual precise introduction of *Cmv5*. Once the F4 generation was complete, the 129S1 genetic background was cleaned via back crosses to the pure breed C57BL/6 strain. After 5 more backcrosses, the final congenics were complete and confirmed via the same markers used to select best male in the F4N2 generation (see material and methods). The congenics had the chromosome 9 region from 40-92 Mb as opposed to 60-92 Mb due to lack of recombinations in that area.

To determine if the congenic mice recapitulated the MCMV susceptibility seen in the F2 generation, mice were infected with 50,000 PFU MCMV for 3 days i.v and spleen and liver (as a control for infection) viral titers were assessed via a classic plaque assay. Compared to the 1 Log difference observed in the F2 mice (Figure 3.3), a more subtle 0.5 Log_{10} increase in MCMCV titer is seen in the congenic mice relative to their C57BL/6 litter mates (Figure 3.4). This was not surprising as the F2 mice still had a lot of additional 129S1 susceptibility loci that could be contributing to the susceptibility.

To better characterize the role of *Cmv5* in the progression of MCMV

infection, we conducted a kinetic experiment where viral titers were assessed at 2, 3, 4 and 5 days p.i. Plaque assays on days 4 and 5 p.i showed no differences in viral titers between the groups. Day 3 p.i always showed a modest (0.5 Log) difference, however this difference was significant only 50% of the time (based on 6 experiments). Day 2 p.i however displayed the strongest effect of *Cmv5*; a 1 Log difference between congenic and C57BL/6 litter mates was confirmed in at least 7 experiments. Thus, based on these results, it is clear that this susceptibility is an early occurrence between the time of infection and approximately day 3, which is the onset of the NK cell specific response mediated via specific Ly49 receptors.

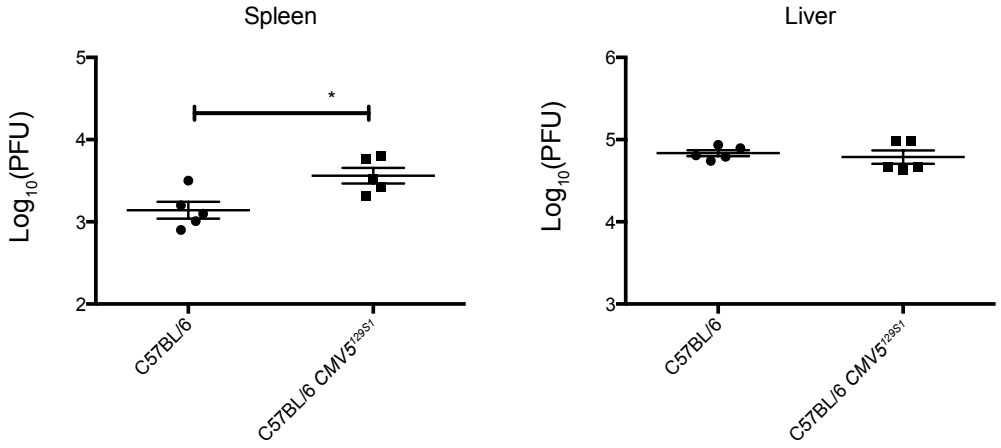

Figure 3.4 – MCMV viral titers 3 days p.i (quantified by plaque assay) in congenic mice (C57BL/6 $Cmv5^{129S1}$) and their C57BL/6 wild type litter mates. Splenic viral titers were calculated per 0.1g of spleen and liver viral titers were calculated per 1g of liver. 5 mice per group were used for the experiments. This result is representative of 3 experiments.
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.

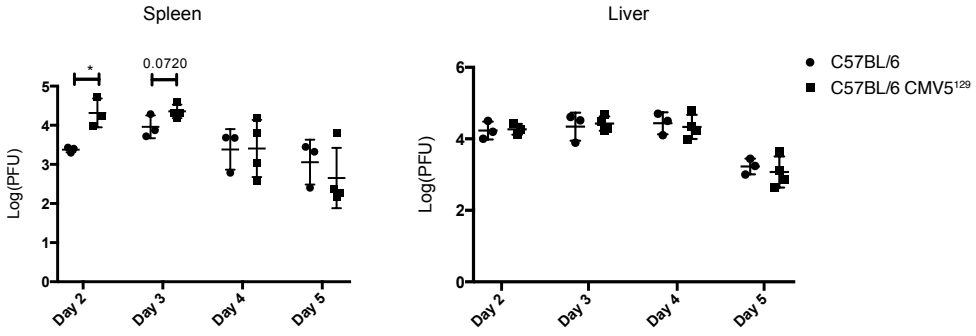

Figure 3.5 – MCMV viral titers 2, 3, 4 and 5 days p.i in congenic mice (C57BL/6 *Cmv5*^{129S1}) and their C57BL/6 wild type litter mates, quantified by plaque assay. The left column represent splenic viral titers and the right column represents liver viral titers (infection control). Splenic viral titers were calculated per 0.1g of spleen and liver viral titers were calculated per 1g of liver. 3-4 mice per group per day were used for this experiment and this result is representative of 2 experiments.
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.
Number above groups indicate the p-value of the difference between the 2 groups as calculated by student's T-test.

3.3 *In vivo* NK Cell MHC$^{-/-}$ Rejection assay

NK cells are well known to be the main players in the clearance of MCMV early after infection through cell-mediated cytotoxicity of infected cells. Since the phenotype was confirmed in the congenic line at at day 2, the next logical step would be to take a look at the general functional capacity of their NK cells. One of the hallmarks of NK cells is their ability to kill non-self cells, and provides a general measure of their ability to elicit their cytotoxic functions. Thus to study the cytotoxic potential and rejection ability of non-self target cells in the congenic mice, an *in vivo* cell rejection assay was set up.

Spleenocytes from congenics, their wild type litter mates and MHC I-/- mice were harvested and stained with a different colour respectively. Subsequently, both congenics and wild typer litter mates were injected with a 1:1 mixture of MHC$^{-/-}$ cells and self cells. A subgroup of mice in both groups had their NK cells depleted as a negative control since there should be no killing of MHC$^{-/-}$ in the absence NK cells. 24 hours post cell mixture injection, the spleens of the injected mice were collected and the ratio of previously stained non-self to self injected cells was then assessed by FACS as a measure of NK cell ability to lyse non-self target cells. No differences were seen between the two mouse groups (Figure 3.6). In fact, a consistent trend of congenic mice having slightly better rejection capacity was consistently seen but never significant.

Although there are no differences between the two groups for this assay,

it does not at all delimit the absence of a defect in NK cells; If the NK cells in the congenic line could reject non-self cells, it does not necessarily translate to an efficient anti-viral response since other aspects such as recognition of viral particles, maturity and activation by many factors are all factors necessary to elicit an efficient immune response.

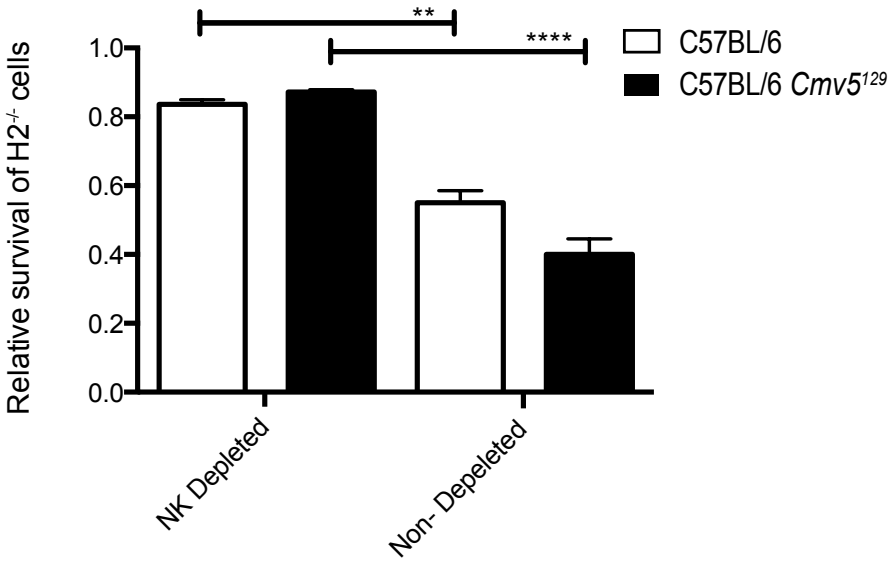

Figure 3.6 – NK cell rejection of MHC$^{-/-}$ cells in congenic mice and their C57BL/6 litter mates. The graph shows the relative survival of MHC$^{-/-}$ cells (Y-axis) in the two mouse groups upon NK cell depletion (as a negative control) and in the presence of the effector NK cells. 4 samples were used in each group. This result is representative of 3 experiments.
** indicates a significant difference where p <0.005 as calculated by student's T-test.
**** indicates a significant difference where p <0.0005 as calculated by student's T-test.

3.4 *Ex vivo* NK Cell Phenotypes

We next examined the capacities of NK cells in response to activating receptor triggering or soluble mediators in order to further asses if any obvious defects are present in the NK cells of the congenic line. Triggering NK activating receptors leads to a downstream signalling that eventually causes NK cells IFNγ production through various pathways depending on the receptor triggered. Upon stimulating total spleenocytes with NK cell activating antibodies (Ly49D, NKp46, Ly49H and NK1.1) for 5 hours, a general trend of increased IFNγ production was seen in the congenics relative to their C57BL/6 litter mates (figure 3.7). Although the increase was very modest and non-significant, the trend was consistent implying that the congenic line's NK cells at the very least can be triggered by activating receptors and have intact downstream signalling via DAP12, CD3ζ and FcϵRI-γ adaptor proteins.

Next, NK cell activation via other factors was assessed in order to determine if the NK cells had any baseline defects in their ability to respond to cellular and soluble mediators. IL-15 secretion by DCs primes NK cells and causes IFNγ secretion and induces effector functions [84, 85]. Knowing this, total spleenocytes were stimulated with IL-15, a mixture of IL-15 and IL-18 (which is more potent due to synergistic effects), poly IC (to stimulate monocytes to indirectly stimulate NK cels) and RMA/s-m157, a cell line expressing the ligand of Ly49H. PMA/ IONO was used a positive con-

trol. Subsequently, their activation and production of perforin, granzyme and IFNγ was assessed at various time points (4, 24 and 48 hours).

The significant differences observed are in NK cell IFNγ (Figure 3.8) production and CD69 surface expression (Figure 3.9). Relative to C57BL/6 litter mates, the congenics have slightly lower levels of IFNγ production after 4 hours, but higher levels after 24 and 48 hours of stimulation with IL 15 and IL 18. CD69 surface expression on NK cells showed no differences except at 48 hours post stimulation with IL 15, where the congenics had higher surface expression levels compared to their C57BL/6 litter mates. All other phenotypes observed such as perforin and granzyme secretion and surface expression of KLRG-1 showed no differences between the two groups (data not shown). IL 12 and IL 18 stimulation was conducted, but only at the 5 hour time point due to lack of availability, and no differences in any activation markers or IFNγ was observed (data not shown).

Thus far, no obvious NK cell defects have been observed in the congenic strain; no differences were observed in NK cell ability to reject and kill non-self cells, moreover, no differences were observed between congenic mice NK cells and their wild type litter mates in IFNγ production upon antibody stimulation and even higher levels of IFNγ production by NK cells was observed in congenics at 24 and 48 hours post simulation with IL15 and IL 18 compared to their C57BL/6 litter mates. NK cell activation as measured by CD69 expression also show no defect in the NK cell ability to be activated via various soluble mediators. The only defect observed thus far is the slightly

lower IFNγ production by NK cells in congenics 4 hours post IL 15 and IL 18 stimulation.

