



uOttawa

L'Université canadienne
Canada's university

**FACULTÉ DES ÉTUDES SUPÉRIEURES
ET POSTDOCTORALES**



**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

Rajen Patel

AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Microbiology and Immunology)

GRADE / DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

The Effects of LY49 Haplotype Divergence on Natural Killer Cell Function

TITRE DE LA THÈSE / TITLE OF THESIS

Andrew Makrigiannis

DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Ashok Kumar

Kathryn Wright

Gary W. Slater

Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

THE EFFECTS OF LY49 HAPLOTYPE DIVERGENCE ON NATURAL KILLER CELL FUNCTION

by

Rajen Patel
Bachelor of Science, McGill University, 2008

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

MASTER OF SCIENCE

In the
Department of Microbiology and Immunology

All rights reserved. However, in accordance with the *Copyright Act of Canada*, this work may be reproduced, without authorization, under the conditions for *Fair Dealing*. Therefore, limited reproduction of this work for the purposes of private study, research, criticism, review and news reporting is likely to be in accordance with the law, particularly if cited appropriately.

© Rajen Patel, Ottawa, Canada, 2010



Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file *Votre référence*
ISBN: 978-0-494-74149-8
Our file *Notre référence*
ISBN: 978-0-494-74149-8

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

■ ■ ■
Canada

APPROVAL

Name: Rajen Patel
Degree: Master of Science
Title of Thesis: The Effects of Ly49 Haplotype Divergence on NK Cell Function

Examining Committee: Dr. Ashok Kumar
Professor
Children's Hospital of Eastern Ontario (CHEO)

Dr. Kathryn Wright
Associate Professor
University of Ottawa

Chair: Dr. Robin Parks
Associate Professor
University of Ottawa

Date Defended/Approved: November 17, 2010

ABSTRACT

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. C57Bl/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-Ly49¹²⁹ 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 *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, *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

I would like to express my appreciation and gratitude to all of those who have made my graduate work possible. I would first like to thank my parents Ashok and Usha and my brother Dharmesh for all of the help and support they have provided throughout my academic life. Whether it was staying up late with me while I study or just being there to listen to my presentations, it made the difference. Thanks!

I would like to give my special thanks and appreciation to Dr. Andrew Makrigiannis for taking me on as a graduate student. Thank you for being patient with me and pushing me throughout my two year in the lab. I believe it helped me transition from an eager science student to a scientific researcher. I had a great time completing my Master's in your lab, especially with such great lab members working beside me. I would like to give my special thanks to Simon for teaching me all the lab techniques and answering all the countless questions I had for him; not to mention helping me edit my thesis. I would also like to thank Lee-Hwa for all the motivation and advice she has given me throughout our time together. And a special thank you to all the rest of the lab: Munir, Nuha, Chloe, Michelle and Laticia for all the laughs and interesting conversations over coffee; not to mention listening to me present countless times.

I would also like to thank my thesis advisory committee members, Dr. Fillion and Dr. Dimock for all their insights and guidance throughout my research at the University of Ottawa. Also a special thanks to the staff at the Animal Care and Veterinary Services: Marie, Wendy, Monique, Christina and Sue for all of their help with the animal models.

I would also like to thank my friends Amit, Sanket, Brijesh and Mehool for helping me relieve my stress from my work at the lab. Whether it was hitting the driving range or going for a movie, it made the difference! Also I would like to give a special thanks to Virja for all her help and support throughout my year in Ottawa. Thanks to you all!

TABLE OF CONTENTS

Approval.....	ii
Abstract.....	iii
Dedication.....	iv
Acknowledgements.....	v
Table of Contents.....	vii
List of Figures	ix
List of Abbreviations.....	xi
1: Introduction.....	1
1.1 Innate Immunity.....	1
1.2 Natural Killer Cells.....	2
1.3 Missing Self Hypothesis	4
1.4 Human Natural Killer Cells	5
1.5 Murine Natural Killer Cells.....	8
1.6 NK Cell Receptors.....	10
1.6.1 Killer Immunoglobulin Receptors	12
1.6.2 FcγRIII (CD16).....	13
1.6.3 Natural Cytotoxicity Receptors	13
1.6.4 NKG2 Receptors.....	14
1.6.5 Nkrp1/Clr	15
1.6.6 2B4	16
1.7 Natural Killer Cell Activation and Development.....	17
1.8 Ly49 Receptors	18
1.9 Structure and Binding of Ly49 Receptors.....	19
1.10 Activating Ly49 Receptors.....	22
1.10.1 Signalling of Activating Ly49 Receptors.....	22
1.10.2 Ly49H and MCMV Resistance.....	23
1.10.3 Ly49D and CHO Killing.....	23
1.11 Inhibitory Ly49 Receptors.....	24
1.11.1 The Inhibitory Signal.....	24
1.11.2 Inhibitory Ly49 and the Education of NK Cells.....	24
1.12 Natural Killer Cell Education.....	25
1.12.1 Educated NK cells	25
1.12.2 Unlicensed NK cells.....	26
1.13 Ly49 Haplotype Divergence	27
1.14 Rationale	30

1.14.1 Purpose	30
1.14.2 Objectives	31
2: Materials and Methods	33
2.1 Mice Strains	33
2.2 DNA Isolation for Genotyping and SNP Analysis	34
2.3 Southern Blot (RFLP Analysis) and Thymic DNA Isolation.....	36
2.4 Cell Lines	37
2.5 Antibodies and Flow Cytometry	38
2.6 MCMV Plaque Assays.....	39
2.7 Cytotoxicity Assay	40
2.8 Assessment of Interferon- γ Production and NK Cell Education	42
2.9 <i>In Vivo</i> Rejection of MHC-I-Deficient Cells	44
2.10 Statistical Analysis.....	45
3: Results.....	46
3.1 Characterization of the B6-Ly49 ¹²⁹ mouse strain.....	46
3.2 The 129-Ly49 Haplotype lacks Activating Receptors for Smith strain MCMV	53
3.3 B6-Ly49 ¹²⁹ NK cells exhibit B6-like cytotoxicity	55
3.4 The 129 Ly49 Haplotype is Sufficient for NK cell Licensing.....	58
3.5 B6-Ly49 ¹²⁹ NK cells Exhibit Enhanced Rejection Capabilities.....	64
4: Discussion.....	70
Appendices	82
Copyright Permission.....	83
Curriculum Vitae	98
Reference List	101

LIST OF FIGURES

Figure 1: Two subsets of human NK cells.....	6
Figure 2: The natural killer gene complex.	9
Figure 3: Activating and inhibitory Ly49 receptors	11
Figure 4: <i>Trans</i> and <i>cis</i> interaction of Ly49 receptors binding to MHC-I	21
Figure 5: Ly49 haplotypes in different inbred mouse strains.....	28
Figure 6: Ly49 haplotypes of B6 and 129 inbred mice.	29
Figure 7: RFLP patterns are identical between 129 and B6-Ly49 ¹²⁹ mice	47
Figure 8: Surface expression of Ly49 receptors on NK cells is similar between 129 and B6-Ly49 ¹²⁹ mice	49
Figure 9: MHC-I expression is similar across all three mice strains.....	51
Figure 10: Surface Expression of NKC and non NKC markers on B6- Ly49 ¹²⁹ NK cells.	52
Figure 11: 129 and B6-Ly49 ¹²⁹ are susceptibility to MCMV infection.....	54
Figure 12: Cytotoxic killing of tumor targets is similar between B6 and B6- Ly49 ¹²⁹ ALAK cells.....	56
Figure 13: Ly49R and Ly49G expressed on B6-Ly49 ¹²⁹ NK cells mediate CHO killing.	57
Figure 14: IFN- γ upon tumor target and antibody stimulation is similar between B6 and B6-Ly49 ¹²⁹ NK cells	59
Figure 15: YAC-I induced IFN-g production in educated subset of NK cells.	61
Figure 16: Responsiveness of the 129 Ly49 haplotype against YAC-1	62
Figure 17: Responsiveness of the 129 Ly49 haplotype upon α NKp46 antibody stimulation.....	63
Figure 18: Enhanced rejection of MHC-I-deficient cells by B6-Ly49 ¹²⁹ mice.....	65

Figure 19: 129 genetic contribution in chromosome 6 of the B6-Ly49 ¹²⁹ and B6.Ly49Q KO mouse strains.	67
Figure 20: Enhanced rejection of MHC-I-deficient cells by B6.Ly49Q KO mice is similar to B6-Ly49 ¹²⁹	69

LIST OF ABBREVIATIONS

129	129S1 mouse strain
ALAK	adherent lymphokine activated killer
APC	allophycocyanin
ATCC	American type culture collection
ACVS	animal care and veterinary services
ADCC	antibody dependant cell mediated cytotoxicity
B2m	B2-microglobulin
BID	BH3 interacting domain death agonist
Clr	C-type lectin related
B6	C57Bl/6 mouse strain
CCAC	Canadian council on animal care
CFSE	carboxyfluorescein succinimidyl ester
DNA	deoxyribonucleic acid
DAP-10	DNAX-activating protien 10
DAP-12	DNAX-activating protien 12
DMEM	Dulbecco's modified eagle's medium
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid

FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
HLA	human leukocyte antigen
ITAM	immune tyrosine-based activating motif
ITIM	immune tyrosine-based inhibiting motif
Ig	immunoglobulin
ILT	immunoglobulin like transcripts
ITSM	immunoreceptor tyrosine based switch motif
IFN	Interferon
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
KIR	killer immunoglobulin receptor
LLT1	lectin like transcript 1
LAIR	leukocyte associated Ig like receptor
LIR	leukocyte Ig like receptor
LRC	leukocyte receptor complex
MIP	macrophage-inflammatory protein
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
MEF	mouse embryonic fibroblast

MULT1	murine UL16-binding protein like transcript 1
NRC	natural cytotoxicity receptors
NK	natural killer
Ocil	osteoclast inhibitory lectin ligands
PAMP	pathogen associated molecular pattern
PMA	phorbol 12-myristate 13-acetate
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase
PLC	phospholipase C
PE	phycoerythrin
PFU	plaque forming unit
pDC	plasmacytoid dendritic cells
Poly I:C	polyinosinic:polycytidylic
PCR	polymerase chain reaction
PI	propidium iodide
RANTES	regulated upon activation normal T cell expressed and secreted
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RPM	rotations per minute
SNP	single nucleotide polymorphisms
SDS	sodium dodecyl sulfate

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- κ B 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 Fc γ RIII 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)-1 α , MIP-1 β 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 Fc γ RIII, no single activating receptor on its own is capable of triggering

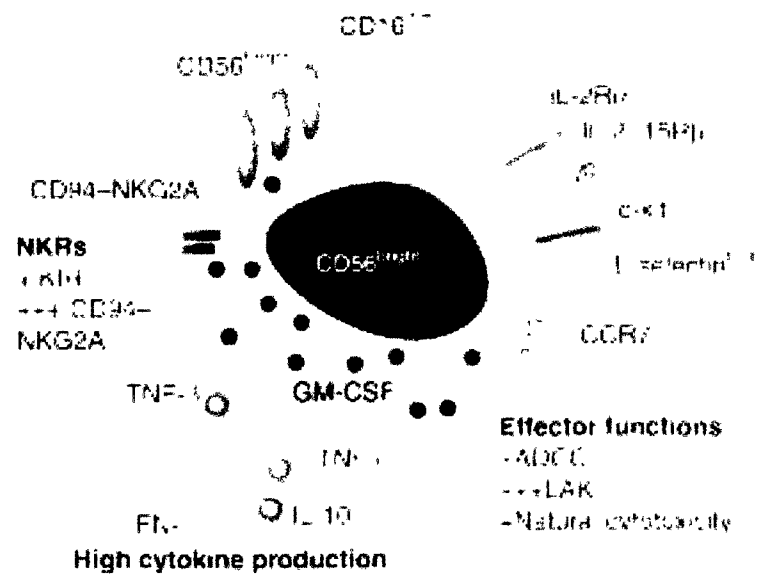
cytotoxicity or cytokine secretion in naïve NK cells (24). As opposed to the dominant B cell or T cell receptors, cross linking of multiple NK cell receptors is required for to reach the threshold for activation (92).

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 CD3 ϵ (35). The expression levels of CD56 and CD16 can

A



B

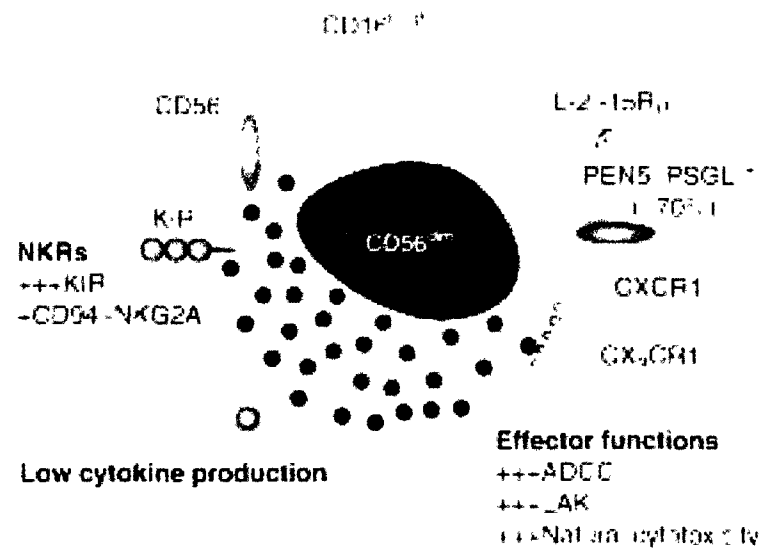


Figure 1: Two subsets of human NK cells. Human NK cells can be characterized by the surface expression of CD56 and CD16. A) NK cells with CD56^{bright}CD16^{dim} surface expression are characterized to be strong producers of cytokines such as IFN γ , TNF α/β and GM-CSF but have little cytotoxic ability. 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^{dim}CD16⁺ are primarily cytotoxic, representing 90% of the NK cells in the peripheral blood (144). In comparison, CD56^{bright}CD16⁻ 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^{dim}CD16⁺ cytotoxic NK cells express a higher density of KIRs while CD56^{bright}CD16⁻ 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^{bright}CD16⁻ NK cells and PEN5 by CD56^{dim}CD16⁺ NK cells are indicative of differential trafficking of each NK cell population (5, 50). Finally, CD56^{high}CD16⁻ 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^{bright}CD16⁻ NK cells with IL-2 or IL-12 enhances cytotoxicity to the level of CD56^{dim}CD16⁺ NK cells (145). Human NK cells express a wide variety of NK cell receptors including NKG2D, CD94/NKG2, CD16, NCR 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

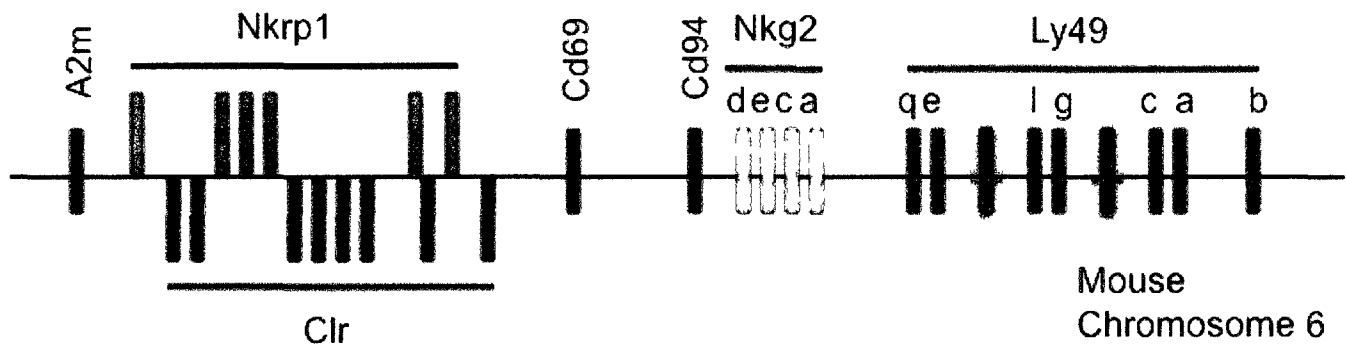
lectin-like NKG2 receptors are encoded in the natural killer cell gene complex (NKC) region at chromosome 12p12-p13 (143).

