The Effects of LY49 Haplotype Divergence on Natural Killer Cell Function

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THE EFFECTS OF LY49 HAPLOTYPE DIVERGENCE ON NATURAL KILLER CELL FUNCTION

by

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Bachelor of Science, McGill University, 2008

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
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Degree: Master of Science
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Natural killer (NK) cells target virally infected cells and tumor cells through Ly49 receptors that recognize MHC-I molecules. Different inbred mouse strains possess disparate Ly49 receptor haplotypes, in addition to NK cell activity. C57BI/6 mice express less Ly49 receptors than 129S1 mice, but have a more robust NK cell population. We intend to determine if the differences in NK cell activity between these inbred mice strains are due to the differences in Ly49 receptor haplotypes. C57Bl/6 mice with the Ly49 gene cluster of 129S1 origin were generated in order to compare 129S1 and B6 Ly49 contribution to NK cell function. These B6-Ly49\textsuperscript{129} congenic mice were confirmed to express the 129 Ly49 receptor pattern by flow cytometry. NK cell activity was assessed by cytotoxicity assays using a panel of NK-resistant and NK-susceptible tumor cell lines, the ability to clear infection with murine cytomegalovirus (MCMV) as well as the ability to reject MHC-I-deficient splenocytes \textit{in vivo}. Susceptibility to MCMV infection by congenic mice was similar to 129 mice. However, there was a significant increase in rejection of MHC-I-deficient cells by the congenic mice in comparison to both the B6 and 129 mice. In addition, \textit{in vitro} tumor cell killing by congenic NK cells was comparable to B6. The current results indicate that expression of an increased number of Ly49 receptors promotes better education of NK cells, which in turn leads to higher rejection of MHC deficient target cells.
I dedicate this thesis to my parents Ashok and Usha Patel and my brother Dharmesh.
ACKNOWLEDGEMENTS

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<tr>
<td>129</td>
<td>129S1 mouse strain</td>
</tr>
<tr>
<td>ALAK</td>
<td>adherent lymphokine activated killer</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>ACVS</td>
<td>animal care and veterinary services</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody dependant cell mediated cytotoxicity</td>
</tr>
<tr>
<td>B2m</td>
<td>B2-microglobulin</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>Clr</td>
<td>C-type lectin related</td>
</tr>
<tr>
<td>B6</td>
<td>C57Bl/6 mouse strain</td>
</tr>
<tr>
<td>CCAC</td>
<td>Canadian council on animal care</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DAP-10</td>
<td>DNAX-activating protien 10</td>
</tr>
<tr>
<td>DAP-12</td>
<td>DNAX-activating protien 12</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>ITAM</td>
<td>immune tyrosine-based activating motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immune tyrosine-based inhibiting motif</td>
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<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>ILT</td>
<td>immunoglobulin like transcripts</td>
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<td>ITSM</td>
<td>immunoreceptor tyrosine based switch motif</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
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<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>KIR</td>
<td>killer immunoglobulin receptor</td>
</tr>
<tr>
<td>LLT1</td>
<td>lectin like transcript 1</td>
</tr>
<tr>
<td>LAIR</td>
<td>leukocyte associated Ig like receptor</td>
</tr>
<tr>
<td>LIR</td>
<td>leukocyte Ig like receptor</td>
</tr>
<tr>
<td>LRC</td>
<td>leukocyte receptor complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage-inflammatory protein</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHC-I</td>
<td>major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC-II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MULT1</td>
<td>murine UL16-binding protein like transcript 1</td>
</tr>
<tr>
<td>NRC</td>
<td>natural cytotoxicity receptors</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>Ocil</td>
<td>osteoclast inhibitory lectin ligands</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cells</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic:polycytidylic</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RANTES</td>
<td>regulated upon activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RPM</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
</tbody>
</table>
**SD** standard deviation

**SEM** standard error mean

**TLR** toll like receptors

**TNF** tumor necrosis factor

**ULBP** UL16 binding protein

**ZAP70** zeta chain (TCR) associated protein kinase 70kDa
1: INTRODUCTION

1.1 Innate Immunity

The innate immune system provides the first line of defence against invading pathogens. Innate immune cells play an essential role in inflammation, immune surveillance and provide a link to the adaptive immune system. An important characteristic of innate immunity is to trigger a rapid immune response against a broad range of pathogens. One way in which this is mediated is by the expression of germ-line receptors, such as the family of toll-like receptors (TLRs) (73). Many phagocytic immune cells, such as macrophages and dendritic cells, express TLRs to detect common pathogen associated molecular patterns (PAMPs). PAMPs are distinct from the host and are specifically associated with pathogens. For example, double stranded RNA is specific to certain virus families, which is recognized by TLR3 (2). Once TLRs recognize their respective ligands, they activate transcription factors such as NF-κB and secretion of pro-inflammatory cytokines leading to inflammation at the site of infection (12).

Presentation of pathogen derived peptides in the context of major histocompatibility complex class I (MHC-I) or class II (MHC-II) is a critical component of the innate immune system. Phagocytic cells can degrade ingested pathogens by fusing the pathogen containing phagosome with lysosomes
containing endosomal proteases. The pathogen is broken down into peptides that are loaded onto MHC-II molecules. Only phagocytic cells express MHC-II, which prime T and B cells to mount a specific response towards the antigen presented (64). Conversely, MHC-I molecules are expressed on all nucleated cells, which normally present self-antigens recognized by CD8+ T cells and natural killer (NK) cells for immune surveillance (20, 37). However, pathogen derived proteins are presented on MHC-I molecules when an intracellular pathogen has infected the cell. Recognition of viral peptides loaded in the context of MHC-I activates CD8+ T cells to kill the infected target cell by the induction of apoptosis (149).

1.2 Natural Killer Cells

Natural killer (NK) cells are a unique lymphocyte population that play an important role in innate immune surveillance. Infection of human cytomegalovirus (CMV) is benign in normal patients but can cause severe recurrent infections in NK deficient hosts (18). NK cells encode germ-line receptors that recognize ubiquitously expressed classical MHC-Ia and non-classical MHC-Ib molecules, contributing to the ability to kill target cells without prior sensitization (37, 63, 101). NK cells target infected host cells with low MHC-I surface expression. Intracellular pathogens down-regulate MHC-I expression on the surface of host cells as a survival mechanism to evade CD8+ T cell responses, however this allows for NK cell recognition. Such pathogens include viruses, intracellular bacteria and parasites (175). NK cells can also recognize malignant cells with aberrant MHC-I expression (79, 188). In addition to surveillance of down-
regulation of MHC-I expression, NK cells recognize antibody coated target cells which leads to antibody dependant cell mediated cytotoxicity (ADCC) (170). Target cells such as extracellular parasites coated with IgG antibodies are recognized by FcγRIII receptors (CD16) on NK cells, which bind to the constant Fc region of the bound antibody (94). Both MHC-I down-regulation and ADCC activates the NK cell to release cytotoxic granules such as perforin and granzymes, which release cytotoxic granules such as perforin and granzymes, which induce apoptosis in the target cell via a mechanism similar to CD8+ T cells (149). It is proposed that perforin polymerizes upon release, creating pore structures on the target cell, allowing granzymes to enter the cell (174). Granzymes can induce apoptosis to the target cell via caspase-3 or BH3 interacting domain death agonist (BID) and subsequent degradation of host DNA (97).

NK cells also secrete many cytokines and chemokines to further activate and recruit other immune cells and augment an immune response. Upon activation, NK cells produce pro-inflammatory cytokines such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (130, 131). Particular cytokine environments can optimize the secretion of different NK cell derived cytokines. For instance, interleukin (IL)-2 and IL-18 promote IFN-γ secretion, while IL-15 and IL-18 favors GM-CSF (36). IFN-γ is an essential cytokine in NK mediated immunity as it up-regulates MHC-I expression for CD8+ T cell recognition, promotes Th1 cytokine production and activation of NK cells (157). Patients with a congenital deficiency
in IFN-γ have a reduced NK cell population (41). NK cells are primed to quickly secrete IFN-γ upon activation as they constitutively express mRNA transcripts for this cytokine (163). NK cells also secrete chemokines such as macrophage-inflammatory protein (MIP)-1α, MIP-1β and RANTES (CCL5) to recruit both NK cells and other immune cells to the site of infection or malignancy (7, 130, 151).

### 1.3 Missing Self Hypothesis

It was first suggested by Ljunggren and Karre that NK cells selectively kill target cells according to “the missing-self hypothesis”, which states that NK cells recognize target cells that have altered expression of MHC-I molecules (98). Healthy “self” cells express sufficient MHC-I molecules that are recognized by inhibitory receptors, and subsequently inhibit an NK cell response (98). However, when an NK cell encounters a target cell with down-regulated MHC-I expression (missing-self), inhibitory receptors are left unbound and an NK cell response ensues (79). This theory did not explain the lack of an NK response against low MHC expressing neuronal cells and MHC-lacking erythrocytes (91). NK cells were later shown to express activating receptors that bind to stress or pathogen associated molecules to further contribute to an NK cell response (119). Ultimately, it was shown that NK cell responses are determined by the integration of signals from both activating and inhibitory receptors found on the surface of each NK cell. The sum of these two opposing signals determines if the NK cell will mediate lysis against the encountered target cell (91). With the exception of FcγRIII, no single activating receptor on its own is capable of triggering
cytotoxicity or cytokine secretion in naïve NK cells (24). As opposed to the dominant B cell or T cell receptors, cross linking of multiple NK cell receptors is required for to reach the threshold for activation (92).

There are two main types of MHC-I receptors found on NK cells, the type I Ig superfamily of receptors such as killer Ig-like receptors (KIRs) expressed in human NK cells and the type II C-type lectin like receptors such as Ly49 receptors expressed in murine NK cells (32, 33, 78, 121). Both are structurally distinct but have similar genetic and functional characteristics in NK cell immunity. A second group of C-type lectin like receptors, the CD94/NKG2 heterodimers, are expressed in both human and murine NK cells (90). The CD94/NKG2 heterodimer recognizes HLA-E in humans and Qa-1 molecules in mice, both are non-classical MHC-Ib molecules which present an MHC-Ia leader peptide (58). This allows CD94/NKG2 receptors to indirectly monitor for MHC-Ia expression. NKG2D is a unique member of this receptor family as it does not associate with CD94, rather it forms a homodimer that triggers cytotoxicity. NKG2D plays a role that is complementary to the natural cytotoxicity receptors (NRCs). NRCs include three activating Ig-like receptors expressed on NK cells: NKp46, NKp44 and NKp30 (118).

1.4 Human Natural Killer Cells

Human NK cells, which make up around 15% of the peripheral lymphocytes in the body, are defined by the differential expression of CD56, CD16 and the lack of CD3ε (35). The expression levels of CD56 and CD16 can
High cytokine production

Low cytokine production

Effector functions
- ADCC
- LAK
- Natural cytotoxicity
Figure 1: Two subsets of human NK cells. Human NK cells can be characterized by the surface expression of CD56 and CD16. A) NK cells with CD56^{bright}CD16^{dim} surface expression are characterized to be strong producers of cytokines such as IFN_{\gamma}, TNF_{\alpha/\beta} and GM-CSF but have little cytotoxic ability. These NK cells have high expression of CD94/NKG2 receptors but low KIR expression. B) In contrast, NK cells with CD56^{dim}CD16^{bright} surface expression are primarily cytotoxic with high expression of KIRs and low expression of CD94/NKG2 receptors. These NK cells produce little cytokines. The expression of high CD16 enables these NK cells to kill via ADCC. Reprinted from (35) with permission from Elsevier.
be used to distinguish different NK cell populations based on their primary effector function (Fig. 1). NK cells that are CD56<sup>dim</sup>CD16<sup>+</sup> are primarily cytotoxic, representing 90% of the NK cells in the peripheral blood (144). In comparison, CD56<sup>bright</sup>CD16<sup>-</sup> NK cells are primarily producers of cytokines, which make up the remaining 10% of NK cells (36). These populations differ in terms of expression of human leukocyte antigen (HLA) receptors, adhesion molecule and cytokine receptors. HLA molecules represent MHC-I complexes expressed in humans. As a consequence of primary effector function, CD56<sup>dim</sup>CD16<sup>+</sup> cytotoxic NK cells express a higher density of KIRs while CD56<sup>bright</sup>CD16<sup>-</sup> cytokine secreting NK cells express a higher density of CD94/NKG2 receptors (5, 183). Unique expression of adhesion molecules, mainly L-selectin (CD62L) by CD56<sup>bright</sup>CD16<sup>-</sup> NK cells and PEN5 by CD56<sup>dim</sup>CD16<sup>+</sup> NK cells are indicative of differential trafficking of each NK cell population (5, 50). Finally, CD56<sup>high</sup>CD16<sup>-</sup> NK cells uniquely express the high affinity IL-2R (expressing α, β and γ chains) and c-Kit receptor tyrosine kinase, allowing the cytokine producers to be more sensitive to IL-2 expansion (113, 122). However, activation of CD56<sup>bright</sup>CD16<sup>-</sup> NK cells with IL-2 or IL-12 enhances cytotoxicity to the level of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells (145).

Human NK cells express a wide variety of NK cell receptors including NKG2D, CD94/NKG2, CD16, NRC receptors: NKp46, NKp44 and NKp30, 2B4 and NKrp1 receptors. Human NK cells have been reported to express TLR 3, 7, 8 and 9 (62, 66, 160). Immunoglobulin-like human NK cell receptors are encoded on the leukocyte receptor complex (LRC) at chromosome 19q13.4 (185). Human C-type
lectin-like NKG2 receptors are encoded in the natural killer cell gene complex (NKC) region at chromosome 12p12-p13 (143).