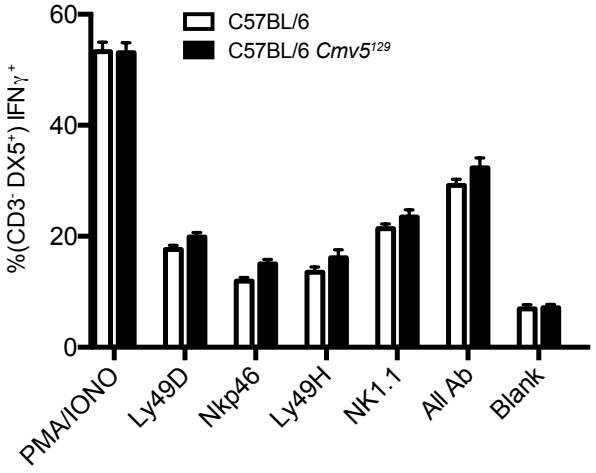

Figure 3.7 – NK cell stimulation using various antibodies and detection of IFNγ production via FACS as a sign of functional activation. The antibody used for stimulation is expressed on the X-axis and the proportion of NK cells ($CD3^-$ $DX5^+$) producing IFNγ is on the Y-axis. PMA/IONO is a positive control. 5 replicate samples are used in each group. These results are representative of 2 experiments.
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.

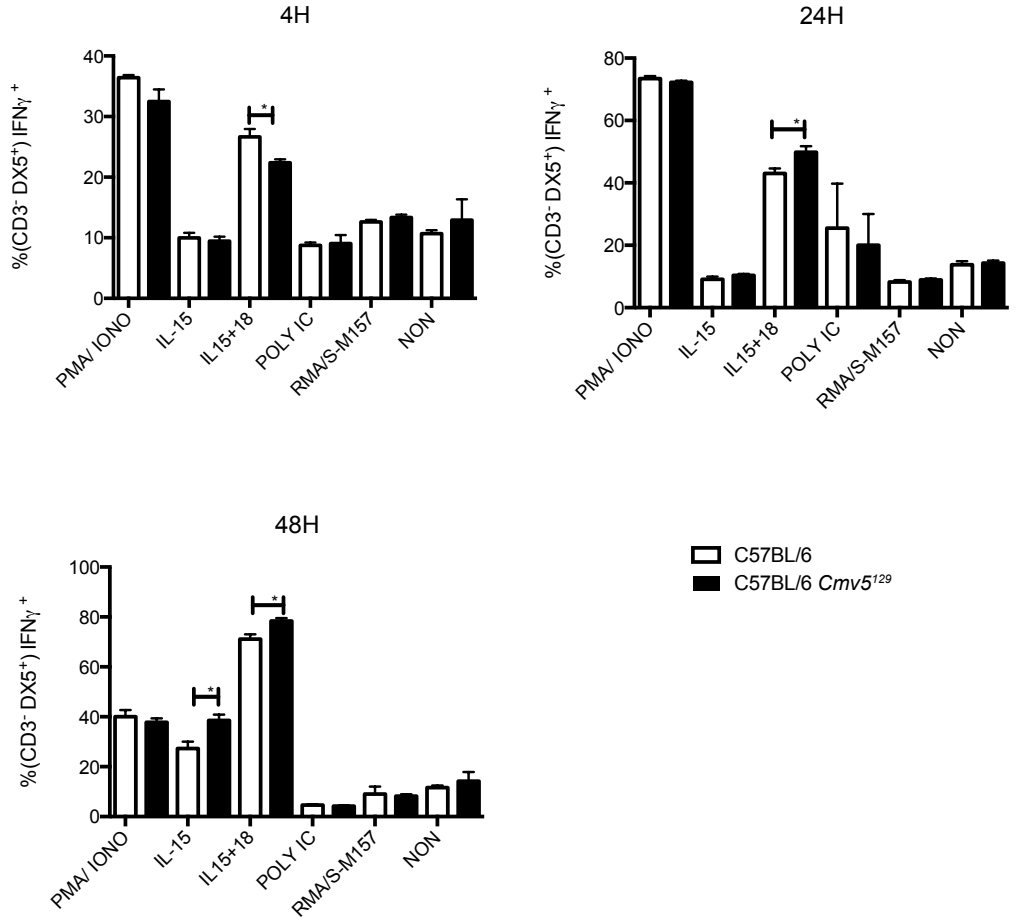

Figure 3.8 – NK cell stimulation using various simtulants (IL15, IL 15 + IL18, polyIC and RMA/s-m157) at 4, 24 and 48 hours and detection of IFNγ production via FACS. The stimulant used is expressed on the X-axis and the proportion of NK cells (CD3$^-$ DX5$^+$) producing IFNγ is on the Y-axis. PMA/IONO is a positive control. 4 samples are used in each group. These results are representative of 2 experiments.
* indicates a significant difference where p <0.05 as calculated by student's T-test.

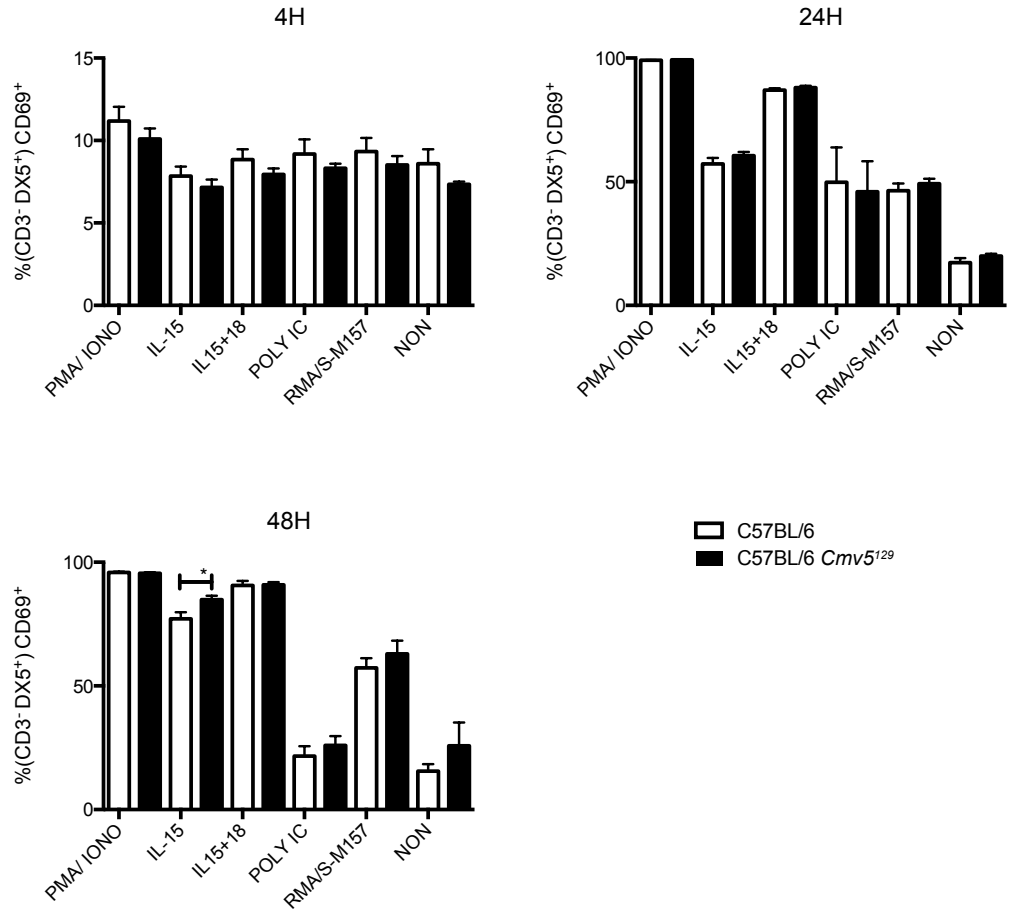

Figure 3.9 – NK cell stimulation using various simtulants (IL15, IL 15 + IL18, polyIC and RMA/s-m157) at 4, 24 and 48 hours and detection of CD69 surface expression as a marker for activation via FACS. The stimulant used is expressed on the X-axis and the proportion of NK cells ($CD3^-$ $DX5^+$) producing IFNγ is on the Y-axis. PMA/IONO is a positive control. 4 samples are used in each group. These results are representative of 2 experiments
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.

3.5 *In vivo* NK cell Phenotypes Upon Infection

Due to the absence of any obvious NK cell defect upon *ex vivo* stimulations, we examined the profile of NK cells during infection. Knowing that the differences in susceptibility were starting to be abolished by day 3, infections at days 1 and 2 were conducted in order to characterize the NK cells in the congenic line.

Since the general activation of NK cells via activating receptors and soluble mediators seemed up to par, the issue could lie in the inability to mature efficiently during infection. In order to test if the NK cell lack of efficacy was due to maturity, congenic mice and their wild type litter mates were infected with MCMV for 1 or 2 days followed by an assessment of their NK cell maturity marker surface expression via FACS.

Interestingly, splenic NK cells from the congenic strain seem to display a lag in maturity upon infection (Figure 3.10). Upon 1 day of infection, this lag seems to happen at stage 3 ($CD11b^+$ $CD27^+$), where we see more stage 3 than stage 4 ($CD11b^+$ $CD27^-$) cells in the congenics relative to their C57BL/6 litter mates. At day 2 of infection, this maturity lag seems to shift a stage lower, where congenics show a lag at stage 2 ($CD11b^-$ $CD27^+$) relative to their wild type litter mates.

In order to asses the impact of this defect in maturity on NK cells, splenic NK cell proportions and their proliferation were assessed via FACS upon the

same infection conditions (day 1 and 2). Although splenic NK cell proportions showed no differences between the two groups in uninfected mice (data not shown), congenic NK cells proportions showed a very modest but significant increase (0.6%) 1 day p.i, which levelled out between the 2 groups by day 2 p.i (Figure 3.11). Surprisingly however, when proliferation was measured via FACS BrdU assay no differences were observed at both days 1 and 2 p.i. between the 2 strains (data not shown).

Next, the effect of the maturity defects on effector functions were assessed, namely IFNγ, granzyme and perforin production upon infection. These effector function are known to correlate with NK cell cytotoxicity and thus measuring their levels would shed light on NK cell cytotoxic potential post infection [86]. Interestingly, IFNγ production was significantly lower in the congenics by day 1 p.i, but reached the same level as the wild type litter mates by day 2 p.i (Figure 3.12). The inverse trend was observed with granzyme, where no differences were observed between the two groups at 1 day p.i but a significant decrease in granzyme production in the congenics was observed 2 days p.i (Figure 3.12). The levels of perforin were also analyzed, however no differences were observed at day 1 or 2 p.i (data not shown).

Thus the defects at the level on NK cells seems to manifest in maturation surface markers, and a lag in their IFNγ production 1 day p.i and granzyme 2 days p.i. Since mild or no defective phenotypes were observed when NK cells were assessed outside the context of infection, this implies that the defect may not lie intrinsically in NK cells, but in another cell type that is vital in

priming the NK cells to produce an efficient anti-viral response.

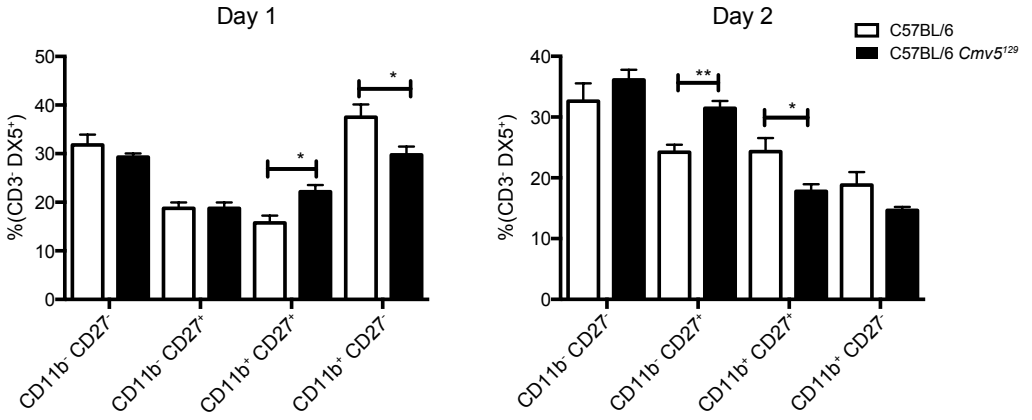

Figure 3.10 – NK cell maturity marker levels quantified via FACS upon 1 or 2 days of MCMV infection in congenic mice and their C57BL/6 litter mates. The Y-axis shows the proportion of NK cells displaying the maturity marker combinations on the X-axis. 4 samples are used in each group. These results are representative of 2 experiments.
* indicates a significant difference where p <0.05 as calculated by student's T-test.
** indicates a significant difference where p <0.005 as calculated by student's T-test.