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 CD11b^{lo}CD16⁻CD69^{high} exhibit similar enhanced cytokine production and lower cytotoxicity, possibly homologous to human CD56^{bright}CD16⁻ 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/Clr*, *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 *Ly49I*, 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*.

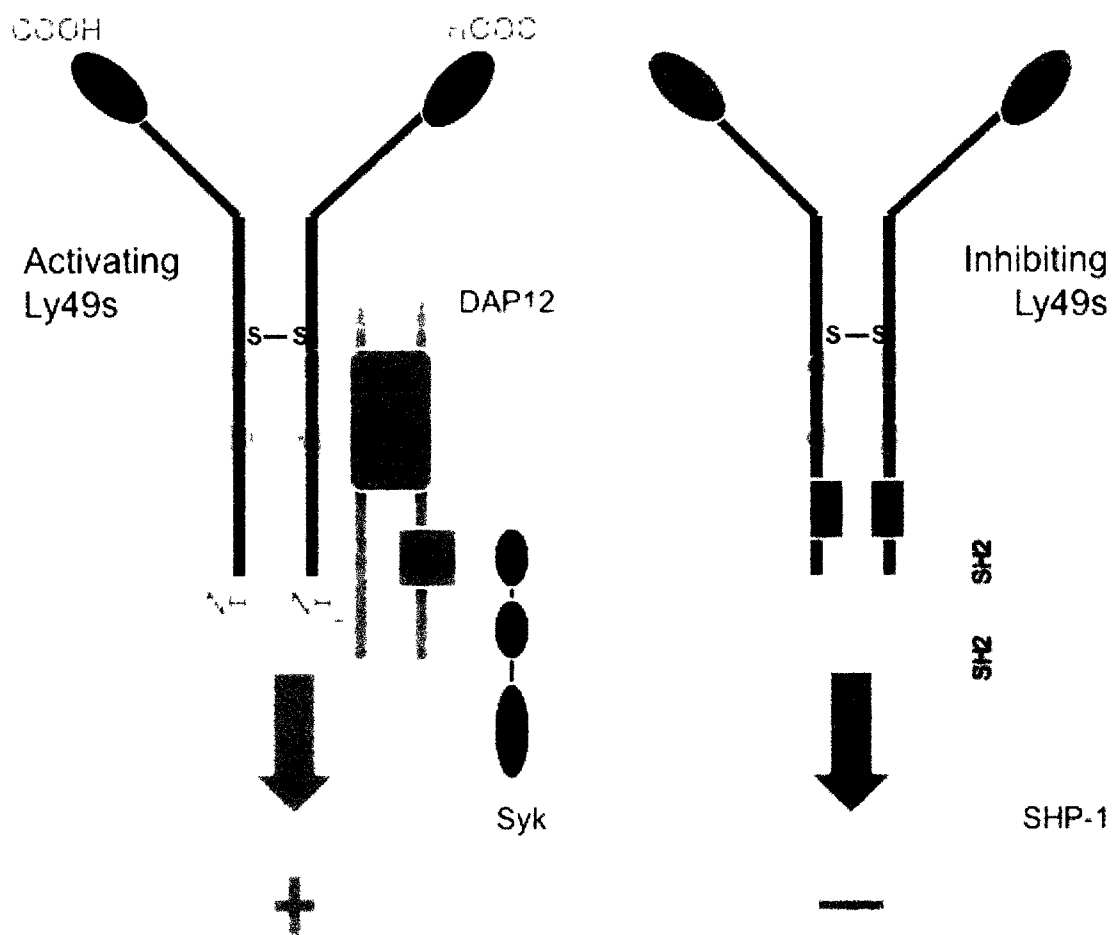
NK Gene Complex



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/IX₆₋₈YXXL/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.



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 α 1 domain and the second extracellular-Ig like domain binds to α 2 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 Bw4-80Ile 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 Fc γ RIII (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 Fc γ RIIIA while murine NK cells express the general Fc γ RIII (136). Human Fc γ RIIIA receptors deliver an activating signal to induce cytotoxicity through the association with a homodimers of CD3 ζ or Fc ϵ RI- γ or in a heterodimer association of CD3 ζ and Fc ϵ RI- γ together (92, 95). In contrast, murine Fc γ RIII only associates with Fc ϵ RI- γ as a homodimer (92). Upon activation, the human Fc γ RIIIA 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 CD3 ζ or Fc ϵ RI γ adapter molecules in either a homodimer or heterodimer (119). NKp44 and NKp30 contain a single Ig-like extracellular domain but associate with CD3 ζ 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 $\alpha\beta$ and $\gamma\delta$ 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 $\alpha 1, \alpha 2$ and $\alpha 3$ MHC-I domains, but lack $\beta 2m$ 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 $\alpha 1$ and $\alpha 2$ MHC-I domains anchored to the membrane by glycosylphosphatidylinositol (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 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-Ia-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: TIYXXV/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 β (CD122) (158). In mice, NKPs also acquire the expression of growth factor receptors such as cKit, Flt3, IL7R α and IL15R α (147). Human NKPs express CD38 in addition to cKit, Flt3, and IL15R α . 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 HSCs 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, NKPs downregulate the expression of Flt3 and IL7R α and upregulate IL2R β , CD2 and 2B4 (34). NKPs 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 NCRs (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 α have little to no NK cells (13,

100). 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 IFN α , 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-15R α 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

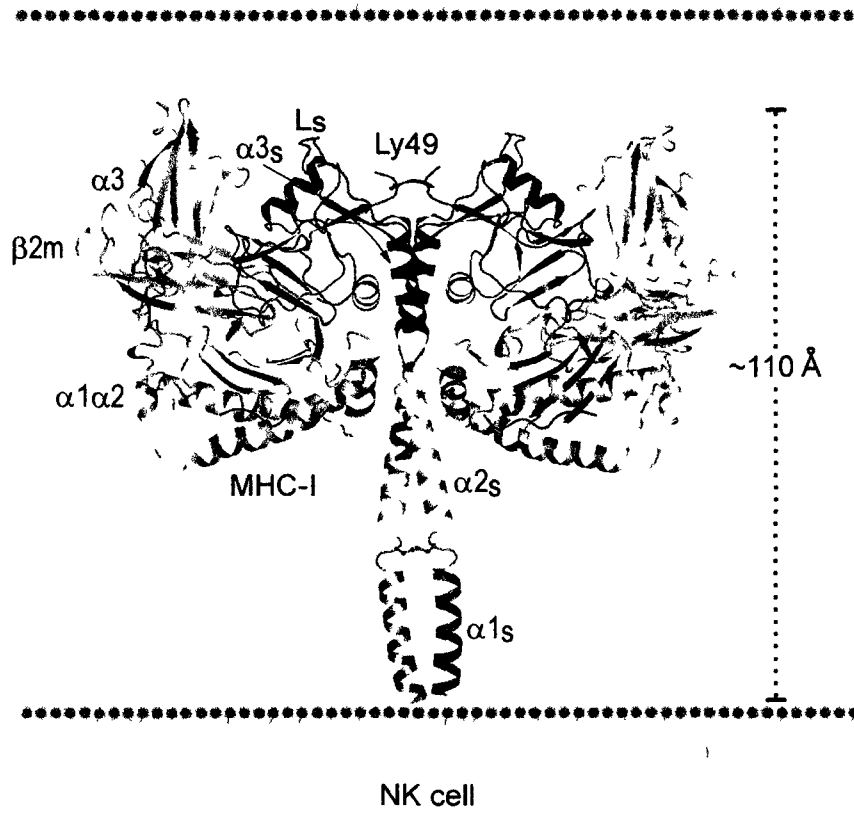
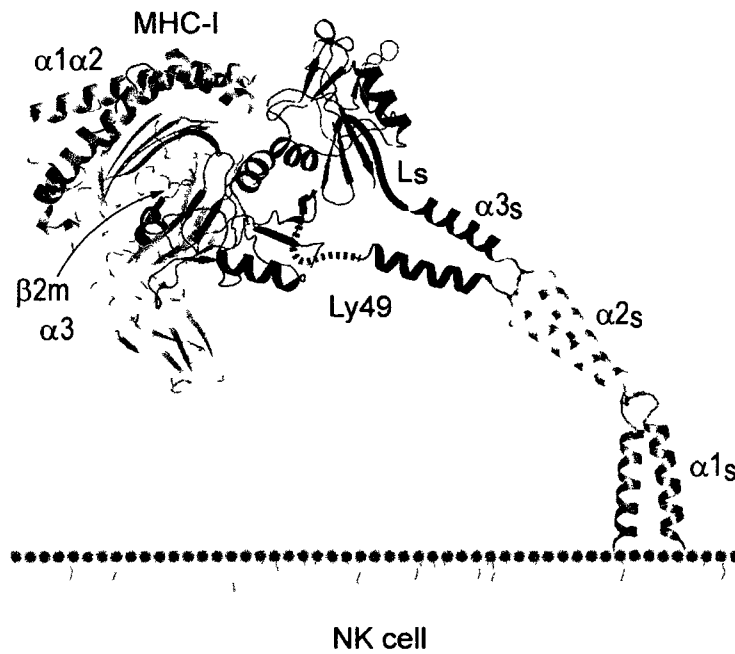
The MHC-I binding Ly49 receptors can interact with its ligand in two distinct conformations. These interactions can either occur in *cis* or in *trans* depending on whether the bound MHC is located on a target cell or the Ly49 bearing NK cell itself (11). A *trans* interaction is postulated to occur 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.

Target cell

**B**

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 (PLC γ) 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 mice 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, *Cmv*^r 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 tumorigenic target cells, as stated by the “missing self” hypothesis (98). NK cells developing in mice deficient in MHC-I expression, using $\beta 2m$ 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^d 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^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 a 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 $\beta 2m$ -deficient mice or the Ly49C/I⁻NKG2A⁻ 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.

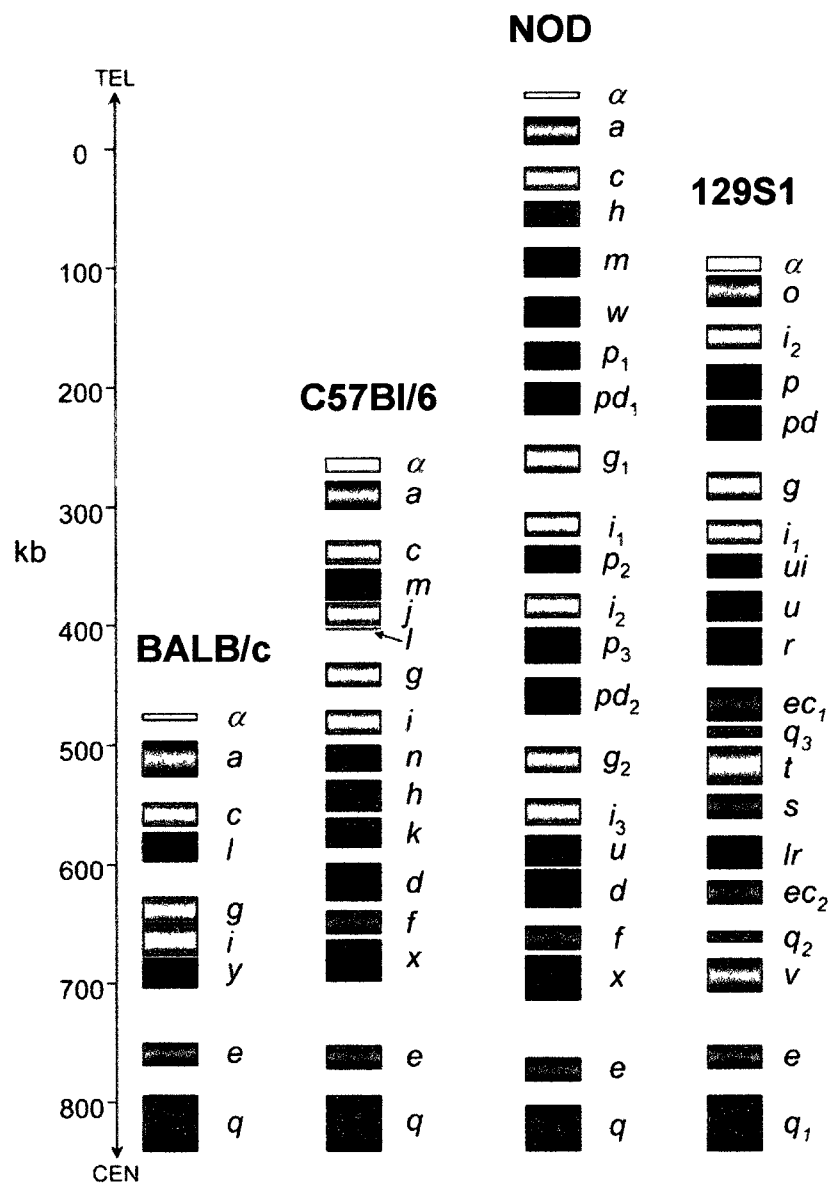
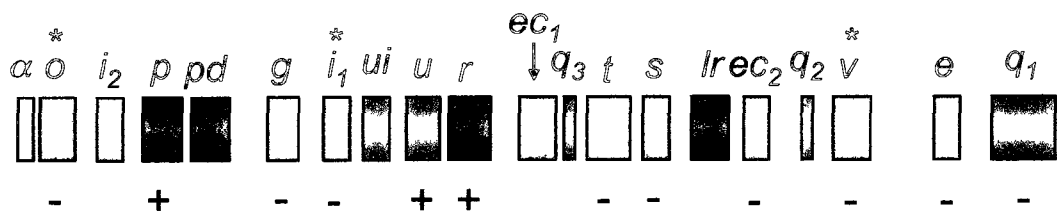


Figure 6: Ly49 haplotype of B6 and 129 inbred mice. The 129 Ly49 haplotype is larger in comparison to the B6 haplotype. The 129 haplotype contains 3 activating and 9 inhibitory receptors, of which Ly49I, O and V are receptors for self MHC-I. In comparison, B6 contain 2 activating and 8 inhibitory receptors with Ly49C and Ly49I as inhibitory receptors towards self MHC-I. Adapted from (15) with permission from Macmillan Publishers Ltd.