1.5 Murine Natural Killer Cells

In contrast to human NK cells, murine NK cells lack the expression of CD56, TLRs and KIRs. Subsequently, no real NK cell subsets can be distinguished between cytotoxic or cytokine producing NK cells in the peripheral blood. However, murine GATA-3+CD127+ thymic NK cells that are CD11bloCD16-CD69high exhibit similar enhanced cytokine production and lower cytotoxicity, possibly homologous to human CD56brightCD16- NK cells (184). Murine NK cells express Ly49 receptors, a C-type lectin like H2 receptor (33, 191). H-2K, H-2D and H-2L represent MHC-Ia molecules in mice. Ly49 receptors bind to MHC-Ia complexes and are functionally and genetically homologous to KIR expression in human NK cells (78). Ly49 genes are encoded in the NKC region of murine chromosome 6, which also include Nkrp1/Cir, Cd94/Nkg2, Cd69 (MAFA) and Klrg1 genes encoding other NK cell markers (Fig. 2) (154). The only Ly49 gene encoded in the human NKC is Ly49l, which is a pseudogene and subsequently not expressed (186). The NKC region has been associated with host resistance to mouse cytomegalovirus (MCMV), herpesvirus simplex virus, Leishmania major and the rejection of the xenogeneic Chinese hamster ovarian (CHO) cell line (14, 43, 70, 135, 155, 156). Murine NK cells also express Ig superfamily receptors such as NKp46 and CD16 encoded on chromosome 19 and 1 respectively (17, 176, 182).
Figure 2: The murine natural killer gene complex. The Natural Killer gene complex is located on chromosome six in mice. This genetic region encodes NK cell receptors such as NKrp1 (red) and their Clr ligand (blue), CD94/NKG2 (yellow) and Ly49s (green). While the NKrp1 and CD94/NKG2 receptors are relatively stable, the Ly49 receptors are highly polymorphic and polygenic. Ly49 variability occurs within the framework genes Ly49q/e, Ly49i/g and Ly49c/a.
NK Gene Complex

Mouse
Chromosome 6
1.6 NK Cell Receptors

When an NK cell encounters a target cell, signals from both the activating and inhibitory receptors integrate to determine the fate of the encountered cell. Inhibitory receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIMs, sequence: I/VXYXXL/V) on the cytoplasmic tail of the receptor, which can recruit various tyrosine phosphatases and diminishes the signal cascade resulting from NK cell activation (Fig. 3) (26). An activating NK cell receptor lacks the ITIM, and instead it contains a charged amino acid residue in the transmembrane region of the receptor, allowing it to associate with adapter proteins containing an immunoreceptor tyrosine-based activating motif (ITAM, sequence: D/EXXYXXL/I_Xb,YXXL/I) (114). These adapter molecules are type-I transmembrane-anchored homodimers, such as DNAX-activating protein 12 (DAP12), FcεRI-γ and CD3ζ, which contain a lysine or arginine that associates with the aspartic acid residue on the transmembrane side of the activating receptor. This interaction is required to stabilize the receptor and activate NK cells (119). Different kinases associate with the ITAM to deliver an activating signal. For instance, DAP12 associated to an activating Ly49 receptor becomes phosphorylated and binds to Zap70 or Syk tyrosine kinases (93, 114). Subsequently, Zap70 and Syk tyrosine kinases will phosphorylate other protein substrates to transduce an activating signal within the NK cell (Fig. 3). An activation signal from an ITAM associated receptor induces cytoskeleton reorganization, degranulation and the secretion of cytokines and chemokines (92).
Figure 3: Activating and inhibitory Ly49 receptors. Activating Ly49 receptors associates with an ITAM containing DAP12 adapter molecule. This complex is stabilized through a charged interaction between an arginine residue on the transmembrane region of the Ly49 receptor and an aspartic acid on the DAP12 adapter molecule. Activation of the Ly49 promotes the phosphorylation of the ITAM and subsequent recruitment of protein kinases such as Syk or Zap70, which propagate an activating signal within the NK cell. Inhibitory receptors contain the ITIM within the cytoplasmic region of the Ly49 receptor. Phosphorylation of the ITIM recruits tyrosine phosphatases such as SHP-1, -2 and SHIP and subsequent dephosphorylation of an activating signal within the NK cell inhibiting NK cell effector function.
Activating Ly49s

Inhibiting Ly49s

COOH

nGlu

DAP12

Syk

SHP-1
1.6.1 Killer Immunoglobulin Receptors

Human NK cells express the Ig-like KIR receptors that recognize classical human MHC-Ia: HLA-A, -B and -C. There are 16 KIR receptors which either have two or three extracellular Ig-like domains that bind to HLA molecules on the surface of target cells. The first extracellular Ig-like domain binds to the HLA α1 domain and the second extracellular-Ig like domain binds to α2 domain of HLA molecules (108). The KIR gene cluster encodes both activating and inhibitory forms of these receptors. Activating KIRs have short intracellular domains, which contains a charged amino acid residue that associates with the ITAM containing adapter molecule DAP12. In comparison, inhibitory KIRs have long cytoplasmic tails that contain two inhibitory motif within the cytoplasmic region (108). For example, both KIR2DS1 (short intracellular domain) and KIR2DL1 (long intracellular domain) both bind to HLA-C molecules on target cells but deliver an activating and inhibitory signal respectively (120). The KIR gene family is highly polymorphic as KIR haplotypes amongst different individuals vary in the number of KIR genes, genetic organization and allelic variations (134, 177). The implications of such high variability can be seen with the association of polymorphisms in the KIR gene family with resistance and susceptibility to many diseases (134). For instance, the activating receptor KIR3DS1 has been associated with delayed progression to AIDS in combination with the HLA-B Bw4-80lle expression (109). In addition, certain KIR/HLA haplotypes have been associated with autoimmune diseases such as diabetes and arthritis (110, 178, 190).
1.6.2 FcγRIII (CD16)

Receptors for the Fc portion of antibodies are expressed on the surface of NK cells and mediate ADCC against an antibody coated target cell (167). Fc receptors exist for many antibody subclasses and are expressed on different cell types. However, NK cells only express Fc receptors that bind to IgG1 and IgG3 antibodies. Each Fc receptor binds to its ligand in a 1:1 stoichiometry (164). Human NK cells express the isoform FcγRIIIA while murine NK cells express the general FcγRIII (136). Human FcγRIIIA receptors deliver an activating signal to induce cytotoxicity through the association with a homodimers of CD3ζ or FcεRI-γ or in a heterodimer association of CD3ζ and FcεRI-γ together (92, 95). In contrast, murine FcγRIII only associates with FcεRI-γ as a homodimer (92). Upon activation, the human FcγRIIIA receptor complex have been shown to be polyubiquitinated and degraded by E3 ubiquitin ligases (133). It has not yet been determined if this is a general mechanism for all activating NK cell receptors.

1.6.3 Natural Cytotoxicity Receptors

The NRCs consists of important activating receptors expressed by human and murine NK cells. The Ig-like receptors NKp46 and NKp30 are expressed on both activated and immature NK cells (118). NKp46 consists of two extracellular domains with an arginine residue at the transmembrane region that associates with either CD3ζ or FcεRIγ adapter molecules in either a homodimer or heterodimer (119). NKp44 and NKp30 contain a single Ig-like extracellular domain but associate with CD3ζ and DAP12 respectively. NKp44 is exclusively
expressed on activated NK cells. NKp46 is the only one of the three NCRs that is conserved in both humans and mice (16). Both NKp46 and NKp44 have been shown to bind to haemagglutinin from influenza (10, 107). In addition, mice genetically deficient for NKp46 are susceptible to influenza infection (54).

1.6.4 NKG2 Receptors

Human and murine NK cells express lectin like NKG2 receptors encoded in the NKC region. NKG2A/B/C/E/F interact with CD94 to form a heterodimer while NKG2D assembles in a homodimer. CD94/NKG2C and NKG2D represent activating receptors, while CD94/NKG2A and CD94/NKG2E are inhibitory. CD94/NKG2B, an isoform of NKG2A and CD94/NKG2F are only expressed in human NK cells (84).

The NKG2D homodimer is expressed on NK cells as well as αβ and γδ T cells (119). Human NKG2D recognizes MICA and MICB, two MHC-Ib stress molecules encoded in the human MHC gene cluster (47). MICA/B contain α1,α2 and α3 MHC-I domains, but lack β2m and do not bind any peptides (16). In addition to MICA/B, a human CMV glycoprotein UL16 binding protein (ULBP) has also been shown to bind to NKG2D. ULBP proteins only contain α1 and α2 MHC-I domains anchored to the membrane by glycophaspatidylinositol (GPI) (38). ULBP proteins are similar in structure to stress induced murine ligands for NKG2D: murine UL16-binding protein-like transcript 1 (MULT1), Rae-1 and H60 (47, 124). Murine NK cells have long been known to efficiently mediate lysis of YAC-1 cells, an NKG2D ligand expressing tumor cell line (72). NKG2D in humans associates with DNAX-activating protein 10 (DAP10), an adapter
molecule which does not encode an ITAM but instead contains a YxNM motif in the cytoplasmic tail that that recruits PI3K for an activating signal (189). In contrast, murine NKG2D is expressed as two different isoforms, NKG2D-long and NKG2D-short, both of which associate with DAP12, but NKG2D-short can also associate with DAP10 (45, 55). Human and murine NKG2D show significant sequence conservation at 60% homology (138).

In contrast to NKG2D, all other NKG2 molecules associate with CD94 to form either an activating or inhibiting receptors. CD94/NKG2 receptors recognize HLA-E molecules in humans and Qa-1 molecules in mice (101). Both HLA-E and Qa-1 present leader peptides from MHC-I molecules. The dominant peptide expressed on Qa-1 is derived from the leader sequence of H-2D and H-2L (1, 39, 42). Receptor binding studies show that inhibitory CD94/NKG2A has higher affinity for HLA-E than the activating CD94/NKG2C (99). CD94/NKG2A recognizes self-MHC-la-leader peptide/HLA-E complexes on target cells resulting in the inhibition of NK activity. It has been theorized that when a pathogenic peptide is displayed on HLA-E, activating CD94/NKG2 receptors may have higher affinity to activate NK cells (58).

1.6.5 Nkrp1/Clr

The NK1.1 antigen (as detected by the antibody clone PK136) has long been used to identify NK cell populations in the C57BL/6 (B6) mouse strain due to its expression in committed NK precursors and mature NK cells (59). It was later identified that the NK1.1 antigen was encoded by the Nkrp1c gene in the B6 genetic background, part of the Nkrp1 family of NK cell receptors (56, 150).
Nkrp1 receptors represent a family of C-type lectin-like receptors expressed by
NK cells and T cells. Nkrp1 receptors and their C-type lectin related (Clr)
ligands, also known as osteoclast inhibitory lectin ligands (Ocil) are both part of
the NKC region (28, 71). Nkrp1 and Clr genes are interspersed within the same
genetic region and are inherited together, with limited polymorphisms within the
gene family (30). The Nkrp1 family consists of 6 members: Nkrp1a, g, c, d/b, f
and e. Nkrp1b and Nkrp1d are allelic variants of the same receptor in the B6 and
Balb/c mice strain respectively (117). Through reporter assay and tetramer
binding staining it has been shown that murine Nkrp1b and Nkrp1f interact with
Clr-b and Clr-g respectively (28, 71). Human NK cells only express Nkrp1A, an
inhibitory Nkrp1 receptor that binds to the ubiquitously expressed lectin-like
transcript-1 (LLT1). Both Nkrp1a and LLT1 are genetically linked and have
sequence homology with rodent Nkrp1b and Clr-b genes, respectively (29).

1.6.6 2B4

Interestingly, not all NK cell receptors require an association with an ITAM
containing adapter molecule to deliver an activating signal. 2B4 receptor (CD122)
represents the SLAM family of transmembrane receptors expressed on both
human and murine NK cells that recognizes CD48 on hematopoietic cells. Unlike
KIRs and Ly49 receptors, 2B4 contain an immunoreceptor tyrosine based switch
motif (ITSM sequence: T1YXXV/I) which allows the receptor to be activating or
inhibitory depending on the adapter molecule with which it is associated (53).
2B4 delivers an activating receptor when bound to a SAP/Fyn complex and
alternatively, a negative signal is delivered when the ITSM associates with EAT2 or ERT (180, 181).

1.7 Natural Killer Cell Activation and Development

NK cell generation begins in the bone marrow from CD34\(^+\) haematopoietic stem cells (HSC) that commit to the NK cell lineage and are subsequently known as NK cell precursors (NKP). NKP cells are associated with the expression of IL2R\(\beta\) (CD122) (158). In mice, NKP\(s\) also acquire the expression of growth factor receptors such as cKit, Flt3, IL7R\(\alpha\) and IL15R\(\alpha\) (147). Human NKP\(s\) express CD38 in addition to cKit, Flt3, and IL15R\(\alpha\). However, these cells have not yet acquired the expression of CD56, CD16 or any NK cell receptors (193). The bone marrow provides an environment rich in cytokines and growth factors needed for HSC\(s\) to develop into NK cells (34). Transcription factors such as PU.1, GATA3, ID2 and ETS1 are essential for NK cell development (147). Upon differentiation into immature NK cells, NKP\(s\) downregulate the expression of Flt3 and IL7R\(\alpha\) and upregulate IL2R\(\beta\), CD2 and 2B4 (34). NKP\(s\) require IL-15 and stromal contact to differentiate into immature NK cells, which acquire the expression of NK1.1 (Nkrp1c) in mice and CD161 (Nkrp1a) in humans, followed by maturation of the NK cell with the acquisition of CD94, CD16, CD11b, KIR/Ly49s and NCR\(s\) (34, 85).

NK cells can remain in circulation for about 5 weeks, however IL-15 is required for NK cell homeostasis within the peripheral blood (50, 139). In addition, mice deficient in either IL-15 or IL-15\(\alpha\) have little to no NK cells (13,
Once NK cells mature in the bone marrow, they are able to migrate to different peripheral organs such as the spleen, thymus, liver, lungs and lymph nodes (68). NK cells express two forms of IL-2R. Naïve NK cells express β and γ chains forming the intermediate affinity IL-2R and activated NK cells express α, β and γ chains, forming the high affinity IL-2R (36). The recognition of IL-2 by IL-2R triggers MKK/ERK pathway and subsequent NK cell activation by the secretion of IFN-γ, and increased cytotoxicity. IL-2 secretion in vivo or stimulation in vitro can activate NK cells independent of NK cell receptor ligation. In addition, in vivo polyinosinic:polycytidylic acid (poly I:C) challenge results in the secretion of IFNα, IL-12 and the trans presentation of IL-15 by dendritic cells and subsequent activation of NK cells (102). The presentation of IL-15 by IL-15Rα on dendritic cells stimulates NK cells to become cytotoxic and secrete IFN-γ (87). It has also been shown that NK cells may require this contact with dendritic cells from the draining lymph nodes in order to prime NK cells to acquire effector functions (102).

1.8 Ly49 Receptors

Murine NK cells express Ly49 receptors, which detect MHC-Ia expression and the presence of pathogen-encoded MHC-like molecules, essential for innate immune responses in the mouse. Ly49 receptors are stochastically expressed on murine NK cells, which results in a variety of NK cell population that express between 0-4 Ly49 receptors. (140). Similar to human KIRs, Ly49 receptors bind to MHC-I complexes, contain activating and inhibitory forms and are highly
polymorphic. The Ly49 gene region in the murine NKC contains a high percentage of LINE1 and other repetitive elements in comparison to the total genome, possibly promoting homologous recombination and subsequent expansion and contraction of the Ly49 family of receptors (4, 29). In addition, homologous recombination within different Ly49 genes can facilitate the creation of hybrid Ly49 receptors made from exons of different Ly49 receptors (103). Different inbred mouse strains contain different Ly49 haplotypes, varying in the number of receptors and the presence of allelic variants (29).