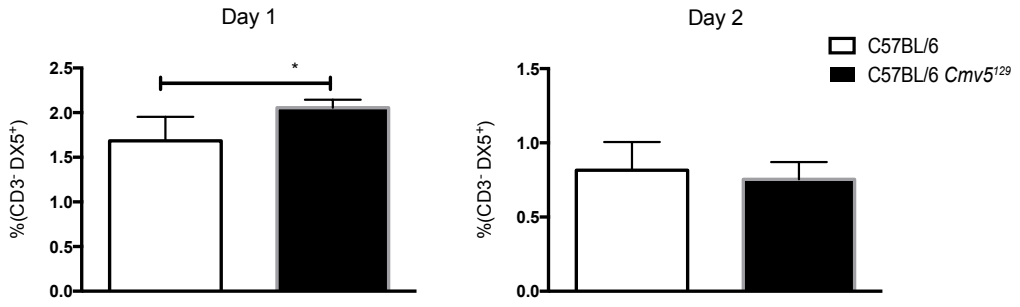

Figure 3.11 – NK cell proportions in the spleen upon 1 or 2 days of MCMV infection in congenic mice and their C57BL/6 litter mates. The Y-axis displays the proportion of NK cells. 4 samples are used in each group. These results are representative of 2 experiments.
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.

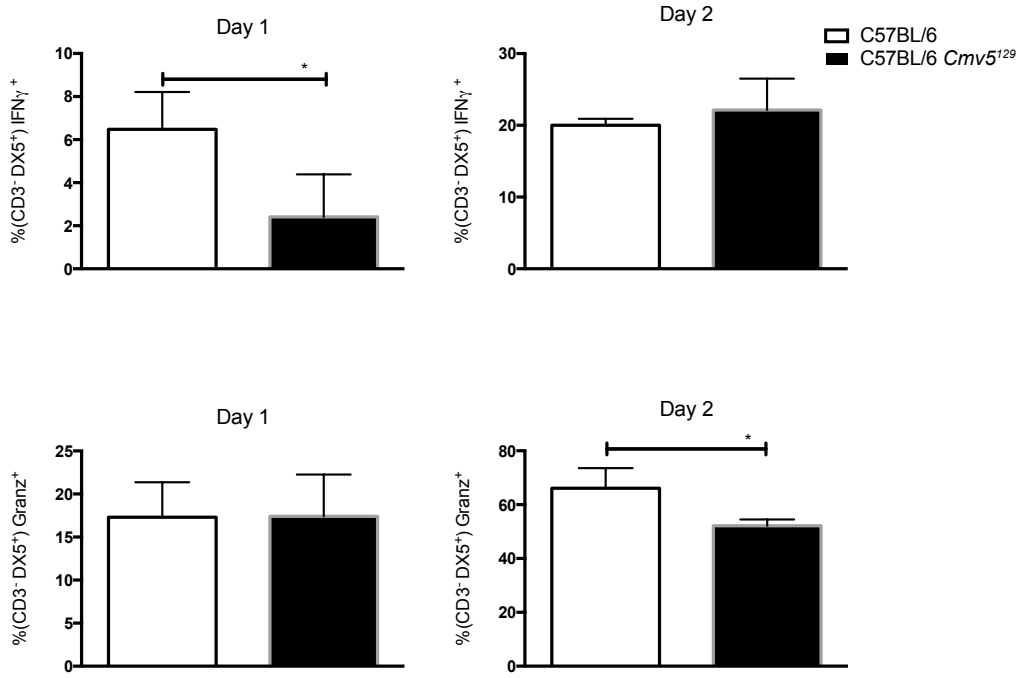

Figure 3.12 – NK cell IFNγ and Granzyme production upon 1 or 2 days of MCMV infection in congenic mice and their C57BL/6 litter mates. The top panels shows IFNγ production 1 and 2 days p.i and the bottom panel shows granzyme production 1 and 2 days p.i. The Y-axis displays the proportion of NK cells producing either IFNγ or granzyme. 4 samples are used in each group. These results are representative of 2 experiments.
* indicates a significant difference where $p < 0.05$ as calculated by student's T-test.

3.6 Early Kinetics of MCMV Infection

Based on previous results (section 3.2), we have found that the window of susceptibility is largest at day 2 and ends at around day 3 to 4. In order to discover the start of this window and get a better idea of the cell types playing a role in this susceptibility, an early kinetics analysis of infection was conducted.

Infections that are less than 2 days, are difficult to quantify by plaque assay due to the lack of sensitivity of the plaque assay protocol. A much more sensitive way to quantify viral levels early during infection is by qPCR of viral genes. One of the first viral mRNA to be transcribed after infection is the IE1 gene, and one of the late genes required for the packaging of the virus is the viral envelope glycoprotein B (gB). Utilizing these 2 viral genes, we observed the start of viral replication (via IE1 expression) and the start of the lytic cycle (via the gB expression) via qPCR 8, 12, 24 and 48 hours p.i. The qPCR results showed a significant 4-5 fold increase 48h p.i in IE1 expression in the congenics relative to their wild type litter mates. The increase in gB expression in the congenics relative to their C57BL/6 litter mates 48 hours p.i showed a 50 fold difference between the 2 groups (Figure 3.13). Other time points showed no difference in viral IE1 or gB mRNA expression levels.

In order to characterize these time points further, we examined a few cytokines known to be necessary for clearing MCMV. Type I IFN mRNA levels showed a consistent trend of differences, where C57BL/6 mice show

higher type I IFN mRNA expression at 36 hours p.i but less at 48 hours p.i as compared to the congenic strain. Although these differences were not significant under the condition of $p<0.05$, the *p-value* was very close to significance. Other cytokine mRNA expression levels such as IL 15, IL 12, IL 18, IFNγ and TNFα were also examined but no differences between the two groups were observed (data not shown).

In conclusion, these experiments show that *Cmv5* imparted susceptibility manifests very early p.i, starting around 36 hours and ending at around 72 hours. Moreover, the trends in type I IFN levels, though not significant, inversely correlate well with viral replication time points implying they may play a role in the lack of congenic NK cell competency at these time points.

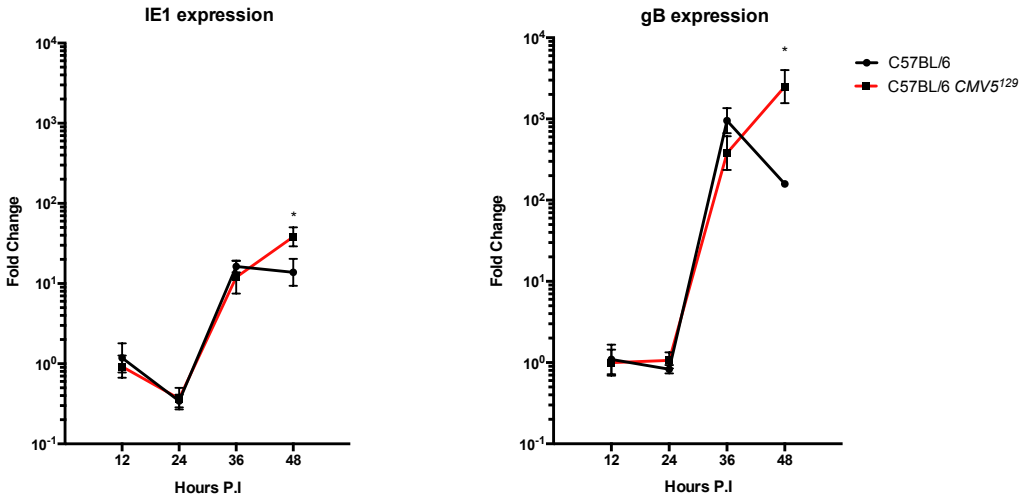

Figure 3.13 – qPCR analysis of the kinetics of MCMV viral IE1 and gB mRNA expression in congenics and their wild type C57BL/6 litter mates. The expression levels are relative to 12H p.i and normalized against the endogenous reference HPRT. 5 samples per group per time point were used. * indicates a significant difference where p <0.05 as calculated by student's T-test.
Numbers on top of data points indicate the p- value (calculated by student's T-test).

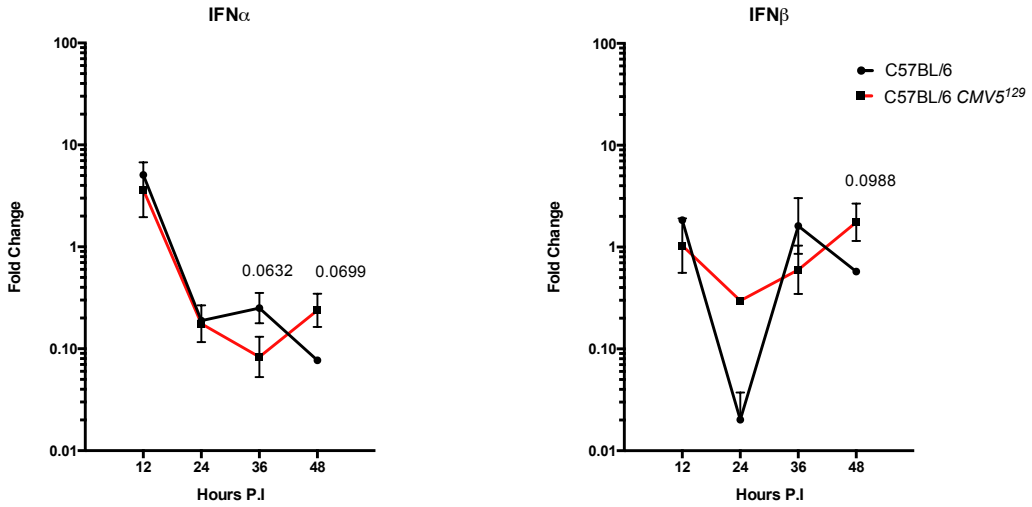

Figure 3.14 – qPCR analysis of the kinetics of Type I IFN mRNA expression levels in congenics and their wild type C57BL/6 litter mates.The expression levels are relative to 12H p.i and normalized against the endogenous reference HPRT. 5 samples per group per time point were used.
Numbers on top of data points indicate the *p*- value (calculated by student's T-test).

3.7 Sub-congenic Strains

Three sub-congenic lines were created to delimit the genetic locus *Cmv5* further. The boundaries of the three lines were at positions 70-92, 76-92 and 85-92 Mb on chromosome 9 (Figure 3.15) (markers used are on Table A.2). This is ultimately needed to define the minimum region of *Cmv5* that causes susceptibility in order to narrow down the interval and aid in finding candidate genes. In addition, a common theme in complex traits is the presence of more than one gene in a QTL which either independently, or through interaction with other genes, contribute to the phenotype. Thus sub congenic strains would also provide information regarding interactions of different regions of a QTL.

The sub-congenic lines were tested for susceptibility at day 2 p.i with MCMV. Surprisingly, all 3 lines had a wide variation in viral titer and their means are around same level. Their titers were also intermediate in relation to the titers of the congenic line and their C57BL/6 litter mates (Figure 3.16). A tentative interpretation of this data is that at least 2 regions in the *Cmv5* region contribute to the susceptibility phenotype; one likely between positions 85 and 92 Mb and another at 40-70 Mb on chromosome 9 that could be interacting in an epistatic manner to bring about the full susceptibility phenotype observed in the full congenics.