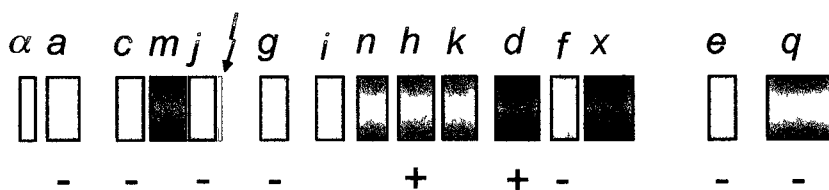
129S1



S:I:Ψ

3:9:7

C57BL/6



2:8:5

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-Ly49¹²⁹ 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 *in vitro* and *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-Ly49¹²⁹ 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-Ly49¹²⁹ 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.Ly49¹²⁹ 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/SvImJ (stock# 002448) and $\beta 2m^{-/-}$ mice (B10.129P2(B6)- $\beta 2m^{tm1Unc}/J$; stock # 002454) on the B6 background were purchased from Jackson Laboratories (Bar Harbor, Maine). Additional B6 mice strains deficient in specific MHC-Ia molecule expression including H-2K $^{b-/-}$ (B6.129P2-H2-Kb $^{tm1}N12$ model # 4216) H-2D $^{b-/-}$ (B6.129P2-H2-Db $^{tm1}N12$ model # 4217) and H-2K $^{b-/-}$ D $^{b-/-}$ double knockout (B6.129P2-H2-Kb $^{tm1}H2-Db^{tm1}N12$ model # 4215) were purchased from Taconic Farms (Hudson, New York).

The B6-Ly49¹²⁹ congenic mouse strain possesses the Ly49¹²⁹ 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-Ly49¹²⁹ 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 100ug/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. 400uL 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 100μL 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*¹²⁹ forward and *Ly49v*¹²⁹ reverse (see above for sequence) were used for genotyping by PCR for the B6-*Ly49*¹²⁹ 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 ddH₂O for 30 minutes, and then treated with depurination solution (0.25 M HCl in ddH₂O) for 20 minutes, denaturation solution (2.5 M NaCl and 0.5 M NaOH in ddH₂O) and neutralization solution (0.15 M NaCl and 0.5 M Tris-HCl pH 8.0 in ddH₂O). 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-Ia-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-TCR β (H57-597), anti-CD94 (18d3), anti-Ly49A/D (12A8), anti-Ly49C//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^{B6} (Cwy-3), anti-Ly49G (4D11), anti-Ly49C/I (5E6), anti-IFN- γ (XMG1.2), anti-H2K^b (AF6-88.5), anti-H2D^b (28-14-8) and anti-Fc γ RIII (2.4G2) were purchased from BD Bioscience (Mississauga, Ontario). Anti-Ly49H^{B6} (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 Fc γ RIII 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 μ g 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 μ L 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 μ L 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 μ M 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/O) were labelled with 80 μ Ci sodium 51-chromate diluted in PBS (MP Biomedicals, Solon, Ohio) for one hour at 37°C with 5% CO₂ in a humidified atmosphere. 5 000 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 μ L 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 μ g/mL of purified rat IgG_{2a}K, 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 μ L/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, 100 μ L 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.

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

2.8 Assessment of Interferon- γ Production and NK Cell Education

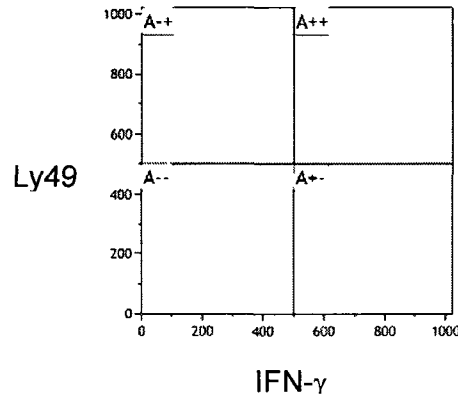
To quantify the production of IFN- γ by NK cells from each mouse strain, splenocytes were collected from mice pretreated with 150 μg of poly(I:C) for 18 hours by crushing the spleen and incubated with 5 mL of ACK lysis buffer for 5 minutes at room temperature, upon which the reaction is stopped by addition 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 $\mu\text{g/mL}$ of anti-NKp46 and 5 $\mu\text{g/mL}$ 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 μL 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 μ L for each well. For pharmacological stimulation, 10 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ g/mL ionomycin was diluted in NK media and 100 μ L 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 μ L 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-TCR β 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 1 μ g PE-IFN- γ diluted in 50 μ L 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⁺TCR β ⁺ 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 (Ly49G¹²⁹), 4E5 (Ly49O/V¹²⁹), 14B11 (Ly49I¹²⁹) and NKG2A (Qa-1) during the staining for CD49b and TCR β 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:



$$\text{fold induction} = \frac{\left(\frac{A++}{A-+} \right)}{\left(\frac{A+-}{A--} \right)}$$

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-2K^{b-/-}, D^{b-/-} and K^{b-/-}D^{b-/-} 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 μ g of poly I:C (Sigma) dissolved in 200 μ L 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 μ M 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 μ M and 0.5 μ M carboxyfluorescein succinimidyl ester (CFSE, Intvitrogen), 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^{high}) to wildtype (CFSE^{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

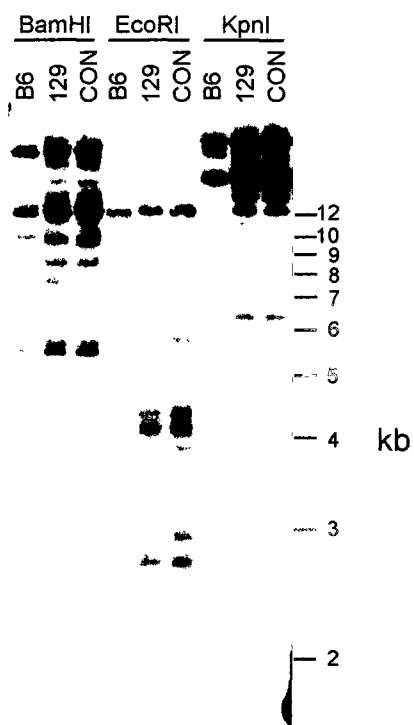
Statistical significance was determined by student t test with a cutoff P value of 0.05 using the Prism software (version 4.0b). Data are presented as mean ±SEM and ±SD .

3: RESULTS

3.1 Characterization of the B6-Ly49¹²⁹ mouse strain

The B6-Ly49¹²⁹ 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 *Ly49v*¹²⁹. 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 *Ly49o* and *Ly49e* (104). The B6-Ly49¹²⁹ mouse exhibits an RFLP pattern similar to the 129 mouse strain. The B6 RFLP pattern differs from both 129 and B6-Ly49¹²⁹ (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-Ly49¹²⁹ mouse strain possesses 129 *Ly49* haplotype. These results were further verified by SNP analysis, which indicated that the NKC region of the B6-Ly49¹²⁹ 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 and 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-Ly49¹²⁹ mouse strain may differ due to the genetic background of the B6 mouse. To test whether the Ly49 expression patterns of B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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 *Ly49g*¹²⁹ and not to *Ly49g*^{B6} (106). The results show that overall B6-Ly49¹²⁹ 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-Ia molecules, specifically H-2K^b and H-2D^b molecules, but not H-2L. MHC-Ia expression can influence the expression of Ly49 receptors as well as the education of mature NK cells (74). The B6-Ly49¹²⁹ mouse should have the same MHC-Ia expression as the B6 and 129 mice strain, however different 14B11 staining patterns may indicate otherwise, as MHC-Ia expression has been shown to influence Ly49 expression (Fig. 9) (48). To verify similar MHC-Ia expression levels in all three

Figure 8: Surface expression of Ly49 receptors on NK cells is similar between 129 and B6-Ly49¹²⁹ mice. NK cells from B6, 129, B6-Ly49¹²⁹ (CON) and B6.Ly49Q KO (Ly49Q^{-/-}) 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 $\pm$ SD.

C57Bl/6

CON

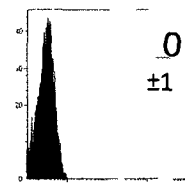
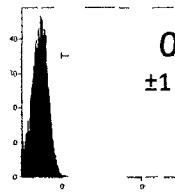
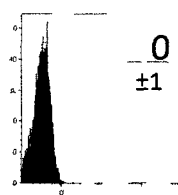
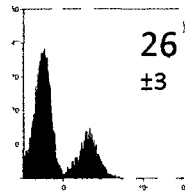
129S1

Ly49Q^{-/-}

Cwy3

B6: Ly49G

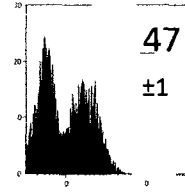
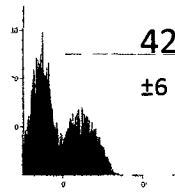
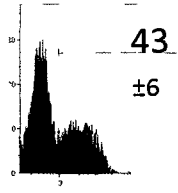
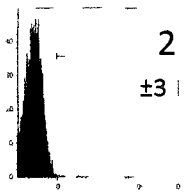
129: ∅



AT8

B6: ∅

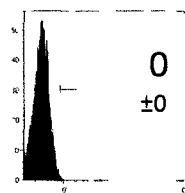
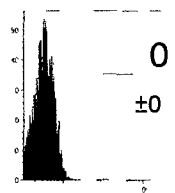
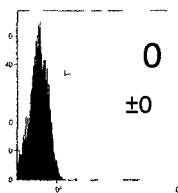
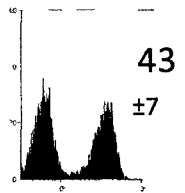
129: Ly49G



3D10

B6: Ly49H

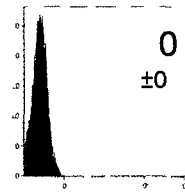
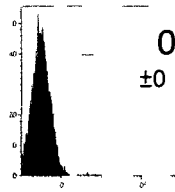
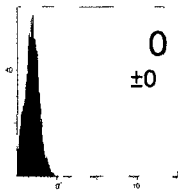
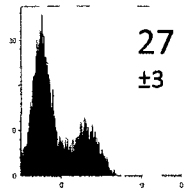
129: ∅



5E6

B6: Ly49C/I

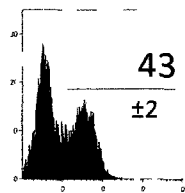
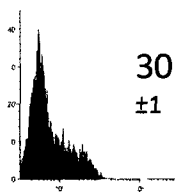
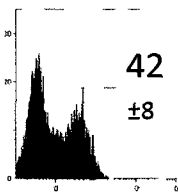
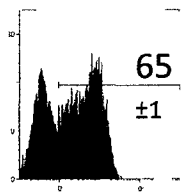
129: ∅



14B11

B6: Ly49C/I/F/H

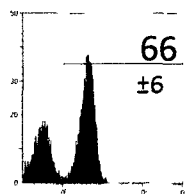
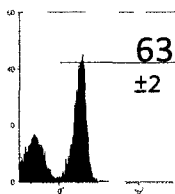
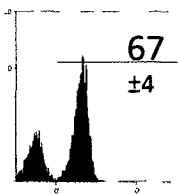
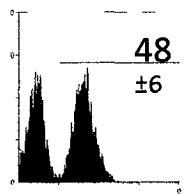
129: Ly49I



12A8

B6: Ly49A/D

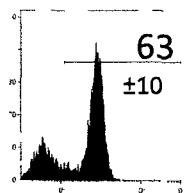
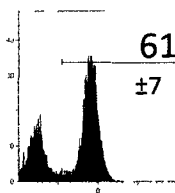
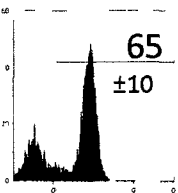
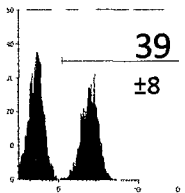
129: Ly49R



4E5

B6: Ly49D

129: Ly49O/V/R



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-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ (CON) mouse strains was assessed for the expression of MHC-I molecules A) H-2K^b and B) H-2D^b. Data shown are means of three separate experiments and is shown with $\pm$ SEM.