Ly49 receptors can be either activating or inhibitory depending on the presence of an ITIM within the cytoplasmic domain or the association with an ITAM containing adapter molecule. Inhibitory Ly49s bind to MHC-I molecules found on normal cells to limit NK effector function via an ITIM contained within the intracellular domain of the receptor itself. The ITIM recruits phosphatases to diminish any activating signals (141). Activating Ly49 receptors do not encode the ITIM but associate with an ITAM containing adaptor protein DAP12 (162). Activating Ly49 receptors recognize surface expression of pathogen-encoded MHC-like molecules such as viral MHC homologues, allogeneic, or even xenogeneic MHC on target cells (70, 161, 194).

1.9 Structure and Binding of Ly49 Receptors

The MHC-I binding Ly49 receptors can interact with its ligand in two distinct conformations. These interactions can either occur in cis or in trans depending on whether the bound MHC is located on a target cell or the Ly49 bearing NK cell itself (11). A trans interaction is postulated to occur when an
Ly49 expressing NK cell encounters an MHC-I molecule on a target cell, delivering an inhibitory signal and lowering the threshold of NK cell activation. In contrast, a cis interaction occurs due to the binding of an Ly49 receptor on an NK cell with an MHC-I molecule on its own surface. This interaction does not deliver an inhibitory signal but lowers the threshold of activation by occupying inhibitory Ly49 receptors and preventing them from binding in trans (31).

The Ly49 receptor gene is composed of seven exon segments, which translate into four domains: the cytoplasmic domain, the transmembrane domain, the long and flexible stalk domain and the C-type lectin like domain (29, 104). A primary crystal structure of Ly49A binding to H-2D$^d$ and Ly49C binding to H-2K$^b$ show two critical sites of interactions. The main site of interaction between the Ly49 receptor and the MHC-I ligand occurs at the $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 2m$ cavity beneath the peptide of the MHC molecule (40, 44, 172). This interaction is similar for both cis and trans (46). The flexible stalk domains allows for two distinct conformations for the Ly49 to interact with MHC-I both in cis and in trans. The Ly49 can interact with two MHC-I molecules on a target cell (in trans) using a back-folded confirmation (Fig. 4A). In contrast, an Ly49 receptor can interact with an MHC-I molecule on the same plane as the NK cell using an extended configuration (Fig. 4B) (46).
Figure 4: *Trans* and *cis* interaction of Ly49 receptors binding to MHC-I. An Ly49 receptor can interact with an MHC-I molecule either on a target cell or on its own surface through a distinct *trans* and *cis* conformation A) Ly49 receptors expressed on NK cells can binds MHC-I molecules on a target cells (*trans* interaction) by adopting a back-folded conformation. B) Ly49 receptors can interact with an MHC-I molecule on the same plane as the NK cell (*cis* interaction) through an extended conformation. Reprinted from (11) with permission from Elsevier.
1.10 Activating Ly49 Receptors

1.10.1 Signalling of Activating Ly49 Receptors

The two main activating Ly49 receptors, as studied in the C57Bl/6 mouse model, are Ly49H and Ly49D. Ly49H was mapped as the locus associated with susceptibility and resistance of mouse cytomegalovirus (MCMV) infection (22). Ly49D was shown to activate NK cells against the xenogeneic CHO cell line (70). Activating Ly49 receptors associate with the ITAM containing DAP12 adapter molecule through a charged aspartic acid in the transmembrane of DAP12 with an arginine residue in the transmembrane of the Ly49 receptor, which consequently stabilizes expression of the receptor complex (162). When the Ly49 receptor binds its ligand, Src family kinases phosphorylate the ITAM motif on the cytoplasmic tail of DAP12 (93, 171). The phosphorylated ITAM recruits Syk or ζ chain (TCR) associated protein kinase (Zap70) and activation of phosphoinositide-3-kinase (PI3K), phospholipase C (PLCγ) and Vav pathways eventually leads to actin reorganization, degranulation and the transcription of different cytokines and chemokines (27, 57, 189).

As shown by knockout models, due to the abundance of many tyrosine kinases, there is redundancy at many signalling steps in the NK cell. For instance, Zap70 can compensate for the lack of Syk and vice versa in NK cell activation (92). Interestingly, Ly49D and H have also been shown to associate with DAP10 in addition to DAP12 (112). However, the relevance of DAP10 association with activating Ly49 receptors is still controversial (129, 169).
1.10.2 Ly49H and MCMV Resistance

MCMV is a double stranded DNA virus similar to human cytomegalovirus, and belongs to the β herpesviridae family, (142). Some mouse strains are resistant to MCMV infection (3) but upon antibody mediated NK depletion become susceptible (25), suggesting the importance of NK cells in the clearance of MCMV infection. B6 mice are known to be resistant to MCMV, while other mouse strains such as Balb/c and 129 succumb to infection (155). This was shown to be due to the presence of Ly49H+ NK cells, as shown by crossing resistant B6 mice with susceptible DBA/2 mice (22), and identifying the resistance locus, Cmvr by genetic mapping (155). It was later shown by Arase et al. that the ligand for Ly49H was m157 as shown by Ly49H+ reporter cells recognizing NIH 3T3 cells transfected with the ligand (8). Ly49P, a different receptor encoded by a gene specifically found in 129-strain mice, has also been shown to bind to another MCMV encoded viral protein m04 (81).

1.10.3 Ly49D and CHO Killing

The activating Ly49 receptor Ly49D has been shown to mediate killing of the xenogeneic CHO cells by NK cells from B6 but not Balb/c mice (69). Idris et al. mapped this phenotype to the chok gene locus located in the NKC region of murine chromosome 6 (69). This locus was subsequently shown to encode Ly49d on the B6 strain (70). Ly49D was shown to bind to a specific ligand on CHO cells, a Chinese hamster class I molecule Hm1-C4 (52). Interestingly, Ly49G was also shown to bind to a ligand on CHO, leading to the inhibition of NK cell killing (111).
1.11 Inhibitory Ly49 Receptors

1.11.1 The Inhibitory Signal

Inhibitory Ly49 receptors contain an ITIM within their cytoplasmic domain. An inhibitory signal is delivered to an NK cell when an inhibitory Ly49 receptor binds to a self MHC-Ia receptor expressed on a host cell. The resultant inhibitory signal diminishes any activating signals initiated by activating receptors. The tyrosine residues in the ITIM is phosphorylated by Src family kinases, which recruits protein tyrosine phosphatases such as SHIP-1, SHP-1 and SHP-2 (146, 168). Different inhibitory receptors favour the recruitment of certain tyrosine phosphatases. For instance, inhibitory Ly49, KIRs and CD94/NKG2 receptor recruit SHP-1 and SHP-2 preferably over SHIP (51, 127). These tyrosine phosphatases diminish activating signals by dephosphorylating protein substrates involved in the activating signal cascade of NK cells (123). This results in reduced calcium influx and the reduction of NK cell effector function.

1.11.2 Inhibitory Ly49 and the Education of NK Cells

In addition to inhibition of NK cell activity, the interaction between inhibitory receptors binding to self MHC-Ia molecules is vital in the development of NK cells. In normal mice, NK cells can recognize target cells lacking MHC-I expression, which can be representative of infected or tumorogenic target cells, as stated by the “missing self” hypothesis (98). NK cells developing in mice deficient in MHC-I expression, using β2m gene-deficient mice, are unable to recognize MHC-I-deficient target cells and thus are tolerant to self (65, 96). In striking contrast, when a transgene for H-2D<sup>d</sup> was introduced into the C57Bl/6
mice, rejection of wildtype B6 cells was augmented (126). This was due to the introduction of a novel self MHC-I allele that is recognized by inhibitory Ly49 receptors during development in the bone marrow, thus wildtype B6 cells have become non-self to the H-2D\textsuperscript{d} transgenic NK cells. The expression of Ly49 receptors that bind to self MHC-I is critical during the development of NK cells for the surveillance of missing self. In addition, some MHC alleles that act as ligands for inhibitory Ly49 receptors have a greater impact on NK cell education than others (21, 76).

1.12 Natural Killer Cell Education

1.12.1 Educated NK cells

As discussed earlier, NK cell education is greatly influenced by the expression of inhibitory Ly49s for self-MHC-I during NK cell development. Currently two models have been proposed to explain the process of NK cell education. Fernandez et al. propose the disarming model, which states that NK cells are chronically activated during development, and that NK cells that express inhibitory receptors for self develop into mature functional NK cells (49), while NK cells which lack inhibitory Ly49 receptors that bind to self-MHC are chronically activated and subsequently become anergic or hyporesponsive. Kim et al. propose the licensing model, which states that NK cells are anergic during development, and that NK cells which express inhibitory Ly49 receptors that bind to self MHC acquire effector functions and develop into properly educated NK cells (86). Both models emphasize the importance of expression of inhibitory Ly49s that bind to self-MHC in the environment to allow for proper NK cell
cytotoxicity and cytokine secretion. In addition to the expression of inhibitory Ly49 receptors, the frequency of Ly49 receptor expression influences the degree of education of each individual NK cell. An NK cell which expresses more inhibitory Ly49 receptors that bind to self MHC-I is more educated in an cumulative fashion resulting in greater cytotoxicity and cytokine production (21, 75). Ly49 interaction with MHC-I for proper education can occur both in cis and in trans (31). In addition to self-Ly49 receptors, CD94/NKG2 receptors also deliver an inhibitory signal when bound to Qa-1 presenting a leader peptide from self-MHC-Ia. NK cells express CD94/NKG2 receptors during development and they also contribute to NK cell education (159).

1.12.2 Unlicensed NK cells

According to the licensing and disarming hypothesis, NK cells that do not express any Ly49 receptors to self are generally hyporesponsive compared to educated NK cells in terms of IFN-γ production as shown by NK cells from β2m-deficient mice or the Ly49C/iNKG2A- NK cell population in B6 mice (49, 65, 86). However, stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin demonstrates that both NK cell populations have the intrinsic capability for proper NK cell effector functions (86). Hyporesponsive NK cells, as defined by the lack of Ly49 to self-MHC, remain in circulation within the host. Orr et al. proposed that a specialized function may exist for these hyporesponsive cells, apart from the rejection of MHC deficient cells (128). For example, NK cells are responsive to virally infected cells that maintain expression of MHC molecules (89). However, licensed NK cells will be inhibited by target cells expressing ligands for activating
receptors, as self-MHC-I recognition by inhibitory receptors delivers a theoretically stronger signal than its activating counterpart (132). In this circumstance, unlicensed cells will have an advantage due to the lack of Ly49 receptors to self-MHC. Orr et al. demonstrated this through the enhanced killing of MHC-sufficient RMA cells transfected with m157 by unlicensed NK cells positive for Ly49H (128).

1.13 Ly49 Haplotype Divergence

The Ly49 receptors are encoded by a family of highly polymorphic and variably polygenic genes (29). This is in contrast to other NKC-encoded receptors such as the \(Nkrp1\) gene family, which appear to be relatively well conserved (30). Early restriction fragment length polymorphism (RFLP) experiments led to the observation that different inbred mouse strains contain variable Ly49 haplotypes (191, 192). More recently, the exact profile of many Ly49 haplotypes has been elucidated. For instance, NOD/ShiLtJ mice have the largest Ly49 repertoire with 21 genes, 129S1 mice possess 19 genes, B6 mice possess 15 genes, and Balb/c mice have the smallest known Ly49 repertoire with only 8 genes (Fig. 5) (15, 29). The consequence of Ly49 haplotype variation on innate immunity is most obvious in the absence of genes encoding activating Ly49 receptors. Inbred mouse strains lacking the expression of Ly49D and Ly49H are deficient in the ability to kill CHO cells and MCMV-infected m157-bearing target cells, respectively (8, 70).

The functional consequences of inhibitory Ly49 repertoire variation for NK cell function are not as well understood, although allelic variation in Ly49A and
Figure 5: Ly49 haplotypes in different Inbred mouse strains Due to the polymorphic and polygenic nature of the Ly49 gene cluster, different inbred mice strain contain different Ly49 haplotypes. The NOD mice have the largest Ly49 repertoire with 21 genes, 129S1 mice possess 19 Ly49 genes, the B6 haplotype contain 15 Ly49 genes, and the smallest Ly49 repertoire is found in Balb/c mice with only 8 genes. Genes with the same color are indicated as allelic variants. Adapted from (15) with permission from Macmillan Publishers Ltd.
Figure 6: Ly49 haplotype of B6 and 129 inbred mice. The 129 Ly49 haplotype is larger in comparison to the B6 haplotype. The 129 haplotype contains 3 activating and 9 inhibitory receptors, of which Ly49I, O and V are receptors for self MHC-I. In comparison, B6 contain 2 activating and 8 inhibitory receptors with Ly49C and Ly49I as inhibitory receptors towards self MHC-I. Adapted from (15) with permission from Macmillan Publishers Ltd.
Ly49G results in different affinities for MHC-I ligand as assessed by soluble MHC-I tetramer binding and killing of MHC-I-transfected target cells (106, 116). It has long been known that different inbred mouse strains exhibit variation in NK cell effector function (82). For instance, B6 mice appear to have a more active NK cell population than 129 (15), despite possessing a smaller Ly49 haplotype than the 129 mouse strain (Fig. 6) (104). One explanation is that activating Ly49 from the B6 strain deliver stronger DAP12-derived signals, as evidenced in studies of Ly49D function (115). However, 129-strain NK cells also exhibit lower Ly49D-independent killing of tumor target cells compared to B6-strain NK cells. The lower cytotoxicity and cytokine production of 129-strain NK cells is indicative of hyporesponsive NK cells.

1.14 Rationale

The educational capacity of inhibitory Ly49 receptors in the 129 mouse strain remains unclear. Similarly and more importantly, it is also unknown if Ly49 haplotype variation affects NK cell function in different inbred mouse strains. I would like to determine whether the Ly49 haplotype expressed by 129 NK cells results in altered functional or educational potential in comparison to the B6 Ly49 haplotype. In addition, I would like to determine if Ly49 haplotype variation can affect NK cell functional potential on a given genetic background.

1.14.1 Purpose

To determine if the variations in Ly49 haplotype amongst different mice strains affects NK cell education and the capacity for NK cell effector functions, a
novel B6-Ly49\textsuperscript{129} congenic mouse strain with the 129 Ly49 gene cluster on the genetic background of the B6 mouse strain has been created and the purpose of this thesis was to assess its NK cell activity relative to inbred B6 and 129 mice \textit{in vitro} and \textit{in vivo}.