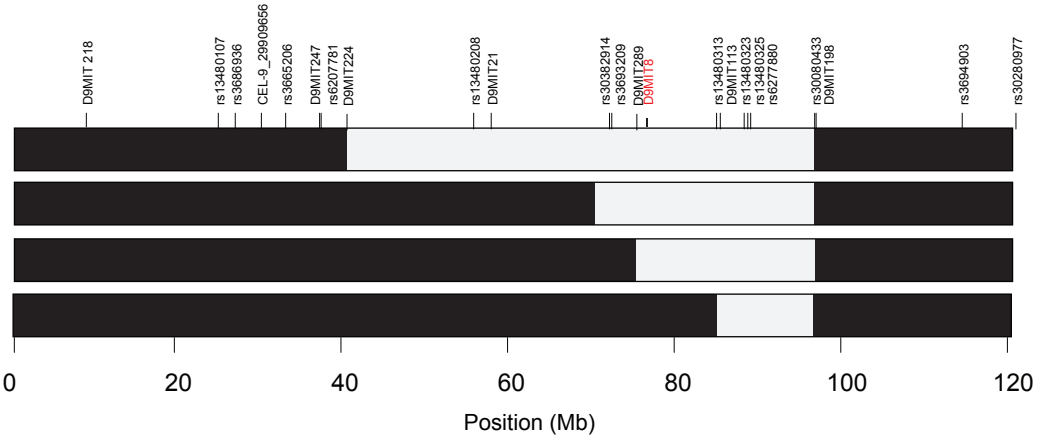

Figure 3.15 – Full congenic and sub-congenic lines' genetic boundaries. The bottom axis displays the position on chromosome 9. The top of the illustration displays the genetic markers used in mapping relative to the position on chromosome 9. D9MIT8 is displayed in red as it is the peak marker in the QTL analysis (Figure 3.2).

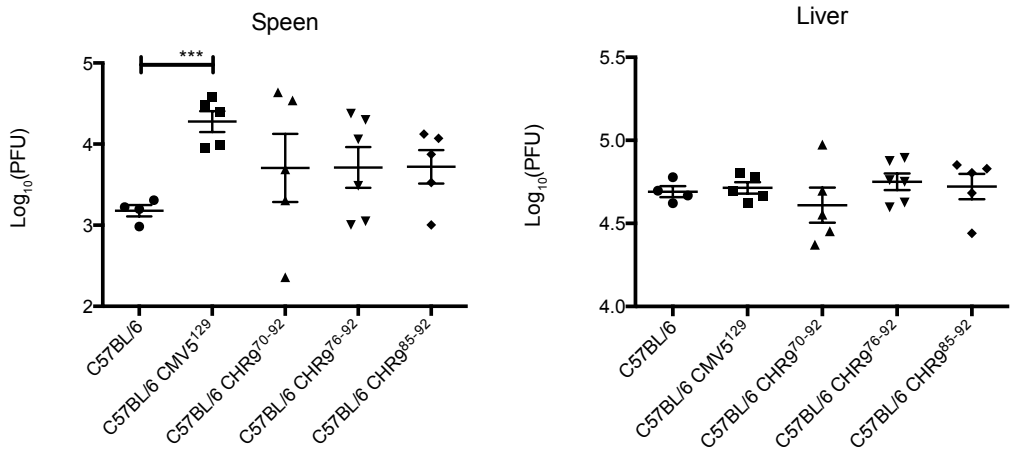

Figure 3.16 – Sub-cogenics lines' MCMV viral titers 2 days p.i quantified via plaque assay. Splenic viral titers were calculated per 0.1g of spleen and liver viral titers were calculated per 1g of liver. 4 samples of C57BL/6 mice and 5 samples of the congenic and sub-congenic groups were used. The results are representative of 3 experiments.
*** indicates a significant difference where p <0.0005 as calculated by student's T-test.

3.8 Exome Sequences

To aid future studies in delimiting the specific susceptibility-causing genes in *Cmv5* we decided to characterize gene coding variants in the *Cmv5* region. Exome sequences of the pure breed C57BL/6 and 129S1 that were previously published in a study by Fairfield et. al. [87], were downloaded from the NCBI sequence read archive and analyzed in Genome Quebec in Dr. Bourque's lab. Although no follow up studies have been conducted to confirm any specific gene involvement in susceptibility, we compiled a list of differential SNPs in the *Cmv5* between C57BL/6 and the 129S1 pure breed strains in protein coding regions that have a predicted high or moderate detrimental effect on protein sequence (Table B.1).

Between positions 40-92 Mb on chromosome 9, we found 25 polymorphic SNPs between 129S1 and C57BL/6 in 21 separate genes that induce coding variations having a predicted high detrimental impact on protein sequence (such as start or stop gain, frame shifts or split site inducing SNPs). 11 of these genes are predicted based on sequence and 10 are documented. 334 other polymorphic SNPs between C57BL/6 and 129S1 in the region are predicted to have moderately detrimental effects on protein sequence (such as a codon insertions or missense inducing SNPs). These 334 SNPs were present in 149 genes, 40 of which were predicted and 109 are documented. Of all the genes in the region, only one, Cd3e, had a documented role in immunity though it was mostly involved in adaptive immunity. Two other genes in

the region could also prove to be interesting candidate genes for follow up studies; Mapk6 and Il10ra (further elaborated on in the discussion). Though using the exome approach only provides genetic defects present in coding sequences, given that most known genetic defects are in protein coding regions it is a good place to begin exploring for genetic factors leading to MCMV susceptibility and will set the stage for future studies to define the specific nature of this genetic defect and its immunological and molecular nature.

Chapter 4

Discussion

4.1 Introductory Remarks

HCMV is one of the most ubiquitous viruses in the human population, and although it is asymptomatic in general, it has been shown to cause life-threatening diseases in newborns and immunocompromised hosts. Therefore, the study of the CMV is crucial however its strict species specificity makes it impossible to study HCMV directly. For this reason, a MCMV- mouse model was developed and proved to be extremely useful in deciphering molecular mechanisms of pathogenesis whose understanding is necessary to elucidate the interactions between the virus and host. The pathologies induced by the two viruses in their respective species is very similar and the host responses in both species follow a similar patters to fight infection. For this reason, the studies utilizing this model have served to make various connections that paved the way for human studies.

Knowing that genetics of the host can modulate resistance to viruses,

many studies have been undertaken to study the genetic factors modulating host resistance. These studies have been crucial to our understanding of virus-host interaction and the causes for susceptibilities. Recently the Vidal lab and others have identified many genetic factors causing susceptibility to CMV in mice and humans. Building on previously generated data in the Vidal lab, this thesis attempted to study yet another genetic cause of susceptibility to MCMV in the 129S1 strain.

The receptor Ly49P has been very well documented and known to need the $H2^k$ MHC-I protein expressing the viral protein m04 in order to successfully triggered. Although strains such as MA/My that express Ly49P but do not have $H2^k$ are susceptible to MCMV, the introduction of $H2^k$ is capable of rescuing the susceptibility phenotype. This was not the case when $H2^k$ was introduced to the background of 129S1 who also express Ly49P (as discussed in section 1.6.1). Moreover, an F1 between 129S1 and C57BL/6 showed that the activating receptor Ly49H (that provides resistance in a dominant fashion in the C57BL/6 background) provided an intermediary resistance relative to the C57BL/6 and the 129S1 pure breeds. These facts taken hand in hand point to a novel modulator of MCMV resistance in the 129S1 strain which this thesis aims to characterize.

4.2 Discussion of Results

Ly49H is known to induce a very strong activating effect on NK cells that usually masks the absence of other activating receptors (such as Ly49P) due to its potency [56]. Moreover, studies have shown that Ly49H is inherited in a dominant fashion in the C57BL/6 background [88]. Interestingly, the susceptibility associated with our QTL seems to modulate resistance even in the presence of Ly49H. We initially got evidence of this upon examining F1 mice (C57BL/6 X 129S1) which display intermediate viral titers relative to C57BL/6 and 129S1 pure breed strains (data not shown). Although this finding alone does not provide evidence that *Cmv5* modulates the activity of Ly49H, the F2 population provided definitive evidence. However, this finding, coupled with the fact that Ly49P activity cannot be recapitulated in the 129S1 strain upon introducing $H2^k$ to their genetic background (Figure 1.2) provided enough evidence to pursue the genetic defects that underlie MCMV susceptibility in the 129S1 strain.

Due to the absence of Ly49H from the 129S1 strain, the F2 population used for mapping would normally have varying combinations of Ly49h alleles. This could be problematic, as the LOD scores obtained from running a QTL analysis on a population that has varying expressions of Ly49H would certainly correlate susceptibility to the absence of that receptor on the NKC on chromosome 6. Thus, it was crucial to control for the effect of the Ly49H activating receptor and special care was taken in order to ensure that its

expression was uniformly homozygous in all F2 mice used in the mapping.

QTL analysis on the Ly49H homozygous F2 population revealed the clear and significant QTL at chromosome 9, *Cmv5* (Figure 3.1). The identification of this region seemed very promising due to the high LOD score and the clear segregation of phenotypes (1 Log) observed when mice used in the mapping were grouped based on the peak marker D9MIT8 (Figure 3.3). Interestingly, when the F2 mice were segregated based the peak *Cm5* marker (D9MIT8), intermediate viral titers in the heterozygous mice relative to mice having either homozygous 129S1 or C57BL/6 alleles at that marker were observed (Figure 3.3). This was the second major evidence that pointed to *Cmv5* being a modulator of Ly49H activity. Based on these results, we decided to create congenic strains bearing *Cmv5* in the C57BL/6 background, since having the QTL in that context could provide further insight into Ly49H regulation. Moreover, congenics bearing the susceptible 129S1 *Cmv5* in a pure C57BL/6 MCMV resistant background would emphasize *Cmv5* susceptibility phenotypes, facilitating their characterization.

The phenotype used for mapping MCMV was viral titers 3 days p.i. 3 days of infection is a common time point to check for MCMV susceptibility since it is known that Ly49H specific antiviral NK cell response is mounted at this time [89]. Initially we hypothesized that 3 days p.i would be the optimal time point since we wanted to observe Ly49H specific defects in MCMV clearance. However, 3 days of infection showed only modest difference in viral titers between the congenics and their CB57BL/6 litter mates (Figure 3.4)

relative to the difference observed in the F2 when segregated by peak marker D9MIT8 (Figure 3.3). This was expected since the F2s had more 129S1 alleles and the *Cmv5* QTL may account for only part of the susceptibility, however it is worth noting that a new virus stock of MCMV was prepared due to the exhaustion of our initial stock. This stock is known to be less virulent as 50,000 PFU/mouse of the virus is necessary to achieve what 12,500 PFU/mouse did in terms of viral titer in C57BL/6 mice. Regardless of the reason for the decrease in susceptibility in the congenic line, the recapitulation of the phenotype proves that the effects of the QTL have been captured in the congenic line.

In order to identify the time range where susceptibility induced by *Cmv5* is maximal, we conducted plaque assays at various days p.i. While day 3 always showed a 0.5 log difference between the congenic line and the C57BL/6 mice, this difference as not always significant and approximately 50% of the variance explained is due to the QTL. 2 days of infection however showed a consistently significant difference of 1 log and the variance explained due to *Cmv5* at this time point was approximately 80%. Later time points such as days 4 and 5 showed no significant differences between the 2 groups. This data implies that the time range the *Cmv5* specific susceptibility manifest maximally is prior to 3 days, at which point the replication of the virus seems to be controlled fairly similarly in both the congenics and their C57BL/6 litter mates likely due to the activity of their Ly49 receptors, including Ly49H, that were shown to be fully intact (Figure 3.7) in the congenics. The fact that

the major difference in viral titers was observed at day 2, prior to the Ly49 specific response, indicates that the *Cmv5* may not affect Ly49H directly, but indirectly through a mechanism responsible for priming NK cells non-specifically early in infection, in turn allowing for an efficient Ly49 specific response. The fact that the 1 Log difference between congenics and their C57BL/6 litter mates is reduced to half its value by day 3 p.i and is reduced to zero by day 4 p.i provides testament to the potency of the Ly49H receptor and its capability to efficiently activate NK cells to quickly clear MCMV in the spleen.