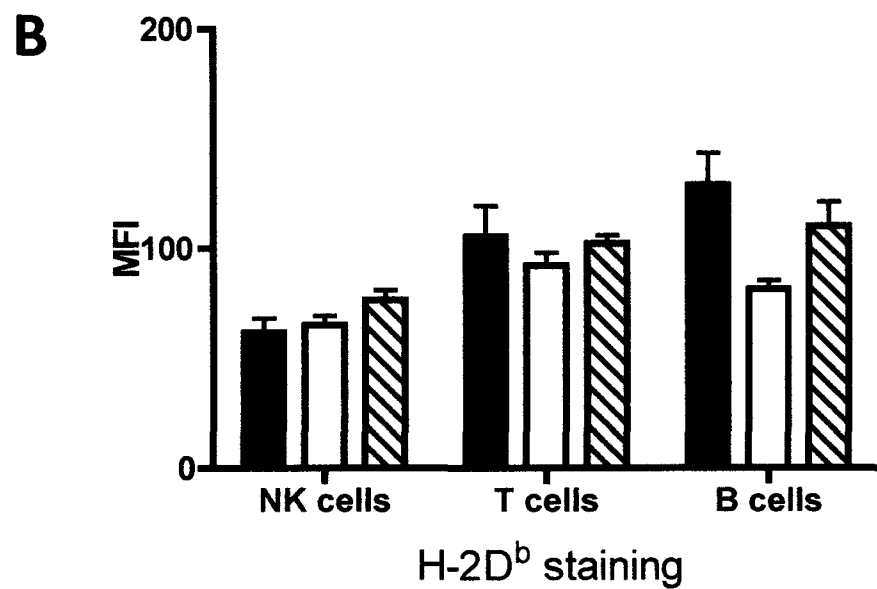
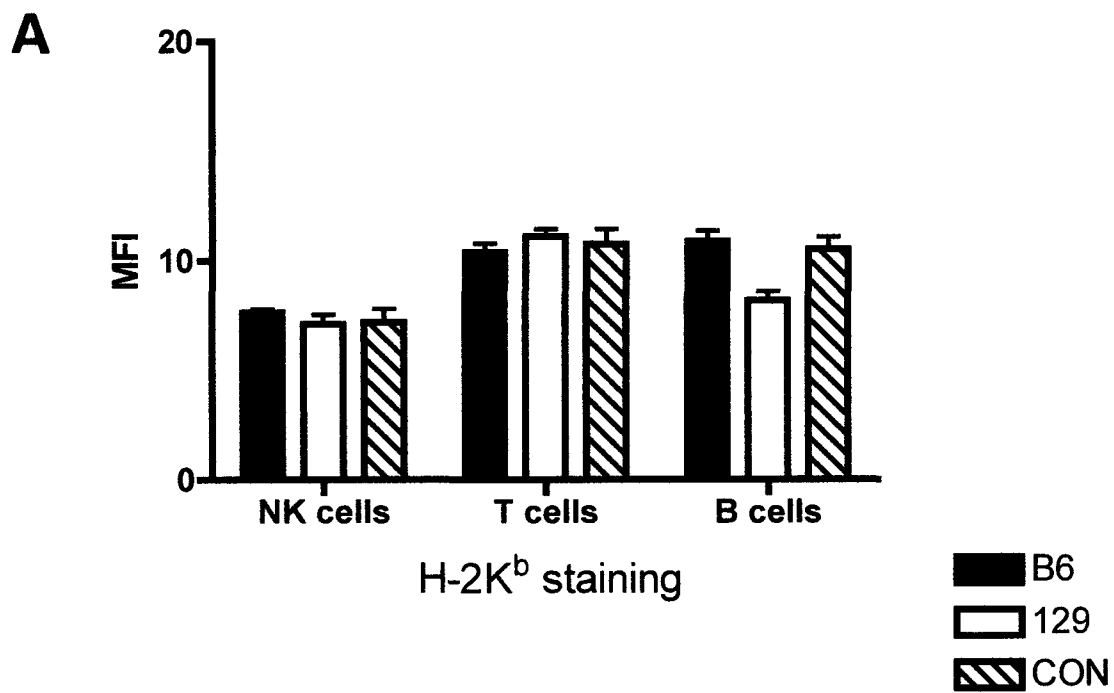
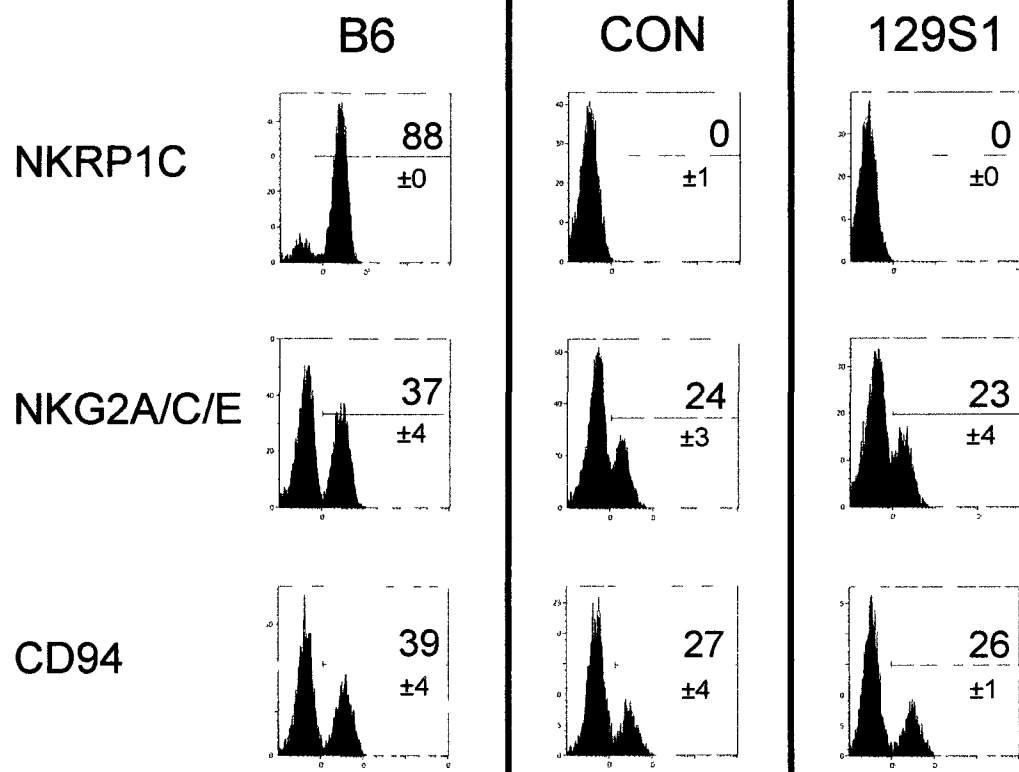
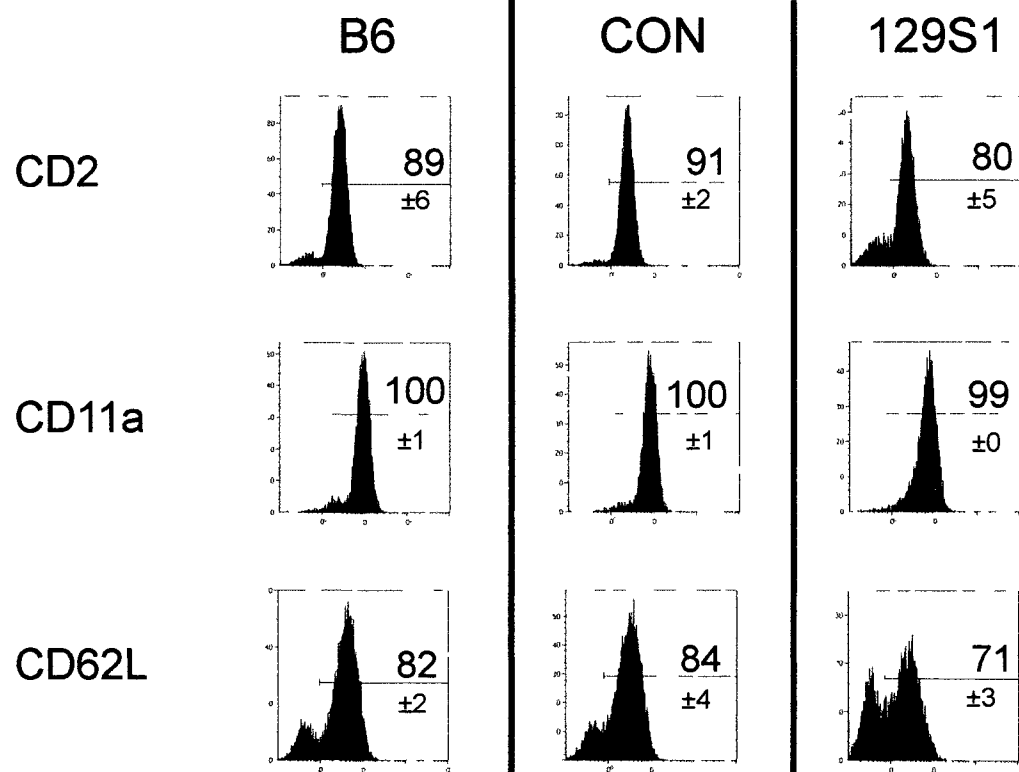


Figure 10: Surface expression of NKC and non NKC markers on B6-Ly49¹²⁹ NK cells. NK cells from B6, 129, and B6-Ly49¹²⁹ (CON) were stained with antibodies against A) markers within the NKC region: NKrp1c^{B6}, NKG2A/C/E and CD94 or B) markers for NK maturation outside the NKC region: CD2, CD11a and CD62L. Data show with mean $\pm$ SD and are representative of three separate experiments.

A



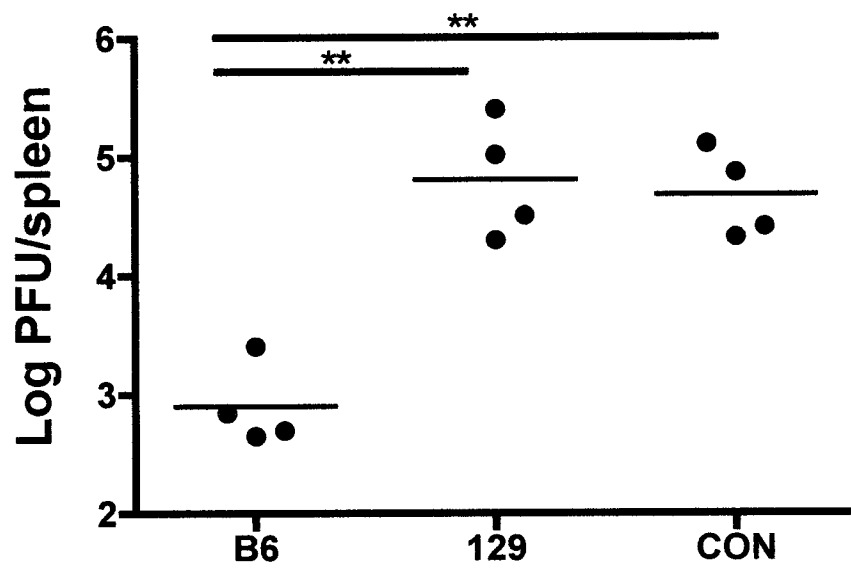
B



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-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ are susceptible to MCMV infection. B6, 129 and B6-Ly49¹²⁹ (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-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ ALAK cells exhibit identical high killing of YAC-1 compared to 129 (Fig. 12). This phenotype was repeated by the xenogenenic target YB2/O, a rat tumor cell line (Fig. 12). Overall the results suggest that the cytotoxic activity of the B6-Ly49¹²⁹ 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-Ly49¹²⁹ and 129 ALAK. The CHO target cells were killed minimally by 129 ALAK. Interestingly, B6-Ly49¹²⁹ 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-Ly49¹²⁹ ALAK cells. ALAK cells were grown in culture for six days from B6, 129 and B6-Ly49¹²⁹ (CON) NK cells. These ALAK were incubated with a panel of NK tumor target cells loaded with ⁵¹Cr 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.

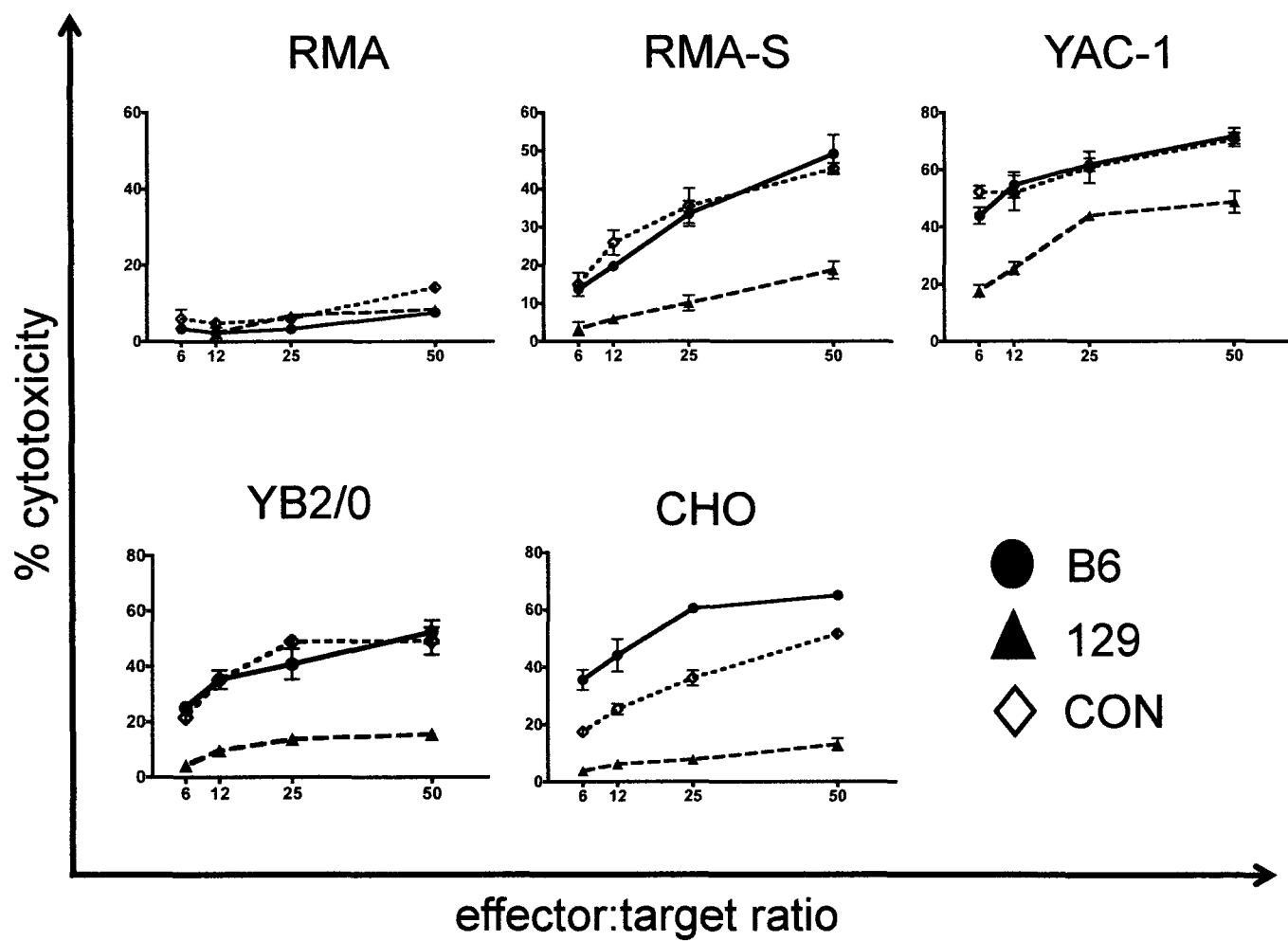
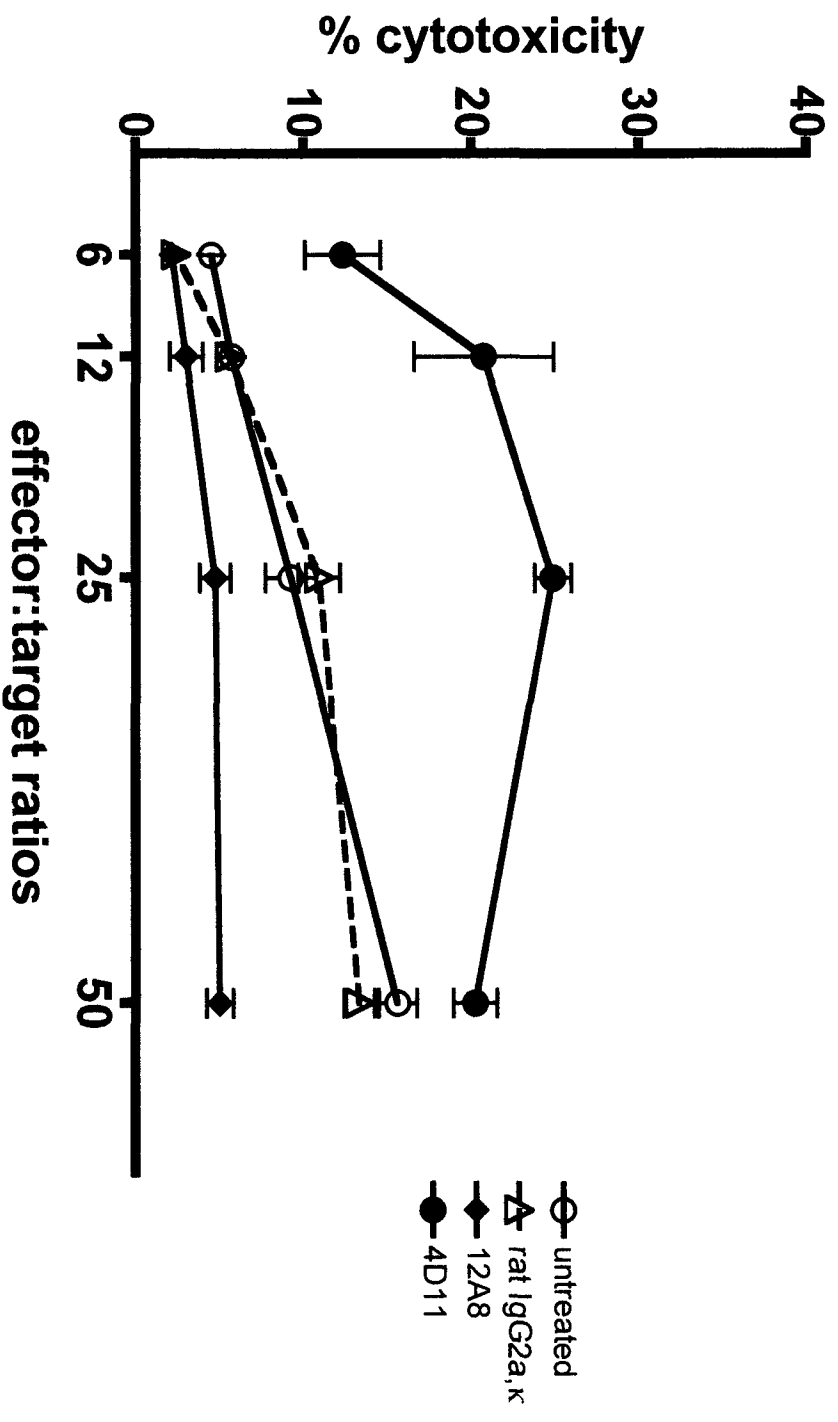