1.14.2 Objectives

1. The first aim is to determine if the 129 Ly49 haplotype contains an activating Ly49 receptor that can recognize m157, a viral MHC-like molecule expressed by MCMV infected cells, or some other MCMV-encoded molecule resulting in NK cell protection from infection. B6 NK cells express Ly49H, which binds to m157 and subsequently kills infected cells conferring resistance to viral infection (9). 129 NK cells contain allelic variants of Ly49H, which have been shown to bind to MCMV viral proteins, but due to defective DAP12 signalling, an activating signal could not be propagated (81, 103, 115). By studying MCMV infection of B6-Ly49\textsuperscript{129} congenic mouse, I can assess if Ly49U and/or Ly49P are able to confer resistance to the congenic mice due to the presence of a functional DAP12 adapter signalling pathway.

2. To determine if differences in Ly49 haplotype can explain the higher effector response of B6 NK cells against target cells in comparison to 129 NK cells. The effects of Ly49 haplotype divergence is more apparent with differences in activating receptors, such as resistance of B6 mice against MCMV due to the expression of Ly49H on NK cells which bind m157, a viral protein expressed on infected cells (155). However, the effects of variations in different inhibitory Ly49 haplotypes are not apparent. Since 129 NK cells are less responsive than B6 NK
cells, due to a defective DAP12 adapter molecule, we cannot compare the effects of Ly49 variations in NK cell function between the two strains (115). By examining the NK cell responsiveness of B6-Ly49$^{129}$ congenic mice, we can directly compare the effects of different Ly49 haplotypes on NK cell function due to the use of the same genetic background.

To determine if the 129 Ly49 haplotype is sufficient to educate NK cells, NK cells that express Ly49 receptors that bind to self MHC-I molecules are educated to respond better than those that express no Ly49 receptors to self MHC-I (49, 86). Additionally, NK cells that express more Ly49 receptors that bind to self MHC-I are more tuned to respond to target cells than those that express less self receptors (21). NK cell education has been so far only studied and reported in the B6 mouse strain, yet not proven in other mouse strains such as 129 mice due to low intrinsic response to target cells. By studying the NK cell response of different subsets expressing various 129 Ly49 self-receptors in the B6.Ly49$^{129}$ congenic mouse, we will be able to characterize NK cell education with a non B6 Ly49 haplotype.
2: MATERIALS AND METHODS

2.1 Mouse Strains

All mice strains were bred and maintained at the Animal Care and Veterinary Services (ACVS) at Roger Guindon Hall (University of Ottawa, Ottawa, Ontario) in compliance with the Canadian council on animal care (CACC). This facility provides a specific pathogen free environment for the housing and breeding of all mice strains used. Wildtype C57BL/6J (stock # 000664), 129S1/SvlmJ (stock# 002448) and β2m<sup>−/−</sup> mice (B10.129P2(B6)-β2m<sup>tm1Unc</sup>/J; stock # 002454) on the B6 background were purchased from Jackson Laboratories (Bar Harbor, Maine). Additional B6 mouse strains deficient in specific MHC-Ia molecule expression including H-2K<sup>b</sup><sup>−/−</sup> (B6.129P2-H2-Kb<sup>tm1</sup>N12 model # 4216) H-2D<sup>b</sup><sup>−/−</sup> (B6.129P2-H2-Db<sup>tm1</sup>N12 model # 4217) and H-2K<sup>b</sup><sup>−/−</sup>-D<sup>b</sup><sup>−/−</sup> double knockout (B6.129P2-H2-Kb<sup>tm1</sup>H2-Db<sup>tm1</sup>N12 model # 4215) were purchased from Taconic Farms (Hudson, New York).

The B6-Ly49<sup>129</sup> congenic mouse strain possesses the Ly49<sup>129</sup> gene cluster on the B6 genetic background and was created by crossing B6 mice with 129 mouse to create a hybrid F1 heterozygous mouse. These F1 mice were then crossed again with B6 mice for 10 generations. The B6-Ly49<sup>129</sup> congenic mouse was created by Dr. Lee-Hwa Tai in the Makrigiannis lab. To ensure that the
progeny maintained a $Ly49^{129}$ haplotype, tail DNA was analyzed by polymerase chain reaction (PCR) for $Ly49v^{129}$, an Ly49 receptor only found in the 129 haplotype (forward primer: 5'-GTG TTC AGT CCC CTG GAA GT-3'; reverse primer: 5'-CTT GGT TTT ATT ATA CAA TCT GTT CTT-3'). After the 10th generation of backcrosses with the B6 mouse strain, two mice heterozygous for the $Ly49^{129}$ haplotype were crossed and tail DNA from the resulting pups was tested for the lack of $Ly49g^{B6}$, to ensure homozygosity for the $Ly49^{129}$ haplotype (forward primer: 5'-GAA GCT CAT TGT GAT AGC TTG-3'; reverse primer: 5'-TGT GTG CTG TGA GGA ATC TG-3'). To determine what proportion of chromosome six was of 129 origin, a single nucleotide polymorphism (SNP) analysis was performed using an Illumina Beadstation 500G mouse medium density linkage panel (The Center for Applied Genomics-Sick Kids Hospital, Toronto, Ontario). The genome of the B6-Ly49$^{129}$ congenic mouse strain was of B6 origin with the exception of a segment on chromosome six spanning nucleotides 79,759,628 to 138,203,431, which contains the $Ly49$ gene cluster. The B6.Ly49Q-KO mouse strain contains the $Ly49^{129}$ haplotype with a disrupted $Ly49q$ gene onto the genetic background of the B6. The genomic contribution of 129 on chromosome six spans from nucleotides 118,022,343 to 149,214,236. The mice used for experiments were at least six weeks old or older. Preference was given to age over gender matching.

### 2.2 DNA Isolation for Genotyping and SNP Analysis

Tails snips were digested with 100µg/mL proteinase K (Invitrogen, Burlington, Ontario) in tail lysis buffer (1M Tris pH 8.5, 0.5M
ethylenediaminetetraacetic acid (EDTA), 10% sodium dodecyl sulfate (SDS), 5M NaCl in aqueous solution) and incubated overnight at 55°C. To prepare deoxyribonucleic acid (DNA) for SNP analysis, tail digests were vortex for 5-10 seconds and spun down at 500 G for 5 minutes. 400 µL of the supernatant was mixed with phenol/chloroform/isoamyl alcohol (Fisher, Toronto, Ontario), vortexed and spun down at 500 G for an additional 5 minutes. The top aqueous phase was carefully removed and to it was added 1/10 volume of 3M sodium acetate and 2X volume of ethanol, mixed by inversion, followed by a spin at top speed for 5 minutes. DNA pellets were washed with 70% ethanol, dried for 10 minutes and resuspended in 100 µL of TE (10mM Tris-Cl, pH 7.5 and 1mM EDTA) by incubating for 1 hour at 65°C.

To prepare DNA for genotyping by PCR, tail digests were spun down for 10 minutes at top speed, 200 µL of the supernatant was transferred into a new eppendorf tube, 600 µL of ethanol was added into each tube and inverted several times to precipitate the DNA. Tubes were spun down for 10 minutes at top speed, and the resulting pellet was washed with 70% ethanol and again centrifuged for 5 minutes at top speed. The supernatant was discarded and spun for 3 minutes at top speed. The remaining supernatant was pipetted out. Each tube was air dried for 10 minutes and dissolved in 100 µL of TE by incubation for 1 hour at 65°C. Primers for Ly49v<sup>129</sup> forward and Ly49v<sup>129</sup> reverse (see above for sequence) were used for genotyping by PCR for the B6-Ly49<sup>129</sup> congenic mouse strain using the following PCR cycle: 94.0°C denaturing temperature (30 seconds/cycle), 58.0°C annealing temperature (30 seconds/cycle), 72.0°C
elongation temperature (30 seconds/cycle and final elongation for 5 minutes) and hold at 10.0°C.

2.3 Southern Blot (RFLP Analysis) and Thymic DNA Isolation

Thymic DNA was used for Southern blot to detect restriction fragment length polymorphism (RFLP) indicative of B6 vs. 129 Ly49 haplotypes. To isolate DNA, the thymus from each mouse strain was anatomically dissected and digested overnight in 3mL tail lysis buffer with 100 µg/mL proteinase K at 55°C in a 15 mL polypropylene tube with rotation. The following morning these tubes were spun down for 5 minutes at 500 G, and the supernatant was transferred into a new 15 mL polypropylene tube with a large bore tip. An equal volume of phenol/chloroform/isoamyl alcohol was added, mixed vigorously and spun down for 5 minutes at 500 G. The upper layer was transferred into a new polypropylene tube. This last step was repeated two more times to remove impurities. After the final transfer, 1/10 volume of sodium acetate and 2 volumes of ethanol was added and inverted several times to precipitate. A large bore tip was used. The DNA was washed with 1 mL of 70% ethanol and air dried for 15 minutes. 100 µL of TE was added and the DNA was dissolved by incubation at 55°C for 1 hour. The concentration of each DNA sample was determined using the Nanodrop ND-100 spectrophotometer (Thermo Scientific). A total of 10 µg of DNA was digested with BamHI, EcoRI and KpnI restriction enzymes (New England BioLabs) and their respective REact buffers (Invitrogen).
The Southern blot was run on a 1% agarose gel overnight at 55-60 volts. The gel was stained with ethidium bromide in ddH$_2$O for 30 minutes, and then treated with depurination solution (0.25 M HCl in ddH$_2$O) for 20 minutes, denaturation solution (2.5 M NaCl and 0.5 M NaOH in ddH$_2$O) and neutralization solution (0.15 M NaCl and 0.5 M Tris-HCl pH 8.0 in ddH$_2$O). The gel was transferred overnight on an Hybond N$^+$ nylon membrane (GE Healthcare), cross-linked onto the membrane with UV exposure and probed with Ly49e and Ly49g cDNA fragments. The membrane was exposed at -80°C for 6 hours with a Kodak bioMax MS film (Perkin Elmer).

2.4 Cell Lines

RMA (murine lymphoma), RMA-S (MHC-la-deficient variant of RMA-S) and YAC-1 (H-2K low lymphoblast) were a generous gift from Dr. A. Veillette (Institut de recherches cliniques de Montreal, Montreal, Quebec). RMA is a mutagenized cell line derived from the RBL-5 line, a Rauscher virus-induced T cell lymphoma from the B6 strain. RMA-S is a cell line derived from RMA selected for H2-K$^b$ and H2-D$^b$ deficiency (125). YAC-1 is a Moloney leukemia virus-induced tumor cell line of the A/Sn mouse strain (83). These cell lines were grown in complete RPMI medium (RPMI supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin). YB2/0 rat cell line was a kind gift from Dr. J. Ryan (University of California, Veterans Affairs Medical Center, San Francisco, California). CHO cells purchased from American Type Culture Collection (ATCC) were grown in complete Dulbecco’s modified Eagle’s medium (DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL
penicillin and 100 μg/mL streptomycin). The CHO cell line is adherent and was harvested using 1mM EDTA in phosphate buffered saline (PBS).

2.5 Antibodies and Flow Cytometry.

Anti-CD49b (DX5), anti-TCRβ (H57-597), anti-CD94 (18d3), anti-Ly49A/D (12A8), anti-Ly49C/I/F/H (14B11), anti-CD107a (ebo1D48) were purchased from eBioscience (San Diego, California). Anti-NKG2A/C/E (20d5), anti-Nkrp1c (PK136), anti-Ly49D (4E5), anti-Ly49G<sup>B6</sup> (Cwy-3), anti-Ly49G (4D11), anti-Ly49C/I (5E6), anti-IFN-γ (XMG1.2), anti-H2K<sup>b</sup> (AF6-88.5), anti-H2D<sup>b</sup> (28-14-8) and anti-FcγRIII (2.4G2) were purchased from BD Bioscience (Mississauga, Ontario). Anti-Ly49H<sup>B6</sup> (3D10) was a kind gift from Dr. Silvia Vidal (McGill, Montreal Quebec). The respective isotype controls were purchased from either eBioscience or BD Bioscience. The above described mAbs were purchased already directly conjugated with one or more of the following fluorescent groups: allophycocyanin (APC), APC eFlour 780, fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

Cells were plated onto a 96-well plates, 0.5-1 million splenocytes in each well. To block FcγRIII receptors and other non-specific mAb binding, blocking was performed before mAb staining using 2 μL of rat serum (Sigma), 1 μL of mouse serum (Sigma) and 0.25 μg of 2.4G2 mAb (BD Bioscience) was added to each well of a 96-well plate, covered and incubated at 4°C for 15 minutes prior to primary mAb staining. All subsequent staining were performed covered at 4°C for 20 minutes. Antibody dilutions and washes between mAb staining were done with FACS Buffer (0.5% bovine serum albumin (BSA) and 0.04% sodium azide.
Dead cells were excluded from flow cytometry analysis by staining with 0.05 µg propidium iodide (PI, BD Biosciences) per well and gating for PI' cells. Flow cytometry was performed on FACScan (BD Bioscience), FACS Calibur (BD Bioscience) and Cytomics FC500 MPL (Beckman Coulter). Acquisition was performed using CellQuest Pro and MXP software. Analysis was performed using CellQuestPro (BD Bioscience), CXP (Beckman Coulter) and Kaluza (Beckman Coulter) software.