Having sufficient confidence in the recapitulation of the susceptibility phenotype in the congenics, and knowing that NK cells are the main effector cells in fighting MCMV even before their Ly49 specific response [11], the next step was be to characterize the congenic line's NK cells. The proportions, numbers and surface expression of activating receptors (NK1.1, NKp46, Ly49H) on splenic NK cells in the congenic and their C57BL/6 litter mates were determined at base conditions, and no differences were observed (data not shown), thus the functional capacity of NK cells was observed.

The NK cell ability to reject allogenic cells compared to self cells is commonly utilized in order to test NK cells' ability to recognize non-self and elicit their killing function and thus provides a general idea of the responsiveness of these cells. The non-self rejection assays also provide an idea of the level of education/ licensing of the NK cell [59]. The lack of difference between the two groups implies that at least the baseline activity of NK cells are intact;

they seem educated enough to be able to recognize non-self cells and elicit efficient target killing. These results were the first to imply that the defect may not reside in NK cells directly.

Previously, a deficiency in NK cell activity was documented in the 129S1 strain that was claimed to be due to DAP12 signalling [90]. In this study by McVicar et. al., 129S1 activating NK cell receptors seemed to be structurally intact, and the protein DAP12 seemed to be functional, but NK cells showed a hypo-responsive phenotype when activated via these activating receptors. In order to determine if this QTL harboured a DAP12 related deficiency, we attempted to stimulate NK cells via activating receptors which utilize the DAP12 adaptor, along with others utilizing different adaptors in the congenics. We examined receptors utilizing the adaptor proteins CD3ζ such as NK1.1 and FcϵRI-γ such as NKp46. No differences in NK cell IFNγ production (Figure 3.7), CD69 and KLRG1 surface expression (data not shown) is observed between both groups when NK cell activating receptors were triggered, regardless of the receptor or the adaptor the activating receptor utilizes (Figure 3.7). This is consistent with the rejection assay results and implies that NK cells in the congenic line are just as competent at eliciting downstream activating signals as C57BL/6 but also implies that we have stumbled upon an underlying cause of susceptibility in 129S1 that is not DAP12 related.

Other studies have shown that essential factors necessary for proper NK cell priming and homeostasis, leading to efficient effector functions are cy-

tokines, a few being IL 15, IL 12 and IL 18 secreted by DCs upon MCMV infection [84, 70]. We examined the congenic strain NK cell production of IFNγ (Figure 3.8) and CD69 (Figure 3.9) and KLRG1 surface expression upon stimulation with soluble cytokines and mediators known to activate NK cells at various time points. Once again NK cells in the congenics are observed to be just as competent, if not more in the congenic line as their C57BL/6 litter mates. The only deficiency observed in the congenic strain NK cells in our *ex vivo* characterizations of NK cells was IFNγ production post stimulation with IL15 and IL 18 for 4 hours, where a smaller proportion of NK cells in the congenic produced IFNγ relative to C57BL/6 litter mates. This deficiency was minor (5%) and was was quickly compensated when NK cells were stimulated for 24 hours. Given the extensive characterizations conducted, and the lack of any major or obvious defect in the congenic strain implies that these NK cells are fully capable of being activated and eliciting their killing function. Thus the evidence provided by our data implies that another cell type that is involved in priming the NK cell to induce these intact functions are the ones bearing the *Cmv5* defect directly.

The lack of obvious NK phenotypes in non-infectious contexts made it obvious that their function during infection must be assessed in order to determine the nature of how *Cmv5* affects NK cells. Knowing that the maximal susceptibility time range happens early on (day 2) and lasts till about day 3, infections at day 1 and 2 were conducted to observe NK functionality. Studies have confirmed that maturation markers, namely CD11b and

CD27 correlate with stimulation thresholds and potency of effector functions elicited by NK cells [34]. Notably, it has been observed that $CD11b^{+}CD27^{+}$ NK cells display the strongest effector functions and have low thresholds of activation [35], however $CD11b^{+}CD27^{-}$ are the main NK cell subset responsible for non-self rejection [33, 34]. Thus, looking at maturity markers in the congenics could provide a good idea of other deficiencies to expect in NK cells.

Congenic NK cells interestingly displayed a lag in stage 3 maturity ($CD11b^{+}$ $CD27^{+}$) after 1 day of infection, and at stage 2 ($CD11b^{-}CD27^{+}$) lag 2 days p.i (Figure 3.10) relative to C57BL/6 litter mates. Upon further characterization of these time points, we observed that a slightly lower proportion of NK cells produced IFNγ in the congenic line but this decrease was modest. The decrease observed 2 days p.i in granzyme secretion in the congenic strain however was significant and large. The granzyme results correlate with the maturity results since $CD11b^{+}CD27^{+}$ NK cells subset was also found to be reduced. Thus the decrease of their proportion in the congenics relative to C57BL/6 litter mates at day 2 post infection goes hand in hand with lower granzyme levels at that time point and could be directly responsible for the higher viral titers observed at this time point. The lower IFNγ levels at day 1 however did not correlate with higher $CD11b^{+}CD27^{+}$ NK cells, though these lower levels could be directly responsible for the lack of activated and mature NK cells at day 2 in the congenics, leading to lower granzyme secretion and susceptibility.

The immaturity of NK cells at days 1 and 2 and the decrease in IFNγ producing NK cells at day 1 p.i all points to the lack of activation of these NK cells early during infection causing them to be functionally deficient in terms of granzyme secretion by day 2. The fact that these cells displayed no obvious deficiency in *ex vivo* assays and *in vivo* non-self rejection assays points to the conclusion that another cell type necessary in activating NK cells is performing sub-optimally early on during infection. Previous maturation studies have also showed that the levels of IFNγ levels responded directly to IL12 and IL 18 secreted by monocytes [33]. Moreover, DCs specifically are known to be important for the priming and homeostasis of NK cells through IL 12, IL 15 and type I IFN production [85, 84] and their contributions through these cytokines in early MCMV infections in relation to NK cells has been documented [91, 58]. Thus DCs could be a major cell type implicated in bearing the *Cmv5* defect. Other cell types that affect maturity and effector functions of NK cells early in MCMV infection are splenic stromal cells, who through an interaction between which are the main producers of type I IFN 8 hours post MCMV infection [11].

Since our data provides evidence that the *Cmv5* defect does not lie directly in NK cells, we conducted an early kinetics of MCMV infection in our congenic mice in order to get a better idea of which cell type may be directly affected by *Cmv5*. Early during MCMV infection, starting at 8 hours to 36 hours, both groups showed no differences in viral mRNA levels in the spleen. The differences start to emerge after 36 hours; while the C57BL/6 mice start

to reduce viral mRNA, the congenic line's viral mRNA levels start to increase (Figure 3.13). This trend was seen with both early and late viral gene expression, however the difference between the congenics and their C57BL/6 litter mates was much more amplified in the gB late gene expression relative to the IE early gene. This implies that viral replication was greatly impaired in C57BL/6 mice while relatively very little control of viral replication was seen in the congenics until day 3. The susceptibility starting point at 36 hours correlates perfectly to the known time of the second wave of type I IFN secretion that DCs are responsible for [70], making this cell type the primary suspect for the susceptibility observed. In order to further clarify this hypothesis, we observed the levels of cytokines known to be secreted by DCs at the 36 hours p.i with MCMV.

Although IL-12 and IL-15 mRNA expression levels did not show any differences in our kinetic study's time points (data not shown) the levels of Type I IFN showed interesting trends. We see a trend of lower levels of type I IFN mRNA expression at 36 hours in the congenic compared with the C57BL/6 litter mates. By 48 hours this trend was reversed and the congenic produced more type I IFN mRNA. This trend was more obvious with IFNα than IFNβ and though not significant, was very close to significance (Figure 3.14) and would need more mice to ensure the data's relevance (we used 4-5 mice/ group). If these type I IFN mRNA expression levels are considered, the lower levels in the congenics at 36 hours could be responsible for the subsequent lack of maturation and effector function deficiency observed in NK cells at

48 hours, leading to the relative susceptibility in the congenic strain. The relatively higher levels of viral replication observed in the congenics at day 2 is likely directly responsible for stimulating DCs, causing them to increase the increase in type I IFN expression observed in the congenics at this time point. Knowing that DCs are the main producers of type I IFN at this time, these findings provide further evidence supporting the hypothesis that DCs are the cell type affected by *Cmv5*. Of course these results will have to be repeated with a larger sample size to ensure significance of these results.

To pave the way for the next stage of this project and allow a finer dissection of *Cmv5* in order to prioritize candidate genes, three sub-congenic lines were created (70-92, 76-82 and 85-92 Mb). These lines were assessed for viral titers in the same conditions that were used to found to display a maximal phenotype between the full congenics and their C57Bl/6 litter mates; 2 days p.i with 50,000 PFU of MCMV. The sub-congenics displayed wide variation in their viral titers, and showed an intermediate phenotype (Figure 3.15) relative to the full congenics and their C57Bl/6 litter mates. The data implies that at least 2 loci within the *Cmv5* locus have susceptibility 129S1 alleles, one between positions 40-70 Mb and another between 85-92 Mb, interacting in a complex fashion to bring the full susceptibility observed in the congenic line. In an attempt to confirm this, a 2 way QTL analysis was conducted using the same F2 population data used for mapping, however due to the resolution of our mapping, it was difficult to define any interactions within the region statistically. It is also worth noting that although the chromo-

some 9 region was carefully genotyped using either an SSLP, RFLP or SNP at every megabase in the *Cmv5* region in these sub-congenic lines, we cannot discount the possibility contaminations of 129S1 background elsewhere in the genome of the sub-congenic mice since the markers used to test their genetic "cleanliness" had a limited resolution (Figure A.1). This could be problematic as other 129S1 alleles in chromosomes that than 9 could be affecting the viral titers of the sub-congenics. This possibility however is likely not the case since no significant region elsewhere in the genome was mapped as susceptibility causing in our QTL analysis, making a complex interaction a more valid possibility.

Finally, we also utilized published exome sequences of the pure breed C57BL/6 and 129S1 strains in order to further characterize the genetic region. These were analyzed in Genome Quebec and filtered to contain only highly or moderately predicted deleterious SNPs in the peak region mapped (Table B.1) in order to aid for future studies that aim to identify specific genes involved. Though these exomes will only shed light on the genetic root for the susceptibility if the defect is in gene coding sequences, it provides a good basis to start the search as most genetic issues occur within protein coding genes. The presence of 170 genes in the region, and the fact that the sub-congenics were not successful in delimiting the region, makes it difficult to prioritize candidate genes. Though only a superficial screening has been done and we have found candidate genes in this region that could prove interesting as candidate genes.

The only gene known to play a function in MCMV infection that was found to have a differential SNP between C57BL/6 and 129S1 in the region is the gene Cd3e, a component of the T cell receptor found at 44.8 Mb on chromosome 9. Through ENU screening, the Biron lab has found that mutations in this gene can induce MCMV susceptibility. Moreover there is evidence in the literature that CD8 T cells do play a part in MCMV clearance, however their involvement was observed only in NK deficient mice, and late during infection [92]. There is no evidence in the literature that proves that Cd3e or T cells are involved in the early clearance of MCMV. We cannot however exclude the possibility that it is involved in our phenotype and thus thus gene will have to be further characterized in the congenics.

No other genes were documented to have any known effect on MCMV infection except Cd3e, although other genes seemed to be interesting. One of these genes which is very close to the peak marker D9MIT8 (76.2 Mb) is Mapk6, also known as Erk3 (75.2 Mb). Very little is known about this specific kinase however a few interaction partners have been identified and a role for it in cancer and T cell activation signalling has been implicated [93, 94, 95]. Though no anti-viral involvement of this kinase have been defined, it is known that its function is affected by MEK1/2, kinases that have also been implicated in being involved the signalling leading to cytotoxic abilities of NK cells [95, 96]. Moreover, the immgen database shows that the mRNA expression of this gene was shown to be extremely unregulated in NK cell specifically 1 day post MCMV infection. Although our data hints that the

Cmv5 defect is likely not directly in NK cells, it does not provide definitive proof, thus genes affecting NK cells directly must also be considered, making Erk3 a candidate that contributes to *Cmv5* susceptibility.