Figure 13: Ly49R and Ly49G expressed on B6-Ly49¹²⁹ NK cells mediate CHO killing. The cytotoxic response of B6-Ly49¹²⁹ (CON) ALAK cells was assessed against CHO target cells treated with monoclonal antibodies 12A8 (anti-Ly49R), 4D11 (anti-Ly49G) and rat IgG2a, κ isotype. The mean percent of target lysis (mean $\pm$ SD) is indicated from triplicate values. Data is representative of three independent experiments.



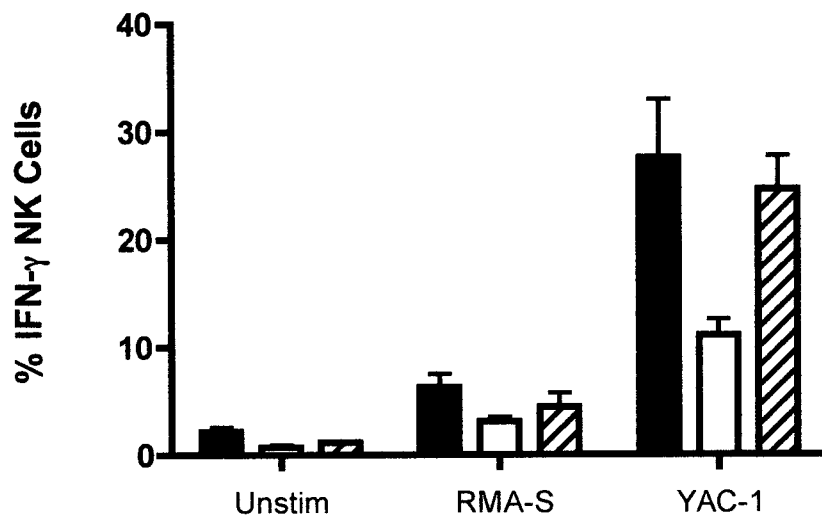
intermediate phenotype may be attributed to the expression of Ly49R¹²⁹, an allelic variant of Ly49D^{B6} with 94.7% sequence homology at the amino acid level (104, 115). To verify that the intermediate CHO killing by B6-Ly49¹²⁹ ALAK cells is Ly49R¹²⁹-mediated, cytotoxicity assays in the presence of an mAb that blocked Ly49R¹²⁹ were performed. CHO cells were killed at a higher rate when B6-Ly49¹²⁹ 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-Ly49¹²⁹ NK cells with 12A8, which blocks the activating receptor Ly49R¹²⁹, yielded the lowest killing of CHO target cells (Fig. 13), suggesting that CHO killing in the B6-Ly49¹²⁹ ALAK cells is mediated by Ly49R¹²⁹.

3.4 The 129 Ly49 Haplotype is Sufficient for NK cell Licensing

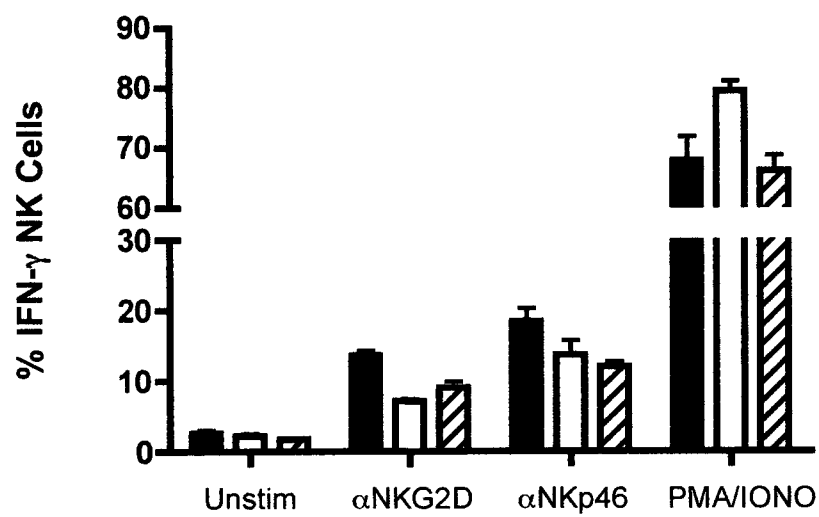
To further assess the functional capacity of B6-Ly49¹²⁹ NK cells, IFN- γ production was tested. NK cells were freshly isolated from all three mouse strains previously activated with poly I:C, and were stimulated with either YAC-1 or RMA-S tumor cells, plate bound antibodies towards NKp46 or NKG2D activating receptors or the pharmacological agents phorbol 12-myristate 13-acetate (PMA) and ionomycin to trigger the production of IFN- γ . Upon YAC-1 stimulation, B6 and B6-Ly49¹²⁹ NK cells exhibited two to three times more IFN- γ producing cells than 129 NK cells (Fig. 14A). RMA-S stimulation did not lead to a high percentage of IFN- γ 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- γ producing NK cells from B6 mice

Figure 14: IFN- γ upon tumor target and antibody stimulation is similar between B6 and B6-Ly49¹²⁹ NK cells. A) Upon stimulation with RMA and YAC-1 tumor cells, IFN- γ ⁺ 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 ⁺ 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 $\pm$ SD of three mice of each strain and is representative of three separate experiments.

A



B



compared to 129 and B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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 express 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 haplotype, B6-Ly49¹²⁹ 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-Ly49¹²⁹ (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.

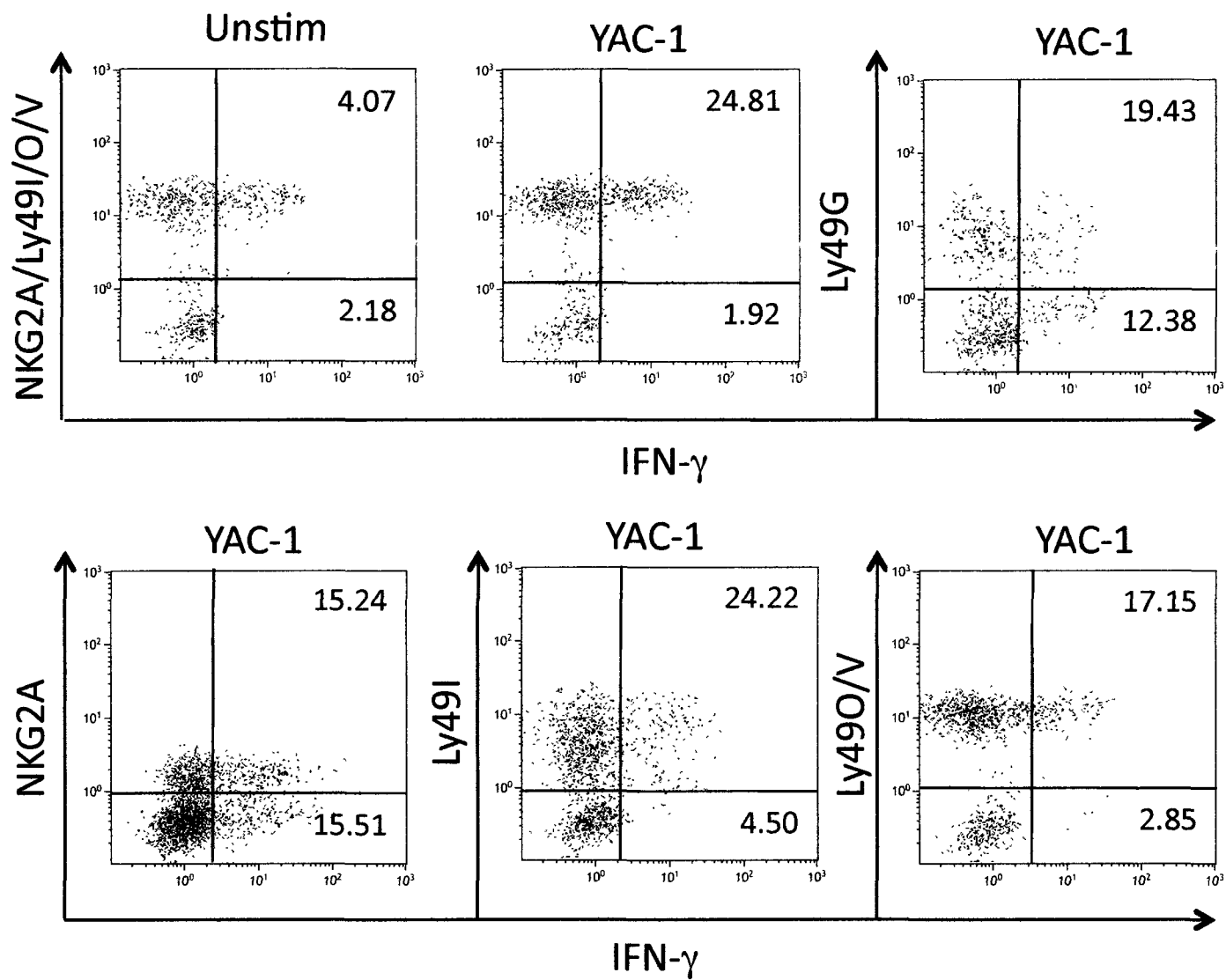
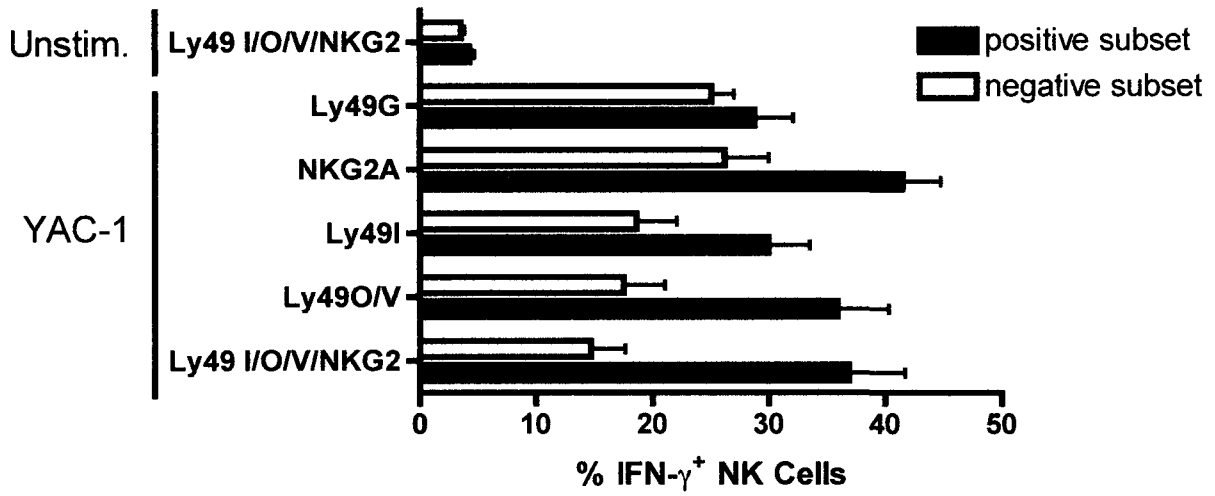


Figure 16: Responsiveness of the 129 Ly49 haplotype to YAC-1 cells. Following YAC-1 stimulation of B6-Ly49¹²⁹ (CON) NK cells A) IFN- γ ⁺ cells were analyzed based on the expression of Ly49I, Ly49O, Ly49V, Ly49G and NKG2A into positive and negative subsets. B) Fold induction of IFN- γ was assessed based on the division of Ly49⁺ over Ly49⁻ of IFN- γ producing NK cells. Data are shown as the mean $\pm$ SD of three mice of each strain and is representative of three separate experiments.