2.6 MCMV Plaque Assays

To assess the resistance or susceptibility to MCMV infection for a given mouse strain, 5,000 plaque forming units (PFU) of mouse cytomegalovirus (MCMV) Smith strain was injected into each recipient mouse by i.p. injection in a total volume of 200 µL of cold PBS. Virus dilutions were kept on ice to maintain viability. Mouse embryonic fibroblasts (MEFs) from Balb/c were a kind gift from Dr. Silvia Vidal (McGill University) and were thawed one day prior to infection of recipient mice. MEFs were grown in DMEM media containing 10% FBS for 3 days to reach confluency. MEF cells were harvested with 0.25% trypsin-EDTA (Gibco), replated using the initial media to preserve growth factors into a 24 well plate. Three days post infection, the spleen and liver from each recipient mouse was collected and homogenized. Spleens were kept in 5 mL cold DMEM containing 2% FBS and livers were kept in 10mL cold DMEM containing 2% FBS. Serial dilutions were made in DMEM containing 2% FBS for the following dilutions: 1:5 for the liver and both 1:2 and 1:50 for the spleen. Media was aspirated from the 24 well plate containing the MEF cells and 200 µL of each
dilution was plated on top of the MEF monolayer. The 24 well plate was then incubated for one hour at 37°C, after which the media from each well was aspirated. Each well was then covered with 2 mL of the following media: 12.5 mL of DMEM 2% low melt agarose and 37.5 mL of DMEM containing 13.5% FBS. The plate was incubated for 3 days at 37°C. Following incubation, cells were stained by flooding the well with 10% formaldehyde for 10 minutes at room temperature. Subsequently, cells were covered with 200 μL of 70% ethanol/1% methylene blue w/v for 10 minutes at room temperature, rinsed with dH2O and air dried. At this point, each well was assessed for plaque formation using a light microscope (Unico, Dayton NJ). The following formula was used to calculate viral titer load per organ:

\[
\# \text{plaques/organ} = (\# \text{plaques/well}) \times (\text{dilution factor}) \times (\text{volume per well}) \times (\text{volume of media per organ})
\]

2.7 Cytotoxicity Assay

Adherent lymphokine-activated killer (ALAK) cells were generated from whole spleens of the indicated mouse strains. Spleens were crushed with a 5 mL syringe plunger (BD Bioscience) into a 70 μM sterile cell strainer (Fisher) and washed with PBS to isolate cells from fat and other structural components of the organ. These cells were then treated with 5 mL/spleen of ACK lysis buffer (PBS with 300 mM NH₄Cl, 20 mM KHCO₃, 0.2 μM Na₂EDTA) for 5 minutes to lyse the red blood cells in solution, followed by adding 10 mL of PBS to stop the reaction. Each spleen was grown in 15 mL of NK media (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 1000 U/mL penicillin and 100 μg/mL streptomycin, 0.1
mM non essential amino acid, 1 mM sodium-pyruvate, 10 mM HEPES and 50 μM 2-mercaptoethanol) supplemented with 1000 U/mL human recombinant IL-2 (Cedarlane, Burlington, Ontario) in a 75 mL flask (Sarstedt) for three days at 37°C at 5% CO₂ in a humidified. After three days, the flask was gently swirled and the old media removed by pipetting, leaving the adherent cells. New NK media supplemented with the same concentration of IL-2 was added. Adherent cells were then incubated for another three days to yield ALAK cells. ALAK cells were harvested by incubating the flask with 1 mM EDTA in PBS for 5 minutes. ALAK cells were kept on ice until used for experiments.

Target cells (RMA, RMA-S, YAC-1, CHO and YB2/0) were labelled with 80 μCi sodium 51-chromate diluted in PBS (MP Biomedicals, Solon, Ohio) for one hour at 37°C with 5% CO₂ in a humidified atmosphere. 5000 ⁵¹Cr labelled target cells were plated in ALAK containing wells at the following effector:target ratios: 50:1, 25:1, 12:1 and 6:1 in a final volume of 200 μL of NK media/well. All conditions were plated in triplicates in a 96-well V-bottom plate. For some experiments, the ALAK used for CHO cytotoxicity assays were pre-treated for 20 minutes at room temperature with 10 μg/mL of purified rat IgG₂ακ, 12A8 and 4D11 antibodies directly into each well before addition of target cells. For maximal release, target cells were incubated with 10% SDS and minimal release was calculated on target cells incubated with media, both to a final volume of 200 μL/well in triplicates. Effector and target cells were allowed to incubate for four hours. Upon incubation, the 96-well plate was centrifuged at 500g for 5 minutes, 100μL of supernatant from each well was access for γ radiation release. All the
cells in the incubation would pellet to the bottom of the Vee bottom well and are not needed for the 51-chromium release measurement. Percent release was measured using the following formula. $\Sigma_{n=3}$ simply denotes the average of three values.

$$% \text{ release} = \frac{(\text{Experimental} - \Sigma_{n=3} \text{ Maximal})}{(\Sigma_{n=3} \text{ Maximal} - \Sigma_{n=3} \text{ Minimal})} \times 100\%$$

2.8 Assessment of Interferon-γ Production and NK Cell Education

To quantify the production of IFN-γ by NK cells from each mouse strain, splenocytes were collected from mice pretreated with 150 μg of poly(I:C) for 18 hours by crushing the spleen and incubated with 5 mL of ACK lysis buffer for 5 minutes at room temperature, upon which the reaction is stopped by addition PBS.

One million splenocytes were incubated with various target cells (RMA-S, YAC-1, etc...) at a ratio of 1:1 in a 96 well plate at 100 μL each suspended in NK media and incubated for 5 hours. To coat each well for antibody stimulation, 1 μg/mL of anti-NKp46 and 5 μg/mL of anti-NKG2D diluted in an enzyme-linked immunosorbent assay (ELISA) coating buffer (BD bioscience) was added to wells in a volume of 50-100 mL in a 96 ELISA plate (Costar) at 4°C overnight. The following day, the ELISA plate was washed with three rounds of PBS. Liquid was removed each time by inverting and vigorously shaking over the sink. 100 μL of NK media is added to each well and plated with one million splenocytes per well.
The unstimulated control consisted of only the splenocytes, which were added to each well supplemented with NK media for a total volume of 200 μL for each well. For pharmacological stimulation, 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1μg/mL ionomycin was diluted in NK media and 100 μL of this mixture was added to each well as a positive control for optimal NK activation. Cells were incubated for a total of five hours with target cells. After the first hour, 1 μL of Golgi Plug (BD Bioscience), which contains a protein transport inhibitor Brefeldin A, was added to each well. After the complete 5 hour incubation, cells were stained for APC-CD49b and APC Alexa Flour 780-TCRβ to gate for NK cells as previously described and subsequently fixed in preparation for intracellular staining of IFN-γ using the cytofix/cytoperm kit (BD Bioscience). Cells were incubated for 20 minutes at 4°C in the dark with fix/perm solution (BD Bioscience), washed twice with perm/wash (BD Bioscience) solution and stained for IFN-γ production with 1μg PE-IFN-γ diluted in 50 μL of fix/perm solution for 30 minutes at 4°C in the dark. After two washes with fix/perm solution, the cells were resuspended in FACS buffer and analyzed by flow cytometry for IFN-γ positive cells after on CD49b^+TCRβ^- cells.

To assess for the percentage of educated cells on different subsets of Ly49-expressing NK cells, splenocyte containing wells were stained with the following mAbs: 4D11 (Ly49G\(^{129}\)), 4E5 (Ly49O/V\(^{129}\)), 14B11 (Ly49I\(^{129}\)) and NKG2A (Qa-1) during the staining for CD49b and TCRβ prior to intracellular staining. Different combinations of 4E5, 14B11 and NKG2A mAbs were used to assess for both single and triple positive cells for each stimulation. The resultant
Ly49+ and Ly49- populations were gated for IFN-γ production. To determine the fold induction of IFN-γ, use the following equation:

\[
\text{fold induction} = \frac{\frac{\text{A++}}{\text{A}-+}}{\frac{\text{A+-}}{\text{A}--}}
\]

2.9 In Vivo Rejection of MHC-I-Deficient Cells

To assess the capability of NK cells to reject MHC-I-deficient cells, splenocytes from H-2Kb/d, Db/d and Kb/Db mice were injected by tail i.v., along with wildtype B6 splenocytes as a control and survival of these injected cells were assessed. Recipient mice were injected with 150 µg of poly I:C (Sigma) dissolved in 200 µL PBS 24 hours prior to injection of MHC-I-deficient cells. Splenocytes from both MHC-I-deficient mice and wildtype B6 mice were prepared from the spleen by homogenizing the spleen as described above, followed by a 7 minute incubation on ice with ACK lysis buffer and subsequent filtration through a 70 µM filter to ensure a single cell suspension. MHC-I-deficient and wildtype splenocytes were differentially labelled by incubation in PBS containing a final concentration of 5 µM and 0.5 µM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen), respectively, for 7 minutes at 37°C. The volume of CFSE can vary between 3-7 mL depending on the pellet size.
Labelling was stopped by adding an equal volume of FBS at room temperature for 2 minutes, followed by flooding the 15 mL tube with RPMI. The tube was spun at 500 G for 5 min at room temperature and the remaining pellet was resuspended and incubated in complete RPMI for 15 minutes. Following incubation, the tube was spun at 500G for 5 min at room temperature and then resuspended in sterile PBS at a final concentration of 50 million cells/mL. Both cell types were mixed at a ratio of 1:1 and injected, this is hereafter referred to as the input. An aliquot of the mixed input was analyzed by flow cytometry to establish the ratio of MHC deficient (CFSE\textsuperscript{high}) to wildtype (CFSE\textsuperscript{low}) cells. A total volume of 200 μL of the input was injected into each mouse strain by i.v. injection. The output was prepared by extracting the spleen of each recipient mouse 16 hours after intravenous injection, homogenizing to obtain a single cell suspension, treating with ACK lysis buffer and measuring for CFSE labelled cells by flow cytometry. The percent rejection was calculated according to the following equation:

\[
\text{% rejection} = 1 - \frac{\left( \frac{\text{CFSE low}}{\text{CFSE high}} \right)_{\text{input}}}{\left( \frac{\text{CFSE low}}{\text{CFSE high}} \right)_{\text{output}}} \times 100%
\]

2.10 Statistical Analysis

Statistical significance was determined by student t test with a cutoff P value of 0.05 using the Prism software (version 4.0b). Data are presented as mean ±SEM and ±SD.
3: RESULTS

3.1 Characterization of the B6-Ly49\textsuperscript{129} mouse strain

The B6-Ly49\textsuperscript{129} congenic mouse was generated by backcrossing the 129 Ly49 gene cluster onto the B6 background for ten generations. Progeny from each generation was verified by PCR for the presence of Ly49\textsuperscript{v129}. However, contribution of B6 genes to the Ly49 cluster can remain undetected in the case of cross-over events within the Ly49 region. B6 and 129 mouse strains encode different Ly49 haplotypes which can be distinguished by distinct RFLP patterns (192). To verify if the entire Ly49 region is 129-derived, Southern blots were performed on genomic thymic DNA from the three mouse strains digested with BamHI using probes for Ly49\textit{o} and Ly49\textit{e} (104). The B6-Ly49\textsuperscript{129} mouse exhibits an RFLP pattern similar to the 129 mouse strain. The B6 RFLP pattern differs from both 129 and B6-Ly49\textsuperscript{129} (Fig. 7). Similar results were obtained using different restriction enzymes such as EcoRI and KpnI (data not shown). Overall, these data indicate that the B6-Ly49\textsuperscript{129} mouse strain possesses 129 Ly49 haplotype. These results were further verified by SNP analysis, which indicated that the NKC region of the B6-Ly49\textsuperscript{129} congenic mouse is derived from the 129 mouse strain (see Materials and Methods).
Figure 7: RFLP patterns are identical between 129 and B6-Ly49129 mice. RFLP analysis was performed on thymic DNA from B6, 129 and B6-Ly49129 (CON) mice any analyzed by Southern blot using BamHI, EcoRI and KpnI restriction enzymes. The blot was probed with Ly49o and Ly49e. Distinct RFLP patterns correlate with different Ly49 haplotypes.
Despite the NKC genetic region being of 129 origin, the expression of Ly49 receptors in the B6-Ly49\textsuperscript{129} mouse strain may differ due to the genetic background of the B6 mouse. To test whether the Ly49 expression patterns of B6-Ly49\textsuperscript{129} NK cells are similar to that of 129 NK cells, an array of mAbs against various Ly49 receptors was used to stain B6, 129 and B6-Ly49\textsuperscript{129} NK cells. Many of these mAbs including Cwy3, 3D10 and 5E6 do not recognize any Ly49 receptors in the 129 haplotype.

In contrast, mAbs 14B11, 12A8 and 4E5 exhibit specificity towards allelic variants of Ly49 receptors from both 129 and B6 mouse strains. AT8 is a mAb that binds to $\text{Ly49g}_{129}$ and not to $\text{Ly49g}_{B6}$ (106). The results show that overall B6-Ly49\textsuperscript{129} NK cells express Ly49 receptors in a pattern similar to that of 129 NK cells as opposed to B6 NK cells (Fig. 8). Interestingly, mAb 14B11 shows a distinct expression pattern in all three mice strains. This is most likely the result of an epigenetic effect caused by the B6 genetic background, since we can rule out a crossing over event within the Ly49 haplotype from both the RFLP and SNP data.

Both B6 and 129 mice strains express the same MHC-I\textsubscript{a} molecules, specifically H-2K\textsuperscript{b} and H-2D\textsuperscript{b} molecules, but not H-2L. MHC-I\textsubscript{a} expression can influence the expression of Ly49 receptors as well as the education of mature NK cells (74). The B6-Ly49\textsuperscript{129} mouse should have the same MHC-I\textsubscript{a} expression as the B6 and 129 mice strain, however different 14B11 staining patterns may indicate otherwise, as MHC-I\textsubscript{a} expression has been shown to influence Ly49 expression (Fig. 9) (48). To verify similar MHC-I\textsubscript{a} expression levels in all three
Figure 8: Surface expression of Ly49 receptors on NK cells is similar between 129 and B6-Ly49\textsuperscript{129} mice. NK cells from B6, 129, B6-Ly49\textsuperscript{129} (CON) and B6.Ly49Q KO (Ly49Q\textsuperscript{-/-}) mice isolated and stained with a panel of antibodies with cross-reactivity against different Ly49 receptors of B6 and 129 origin. Data shown are means of three separate experiments and is shown with ±SD.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>B6 Strain</th>
<th>129 Strain</th>
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<tbody>
<tr>
<td>Cwy3</td>
<td>Ly49G</td>
<td></td>
</tr>
<tr>
<td>AT8</td>
<td>Ly49G</td>
<td></td>
</tr>
<tr>
<td>3D10</td>
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<tr>
<td>4E5</td>
<td>Ly49D</td>
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**C57Bl/6**
- Cwy3: 26 ±3
- AT8: 2 ±3
- 3D10: 43 ±7
- 5E6: 27 ±3
- 14B11: 65 ±1
- 12A8: 48 ±6
- 4E5: 39 ±8

**CON**
- Cwy3: 0 ±1
- AT8: 43 ±6
- 3D10: 0 ±0
- 5E6: 0 ±0
- 14B11: 42 ±8
- 12A8: 67 ±4
- 4E5: 65 ±10

**129S1**
- Cwy3: 0 ±1
- AT8: 42 ±6
- 3D10: 0 ±0
- 5E6: 0 ±0
- 14B11: 30 ±1
- 12A8: 63 ±2
- 4E5: 61 ±7

**Ly49Q⁻⁻⁻**
- Cwy3: 0 ±1
- AT8: 47 ±1
- 3D10: 0 ±0
- 5E6: 0 ±0
- 14B11: 43 ±2
- 12A8: 66 ±6
- 4E5: 63 ±10
mice strains, splenocytes from the three mouse strains were stained for H-2K\(^b\)
and H-2D\(^b\) (Fig. 9). We found that the overall MFI from different splenocyte
populations were similar in the three mouse strains.