The involvement of IL 10 in MCMV infections has been documented and found to inhibit viral replication late during the infection through a complex interaction including DC, NK and CD4 cells [97]. Moreover, IL 10 has been implicated as a cytokine necessary in providing protection from collateral injury by dampening immune responses associated with MCMV infection [98]. We found moderately deleterious polymorphic SNPs between 129S1 and C57BL/6 on the Il10ra gene (45 Mb), the receptor of IL 10 in our *Cmv5* QTL, yet another gene that could prove to be interesting if followed up in the congenics.

All in all, our data hints that DCs are the main candidate bearing the *Cmv5* deficiency. The lack of any major phenotypes observed during the characterization of the congenic NK cells outside the context of infection, coupled with the fact that they displayed deficiencies upon infection provides evidence that they may not be the main cell type directly bearing the *Cmv5* induced defect. Moreover, it is known that at 36 hours p.i with MCMV, DCs are the main producers of cytokines that activate NK cells, including the type I IFNs. The type I IFN mRNA expression deficiency observed during the early kinetics of infection and their correlations with the start of susceptibility at 36 hours in the congenics all seems to fit in the context of defective DCs at this time point. This would lead to NK cells that are not efficiently mature

or activated pre Ly49H response, causing the susceptibility observed in the congenics which is rescued once the Ly49H response is mounted. Although this is not directly confirmed and will have to be tested with assays that study DCs and NK cells more closely in the congenics, the data looks promising and, with the aid of the exome sequences analyzed, will lead the way to further dissect the genes or genetic aspects responsible for the susceptibility in the *Cmv5* locus and understanding how it affects NK cells.

4.3 Future Studies

Confirmation of the cell type would be the first step in starting to fully understand the nature of the *Cmv5* QTL. The observation that the mRNA expression levels of intereukin (IL-12 and IL-15) are not changed between both groups and the lack of significance in the trends seen in type I IFN mRNA levels is surprising. A better and cleaner approach however would be to test these DCs separately as opposed to observing the total mRNA level of ILs in the spleen, and more importantly to detect he protein levels of the type I IFN and these ILs as opposed to their mRNA levels as they may be regulated translationally. This could be easily done by using bone marrow derived dendritic cells (BMDCs) which can be stimulated using an array of stimulants and quantify their secreted protein levels of cytokines via ELISA.

Since the sub-congenics did not aid in the delimiting the genetic region responsible for susceptibility, a good approach would be to conduct a micro

array during the susceptibility time range. The genes seen to be up or down regulated would then be cross-referenced to the exome sequence list already generated in order to identify the genes responsible. If genes showng differential regulation between the groups in the micro array do not correlate to any SNPs in tabs B.1, then the defect probably lies in a non-coding region of *Cmv5* in which case a genome sequence would have to be looked at in order to define regulatory regions or other intronic differences that contribute to the susceptibility.

Knowing the cell type and the genes responsible, this study can then progress to its final stage where the mechanistic nature of the *Cmv5* defect could be deciphered leading to the discovery of a novel mechanism of MCMV susceptibility. This will aid us in further understanding the complex interactions between the host and CMV and will hopefully aid to extrapolate our findings to the human context.

List of Abbreviations

BAC	Bacterial Artificial Chromosome
BMDC	Bone Marrow Derived Dendritic Cell
BrDU	5-bromo-2'-deoxyuridine
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cell
cNK	Conventional NK cells
CXCL	C-X-C Motif Chemokine
DC	Dendritic Cell
gB	glycoprotein B
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
i.p	Intraperitoneal
i.v	Intravenous
IE	Immediate-Early
IFN	Interferon
IL	Interleukin
IONO	Ionomycine

ITAM	Immunotyrosine Activating Motif
ITIM	Immunotyrosine Inhibitory Motif
KIR	Killer Cell Immunoglobulin-like Receptor
KO	Knock Out
LOD	Logarithm of Odds
Mb	Mega Base pairs
MCMV	Murine Cytomegalovirus
MEF	Mouse Embryonic FIbroblasts
MHC	Major Histocompatibility Complex
NK	Natural Killer
NKC	Natural Killer Gene Complex
NKp46	Natural Killer cell p46 related protein
ORF	Open Reading Frame
p.i	Post Infection
pDC	Plasmacytoid Dendritic Cell
PMA	Phorbol 12-Myristate 13-Acetate
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RFLP	Restriction Fragment Length Polymorphism
SHP 1	SH2-domain-containing protein tyrosine phosphatase 1
SHP 2	SH2-domain-containing protein tyrosine phosphatase 2
SNP	Single Nucleotide Polymorphism
SSLP	Simple Sequence Length Polymorphism

TLR	Toll-Like Receptor
TNFα	Tumor Necrosis Factor α
TRAIL	TNF-Related Apoptosis-Inducing Ligand

Appendix A

Markers Used for Genotyping

Table A.1 – Chr9 markers used in QTL mapping

Marker ID	Position (Mb)
D9MIT 218	8.90
rs13480107	24.74
rs3686936	26.77
CEL929909656	29.91
rs3665206	32.83
D9MIT247	36.90
rs6207781	37.08
rs13480208	55.40
D9MIT21	57.50
rs30382914	71.70
rs3693209	71.97
D9MIT8	76.20
rs13480313	84.55
D9MIT269	85.00
rs13480323	87.87
rs13480325	88.30
rs6277880	88.65
rs30080433	96.33
rs3694903	114.04
rs30280977	120.49

Table A.2 – Chr9 markers used in for genotyping congenics and sub-congenic strains

Marker ID	Position (Mb)
D9MIT224	36.574618
D9MIT21	57.482346
D9MIT289	69.159629
D9MIT261	76.619854
D9MIT113	85.593452
D9MIT198	91.176808

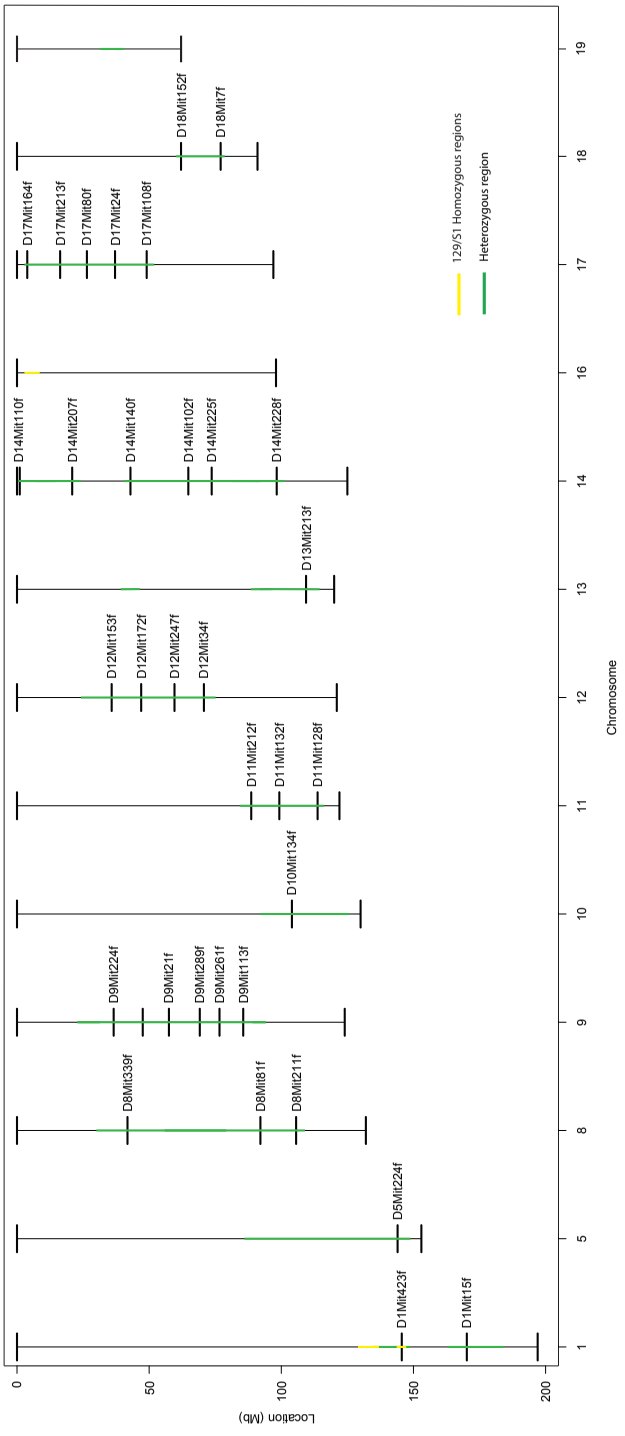

Figure A.1 – Genotyping markers used in the cleanup of the F4N3 generation. The green regions are heterozygous regions in the F4N2 best male chosen for cleanup in order to create the *Cmv5* congenic line. The green lines on the chromosomes are heterozygous regions and the yellow lines are homozygous 129S1 regions in the F2N2 best male chosen. Unmarked regions of the chromosomes (black line) and missing chromosomes are clean C57BL/6 regions.

Appendix B

Cmv5 Coding Variants Identified by Exome Sequencing

Table B.1 – Differential SNPs between C57BL/6 and 129S1 in chromosome 9 (71-92 Mb) that lead to deleterious coding variants

Position (bp)	*Gene*	Impact	Effect	SNP ID
40338825	*Gm17540*	High	Splice site donor	rs37355412
40613208	*Hspa8*	Moderate	Codon change/ insertion	.
40725310	*4931429I11Rik*	Moderate	Non-synonymous	rs36685920
40725380	*4931429I11Rik*	Moderate	Non-synonymous	.
40781684	*Crtam*	Moderate	Non-synonymous	rs29787313
40792434	*Crtam*	Moderate	Codon insertion	.
40795971	*Crtam*	Moderate	Non-synonymous	rs46436456
40796017	*Crtam*	Moderate	Non-synonymous	rs37955209