A



B

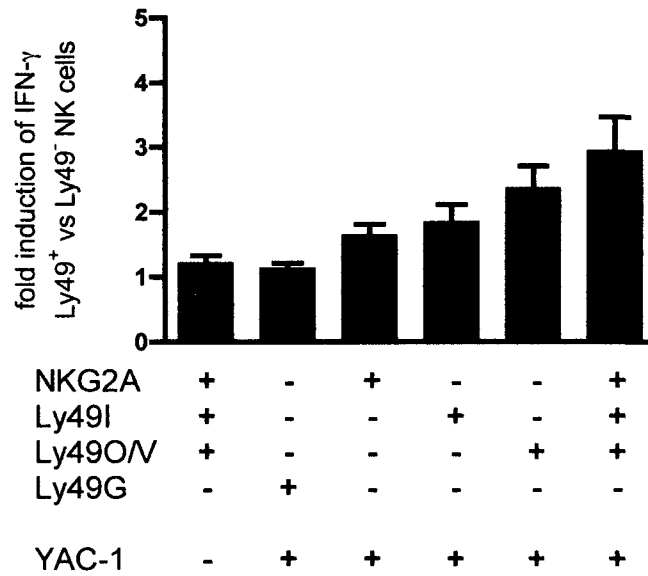
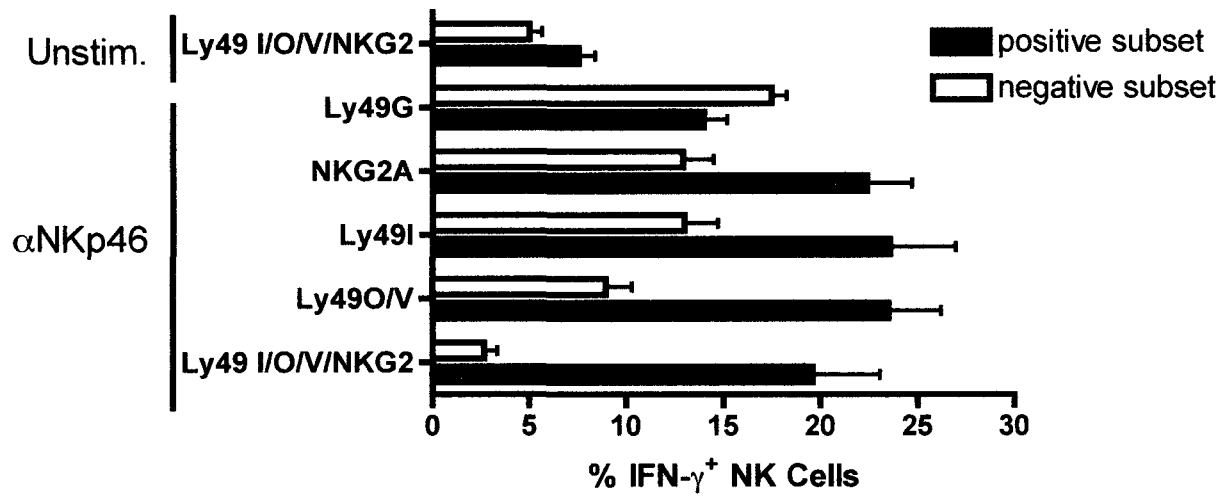
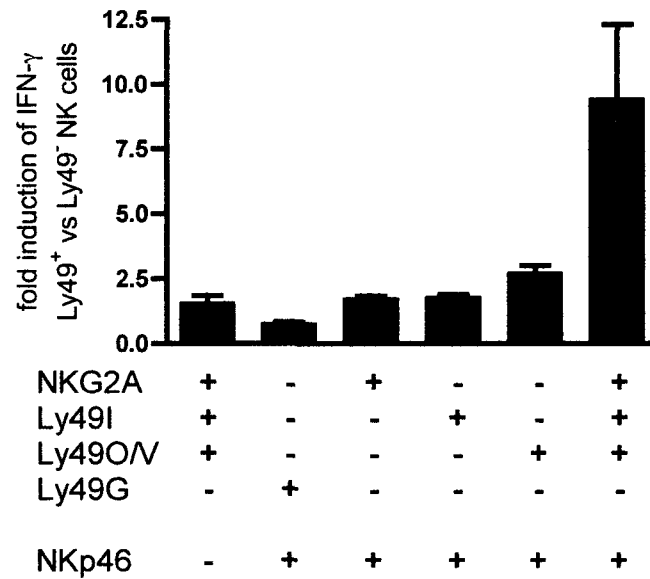


Figure 17: Responsiveness of the 129 Ly49 haplotype upon α NKp46 antibody stimulation. Following α NKp46 stimulation of B6-Ly49¹²⁹ (CON) NK cells A) IFN- γ ⁺ NK cells were analyzed based on the expression of Ly49I, Ly49O, Ly49V, Ly49G and NKG2A into positive and negative subsets. B) Fold induction of IFN- γ was assessed based on the division of Ly49⁺ over Ly49⁻ of IFN- γ producing NK cells. Data is shown as the mean $\pm$ SD of three mice of each strain and is representative of three separate experiments.

A



B



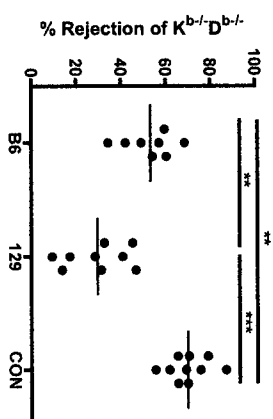
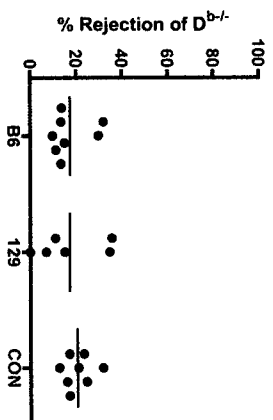
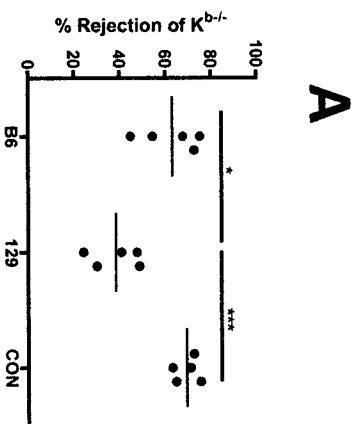
B). Ly49G was used as a negative control as it does not bind to H-2K^b or H-2D^b MHC molecules and is thus a non-self receptor. With both YAC-1 and NKp46 stimulation, Ly49G positive NK cells are not more likely to produce IFN- γ than the negative population, similar to the unstimulated NKG2A/Ly49I, O, 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- γ than Ly49I-negative or Ly49O/V-negative NK cells (Fig. 17). In contrast, NKG2A/Ly49I,O or V positive NK cells were 10-fold more likely to produce IFN- γ 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.

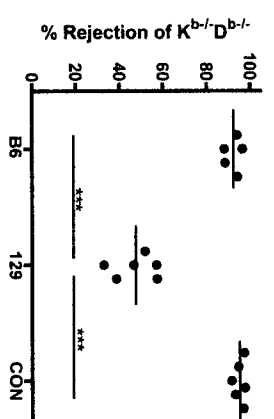
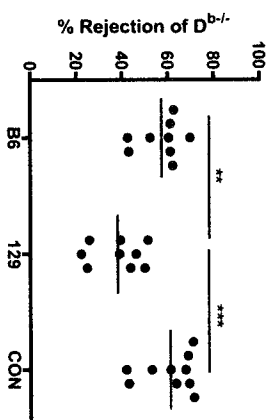
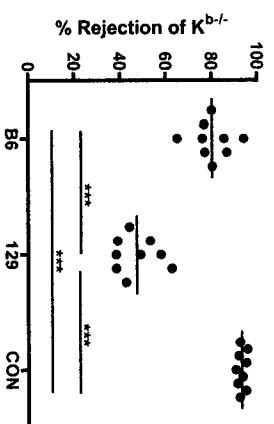
3.5 B6-Ly49¹²⁹ NK cells Exhibit Enhanced Rejection Capabilities

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-Ly49¹²⁹ mice A) Unstimulated or B) poly(I:C) treated mice were injected i.v. with wildtype an MHC-I-deficient cells (H-2K^{b-/-}, H-2D^{b-/-} and H-2K^{b-/-}H-2D^{b-/-}) 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.



B



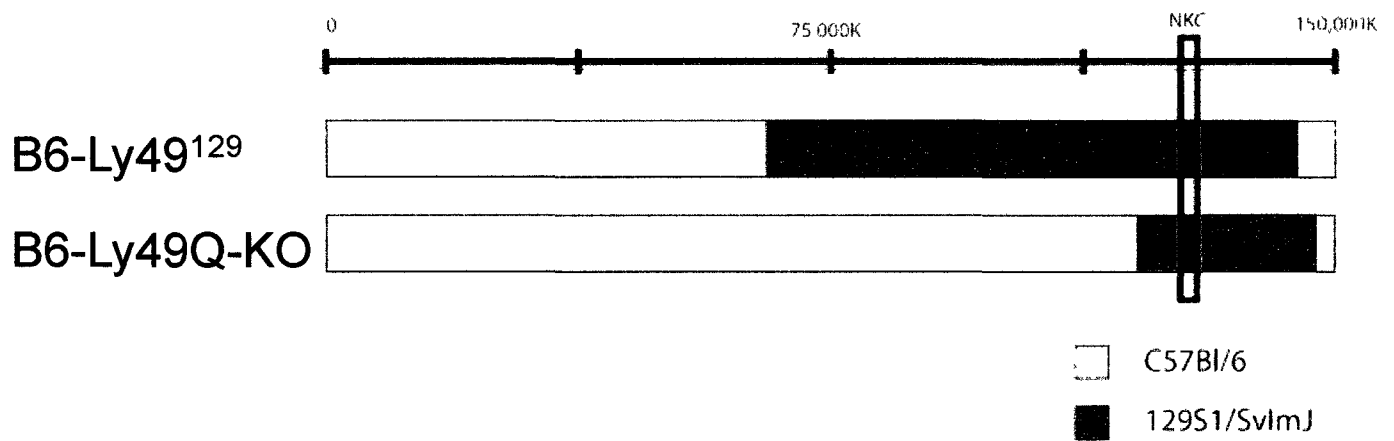
was based on any changes from the input ratio of MHC-sufficient and –deficient injected cells. MHC-sufficient cells serve as a reference for the relative rejection of MHC-I deficient cells. Rejection of $K^{b-/-}$ cells was higher by 20% in both the B6 and B6-Ly49¹²⁹ mouse strains in comparison to the low rejection of 129 mice (Fig. 18A). Rejection of $D^{b-/-}$ cells was too low to assess any differences between the mouse strains. Strikingly, rejection of the double knockout $K^{b-/-}D^{b-/-}$ cells was highest in B6-Ly49¹²⁹ 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 $D^{b-/-}$ cells, recipient mice were injected with poly(I:C) to stimulate the NK cell population and increase the rejection of MHC-deficient cells. Poly(I:C) primes NK cells by stimulating dendritic cells to secrete IL-12, IFN α and the trans-presentation of IL-15 (102). Rejection of $D^{b-/-}$ cells was higher in B6 and B6-Ly49¹²⁹ mice, both of which exhibit 20% more rejection of MHC-deficient cells than 129 mice (Fig. 18B). However, rejection of $K^{b-/-}$ cells with poly(I:C) stimulation was highest in B6-Ly49¹²⁹ mice, which showed, 10% higher rejection than B6 mice and 40% higher rejection than 129 mice, similar to the rejection of $K^{b-/-}D^{b-/-}$ cells by untreated mice. B6 and B6-Ly49¹²⁹ mice exhibited no significant difference in their ability to reject $K^{b-/-}D^{b-/-}$ cells, however both were about 40% higher than 129 mice (Fig. 18).

Higher rejection of MHC-I-deficient cells by B6-Ly49¹²⁹ 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-Ly49¹²⁹ and B6.Ly49Q KO mouse strains. Using SNP analysis, the genetic origin of the NKC encoding chromosome 6 was analyzed for both B6-Ly49¹²⁹ and B6.Ly49Q KO mouse strains. The 129 genetic content spans from nucleotides 79,759,628 to 138,203,431 for the B6-Ly49¹²⁹ mouse and from nucleotides 118,022,343 to 149,214,236 for the B6.Ly49Q-KO mouse.

Mus Musculus Chromosome 6

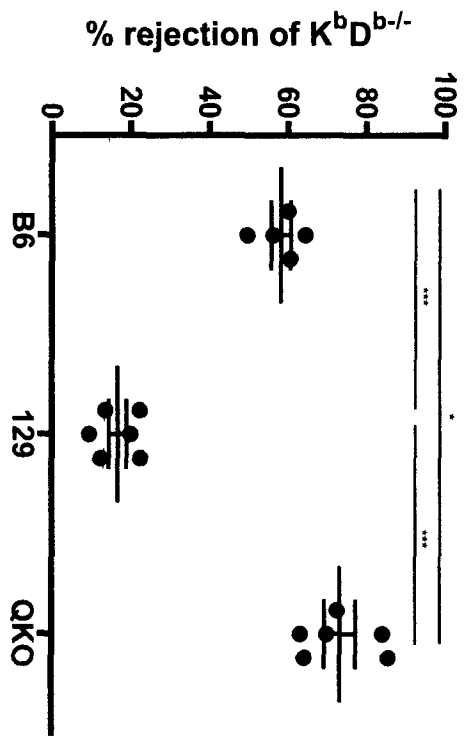


with the *Ly49* gene cluster, one may argue that these genes also contribute to the effector functions of the NK cell population. To address this possibility, *in vivo* rejection of $K^{b-/-}D^{b-/-}$ and $Kb^{-/-}$ cells by untreated and poly(I:C) treated mice respectively was assessed using B6.Ly49Q-KO mice. These mice also express the 129 Ly49 haplotype on the B6 genetic background at similar levels to the B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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).