We next tested the expression pattern of non-Ly49 NK cell receptors
within the NKC region to test if the expression on B6-Ly49\(^{129}\) NK cell matches
that of the 129. B6 and 129 NK cells both express CD94/NKG2 family members,
which are relatively conserved in both mice strains (179), but have distinct level
of expression (Fig. 10A). The antibody PK136 is specific for NKR-PIC\(^{B6}\) and has
no cross reactivity with 129-mouse strain NK cells. B6-Ly49\(^{129}\) NK cells do not
stain with PK136 and exhibit CD94 and NKG2A staining patterns similar to the
129 mouse strain (Fig. 10A). These results along with the Ly49 RFLP and SNP
analyses prove that the NKC region of B6-Ly49\(^{129}\) is derived from the 129 mouse
strain.

Lastly we tested the expression patterns of NK cell markers CD2, CD11a
and CD62L, all of which are encoded outside of chromosome 6. The NK cell
expression levels of CD2 and CD11a were similar in all three mouse strains (Fig.
10B). Interestingly, the level of expression of CD62L was distinct between B6
and 129 NK cells. B6-Ly49\(^{129}\) NK cells exhibited CD62L levels similar to B6 NK
cells. This was expected as this receptor is not encoded on chromosome 6 (Fig.
10B).
Figure 9: MHC-I Expression is similar across all three mice strains. Splenocytes from B6, 129 and B6-Ly49^{129} (CON) mouse strains was assessed for the expression of MHC-I molecules A) H-2K\textsuperscript{b} and B) H-2D\textsuperscript{b}. Data shown are means of three separate experiments and is shown with ±SEM.
A

MFI

20

10

0

NK cells
T cells
B cells

H-2K\textsuperscript{b} staining

B

MFI

200

100

0

NK cells
T cells
B cells

H-2D\textsuperscript{b} staining

- B6
- 129
- CON
Figure 10: Surface expression of NKC and non NKC markers on B6-Ly49^{129} NK cells. NK cells from B6, 129, and B6-Ly49^{129} (CON) were stained with antibodies against A) markers within the NKC region: NKp1c^{66}, NKG2A/C/E and CD94 or B) markers for NK maturation outside the NKC region: CD2, CD11a and CD62L. Data show with mean ±SD and are representative of three separate experiments.
A

<table>
<thead>
<tr>
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<th>B6</th>
<th>CON</th>
<th>129S1</th>
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<tbody>
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<td>0 ± 0</td>
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<td>37 ± 4</td>
<td>24 ± 3</td>
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<tr>
<td>CD94</td>
<td>39 ± 4</td>
<td>27 ± 4</td>
<td>26 ± 1</td>
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B

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<th></th>
<th>B6</th>
<th>CON</th>
<th>129S1</th>
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<tbody>
<tr>
<td>CD2</td>
<td>89 ± 6</td>
<td>91 ± 2</td>
<td>80 ± 5</td>
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<tr>
<td>CD11a</td>
<td>100 ± 1</td>
<td>100 ± 1</td>
<td>99 ± 0</td>
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<tr>
<td>CD62L</td>
<td>82 ± 2</td>
<td>84 ± 4</td>
<td>71 ± 3</td>
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3.2 The 129-Ly49 Haplotype lacks Activating Receptors for Smith strain MCMV

129 mice are susceptible to MCMV infection. This may be due to the absence of a functional activating Ly49H that can detect infected cells or a defective DAP12 signalling pathway used by these activating Ly49 (104, 115). 129 NK cells express Ly49U (105), an activating receptor similar to Ly49H in the B6 haplotype that confers MCMV resistance by binding to the MHC-like m157 protein on MCMV-infected cells resulting in NK cell activation (9, 22). In addition, the 129 Ly49 haplotype also contains Ly49P (105), a receptor that has been shown to bind to the MCMV encoded protein m04 in the MaMy mouse strain (81). However, due to the presence of defective DAP12 signalling in 129 strain mice (115), it is unclear if Ly49 receptors in 129 mice can recognize or activate MCMV-infected cells via Ly49 expression. The B6-Ly49\(^{129}\) mouse strains provide the means to verify if the 129 Ly49 haplotype does indeed contain any activating receptor that is capable of binding to a virally encoded ligand. Recipient mice were infected with 5 000 PFU of MCMV Smyth strain in cold PBS by i.p. injection and assessed for viral titer 3 days post infection. As previously reported, B6 mice showed resistance to MCMV in comparison to 129 mice with a 100-fold difference in splenic viral titer (Fig. 11). Despite the expression of the 129 Ly49 haplotype with B6-derived DAP12 protein, the B6-Ly49\(^{129}\) mouse strain exhibited MCMV titers similar to 129 mice (Fig. 11). These results indicate that the 129 Ly49 haplotype does not encode any activating receptors capable of recognizing MCMV-infected cells on a H-2\(^b\) background capable of limiting virus replication.
Figure 11: 129 and B6-Ly49<sup>129</sup> are susceptible to MCMV infection. B6, 129 and B6-Ly49<sup>129</sup> (CON) mice were injected with 5 000 PFU of MCMV Smyth strain i.p. Following a 3-day infection, viral titers were assessed by homogenizing spleens from each recipient mouse, plating over a monolayer of MCMV susceptible Balb/c MEFs and counting for PFUs. Data are shown as the mean of three mice of each strain and is pooled from two separate experiments. ***p < 0.001
3.3 B6-Ly49^{129} NK cells exhibit B6-like cytotoxicity

To compare the killing capacity of the different NK cell populations, the cytotoxic response of adherent lymphokine activated killer (ALAK) cells from each strain of mice was tested against a panel of tumor target cells. ALAK cells were grown by culturing splenocytes for 6 days with IL-2 (see Materials and Methods). Due to the expression of H2^b MHC-I molecules, RMA tumor cells were not subject to NK cytotoxicity from all three mouse strains (Fig. 12). In contrast, we observed differential killing of RMA-S, an MHC-I deficient variant of RMA. 129 ALAK exhibited minimal killing in comparison to B6 and B6-Ly49^{129} ALAK, which both exhibited identical high killing of this tumor target (Fig. 12). Similar results were seen with YAC-1 tumor cells, a classic MHC-I low NK target which expresses ligands for the activating NKG2D receptor (72). B6 and B6-Ly49^{129} ALAK cells exhibit identical high killing of YAC-1 compared to 129 (Fig. 12). This phenotype was repeated by the xenogenenic target YB2/0, a rat tumor cell line (Fig. 12). Overall the results suggest that the cytotoxic activity of the B6-Ly49^{129} NK cells is comparable to B6 NK cells. This in turn suggests that the 129-Ly49 haplotype is sufficient for proper NK cell education.

CHO cells provide an example of tumor cell cytotoxicity mediated by an activating Ly49 receptor. Ly49D expressed on B6 NK cells is able to bind to Hm-1C4, an MHC ligand expressed by CHO cells (52). B6 ALAK exhibited efficient CHO killing in comparison to B6-Ly49^{129} and 129 ALAK. The CHO target cells were killed minimally by 129 ALAK. Interestingly, B6-Ly49^{129} ALAK were able to kill CHO target cells better than 129 but less than B6 NK cells (Fig. 12). This
Figure 12: Cytotoxic killing of tumor targets is similar between B6 and B6-Ly49<sup>129</sup> ALAK cells. ALAK cells were grown in culture for six days from B6, 129 and B6-Ly49<sup>129</sup> (CON) NK cells. These ALAK were incubated with a panel of NK tumor target cells loaded with <sup>51</sup>Cr at an effector:target ratio of 50:1, 25:1, 12:1 and 6:1 and assessed for cytotoxicity. The mean percent of target lysis (mean ±SD) is indicated from triplicate values. Data are representative of three independent experiments.
Figure 13: Ly49R and Ly49G expressed on B6-Ly49^{129} NK cells mediate CHO killing. The cytotoxic response of B6-Ly49^{129} (CON) ALAK cells was assessed against CHO target cells treated with monoclonal antibodies 12A8 (anti-Ly49R), 4D11 (anti-Ly49G) and rat IgG2a, \kappa isotype. The mean percent of target lysis (mean ±SD) is indicated from triplicate values. Data is representative of three independent experiments.
intermediate phenotype may be attributed to the expression of Ly49R\textsubscript{129}, an allelic variant of Ly49D\textsuperscript{B6} with 94.7\% sequence homology at the amino acid level (104, 115). To verify that the intermediate CHO killing by B6-Ly49\textsuperscript{129} ALAK cells is Ly49R\textsuperscript{129}-mediated, cytotoxicity assays in the presence of an mAb that blocked Ly49R\textsuperscript{129} were performed. CHO cells were killed at a higher rate when B6-Ly49\textsuperscript{129} ALAK cells were incubated with 4D11, which blocks the inhibitory Ly49G, known to bind a ligand on CHO cells (112) (Fig. 13). In contrast, incubating B6-Ly49\textsuperscript{129} NK cells with 12A8, which blocks the activating receptor Ly49R\textsuperscript{129}, yielded the lowest killing of CHO target cells (Fig. 13), suggesting that CHO killing in the B6-Ly49\textsuperscript{129} ALAK cells is mediated by Ly49R\textsuperscript{129}.

3.4 The 129 Ly49 Haplotype is Sufficient for NK cell Licensing

To further assess the functional capacity of B6-Ly49\textsuperscript{129} NK cells, IFN-\gamma production was tested. NK cells were freshly isolated from all three mouse strains previously activated with poly I:C, and were stimulated with either YAC-1 or RMA-S tumor cells, plate bound antibodies towards NKp46 or NKG2D activating receptors or the pharmacological agents phorbol 12-myristate 13-acetate (PMA) and ionomycin to trigger the production of IFN-\gamma. Upon YAC-1 stimulation, B6 and B6-Ly49\textsuperscript{129} NK cells exhibited two to three times more IFN-\gamma producing cells than 129 NK cells (Fig. 14A). RMA-S stimulation did not lead to a high percentage of IFN-g producing NK cells but a trend similar to YAC-1 stimulation was observed among the NK cells from the three strains (Fig. 14A). Plate bound mAb-mediated crosslinking of NKG2D and NKp46 activating receptors yielded a higher percentage of IFN-\gamma producing NK cells from B6 mice
Figure 14: IFN-γ upon tumor target and antibody stimulation is similar between B6 and B6-Ly49<sup>129</sup> NK cells. A) Upon stimulation with RMA and YAC-1 tumor cells, IFN-γ<sup>+</sup> NK cells were assessed using flow cytometry. B) NK cells were stimulated using plate bound antibodies against NKG2D and NKp46 and assessed for IFN-γ<sup>+</sup> NK cells. To verify the maximal activity of each NK cell population, cells were stimulated with PMA and ionomycin. Data are shown as the mean ±SD of three mice of each strain and is representative of three separate experiments.
compared to 129 and B6-Ly49^{129} NK cells (Fig. 14B). This could be attributed to
differences in antibody specificity for the receptors on B6 and 129 mice strains
(Fig. 14B). The pharmacological stimulation of PMA and ionomycin for B6, 129
and B6-Ly49^{129} NK cells yielded approximately 80% IFN-γ positive cells and
confirmed that all three mouse strains are capable of producing IFN-γ with no
intrinsic defects in any of the three mouse strains (Fig. 14B). Analysis of bulk NK
cells does not take into account the variegated expression of many NK receptors
and the different functional potential of these subsets within the NK cell
population. NK cells that expresses Ly49 receptors specific for self-MHC are
more responsive than those that express Ly49 receptors that do not bind the
murine MHC-I molecules and are thus variously termed educated, licensed or
armed (49, 86). To verify if the education hypothesis is valid in NK cells
expressing a 129-derived Ly49 haploype, B6-Ly49^{129} NK cells were stimulated
with YAC-1 or plate bound anti-NKp46 antibodies and multiparameter flow
cytometry was used to identify the contribution of different NK cell populations to
the overall response. NK cells that express NKG2A, a self-receptor that binds to
Qa-1, or Ly49I, O or V were much more likely to produce IFN-γ than the
respective negative population (Fig. 15). NK cells expressing NKG2A or Ly49I
were twice as likely to produce IFN-γ compared to their respective negative NK
subsets (Fig. 16A and B). NK cells expressing Ly49O and Ly49V were three
times as likely to produce IFN-γ than the negative population (Fig. 16A and B).
When IFN-γ production was tested in NK cells expressing NKG2A/Ly49I, O or V,
a four-fold difference over the negative population was observed (Fig. 16A and
Figure 15: YAC-I induced IFN-γ production in educated subset of NK cells. Upon YAC-1 stimulation of B6-Ly49^{129} (CON) NK cells, we observe that a higher percentage of IFN-γ^{+} cells in subsets that express inhibitory self MHC-I receptors Ly49I, Ly49O, LY49V and CD94/NKG2A irrespective of Ly49G expression, a non-self MHC-I receptor. The numbers indicate the percentage of cells present in each quadrant. Data shown are representative of three separate experiments.
Figure 16: Responsiveness of the 129 Ly49 haplotype to YAC-1 cells.
Following YAC-1 stimulation of B6-Ly49<sup>129</sup> (CON) NK cells A) IFN-γ<sup>+</sup> cells were analyzed based on the expression of Ly49I, Ly49O, Ly49V, Ly49G and NKG2A into positive and negative subsets. B) Fold induction of IFN-γ was assessed based on the division of Ly49<sup>+</sup> over Ly49<sup>−</sup> of IFN-γ producing NK cells. Data are shown as the mean ±SD of three mice of each strain and is representative of three separate experiments.
Figure 17: Responsiveness of the 129 Ly49 haplotype upon αNKp46 antibody stimulation. Following αNKp46 stimulation of B6-Ly49<sup>129</sup> (CON) NK cells A) IFN-γ<sup>+</sup> NK cells were analyzed based on the expression of Ly49I, Ly49O, Ly49V, Ly49G and NKG2A into positive and negative subsets. B) Fold induction of IFN-γ was assessed based on the division of Ly49<sup>+</sup> over Ly49<sup>-</sup> of IFN-γ producing NK cells. Data is shown as the mean ±SD of three mice of each strain and is representative of three separate experiments.
A

Unstim. | Ly49 I/O/V/NKG2 | Ly49G | NKG2A | Ly49I | Ly49O/V | Ly49 I/O/V/NKG2

αNKp46

% IFN-γ⁺ NK Cells

positive subset

negative subset

B

fold induction of IFN-γ

Ly49⁺ vs Ly49⁻ NK cells

NKG2A + - + - - - +
Ly49I + - - + - - +
Ly49O/V + - - - + + +
Ly49G - + - - - - -
NKp46 - + + + + + +
B). Ly49G was used as a negative control as it does not bind to H-2K\(^b\) or H-2D\(^b\) MHC molecules and is thus a non-self receptor. With both YAC-1 and NKp46 stimulation, Ly49G positive NK cells are not more likely to produce IFN-\(\gamma\) than the negative population, similar to the unstimulated NKG2A/Ly49I, O, V positive vs. negative NK cells (Fig. 16 and 17). In comparison to YAC-1, anti-NKp46 stimulation showed an overall smaller fold difference but a similar trend with Ly49I or Ly49O/V positive being more likely to produce IFN-\(\gamma\) than Ly49I-negative or Ly40O/V-negative NK cells (Fig. 17). In contrast, NKG2A/Ly49I,O or V positive NK cells were 10-fold more likely to produce IFN-\(\gamma\) than the negative subset (Fig. 17).