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
40812656	*Crtam*	Moderate	Non-synonymous	rs37634497
41389434	*2610203C20Rik*	Moderate	Non-synonymous	rs30317864
41389440	*2610203C20Rik*	Moderate	Non-synonymous	rs51536939
41389469	*2610203C20Rik*	High	Stop gained	rs30471965
41398380	*2610203C20Rik*	Moderate	Non-synonymous	rs30174515
41792521	*Sorl1*	Moderate	Non-synonymous	rs48859022
41797509	*Sorl1*	Moderate	Non-synonymous	rs48327489
41804371	*Sorl1*	Moderate	Non-synonymous	rs45639841
41808422	*Sorl1*	Moderate	Non-synonymous	rs36294187
41907338	*Sorl1*	Moderate	Non-synonymous	rs37338110
42063454	*Sc5d*	Moderate	Non-synonymous	rs36310583
42138334	*Tecta*	Moderate	Non-synonymous	rs33756006
42167409	*Tecta*	Moderate	Non-synonymous	rs50440510
42175200	*Tecta*	Moderate	Non-synonymous	rs30224599
42181464	*Tecta*	Moderate	Non-synonymous	rs47344519
42779288	*Arhgef12*	Moderate	Non-synonymous	rs29892489
43910600	*Mfrp*	Moderate	Non-synonymous	.
43910642	*Mfrp*	Moderate	Codon change/ insertion	.
43916570	*C1qtnf5*	Moderate	Non-synonymous	rs32590510
43920777	*Rnf26*	Moderate	Non-synonymous	.
43945348	*Mcam*	Moderate	Non-synonymous	rs29698032
43946961	*Mcam*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
43948723	*Mcam*	Moderate	Codon change/ deletion	.
44041850	*Cbl*	Moderate	Codon change/ insertion	.
44054937	*Ccdc153*	Moderate	Non-synonymous	rs30614950
44056719	*Pdzd3*	Moderate	Non-synonymous	rs31005557
44056728	*Pdzd3*	Moderate	Non-synonymous	rs30832814
44072101	*Nlrx1*	Moderate	Non-synonymous	rs30527605
44072861	*Nlrx1*	Moderate	Non-synonymous	.
44087084	*Abcg4*	Moderate	Non-synonymous	.
44104392	*Hinfp*	Moderate	Non-synonymous	rs32606720
44110643	*Hinfp*	Moderate	Non-synonymous	rs30283546
44121741	*C2cd2l*	Moderate	Non-synonymous	.
44123252	*C2cd2l*	Moderate	Non-synonymous	rs32609280
44141014	*Dpagt1*	Moderate	Non-synonymous	rs32608944
44147581	*Hmbs*	Moderate	Non-synonymous	rs32607118
44196156	*Hyou1*	Moderate	Non-synonymous	rs51288525
44196224	*Hyou1*	Moderate	Non-synonymous	rs29687128
44221229	*Ccdc84*	Moderate	Non-synonymous	rs32608788
44243294	*Foxr1*	High	Frame shift	.
44243349	*Foxr1*	Moderate	Non-synonymous	rs3678819
44243376	*Foxr1*	Moderate	Non-synonymous	rs3678858
44244167	*Foxr1*	Moderate	Non-synonymous	rs29589588
44316486	*Bcl9l*	Moderate	Codon change/ deletion	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
44415599	*Ddx6*	Moderate	Non-synonymous	rs30403127
44415615	*Ddx6*	Moderate	Codon change/ deletion	.
44489398	*Treh*	Moderate	Non-synonymous	rs32620090
44489427	*Treh*	Moderate	Non-synonymous	rs32620092
44489461	*Treh*	Moderate	Non-synonymous	rs52032317
44492743	*Treh*	Moderate	Non-synonymous	rs30383290
44496509	*Phldb1*	Moderate	Non-synonymous	rs46400871
44502543	*Phldb1*	Moderate	Non-synonymous	.
44506196	*Phldb1*	Moderate	Non-synonymous	rs32621093
44524689	*Phldb1*	Moderate	Non-synonymous	rs30090974
44526392	*Phldb1*	Moderate	Non-synonymous	rs33641253
44534176	*Phldb1*	Moderate	Non-synonymous	rs32621052
44590248	*Ift46*	Moderate	Non-synonymous	.
44625856	*Mll1*	Moderate	Non-synonymous	rs32628915
44630153	*Mll1*	Moderate	Non-synonymous	rs29981133
44656172	*Mll1*	Moderate	Non-synonymous	rs30136208
44686822	*Mll1*	Moderate	Non-synonymous	rs51490545
44741351	*Ube4a*	High	Stop lost	rs47003410
44761112	*Ube4a*	Moderate	Non-synonymous	rs32638708
44764810	*Ube4a*	High	Splice site acceptor	rs32642139
44793242	*Cd3d*	Moderate	Non-synonymous	rs30180211
44793244	*Cd3d*	Moderate	Non-synonymous	rs30091366

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
44793250	*Cd3d*	Moderate	Non-synonymous	rs29603950
44793815	*Cd3d*	Moderate	Non-synonymous	rs8259412
44810252	*Cd3e*	Moderate	Non-synonymous	rs46391226
44855368	*Mpzl2*	Moderate	Non-synonymous	rs51358124
44870127	*Mpzl3*	Moderate	Non-synonymous	rs33712315
44870297	*Mpzl3*	Moderate	Non-synonymous	rs29995555
44870312	*Mpzl3*	Moderate	Non-synonymous	rs30184066
44901829	*Amica1*	Moderate	Non-synonymous	rs47927111
44901931	*Amica1*	Moderate	Non-synonymous	rs46109104
44906019	*Amica1*	Moderate	Non-synonymous	rs30322647
44915754	*Gm10684*	High	Frame shift	.
44915807	*Gm10684*	High	Frame shift	.
44915851	*Gm10684*	Moderate	Non-synonymous	rs29736455
44918086	*Gm10684*	High	Frame shift	.
44918142	*Gm10684*	High	Frame shift	.
44918158	*Gm10684*	Moderate	Non-synonymous	rs50838068
44918261	*Gm10684*	Moderate	Non-synonymous	rs51081523
44983235	*Tmprss4*	Moderate	Non-synonymous	rs50708380
44987494	*Tmprss4*	Moderate	Non-synonymous	rs49103446
45052306	*BC049352*	Moderate	Non-synonymous	rs50373811
45052314	*BC049352*	High	Stop gained	.
45064274	*Il10ra*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
45064323	*Il10ra*	Moderate	Non-synonymous	rs30483633
45073701	*Il10ra*	Moderate	Non-synonymous	rs49484941
45073702	*Il10ra*	Moderate	Non-synonymous	.
45074633	*Il10ra*	Moderate	Non-synonymous	rs29639715
45136608	*Tmprss13*	Moderate	Non-synonymous	rs52431095
45136678	*Tmprss13*	Moderate	Codon deletion	.
45136693	*Tmprss13*	Moderate	Non-synonymous	rs51091355
45207961	*Fxyd2*	Moderate	Non-synonymous	rs48458585
45256013	*Dscaml1*	Moderate	Non-synonymous	rs30331523
45258677	*Dscaml1*	Moderate	Non-synonymous	rs47527715
45258695	*Dscaml1*	Moderate	Non-synonymous	rs51550585
45548571	*Dscaml1*	High	Stop gained	rs32646400
45578957	*Cep164*	Moderate	Non-synonymous	rs48502272
45578958	*Cep164*	Moderate	Non-synonymous	rs49802223
45580393	*Cep164*	Moderate	Non-synonymous	rs32641936
45581921	*Cep164*	Moderate	Non-synonymous	rs30478988
45581932	*Cep164*	Moderate	Non-synonymous	rs30043307
45581945	*Cep164*	Moderate	Non-synonymous	rs30422146
45583398	*Cep164*	Moderate	Non-synonymous	rs3697381
45583817	*Cep164*	Moderate	Non-synonymous	rs48194402
45583997	*Cep164*	Moderate	Non-synonymous	rs32647207
45584889	*Cep164*	Moderate	Non-synonymous	rs32647965

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
45586562	*Cep164*	Moderate	Non-synonymous	rs32640978
45587230	*Cep164*	Moderate	Non-synonymous	rs30242646
45587487	*Cep164*	Moderate	Non-synonymous	.
45587879	*Cep164*	Moderate	Non-synonymous	.
45590235	*Cep164*	Moderate	Non-synonymous	rs32645877
45595527	*Cep164*	Moderate	Non-synonymous	rs32645146
45601065	*Cep164*	Moderate	Non-synonymous	rs32648770
45602206	*Cep164*	Moderate	Codon deletion	.
45602218	*Cep164*	Moderate	Non-synonymous	rs52494800
45602415	*Cep164*	Moderate	Non-synonymous	rs32649996
45617915	*Cep164*	Moderate	Non-synonymous	rs30220443
45677253	*Rnf214*	High	Splice site donor	.
45708250	*Rnf214*	Moderate	Non-synonymous	rs29798005
45755949	*Sidt2*	Moderate	Non-synonymous	rs30180984
46019895	*Sik3*	Moderate	Non-synonymous	rs32676144
46019961	*Sik3*	Moderate	Non-synonymous	rs32676146
46020140	*Sik3*	Moderate	Codon change/ insertion	.
46020257	*Sik3*	Moderate	Non-synonymous	rs32676148
46038435	*Apoa1*	Moderate	Non-synonymous	rs13462139
46042805	*Apoc3*	Moderate	Non-synonymous	rs29889677
46043266	*Apoc3*	Moderate	Non-synonymous	rs29881777
46051313	*Apoa4*	Moderate	Codon change/ deletion	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
48128000	*Fam55b*	Moderate	Non-synonymous	rs29976443
48131135	*Fam55b*	Moderate	Non-synonymous	rs51271082
48134511	*Fam55b*	Moderate	Non-synonymous	rs30183699
48134553	*Fam55b*	Moderate	Non-synonymous	rs30361821
48134565	*Fam55b*	Moderate	Non-synonymous	rs30183537
48134576	*Fam55b*	Moderate	Non-synonymous	rs48981003
48134578	*Fam55b*	Moderate	Non-synonymous	rs29689918
48147637	*Fam55b*	Moderate	Non-synonymous	rs46739467
48147670	*Fam55b*	Moderate	Non-synonymous	rs48286201
48201106	*Fam55d*	Moderate	Non-synonymous	rs29927469
48201122	*Fam55d*	Moderate	Non-synonymous	rs30136314
48201214	*Fam55d*	Moderate	Non-synonymous	rs51400475
48204575	*Fam55d*	Moderate	Non-synonymous	rs33702650
48204614	*Fam55d*	Moderate	Non-synonymous	rs33732329
50302674	*1600029D21Rik*	Moderate	Non-synonymous	rs29794929
50309207	*1600029D21Rik*	Moderate	Non-synonymous	rs32735778
50312522	*1600029D21Rik*	Moderate	Non-synonymous	rs33759675
50336725	*Pts*	Moderate	Non-synonymous	rs30353028
50344404	*Bco2*	Moderate	Non-synonymous	rs6386942
50344407	*Bco2*	Moderate	Non-synonymous	rs6386944
50427921	*Pih1d2*	Moderate	Non-synonymous	.
50429813	*Pih1d2*	Moderate	Non-synonymous	rs48262782

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
50429893	*Pih1d2*	Moderate	Non-synonymous	.
50433071	*Pih1d2*	Moderate	Non-synonymous	rs32747743
50444648	*Dlat*	Moderate	Non-synonymous	rs48577256
50477631	*Dixdc1*	Moderate	Non-synonymous	rs47690621
50490709	*Dixdc1*	Moderate	Non-synonymous	rs29887554
50559773	*Hspb2*	Moderate	Non-synonymous	rs29794528
50571963	*1110032A03Rik*	High	Stop lost	rs32749244
50571990	*1110032A03Rik*	Moderate	Non-synonymous	rs29601109
50578796	*Fdxacb1*	Moderate	Non-synonymous	rs49522820
50703788	*Sik2*	Moderate	Non-synonymous	rs29881697
50704812	*Sik2*	Moderate	Non-synonymous	.
50865748	*Layn*	Moderate	Non-synonymous	rs33773426
50870348	*Layn*	Moderate	Non-synonymous	.
50871351	*Layn*	Moderate	Non-synonymous	.
50924745	*Btg4*	Moderate	Non-synonymous	rs13461391
50926093	*Btg4*	Moderate	Non-synonymous	rs13461390
50927311	*Btg4*	Moderate	Non-synonymous	rs32767530
50927526	*Btg4*	Moderate	Non-synonymous	rs3704452
51078732	*Gm684*	Moderate	Non-synonymous	.
51078954	*Gm684*	Moderate	Non-synonymous	rs29872866
51088487	*2010007H06Rik*	High	Frame shift	.
51755301	*Fdx1*	Moderate	Non-synonymous	rs30035791