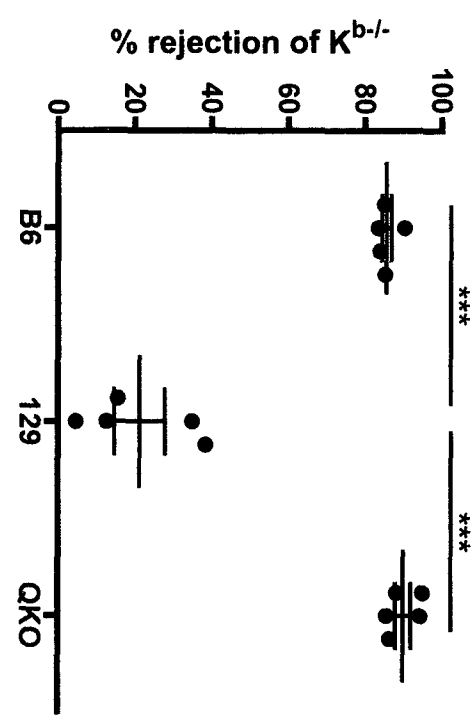
Collectively, these results indicate that the B6-Ly49¹²⁹ mouse strain is capable of discriminating and rejecting MHC-I deficient cells. Therefore, the 129 Ly49 haplotype is sufficient to educate NK cells to reject MHC-I deficient cells *in vivo*, possibly even to a greater extent than the B6 Ly49 haplotype.

Figure 20: Enhanced rejection of MHC-I-deficient cells by B6.Ly49Q KO mice is similar to B6-Ly49¹²⁹. A) Rejection of H-2Kb^{-/-}H-2Db^{-/-} by unstimulated mice and B) rejection of H-2Kb^{-/-} 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

A



B



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-Ly49¹²⁹ congenic model provides a unique opportunity to study Ly49 haplotype variations and its effects on NK cell functions. Our results show that the B6-Ly49¹²⁹ congenic NK cells were able to kill tumor targets *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 haplotype is sufficient to educate NK cells, a process only previously validated in the B6 mouse strain (49, 86). Strikingly, the B6-Ly49¹²⁹ NK mice were able to kill MHC deficient target cells more efficiently than both the B6 and 129 *in vivo* (Fig. 18).

The B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ 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 *Ly49q₁* 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-Ly49¹²⁹ (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-Ly49¹²⁹ strain. Both the B6.Ly49Q-KO and the B6-Ly49¹²⁹ 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-Ib expression differs between the two mouse strains (88). The expression of the 129 Ly49 haplotype with MHC-Ib expression from B6 may influence expression of 14B11 staining Ly49 receptors in the B6-Ly49¹²⁹ mouse, although no binding by any LY49 to MHC-Ib 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-Ly49¹²⁹ congenic mice are mature, as indicated by the expression of the NK markers CD2, CD11a and CD62L (Fig. 10).

The B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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-Ly49¹²⁹ 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- γ production upon NKG2D antibody stimulation. B6 NK cells were stimulated to produce about 5% more IFN- γ than 129 or B6-Ly49¹²⁹ NK cells (Fig. 14B). This result may be due to the nature of the α NKG2D mAb, which was created against the B6 NKG2D allele. As a result, the α NKG2D antibody may not bind to NKG2D¹²⁹ with the same affinity and result in less activation of NK cells and IFN- γ secretion. Killing of YAC-1 by B6-Ly49¹²⁹ 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-Ly49¹²⁹ 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-2D^k 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-*Ly49*¹²⁹ mice show that both 129 and B6-*Ly49*¹²⁹ 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-2^b 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-*Ly49*¹²⁹ NK cells exhibit an intermediate phenotype that is higher than 129 but

lower than B6 NK cells (Fig. 12). In addition, B6-Ly49¹²⁹ 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 Ly49G¹²⁹ 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^{B6} 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^b-deficient and H-2K^bD^b-deficient splenocytes was higher in B6-Ly49¹²⁹ 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^b-deficient cells was higher than of D^b-deficient cells in both unstimulated and poly(I:C) activated recipient mice. This result is consistent with reports that H-2K^b has a stronger educational impact than H-2D^b meaning that H-2K^b-deficient cells should be rejected more strongly than H-2D^b-deficient cells (74). Splenocytes lacking H-2K^b were shown to be killed approximately 40% more than D^b-deficient cells (Fig. 18). To ensure the higher rejection of H-2K^b and H-2K^b H-2D^b by the congenic mice was not a result of the presence of the 129 derived gene located outside the NKC in the B6-Ly49¹²⁹ mice, similar experiments were repeated with the B6.Ly49Q^{-/-} mouse model, yielding similar results (Fig. 20).

The results are consistent with the findings that expression of H-2K^b molecules confers stronger rejection of MHC-I-deficient cells than H-2D^b (74). Target cells lacking expression of H-2K^b are unable to bind to inhibitory Ly49 receptors on host NK cells and are consequently rejected, to a higher degree than H-2D^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-Ly49¹²⁹ 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^{high} NK cells or cytotoxic CD56^{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, Ly49I, 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 Ly49I, O, or V receptors, all of which bind the self-MHC-I in H-2^b 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 Ly49I/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-Ly49¹²⁹ 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-Ly49¹²⁹ NK cells may be a result of a slower response due to 129 gene contribution outside the NKC. Overall, the B6-Ly49¹²⁹ 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-Ly49¹²⁹

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-Ly49¹²⁹ mouse strain with *in vitro* effector functions and *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-Ly49¹²⁹ 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-Ly49¹²⁹ mouse showed enhanced rejection of MHC-I deficient cells *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.

APPENDICES

ELSEVIER LICENSE TERMS AND CONDITIONS

This is a License Agreement between Rajen Patel ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Rajen Patel
Customer address	451 Smyth Road Ottawa, ON K1H8M5
License number	2510240262149
License date	Sep 15, 2010
Licensed content publisher	Elsevier
Licensed content publication	Trends in Immunology
Licensed content title	The biology of human natural killer-cell subsets
Licensed content author	Megan A. Cooper, Todd A. Fehniger, Michael A. Caligiuri
Licensed content date	1 November 2001
Licensed content volume number	22
Licensed content issue number	11
Number of pages	8
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	print
Are you the author of this	No

Elsevier article?

Will you be translating? No

Order reference number

Title of your thesis/dissertation effect of Ly49 Haplotype Variance on Natural Killer cell Function and Education

Expected completion date Sep 2010

Estimated size (number of 100 pages)

Elsevier VAT number GB 494 6272 12

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

“Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. **Revocation:** Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website:** The following terms and conditions apply to electronic reserve and author websites:

Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:

This license was made in connection with a course,

This permission is granted for 1 year only. You may obtain a license for future website posting,

All content posted to the web site must maintain the copyright information line on the bottom of each image,

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com> , and

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and

the permission granted is limited to the personal version of your paper. You are not allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> , As part of our normal

production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. Author website for books with the following additional clauses:

Authors are permitted to place a brief summary of their work online only.

A hyper-text must be included to the Elsevier homepage at <http://www.elsevier.com>

All content posted to the web site must maintain the copyright information line on the bottom of each image

You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. Website (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx>. or for books to the Elsevier homepage at <http://www.elsevier.com>

20. Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. Other Conditions:

v1.6

Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10849143.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2510240262149 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE TERMS AND CONDITIONS

This is a License Agreement between Rajen Patel ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard, Langford Lane Kidlington, Oxford, OX5 1GB, UK
Registered Company Number	1982084
Customer name	Rajen Patel
Customer address	451 Smyth Road Ottawa, ON K1H8M5
License number	2510231176970
License date	Sep 15, 2010
Licensed content publisher	Elsevier
Licensed content publication	Immunity
Licensed content title	Distinct Conformations of Ly49 Natural Killer Cell Receptors Mediate MHC Class I Recognition in <i>Trans</i> and <i>Cis</i>
Licensed content author	Jonathan Back, Emilio L. Malchiodi, Sangwoo Cho, Leonardo Scarpellino, Pascal Schneider, Melissa C. Kerzic, Roy A. Mariuzza, Werner Held
Licensed content date	16 October 2009
Licensed content volume number	31
Licensed content issue number	4
Number of pages	11
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations

Number of figures/tables/illustrations 1
 Format print
 Are you the author of this Elsevier article? No
 Will you be translating? No
 Order reference number
 Title of your thesis/dissertation effect of Ly49 Haplotype Variance on Natural Killer cell Function and Education
 Expected completion date Sep 2010
 Estimated size (number of pages) 100
 Elsevier VAT number GB 494 6272 12
 Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

“Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].” Also Lancet special credit - “Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier.”

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. **Altering/Modifying Material: Not Permitted.** However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. **Reservation of Rights:** Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

8. **License Contingent Upon Payment:** While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. **Warranties:** Publisher makes no representations or warranties with respect to the licensed material.

10. **Indemnity:** You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. **No Transfer of License:** This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. **No Amendment Except in Writing:** This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. **Objection to Contrary Terms:** Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire

agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. **Revocation:** Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. **Website:** The following terms and conditions apply to electronic reserve and author websites:

Electronic reserve: If licensed material is to be posted to website, the web site is to be password-protected and made available only to bona fide students registered on a relevant course if:

This license was made in connection with a course,

This permission is granted for 1 year only. You may obtain a license for future website posting,

All content posted to the web site must maintain the copyright information line on the bottom of each image,

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com> , and

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

17. **Author website** for journals with the following additional clauses:

All content posted to the web site must maintain the copyright information line on the bottom of each image, and

the permission granted is limited to the personal version of your paper. You are not

allowed to download and post the published electronic version of your article (whether PDF or HTML, proof or final version), nor may you scan the printed edition to create an electronic version,

A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> , As part of our normal production process, you will receive an e-mail notice when your article appears on Elsevier's online service ScienceDirect (www.sciencedirect.com). That e-mail will include the article's Digital Object Identifier (DOI). This number provides the electronic link to the published article and should be included in the posting of your personal version. We ask that you wait until you receive this e-mail and have the DOI to do any posting.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

18. Author website for books with the following additional clauses:

Authors are permitted to place a brief summary of their work online only.

A hyper-text must be included to the Elsevier homepage at <http://www.elsevier.com>

All content posted to the web site must maintain the copyright information line on the bottom of each image

You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version.

Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

19. Website (regular and for author): A hyper-text must be included to the Homepage of the journal from which you are licensing at

<http://www.sciencedirect.com/science/journal/xxxxx>. or for books to the Elsevier homepage at <http://www.elsevier.com>

20. Thesis/Dissertation: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

21. Other Conditions:

v1.6

Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10849129.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2510231176970 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

This is a License Agreement between Rajen Patel ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2510230631430
License date	Sep 15, 2010
Licensed content publisher	Nature Publishing Group
Licensed content publication	Genes and Immunity
Licensed content title	Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome
Licensed content author	S Belanger, L-H Tai, S K Anderson and A P Makrigiannis
Volume number	9
Issue number	6
Pages	
Year of publication	2008
Portion used	Figures / tables
Number of figures / tables	1
Requestor type	Student
Type of Use	Thesis / Dissertation
Billing Type	Invoice
Company	Rajen Patel
Billing Address	451 Smyth Road Roger Guindon Hall Room 4226 Ottawa, ON K1H8M5 Canada
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.
2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.
3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).
4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]
(reference citation), copyright (year of publication)

For AOP papers, the credit line should read:

Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME],
advance online publication, day month year (doi: 10.1038/sj.[JOURNAL
ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME]
(reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit <http://www.macmillanmedicalcommunications.com> for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME]
(reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

Gratis licenses (referencing \$0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK10849123.

Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

**Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006**

If you find copyrighted material related to this license will not be used and wish to cancel, please contact us referencing this license number 2510230631430 and noting the reason for cancellation.

Questions? customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

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

Patel, R, Belanger S, Tai L-H, Troke AD, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. 23rd Annual Meeting for the Canadian Society for Immunology, Workshop I: Leukocyte Development, Activation and Signalling, April 2010, Niagara Falls, Ontario.

Publications

Patel, R, Belanger S, Tai L-H, Troke AD, Makrigiannis A, Effects of Ly49 Haplotype Variance on Natural Killer Cell Function and Education. Journal of Immunology, 185(8); 4783-4792, October 15, 2010.

Poster Abstracts

- **Patel, R, Belanger S, Makrigiannis AP, Effects of Ly49 Haplotype Divergence on Natural Killer Cell Function. BMI Poster Day, May 2010, Ottawa, Ontario (2nd poster prize awarded - Microbiology and Immunology).**
- **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. 2nd Annual Ottawa Regional Microbiology Symposium, April 2010, Ottawa, 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
McGill University, Montreal, Quebec

REFERENCE LIST

1. **Aldrich, C. J., A. DeCloux, A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman.** 1994. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* **79**:649-658.
2. **Alexopoulou, L., A. C. Holt, R. Medzhitov, and R. A. Flavell.** 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**:732-738.
3. **Allan, J. E., and G. R. Shellam.** 1984. Genetic control of murine cytomegalovirus infection: virus titres in resistant and susceptible strains of mice. *Arch Virol* **81**:139-150.
4. **Anderson, S. K., K. Dewar, M. L. Goulet, G. Leveque, and A. P. Makrigiannis.** 2005. Complete elucidation of a minimal class I MHC natural killer cell receptor haplotype. *Genes Immun* **6**:481-492.
5. **Andre, P., O. Spertini, S. Guia, P. Rihet, F. Dignat-George, H. Brailly, J. Sampol, P. J. Anderson, and E. Vivier.** 2000. Modification of P-selectin glycoprotein ligand-1 with a natural killer cell-restricted sulfated lactosamine creates an alternate ligand for L-selectin. *Proc Natl Acad Sci U S A* **97**:3400-3405.
6. **Anfossi, N., P. Andre, S. Guia, C. S. Falk, S. Roetynck, C. A. Stewart, V. Breso, C. Frassati, D. Revirion, D. Middleton, F. Romagne, S. Ugolini, and E. Vivier.** 2006. Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25**:331-342.
7. **Appay, V., and S. L. Rowland-Jones.** 2001. RANTES: a versatile and controversial chemokine. *Trends Immunol* **22**:83-87.
8. **Arase, H., and L. L. Lanier.** 2002. Virus-driven evolution of natural killer cell receptors. *Microbes Infect* **4**:1505-1512.
9. **Arase, H., E. S. Mocarski, A. E. Campbell, A. B. Hill, and L. L. Lanier.** 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* **296**:1323-1326.