Collectively, these data suggest that the 129 Ly49 receptors are able to adequately educate NK cells resulting in functional cytotoxicity and cytokine responses. Furthermore, these results indicate that the hyporesponsive nature of NK cells from the 129-mouse strain is not due to a divergent 129 Ly49 haplotype but rather a non-Ly49 genetic defect.

### 3.5 B6-Ly49\(^{129}\) NK cells Exhibit Enhanced Rejection Capabilities

In vivo rejection of MHC class I-deficient cells is thought to be a consequence of NK cell education (19). To test the ability of each mouse strain to recognize and lyse MHC-deficient cells, splenocytes from MHC-sufficient and -deficient mice were differentially labelled with fluorescent dye, mixed at a 1:1 ratio and injected into each recipient mouse strain. After 16 hours splenocytes were analyzed by flow cytometry for rejection of MHC-I deficient cells. Rejection
Figure 18: Enhanced rejection of MHC-I-deficient cells by B6-Ly49<sup>129</sup> mice

A) Unstimulated or B) poly(I:C) treated mice were injected i.v. with wildtype an MHC-I-deficient cells (H-2K<sup>b</sup>/H-2D<sup>b</sup>/ and H-2K<sup>b</sup>/H-2D<sup>b</sup>/) differentially labelled with CFSE. After 16 hours, recipient mice were sacrificed and assessed for the presence of MHC-sufficient and -deficient cells in the spleen. Data are pooled from three separate experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
% Rejection of $K^{b,c}$

% Rejection of $D^{b,c}$

% Rejection of $K^{b,c}$

% Rejection of $D^{b,c}$

% Rejection of $K^{b,c}$

% Rejection of $D^{b,c}$
was based on any changes from the input ratio of MHC-sufficient and -deficient injected cells. MHC-sufficient cells serve as a reference for the relative rejection of MHC-I deficient cells. Rejection of K\textsuperscript{b-/-} cells was higher by 20% in both the B6 and B6-Ly49\textsuperscript{129} mouse strains in comparison to the low rejection of 129 mice (Fig. 18A). Rejection of D\textsuperscript{b-/-} cells was too low to assess any differences between the mouse strains. Strikingly, rejection of the double knockout K\textsuperscript{b-/-}D\textsuperscript{b-/-} cells was highest in B6-Ly49\textsuperscript{129} mice, about 10% higher from the B6 mice and 30% higher than the 129 (Fig. 18A). These differences were all statistically significant.

To augment the rejection of D\textsuperscript{b-/-} cells, recipient mice were injected with poly(l:C) to stimulate the NK cell population and increase the rejection of MHC-deficient cells. Poly(l:C) primes NK cells by stimulating dendritic cells to secrete IL-12, IFN\textalpha and the trans-presentation of IL-15 (102). Rejection of D\textsuperscript{b-/-} cells was higher in B6 and B6-Ly49\textsuperscript{129} mice, both of which exhibit 20% more rejection of MHC-deficient cells than 129 mice (Fig. 18B). However, rejection of K\textsuperscript{b-/-} cells with poly(l:C) stimulation was highest in B6-Ly49\textsuperscript{129} mice, which showed, 10% higher rejection than B6 mice and 40% higher rejection than 129 mice, similar to the rejection of K\textsuperscript{b-/-}D\textsuperscript{b-/-} cells by untreated mice. B6 and B6-Ly49\textsuperscript{129} mice exhibited no significant difference in their ability to reject K\textsuperscript{b-/-}D\textsuperscript{b-/-} cells, however both were about 40% higher than 129 mice (Fig. 18).

Higher rejection of MHC-I-deficient cells by B6-Ly49\textsuperscript{129} NK cells indicates enhanced education of NK cells within the congenic mouse possibly due to the expression of the 129 Ly49 haplotype. However, due to the expression of additional 129 genes outside the NKC region as a result of linkage disequilibrium
Figure 19: 129 genetic contribution in chromosome 6 of the B6-Ly49\textsuperscript{129} and B6.Ly49Q KO mouse strains. Using SNP analysis, the genetic origin of the NKC encoding chromosome 6 was analyzed for both B6-Ly49\textsuperscript{129} and B6.Ly49Q KO mouse strains. The 129 genetic content spans from nucleotides 79,759,628 to 138,203,431 for the B6-Ly49\textsuperscript{129} mouse and from nucleotides 118,022,343 to 149,214,236 for the B6.Ly49Q-KO mouse.
Mus Musculus Chromosome 6

B6-Ly49\textsuperscript{129}

B6-Ly49Q-KO

\begin{itemize}
\item C57Bl/6
\item 129S1/SvlmJ
\end{itemize}
with the Ly49 gene cluster, one may argue that these genes also contribute to the effector functions of the NK cell population. To address this possibility, in vivo rejection of K\(^{b^{-}}\)D\(^{b^{-}}\) and Kb\(^{-}\) cells by untreated and poly(I:C) treated mice respectively was assessed using B6.Ly49Q-KO mice. These mice also express the 129 Ly49 haplotype on the B6 genetic background at similar levels to the B6-Ly49\(^{129}\) mouse strain (Fig. 8) with significantly less contribution of 129 genes outside the NKC region (165, 166) (Fig. 19). Ly49Q is disrupted in this mouse strain, but this particular Ly49 is expressed in plasmacytoid dendritic cells (pDCs), neutrophils, macrophages and osteoclasts and not on NK cells (77, 153, 173). Rejection levels of K\(^{b^{-}}\)D\(^{b^{-}}\) cells by B6.Ly49Q-KO mice were similar to that shown by B6-Ly49\(^{129}\) mice, with about 10% higher rejection than B6 and 50% higher rejection than the 129 mouse strain (Fig. 20A). A similar trend was seen with rejection of K\(^{b^{-}}\) cells upon poly(I:C) stimulation (Fig. 20B).

Collectively, these results indicate that the B6-Ly49\(^{129}\) mouse strain is capable of discriminating and rejecting MHC-I deficient cells. Therefore, the 129 Ly49 haplotype is sufficient to educate NK cells to reject MHC-I deficient cells in vivo, possibly even to a greater extent than the B6 Ly49 haplotype.
Figure 20: Enhanced rejection of MHC-I-deficient cells by B6.Ly49Q KO mice is similar to B6-Ly49\textsuperscript{129}. A) Rejection of H-2K\textsuperscript{b}/H-2Db\textsuperscript{b} by unstimulated mice and B) rejection of H-2K\textsuperscript{b} by poly(I:C) treated mice was performed as previously described using B6, 129 and B6.Ly49Q KO (QKO) recipient mouse strains. *p < 0.05; ***p < 0.001
4: DISCUSSION

The Ig-like KIRs and C-type lectin-like Ly49s are both NK receptors that are structurally distinct but share functional characteristics and exhibit convergent evolution (29). Both receptors are stochastically expressed on NK cells, contain activating and inhibitory counterparts and are inherited as variable haplotypes within a set of framework genes (67, 137, 187). Throughout their evolution, animals such as mice, rats and horses have evolved to use Ly49 receptors as MHC-I receptors on NK cells, in contrast to KIRs expressed on NK cells from humans, other primates and cattle (134). Within a given species, individual genomes contain different KIR/Ly49 gene content due to their highly polymorphic and polygenic nature. As a result, we observe high variability both in gene duplication and allelic variants of the same ancestral gene. Through gene duplication, deletion and crossing over events within the gene cluster, we observe differences in the Ly49 gene cluster. Interestingly, different KIR/HLA patterns have been associated with autoimmunity and pathogenic diseases (109, 110). Due to the aforementioned similarities, murine Ly49 receptors provide an ideal model to study KIRs on NK cell and their influence on human health and disease. Different inbred mouse strains express various haplotypes of Ly49 receptors, similar to KIR expression amongst different people. By backcrossing the 129 Ly49 haplotype onto the B6 genetic background, we were able to
determine if the relatively divergent Ly49 receptors of the 129 haplotype are responsible for low NK cell effector function observed in the 129 inbred mice strain.

The B6-Ly49\(^{129}\) congenic model provides a unique opportunity to study Ly49 haplotype variations and its effects on NK cell functions. Our results show that the B6-Ly49\(^{129}\) congenic NK cells were able to kill tumor targets \textit{in vitro} as effectively as B6 NK cells (Fig. 12). The expression of the 129 Ly49 haplotype on the genetic background of B6, essentially restoring any defective DAP12 signalling as previously reported (115) verified that no activating receptor against MCMV is present in the 129 haplotype that could potentially confer resistance to infection in an H-2\(^b\) background (Fig. 11). Ly49P has been reported to impart MCMV resistance via an H-2D\(^k\)-restricted mechanism (81). In addition, we were able to demonstrate that the 129 Ly49 haploype is sufficient to educate NK cells, a process only previously validated in the B6 mouse strain (49, 86). Strikingly, the B6-Ly49\(^{129}\) NK mice were able to kill MHC deficient target cells more efficiently than both the B6 and 129 \textit{in vivo} (Fig. 18).

The B6-Ly49\(^{129}\) congenic mouse strain was verified to express 129 derived Ly49 receptors by SNP analysis, RFLP and flow cytometry (Figs. 7, 8, 10 and 19). However, staining by the 14B11 monoclonal antibody, specific for Ly49I in 129 and Ly49C, I, F and H in B6 mice, exhibited a distinct expression pattern between the B6-Ly49\(^{129}\) and wildtype mouse strains (Fig. 8). This may be an effect of additional genes of 129 origin outside the NKC that are present in the B6-Ly49\(^{129}\) mice strain (Fig. 19). To investigate this anomaly, we stained NK cells
from the B6.Ly49Q-KO mouse strain with 14B11. The B6.Ly49Q-KO mouse contains the 129 Ly49 haplotype with a disruption in Ly49q, on the B6 genetic background, which was also achieved by backcrossing (165, 166). The 129 genetic contribution in B6.Ly49Q-KO mice is approximately half of that in B6-Ly49^{129} (Fig. 19). Ly49Q is expressed in pDC, neutrophils, macrophages and osteoclasts, but not NK cells, making B6.Ly49Q-KO mice a good model to compare staining of 14B11 on NK cells with the B6-Ly49^{129} strain. Both the B6.Ly49Q-KO and the B6-Ly49^{129} mouse strain yielded similar expression patterns for 14B11 staining (Fig. 8). The results indicate that epigenetic effects may be a possible cause of the abnormal 14B11 staining, which are likely encoded in the region outside the NKC. In addition, differential expression of MHC-I expression is known to influence the expression of Ly49 receptors on NK cells (61, 148). Despite B6 and 129 mice having the same MHC-Ia molecules, MHC-1b expression differs between the two mouse strains (88). The expression of the 129 Ly49 haplotype with MHC-1b expression from B6 may influence expression of 14B11 staining Ly49 receptors in the B6-Ly49^{129} mouse, although no binding by any LY49 to MHC-1b has yet been reported.

Surface expression of NKG2D and CD94, both encoded within the NKC, are consistent with the 129 expression pattern. In contrast, known NK markers outside the NKC are consistent with the B6 profile (Fig. 10). It is interesting to note that despite both mouse strains expressing the same receptors, there is a distinct expression pattern associated with each mouse strain due to background differences in expression levels and allelic variation contributing to mAb binding...
affinity. These results also indicate that the NK cell population in the B6-Ly49\textsuperscript{129} congenic mice are mature, as indicated by the expression of the NK markers CD2, CD11a and CD62L (Fig. 10).

The B6-Ly49\textsuperscript{129} mouse model possesses the entire NKC region from the 129S1 inbred mouse strain, which contains NKG2D, a receptor known to mediate killing of YAC-1 target cells (72). B6 and B6-Ly49\textsuperscript{129} ALAK both exhibit similar killing of YAC-1, despite expressing NKG2D alleles of B6 and 129 origin, respectively. The 129 Ly49 haplotype expressed in the B6-Ly49\textsuperscript{129} ALAK is sufficient to educate NK cells to kill YAC-1 as effectively as B6 ALAK cells (Fig. 12). A similar phenotype was not seen with IFN-\(\gamma\) production upon NKG2D antibody stimulation. B6 NK cells were stimulated to produce about 5% more IFN-\(\gamma\) than 129 or B6-Ly49\textsuperscript{129} NK cells (Fig. 14B). This result may be due to the nature of the \(\alpha\)NKG2D mAb, which was created against the B6 NKG2D allele. As a result, the \(\alpha\)NKG2D antibody may not bind to NKG2D\textsuperscript{129} with the same affinity and result in less activation of NK cells and IFN-\(\gamma\) secretion. Killing of YAC-1 by B6-Ly49\textsuperscript{129} and B6 ALAK was similar to the rat myeloma cell line YB2/0, which lack known ligands for Ly49 activating receptors. Similar killing of YAC-1 and YB2/0 target cells by both the B6 and B6-Ly49\textsuperscript{129} ALAK indicates sufficient education of NK cells irrespective of the expression of B6 or 129 Ly49 haplotypes (Fig. 12).

B6 mice are resistant to MCMV due to the presence of Ly49H, a receptor that is able to detect and kill infected cells through binding of the viral ligand m157 (9). In contrast, 129 mice are susceptible to MCMV infection that leads to a
fatal outcome of the disease. Two possible issues can contribute to this phenotype. First, the 129 haplotype does not encode Ly49h. However the 129 haplotype contains Ly49u (105), an allelic variant of Ly49h. The 129 Ly49 haplotype also contains Ly49P, a receptor that has been reported to bind to the MCMV viral protein m04 in the context of H-2Dk in the MaMy mouse strain (81). Secondly, the 129 mouse strain has been reported to have defective signalling from the DAP12 adapter molecule (115), necessary for signalling by activating Ly49. Therefore, it is formally possible that Ly49U and Ly49P receptors may bind to the m157 and m04 ligands respectively, but due to defective signalling, there will be no activating signal leading to NK cell activation and subsequent killing of infected target cells. MCMV infection of B6, 129 and B6-Ly49129 mice show that both 129 and B6-Ly49129 exhibit high viral titers as opposed to the resistant B6 mice strain (Fig. 11). This result verifies that no counterpart to Ly49H exists in the 129 haplotype. However, it is possible that the role of Ly49U is to bind undiscovered m157 ligand expressed on infected cells. In addition, Ly49P may be potentially functional, but not in an H-2b haplotype.