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
53185532	*Exph5*	Moderate	Non-synonymous	rs29592244
53262855	*Atm*	Moderate	Non-synonymous	rs29639170
53267036	*Atm*	Moderate	Non-synonymous	rs48455466
53332548	*Atm*	Moderate	Non-synonymous	.
53731919	*Gm16380*	Moderate	Non-synonymous	rs30477887
53731926	*Gm16380*	Moderate	Non-synonymous	rs33645468
53732166	*Gm16380*	Moderate	Non-synonymous	rs29638983
53732225	*Gm16380*	Moderate	Non-synonymous	rs29645267
54134577	*Gldn*	Moderate	Non-synonymous	rs29736523
54768304	*Agphd1*	Moderate	Non-synonymous	rs30234701
54877916	*Chrnb4*	Moderate	Non-synonymous	rs30375940
54997504	*Ube2q2*	Moderate	Non-synonymous	.
54997537	*Ube2q2*	Moderate	Non-synonymous	.
54997563	*Ube2q2*	Moderate	Non-synonymous	.
54997569	*Ube2q2*	Moderate	Non-synonymous	.
55056883	*Fbxo22*	Moderate	Non-synonymous	.
55056908	*Fbxo22*	Moderate	Non-synonymous	.
55056964	*Fbxo22*	Moderate	Non-synonymous	.
55056985	*Fbxo22*	Moderate	Non-synonymous	.
55057167	*Fbxo22*	Moderate	Non-synonymous	.
55057189	*Fbxo22*	Moderate	Non-synonymous	.
55068877	*Fbxo22*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
55068958	*Fbxo22*	Moderate	Non-synonymous	.
55068995	*Fbxo22*	High	Splice site donor	.
55821553	*Rfpl3s*	Moderate	Non-synonymous	rs38338204
55822903	*Rfpl3s*	Moderate	Non-synonymous	rs29642778
55828606	*Rfpl3s*	Moderate	Non-synonymous	rs36340363
55974451	*Pstpip1*	Moderate	Non-synonymous	rs40216839
56089014	*C230081A13Rik*	Moderate	Non-synonymous	rs38848454
56108260	*C230081A13Rik*	Moderate	Non-synonymous	.
57089969	*Trcg1*	Moderate	Non-synonymous	rs38051758
57091996	*Trcg1*	Moderate	Non-synonymous	rs29880999
57093650	*Trcg1*	Moderate	Non-synonymous	rs33771359
57101816	*1700017B05Rik*	Moderate	Non-synonymous	rs30485901
57104690	*1700017B05Rik*	Moderate	Non-synonymous	rs33628338
57104940	*1700017B05Rik*	Moderate	Non-synonymous	rs29739966
57106209	*1700017B05Rik*	Moderate	Non-synonymous	rs13465819
57106276	*1700017B05Rik*	Moderate	Non-synonymous	rs30300255
57182259	*Gm5121*	Moderate	Non-synonymous	rs48610520;rs29589024
57182485	*Gm5121*	Moderate	Non-synonymous	rs30498010
57638302	*Arid3b*	High	Frame shift	.
57681530	*Arid3b*	Moderate	Non-synonymous	.
57777521	*Ubl7*	Moderate	Non-synonymous	rs30066215
57802339	*Sema7a*	Moderate	Non-synonymous	rs48774862

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
57802646	*Sema7a*	Moderate	Non-synonymous	rs51784465
58376295	*Cd276*	Moderate	Non-synonymous	rs30129594
58430286	*Nptn*	Moderate	Non-synonymous	.
58993581	*Gm7589*	Moderate	Non-synonymous	rs46951303;rs29745656
58993637	*Gm7589*	High	Frame shift	.
58993650	*Gm7589*	Moderate	Non-synonymous	rs33643125
58993725	*Gm7589*	Moderate	Non-synonymous	rs29596990
58993972	*Gm7589*	Moderate	Non-synonymous	rs49703292
58994013	*Gm7589*	Moderate	Non-synonymous	rs50521424
59162550	*Adpgk*	Moderate	Non-synonymous	rs13480222
59162619	*Adpgk*	Moderate	Non-synonymous	rs29744020
59162715	*Adpgk*	Moderate	Non-synonymous	rs29884714
59561992	*Gramd2*	Moderate	Non-synonymous	rs29794257
59690888	*Myo9a*	Moderate	Non-synonymous	.
59830601	*Thsd4*	Moderate	Non-synonymous	rs37565778
59835173	*Thsd4*	Moderate	Non-synonymous	rs6224703
60358058	*Thsd4*	Moderate	Non-synonymous	rs29736591
60358262	*Thsd4*	Moderate	Non-synonymous	rs30470183
60685812	*Gm9869*	Moderate	Non-synonymous	rs29782936
60717414	*Uaca*	Moderate	Non-synonymous	rs3667578
60718415	*Uaca*	Moderate	Non-synonymous	rs37765393
60718477	*Uaca*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
61772881	*Kif23*	Moderate	Non-synonymous	.
61774853	*Kif23*	Moderate	Non-synonymous	rs29593069
61775145	*Kif23*	Moderate	Non-synonymous	rs48889424
64025553	*Rpl4*	Moderate	Non-synonymous	rs50793796
64853575	*Ptplad1*	Moderate	Non-synonymous	.
65127937	*Cilp*	High	Frame shift	.
65147737	*Clpx*	Moderate	Non-synonymous	rs30333818
65284799	*Mtfmt*	Moderate	Non-synonymous	rs30171474
65706061	*Trip4*	Moderate	Non-synonymous	rs33753128
65952731	*Snx1*	Moderate	Codon insertion	.
65953378	*Snx1*	Moderate	Non-synonymous	rs30280035
65972481	*Snx1*	Moderate	Non-synonymous	rs30180376
66611083	*Car12*	Moderate	Non-synonymous	rs30231210
66646069	*Gm10647*	Moderate	Non-synonymous	rs36796671
66646297	*Gm10647*	High	Frame shift	.
66875970	*Tpm1*	Moderate	Non-synonymous	rs30287498
67172607	*Tln2*	Moderate	Non-synonymous	rs30231760
67194311	*Tln2*	Moderate	Non-synonymous	rs38285632
67607909	*C2cd4b*	Moderate	Non-synonymous	rs3721766
67678921	*C2cd4a*	Moderate	Non-synonymous	rs52199156
67678963	*C2cd4a*	Moderate	Non-synonymous	rs52224504
67731354	*Vps13c*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
67769701	*Vps13c*	Moderate	Non-synonymous	rs3722443
69870363	*Gtf2a2*	Moderate	Non-synonymous	.
70145143	*Myo1e*	Moderate	Non-synonymous	rs30226946
70226548	*Myo1e*	Moderate	Non-synonymous	rs37761940
70258022	*Ccnb2*	Moderate	Non-synonymous	.
70390795	*Sltm*	Moderate	Non-synonymous	.
71010272	*Aqp9*	Moderate	Non-synonymous	.
71572293	*Cgnl1*	Moderate	Non-synonymous	rs33742548
71572523	*Cgnl1*	Moderate	Codon insertion	.
71697651	*Tcf12*	Moderate	Non-synonymous	rs29837273
71909075	*Rpl15-ps2*	Moderate	Non-synonymous	rs48426154
72022423	*Gm7866*	Moderate	Non-synonymous	rs46633605
72022784	*Gm7866*	High	Stop gain	rs47966639
72160565	*Zfp280d*	Moderate	Non-synonymous	rs29642719
72320279	*Tex9*	Moderate	Non-synonymous	rs33689583
72320360	*Tex9*	Moderate	Non-synonymous	rs33729587
72320415	*Tex9*	High	Frame shift	.
72321566	*Tex9*	Moderate	Non-synonymous	rs37269586
72465009	*Rfx7*	Moderate	Non-synonymous	rs48028757
72465758	*Rfx7*	Moderate	Non-synonymous	rs6169454
72467414	*Rfx7*	Moderate	Non-synonymous	rs36250468
72877512	*Pigb*	Moderate	Non-synonymous	rs33732168

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
72887364	*Pigb*	Moderate	Non-synonymous	rs33776652
73779768	*Unc13c*	Moderate	Non-synonymous	rs32916913
73780313	*Unc13c*	Moderate	Non-synonymous	rs29938276
74904518	*Arpp19*	Moderate	Non-synonymous	.
74904575	*Arpp19*	Moderate	Non-synonymous	.
75138735	*Myo5c*	Moderate	Non-synonymous	rs29927685
75236302	*Mapk6*	Moderate	Non-synonymous	rs50383439
75236487	*Mapk6*	Moderate	Non-synonymous	.
75524543	*Scg3*	Moderate	Non-synonymous	rs46539645
75531505	*Scg3*	Moderate	Non-synonymous	rs33722005
75862811	*Gm16662*	High	Splice site donor	.
76046785	*Gfral*	Moderate	Non-synonymous	rs32986219
76094198	*Hcrtr2*	Moderate	Non-synonymous	rs29735660
76340746	*Fam83b*	Moderate	Non-synonymous	.
77830544	*Elovl5*	Moderate	Non-synonymous	rs47617099
78243776	*4930542C12Rik*	Moderate	Non-synonymous	.
78243855	*Ddx43*	Moderate	Non-synonymous	rs33708567
78243964	*4930542C12Rik*	High	Start lost	rs33757053
78296185	*Mto1*	Moderate	Non-synonymous	rs13473312
78296327	*Gm17324*	Moderate	Non-synonymous	rs51510867
78297561	*Mto1*	Moderate	Non-synonymous	rs46625372
79540098	*Col12a1*	Moderate	Non-synonymous	.

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
79603353	*Cox7a2*	High	Frame shift	.
79964407	*Senp6*	Moderate	Non-synonymous	rs29633357
79978651	*Senp6*	Moderate	Non-synonymous	rs29936511
79991493	*Senp6*	Moderate	Non-synonymous	rs29838871
80163983	*Impg1*	Moderate	Non-synonymous	rs52051539
80242010	*Impg1*	Moderate	Non-synonymous	rs46161597
80358861	*Impg1*	Moderate	Non-synonymous	rs49899450
80358888	*Impg1*	Moderate	Non-synonymous	rs46905549
82820092	*Phip*	Moderate	Non-synonymous	.
85590799	*Ibtk*	Moderate	Non-synonymous	rs29590417
85614427	*Ibtk*	Moderate	Non-synonymous	rs33755021
85738068	*Tpbg*	Moderate	Non-synonymous	.
85738450	*Tpbg*	Moderate	Non-synonymous	rs4227810
86413863	*Dopey1*	Moderate	Non-synonymous	rs47913613
86414527	*Dopey1*	Moderate	Non-synonymous	rs47435941
86452104	*Pgm3*	Moderate	Non-synonymous	rs46811673
86456296	*Pgm3*	Moderate	Non-synonymous	rs13480319
86480595	*Me1*	Moderate	Non-synonymous	rs45995457
86687379	*Snap91*	High	Splice site acceptor	.
86935286	*Cyb5r4*	Moderate	Non-synonymous	rs46143602
86950662	*Cyb5r4*	Moderate	Non-synonymous	rs45634827
86952067	*Cyb5r4*	Moderate	Non-synonymous	rs29974382

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Table B.1 – continued from previous page

Position (bp)	*Gene*	Impact	Effect	SNP ID
86953792	*Cyb5r4*	Moderate	Non-synonymous	rs29588640
87098859	*4922501C03Rik*	Moderate	Non-synonymous	rs29735336
87099043	*4922501C03Rik*	Moderate	Non-synonymous	rs30479538
87107699	*4922501C03Rik*	Moderate	Non-synonymous	rs51077365
87111988	*4922501C03Rik*	Moderate	Non-synonymous	rs51436682
87116036	*4922501C03Rik*	Moderate	Non-synonymous	rs46430546
87116913	*4922501C03Rik*	Moderate	Non-synonymous	rs51274420
87120555	*4922501C03Rik*	Moderate	Non-synonymous	rs3690414
87122105	*4922501C03Rik*	Moderate	Non-synonymous	rs29588614
87122249	*4922501C03Rik*	Moderate	Non-synonymous	rs30121847
87126243	*4922501C03Rik*	Moderate	Non-synonymous	rs30431679
87126832	*4922501C03Rik*	Moderate	Non-synonymous	rs3665877
87134697	*4922501C03Rik*	Moderate	Non-synonymous	rs51786635
87143266	*4922501C03Rik*	Moderate	Non-synonymous	rs29980857
88464262	*Zfp949*	Moderate	Non-synonymous	rs29889052
88986353	*Trim43b*	Moderate	Non-synonymous	rs46737732
89094291	*Bcl2a1b*	Moderate	Non-synonymous	rs47268553
89110221	*Mthfs*	Moderate	Non-synonymous	rs45779795
90088299	*Adamts7*	Moderate	Non-synonymous	rs29987966

NOTE: SNP IDs that are "." are putative SNPs.

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