CHO tumor cell killing is mediated by the activating Ly49D on B6 NK cells, which binds to the MHC-I ligand Hm1-C4 (52, 70). The 129 Ly49 haplotype contains Ly49R, an allelic variant of Ly49D with 95% amino acid sequence homology (115), which has been shown to mediate CHO killing. Despite the expression of Ly49R, 129 NK cells exhibit low in vitro killing of CHO targets (Fig. 13), possibly due to defective DAP12 signalling (115). Interestingly, the B6-Ly49129 NK cells exhibit an intermediate phenotype that is higher than 129 but
lower than B6 NK cells (Fig. 12). In addition, B6-Ly49<sup>129</sup> NK cell were treated with blocking antibodies against the activating Ly49R and the inhibitory Ly49G, both of which have been shown to influence killing of CHO (70, 112). Blocking of the inhibitory Ly49G<sup>129</sup> to its as yet unknown CHO-ligand favors activation of NK killing resulting in higher killing of CHO (Fig. 13), as reported for the Ly49G<sup>B6</sup> allele (111). Blocking Ly49R resulted in minimal killing, compared to untreated or blocking with the isotype control (Fig. 13). These results support our hypothesis that the low killing of CHO by 129 NK cells is due in part to the expression of Ly49R on NK cells with defective DAP12 signalling. Allelic differences in Ly49D/R and Ly49G may also contribute to the lower killing of CHO cells by 129 haplotype-containing NK cells.

It is interesting to note that rejection of H-2K<sup>b</sup>-deficient and H-2K<sup>b</sup> D<sup>b</sup>-deficient splenocytes was higher in B6-Ly49<sup>129</sup> than B6 or 129 mice (Fig. 18). However the in vitro cytotoxicity data show that killing of MHC low or allogenic tumor targets is the same as B6 (Fig. 12). This discrepancy can be explained by the activation state of the NK cells used in each experiment. The in vitro killing experiment uses ALAK cells, which are generated by culture of splenocytes in the presence of IL-2 for 6 days. It has been shown that hyporesponsive NK cells can be activated by IL-2 treatment, essentially reversing the hyporesponsive phenotype (49, 152). In vitro killing of RMA and RMA-S was repeated with isolated NK cells from the spleens of poly(I:C) injected mice to maintain NK cell education. However, due to low yield of NK cells, differences in cytotoxicity could not be assessed (data not shown).
In vivo killing of MHC-I deficient cells was assessed in both untreated and poly(I:C) activated recipient mice. Rejection of K\textsuperscript{b}-deficient cells was higher than of D\textsuperscript{b}-deficient cells in both unstimulated and poly(I:C) activated recipient mice. This result is consistent with reports that H-2K\textsuperscript{b} has a stronger educational impact than H-2D\textsuperscript{b} meaning that H-2K\textsuperscript{b}-deficient cells should be rejected more strongly than H-2D\textsuperscript{b}-deficient cells (74). Splenocytes lacking H-2K\textsuperscript{b} were shown to be killed approximately 40% more than D\textsuperscript{b}-deficient cells (Fig. 18). To ensure the higher rejection of H-2K\textsuperscript{b} and H-2K\textsuperscript{b} H-2D\textsuperscript{b} by the congenic mice was not a result of the presence of the 129 derived gene located outside the NKC in the B6-Ly49\textsuperscript{129} mice, similar experiments were repeated with the B6.Ly49Q\textsuperscript{i-} mouse model, yielding similar results (Fig. 20).

The results are consistent with the findings that expression of H-2K\textsuperscript{b} molecules confers stronger rejection of MHC-I-deficient cells than H-2D\textsuperscript{b} (74). Target cells lacking expression of H-2K\textsuperscript{b} are unable to bind to inhibitory Ly49 receptors on host NK cells and are consequently rejected, to a higher degree than H-2D\textsuperscript{b} lacking target cells (Fig. 18). The expression of an additional inhibitory Ly49 receptor towards self MHC-I in the 129 haplotype allows for higher education of NK cells from the B6-Ly49\textsuperscript{129} congenic mice which in turn leads to higher rejection of MHC-I-deficient cells in vivo.

Human NK cells express varying levels of CD56 that are indicative of their primary effector function, either cytokine producing CD56\textsuperscript{high} NK cells or cytotoxic CD56\textsuperscript{dim} NK cells (35). In regards to our study, we chose to investigate the murine splenic NK cell population due to their high relative abundance (about 5%
of the lymphocytes in the spleen) in comparison to other NK sources such as the liver, lungs or lymph nodes (34). Splenic NK cells are capable of cytotoxicity and IFN-γ production upon cross-linking activating receptors via mAbs or tumor stimulation (Fig. 14). In addition, the subset of NK cells that express any or all of the receptors for self MHC-I, Ly49l, O, V and NKG2A/CD94, are capable of responding significantly better than a subset lacking these receptors (Fig. 15-17).

Two mechanisms of NK cell education have been proposed. Fernandez et al. propose that NK cells are hyperresponsive during development and the expression of inhibitory Ly49 receptors to self MHC-I prevents NK cells from being chronically activated and as a result hyporesponsive due to exhaustion (49). In contrast, Kim et al. believe that NK cells are intrinsically hyporesponsive during development and the expression of inhibitory Ly49 receptors towards self MHC-I allows the NK cells to acquire NK effector functions (86).

NK cells from B6-Ly49129 mouse that express Ly49l, O, or V receptors, all of which bind the self-MHC-I in H-2b mice including both 129 and B6 (60), produce more IFN-γ than those that express no Ly49 receptors to self in the B6-Ly49129 mouse (Fig. 15). In addition, NK cells expressing the NKG2A inhibitory receptor that binds to Qa-1 also contribute to this educational effect (74). NK cells that express more receptors to self are also more responsive, as seen in B6 mice according to the rheostat model (75). Most NK cells from the three mouse strains have the intrinsic ability to produce IFN-γ as shown by the similar IFN-γ production by NK cells (~80%) from all mouse strains upon pharmacological PMA and ionomycin stimulation (Fig. 14). A large subset of Ly49l/O/V+ NK cells
do not produce IFN-γ upon stimulation (Fig. 15). These results may indicate that not all splenic NK cells are primed to produce cytokines, similar to human NK cells.

Also, we show that B6 and B6-Ly49^{129} mice have similar killing of tumor target cells by cytotoxicity yet the IFN-γ production of congenic mice is slightly lower than that of B6. This may be due to the fact that the cytotoxic response of NK cells is a much more rapid response than cytokine production. Brodin et al. show that NK cells producing IFN-γ upon stimulation are always CD107^{+}, a marker for exocytosis of granules containing perforin and granzyme (21, 23). They show that no subset is IFN-γ^{−}CD107a^{−}, indicating that NK cells are more readily cytotoxic before they are able to secrete IFN-γ. The lower production of IFN-γ by the B6-Ly49^{129} NK cells may be a result of a slower response due to 129 gene contribution outside the NKC. Overall, the B6-Ly49^{129} NK cells are able to produce IFN-γ at a significantly higher rate than the 129 NK cells, confirming that the 129 Ly49 haplotype is sufficient to educate NK cells as shown by cytokine production in addition to cytotoxicity.

Uneducated NK cells have recently been shown to play an important role against NK cell targets, such as infected host cells with normal MHC expression. According to Orr et al, uneducated Ly49H^{+} are better responders than the educated Ly49H^{+} in the presence of m157-bearing RMA cells (128). These results indicate a specific role for the uneducated population of NK cells, which express no Ly49 receptors to self MHC-I and are not subject to inhibition from MHC-sufficient infected cells. Uneducated Ly49R^{+} NK cells from B6-Ly49^{129}
should show a similar effect against CHO. However, Orr et al. neglect the educational impact of CD94/NKG2A receptors and consider Ly49 C/i' NK cells as uneducated NK cells. In order to properly assess the impact of uneducated NK cells, CD94/NKG2A' NK cells must be considered.

However, due to the limitations of commercial antibodies available, in particular the lack of an antibody that only binds to Ly49R, we were unable to study uneducated NK cells in the B6-Ly49129 congenic mice. Most antibodies against murine Ly49 were made against the B6 strain, which have different specificities than the 129 strain. Cross reactivity is present between the B6 and 129 mouse strains due to high sequence homology between receptors in the two Ly49 haplotypes. A better antibody repertoire is needed against 129 Ly49 receptors to better compare the two NK populations, in particular the education of NK cells in these two mice strain.

Phylogenetic analysis of Ly49 receptors from B6, 129, Balb/c and NOD mice categorizes three main groups of receptors based on sequence homology (104). These groups can be represented by Ly49q, Ly49o and Ly49e, the latter two which were used as probes in the RFLP analysis (Fig. 8). Studying different Ly49 receptor haplotypes amongst these inbred mouse strains provides a unique opportunity to study KIR haplotype divergence in the human population. In order further support our hypothesis, we can test for the opposite effect of the B6-Ly49129 mouse strain by creating a congenic for the Balb/c Ly49 haplotype onto the B6 genetic background. Effectively, this mouse strain would contain fewer Ly49 receptors to self than the B6 haplotype. We would expect to see the
opposite effect of the B6-Ly49\textsuperscript{129} mouse strain with \textit{in vitro} effector functions and \textit{in vivo} rejection of MHC-deficient cells.

It is evident that activating Ly49 receptors have a profound effect on NK cell function when considering these receptors bind to pathogen-encoded ligands. However, the role of inhibitory Ly49 receptors, in particular differences in Ly49 haplotypes, in regards to NK cell effector function and education is not apparent. Inbred mouse strains such as B6 and 129 have disparate NK cell function, which cannot be an effect of differences in Ly49 haplotype, as our B6-Ly49\textsuperscript{129} mouse strain, which contains the 129 Ly49 haplotype on the B6 genetic background, has strong NK cell activity similar to the B6. Interestingly, the B6-Ly49\textsuperscript{129} mouse showed enhanced rejection of MHC-I deficient cells \textit{in vivo}, possibly suggesting enhanced education of NK cells due to expression of an additional Ly49 receptor that binds to self MHC-I encoded in the 129 haplotype compared to the B6 haplotype. The process of NK cell education has also been reported in human NK cells (6). Human KIRs are associated with two main haplotypes that are distributed in different ethnic populations, which are highly variable among individuals (177). Considering the functional and evolutionary similarities between murine Ly49 receptors and human KIRs, we can assume that the effects of different Ly49 haplotypes on NK cell function and education can correlate to differences in KIR haplotypes in humans and their association with autoimmunity and clearance of infectious diseases such as HIV-1 and hepatitis C (80, 109). Our studies of the Ly49 haplotype divergence in B6 and 129 inbred mouse strains, in particular differences in inhibitory Ly49 expression,
suggest similar effects on NK cell education for the recognition of missing self-MHC-I, as both Ly49 haplotypes are adequate to educate NK cells, but more dramatic effects are seen for activating Ly49 variation.
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Curriculum Vitae

Name: Rajen Patel
Birth Place: Montreal, Quebec, Canada

Laboratory Skills

- Experience working with tumor cell lines and bacterial cultures
- Knowledge of molecular biology including cloning and sequencing
- Experience with cell sorting (MACS) and flow cytometry (BD Calibur, BC Cyan)
- Skilled to work with various mouse strains including i.p and i.v procedures
- Experience with preparation of manuscripts for publication in scholarly journals

Research Experience

University of Ottawa: Biochemistry, Microbiology and Immunology
2010 - Present
National Research Council of Canada, Ottawa, Ontario
PhD Thesis Title: Effects of Ly49 Haplotype Variation on Natural Killer Cell Function
Supervisor: Dr. Subash Sad

University of Ottawa: Biochemistry, Microbiology and Immunology
2008 - 2010
Roger Guindon Hall, Ottawa, Ontario
M.Sc Thesis Title: Effects of Ly49 Haplotype Variation on Natural Killer Cell Function
Supervisor: Dr. Andrew Makrigiannis

McGill Microbiology and Immunology Honors Project
2007 - 2008
Montreal General Hospital, Montreal, Quebec
Honors Project Title: Mycobacterium avium Complex and Variations in Macrophage Response
Supervisor: Dr. Marcel Behr

McGill University - Sentinel Bioactive Paper Network (NSERC)
Summer Session 2006 & 2007
Pulp and Paper Research Building, Montreal, Quebec
Project Title: Antimicrobial Filter for Treating Contaminated Drinking Water
Co-Supervisors: Dr. Derek Gray and Dr. Mike Paice
Academic Training

Doctor of Philosophy Candidate: Biochemistry, Microbiology and Immunology
2010 – Present
National Research Council of Canada, Ottawa, Ontario, Canada
Lab Supervisor Dr. Subash Sad

Masters of Science Candidate: Biochemistry, Microbiology and Immunology
2008 – 2010
University of Ottawa, Ottawa, Ontario, Canada (2009-2010)
McGill University, Montreal, Quebec, Canada (2008-2009)
Lab Supervisor Dr. Andrew Makrigiannis

Bachelors of Science: Major in Microbiology and Immunology
2005 – 2008
McGill University, Montreal, Quebec, Canada
Interdepartmental Honors Immunology Supervisor Dr. Marcel Behr

Diploma of Collegial Studies, Health Science
2003 – 2005
Marianopolis College, Montreal, Quebec, Canada

Scholarships and Awards

Fisher Scientific
Award of Excellence in Graduate Studies (M.Sc) (Value: $500) November 2010

University of Ottawa: Department of Microbiology and Immunology
Admission Scholarship (Value: $9,000/year for 4 years) August 2010

University of Ottawa: Department of Microbiology and Immunology
Entrance Scholarship (Value: $2,500) August 2009

McGill University: Department of Experimental Medicine
Principal’s Graduate Fellowship (Value: $2,500 – Personally Declined) August 2009

McGill University: Department of Experimental Medicine
Provost’s Graduate Fellowship (Value: $2,000) August 2008

Quebec India Business Council
QIBC Bursary Award (Value: $2,000) May 2008

Millennium Excellence Award – National in-course
Canadian Millennium Scholarships (Value: $4,000) May 2006
Conference Presentations


Publications


Poster Abstracts


- Patel, R, Belanger S, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. 23rd Annual Meeting for the Canadian Society for Immunology, April 2010, Niagara Falls, Ontario.


- Patel, R, Belanger S, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. IRCM Research Day, June 2009, Montreal, Quebec

- Patel, R, Belanger S, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. 22nd Annual Meeting for the Canadian Society for Immunology April 2009, Whistler, B.C.

- Patel, R, Belanger S, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. 9th Annual McGill Biomedical Graduate Conference, February 2009, Montreal, Quebec.

Affiliations

Canadian Society for Immunology – Student Member 2007-Present
University of Ottawa, Ottawa, Ontario

Coaching Association of Canada - Assistant Coach Soccer Program 2002-Present
Special Olympics Ottawa, Ottawa, Ontario
Special Olympics North Island, Montreal, Quebec

Microbiology Immunology Student Association – VP Social 2007-2008
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REFERENCES