10. **Arnon, T. I., M. Lev, G. Katz, Y. Chernobrov, A. Porgador, and O. Mandelboim.** 2001. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* **31**:2680-2689.
11. **Back, J., E. L. Malchiodi, S. Cho, L. Scarpellino, P. Schneider, M. C. Kerzic, R. A. Mariuzza, and W. Held.** 2009. Distinct conformations of Ly49 natural killer cell receptors mediate MHC class I recognition in trans and cis. *Immunity* **31**:598-608.
12. **Barton, G. M., and R. Medzhitov.** 2002. Toll-like receptors and their ligands. *Curr Top Microbiol Immunol* **270**:81-92.
13. **Barton, K., N. Muthusamy, C. Fischer, C. N. Ting, T. L. Walunas, L. L. Lanier, and J. M. Leiden.** 1998. The Ets-1 transcription factor is required for the development of natural killer cells in mice. *Immunity* **9**:555-563.
14. **Beebe, A. M., S. Mauze, N. J. Schork, and R. L. Coffman.** 1997. Serial backcross mapping of multiple loci associated with resistance to *Leishmania major* in mice. *Immunity* **6**:551-557.
15. **Belanger, S., L. H. Tai, S. K. Anderson, and A. P. Makrigiannis.** 2008. Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes Immun* **9**:509-521.
16. **Biassoni, R., C. Cantoni, D. Marras, J. Giron-Michel, M. Falco, L. Moretta, and N. Dimasi.** 2003. Human natural killer cell receptors: insights into their molecular function and structure. *J Cell Mol Med* **7**:376-387.
17. **Biassoni, R., A. Pessino, C. Bottino, D. Pende, L. Moretta, and A. Moretta.** 1999. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur J Immunol* **29**:1014-1020.
18. **Biron, C. A., K. S. Byron, and J. L. Sullivan.** 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med* **320**:1731-1735.
19. **Bix, M., N. S. Liao, M. Zijlstra, J. Loring, R. Jaenisch, and D. Raulet.** 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature* **349**:329-331.
20. **Bouvier, M.** 2003. Accessory proteins and the assembly of human class I MHC molecules: a molecular and structural perspective. *Mol Immunol* **39**:697-706.

21. **Brodin, P., T. Lakshmikanth, S. Johansson, K. Karre, and P. Hoglund.** 2009. The strength of inhibitory input during education quantitatively tunes the functional responsiveness of individual natural killer cells. *Blood* **113**:2434-2441.
22. **Brown, M. G., A. O. Dokun, J. W. Heusel, H. R. Smith, D. L. Beckman, E. A. Blattenberger, C. E. Dubbelde, L. R. Stone, A. A. Scalzo, and W. M. Yokoyama.** 2001. Vital involvement of a natural killer cell activation receptor in resistance to viral infection. *Science* **292**:934-937.
23. **Bryceson, Y. T., M. E. March, D. F. Barber, H. G. Ljunggren, and E. O. Long.** 2005. Cytolytic granule polarization and degranulation controlled by different receptors in resting NK cells. *J Exp Med* **202**:1001-1012.
24. **Bryceson, Y. T., M. E. March, H. G. Ljunggren, and E. O. Long.** 2006. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**:159-166.
25. **Bukowski, J. F., B. A. Woda, S. Habu, K. Okumura, and R. M. Welsh.** 1983. Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J Immunol* **131**:1531-1538.
26. **Burshtyn, D. N., A. M. Scharenberg, N. Wagtmann, S. Rajagopalan, K. Berrada, T. Yi, J. P. Kinet, and E. O. Long.** 1996. Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor. *Immunity* **4**:77-85.
27. **Caraux, A., N. Kim, S. E. Bell, S. Zompi, T. Ranson, S. Lesjean-Pottier, M. E. Garcia-Ojeda, M. Turner, and F. Colucci.** 2006. Phospholipase C-gamma2 is essential for NK cell cytotoxicity and innate immunity to malignant and virally infected cells. *Blood* **107**:994-1002.
28. **Carlyle, J. R., A. M. Jamieson, S. Gasser, C. S. Clingan, H. Arase, and D. H. Raulet.** 2004. Missing self-recognition of Ocil/Cir-b by inhibitory NKR-P1 natural killer cell receptors. *Proc Natl Acad Sci U S A* **101**:3527-3532.
29. **Carlyle, J. R., A. Mesci, J. H. Fine, P. Chen, S. Belanger, L. H. Tai, and A. P. Makrigiannis.** 2008. Evolution of the Ly49 and Nkrp1 recognition systems. *Semin Immunol* **20**:321-330.
30. **Carlyle, J. R., A. Mesci, B. Ljutic, S. Belanger, L. H. Tai, E. Rousselle, A. D. Troke, M. F. Proteau, and A. P. Makrigiannis.** 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J Immunol* **176**:7511-7524.
31. **Chalifour, A., L. Scarpellino, J. Back, P. Brodin, E. Devedre, F. Gros, F. Levy, G. Leclercq, P. Hoglund, F. Beermann, and W. Held.** 2009. A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity* **30**:337-347.

32. **Chambers, W. H., T. Adamkiewicz, and J. P. Houchins.** 1993. Type II integral membrane proteins with characteristics of C-type animal lectins expressed by natural killer (NK) cells. *Glycobiology* **3**:9-14.
33. **Chan, P. Y., and F. Takei.** 1986. Expression of a T cell receptor-like molecule on normal and malignant murine T cells detected by rat monoclonal antibodies to nonclonotypic determinants. *J Immunol* **136**:1346-1353.
34. **Colucci, F., M. A. Caligiuri, and J. P. Di Santo.** 2003. What does it take to make a natural killer? *Nat Rev Immunol* **3**:413-425.
35. **Cooper, M. A., T. A. Fehniger, and M. A. Caligiuri.** 2001. The biology of human natural killer-cell subsets. *Trends Immunol* **22**:633-640.
36. **Cooper, M. A., T. A. Fehniger, S. C. Turner, K. S. Chen, B. A. Ghaheri, T. Ghayur, W. E. Carson, and M. A. Caligiuri.** 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood* **97**:3146-3151.
37. **Correa, I., and D. H. Raulet.** 1995. Binding of diverse peptides to MHC class I molecules inhibits target cell lysis by activated natural killer cells. *Immunity* **2**:61-71.
38. **Cosman, D., J. Mullberg, C. L. Sutherland, W. Chin, R. Armitage, W. Fanslow, M. Kubin, and N. J. Chalupny.** 2001. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14**:123-133.
39. **Cotterill, L. A., H. J. Stauss, M. M. Millrain, D. J. Pappin, D. Rahman, B. Canas, P. Chandler, A. Stackpoole, E. Simpson, P. J. Robinson, and P. J. Dyson.** 1997. Qa-1 interaction and T cell recognition of the Qa-1 determinant modifier peptide. *Eur J Immunol* **27**:2123-2132.
40. **Dam, J., R. Guan, K. Natarajan, N. Dimasi, L. K. Chlewicki, D. M. Kranz, P. Schuck, D. H. Margulies, and R. A. Mariuzza.** 2003. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K(b). *Nat Immunol* **4**:1213-1222.
41. **Davies, E. G., D. Isaacs, and R. J. Levinsky.** 1982. Defective immune interferon production and natural killer activity associated with poor neutrophil mobility and delayed umbilical cord separation. *Clin Exp Immunol* **50**:454-460.
42. **DeCloux, A., A. S. Woods, R. J. Cotter, M. J. Soloski, and J. Forman.** 1997. Dominance of a single peptide bound to the class I(B) molecule, Qa-1b. *J Immunol* **158**:2183-2191.

43. **Delano, M. L., and D. G. Brownstein.** 1995. Innate resistance to lethal mousepox is genetically linked to the NK gene complex on chromosome 6 and correlates with early restriction of virus replication by cells with an NK phenotype. *J Virol* **69**:5875-5877.
44. **Deng, L., S. Cho, E. L. Malchiodi, M. C. Kerzic, J. Dam, and R. A. Mariuzza.** 2008. Molecular architecture of the major histocompatibility complex class I-binding site of Ly49 natural killer cell receptors. *J Biol Chem* **283**:16840-16849.
45. **Diefenbach, A., E. Tomasello, M. Lucas, A. M. Jamieson, J. K. Hsia, E. Vivier, and D. H. Raulet.** 2002. Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D. *Nat Immunol* **3**:1142-1149.
46. **Doucey, M. A., L. Scarpellino, J. Zimmer, P. Guillaume, I. F. Luescher, C. Bron, and W. Held.** 2004. Cis association of Ly49A with MHC class I restricts natural killer cell inhibition. *Nat Immunol* **5**:328-336.
47. **Eagle, R. A., J. A. Traherne, O. Ashiru, M. R. Wills, and J. Trowsdale.** 2006. Regulation of NKG2D ligand gene expression. *Hum Immunol* **67**:159-169.
48. **Fahlen, L., U. Lendahl, and C. L. Sentman.** 2001. MHC class I-Ly49 interactions shape the Ly49 repertoire on murine NK cells. *J Immunol* **166**:6585-6592.
49. **Fernandez, N. C., E. Treiner, R. E. Vance, A. M. Jamieson, S. Lemieux, and D. H. Raulet.** 2005. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood* **105**:4416-4423.
50. **Frey, M., N. B. Packianathan, T. A. Fehniger, M. E. Ross, W. C. Wang, C. C. Stewart, M. A. Caligiuri, and S. S. Evans.** 1998. Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets. *J Immunol* **161**:400-408.
51. **Fry, A. M., L. L. Lanier, and A. Weiss.** 1996. Phosphotyrosines in the killer cell inhibitory receptor motif of NKB1 are required for negative signaling and for association with protein tyrosine phosphatase 1C. *J Exp Med* **184**:295-300.
52. **Furukawa, H., K. Iizuka, J. Poursine-Laurent, N. Shastri, and W. M. Yokoyama.** 2002. A ligand for the murine NK activation receptor Ly-49D: activation of tolerized NK cells from beta 2-microglobulin-deficient mice. *J Immunol* **169**:126-136.

53. **Garni-Wagner, B. A., A. Purohit, P. A. Mathew, M. Bennett, and V. Kumar.** 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J Immunol* **151**:60-70.
54. **Gazit, R., R. Gruda, M. Elboim, T. I. Arnon, G. Katz, H. Achdout, J. Hanna, U. Qimron, G. Landau, E. Greenbaum, Z. Zakay-Rones, A. Porgador, and O. Mandelboim.** 2006. Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nat Immunol* **7**:517-523.
55. **Gilfillan, S., E. L. Ho, M. Cella, W. M. Yokoyama, and M. Colonna.** 2002. NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation. *Nat Immunol* **3**:1150-1155.
56. **Giorda, R., and M. Trucco.** 1991. Mouse NKR-P1. A family of genes selectively coexpressed in adherent lymphokine-activated killer cells. *J Immunol* **147**:1701-1708.
57. **Graham, D. B., M. Cella, E. Giurisato, K. Fujikawa, A. V. Miletic, T. Kloeppel, K. Brim, T. Takai, A. S. Shaw, M. Colonna, and W. Swat.** 2006. Vav1 controls DAP10-mediated natural cytotoxicity by regulating actin and microtubule dynamics. *J Immunol* **177**:2349-2355.
58. **Gunturi, A., R. E. Berg, and J. Forman.** 2004. The role of CD94/NKG2 in innate and adaptive immunity. *Immunol Res* **30**:29-34.
59. **Hackett, J., Jr., M. Tutt, M. Lipscomb, M. Bennett, G. Koo, and V. Kumar.** 1986. Origin and differentiation of natural killer cells. II. Functional and morphologic studies of purified NK-1.1+ cells. *J Immunol* **136**:3124-3131.
60. **Hanke, T., H. Takizawa, C. W. McMahon, D. H. Busch, E. G. Pamer, J. D. Miller, J. D. Altman, Y. Liu, D. Cado, F. A. Lemonnier, P. J. Bjorkman, and D. H. Raulet.** 1999. Direct assessment of MHC class I binding by seven Ly49 inhibitory NK cell receptors. *Immunity* **11**:67-77.
61. **Hanke, T., H. Takizawa, and D. H. Raulet.** 2001. MHC-dependent shaping of the inhibitory Ly49 receptor repertoire on NK cells: evidence for a regulated sequential model. *Eur J Immunol* **31**:3370-3379.
62. **Hart, O. M., V. Athie-Morales, G. M. O'Connor, and C. M. Gardiner.** 2005. TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production. *J Immunol* **175**:1636-1642.
63. **Held, W., J. Roland, and D. H. Raulet.** 1995. Allelic exclusion of Ly49-family genes encoding class I MHC-specific receptors on NK cells. *Nature* **376**:355-358.

64. **Hiltbold, E. M., and P. A. Roche.** 2002. Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol* **14**:30-35.
65. **Hoglund, P., C. Ohlen, E. Carbone, L. Franksson, H. G. Ljunggren, A. Latour, B. Koller, and K. Karre.** 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. *Proc Natl Acad Sci U S A* **88**:10332-10336.
66. **Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann.** 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* **168**:4531-4537.
67. **Hsu, K. C., X. R. Liu, A. Selvakumar, E. Mickelson, R. J. O'Reilly, and B. Dupont.** 2002. Killer Ig-like receptor haplotype analysis by gene content: evidence for genomic diversity with a minimum of six basic framework haplotypes, each with multiple subsets. *J Immunol* **169**:5118-5129.
68. **Huntington, N. D., C. A. Voshenrich, and J. P. Di Santo.** 2007. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat Rev Immunol* **7**:703-714.
69. **Idris, A. H., K. Iizuka, H. R. Smith, A. A. Scalzo, and W. M. Yokoyama.** 1998. Genetic control of natural killing and in vivo tumor elimination by the Chok locus. *J Exp Med* **188**:2243-2256.
70. **Idris, A. H., H. R. Smith, L. H. Mason, J. R. Ortaldo, A. A. Scalzo, and W. M. Yokoyama.** 1999. The natural killer gene complex genetic locus Chok encodes Ly-49D, a target recognition receptor that activates natural killing. *Proc Natl Acad Sci U S A* **96**:6330-6335.
71. **Iizuka, K., O. V. Naidenko, B. F. Plougastel, D. H. Fremont, and W. M. Yokoyama.** 2003. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. *Nat Immunol* **4**:801-807.
72. **Jamieson, A. M., A. Diefenbach, C. W. McMahon, N. Xiong, J. R. Carlyle, and D. H. Raulet.** 2002. The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17**:19-29.
73. **Janeway, C. A., Jr., and R. Medzhitov.** 2002. Innate immune recognition. *Annu Rev Immunol* **20**:197-216.
74. **Johansson, S., M. Johansson, E. Rosmaraki, G. Vahlne, R. Mehr, M. Salmon-Divon, F. Lemonnier, K. Karre, and P. Hoglund.** 2005. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *J Exp Med* **201**:1145-1155.

75. **Joncker, N. T., N. C. Fernandez, E. Treiner, E. Vivier, and D. H. Raulet.** 2009. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* **182**:4572-4580.
76. **Jonsson, A. H., L. Yang, S. Kim, S. M. Taffner, and W. M. Yokoyama.** 2010. Effects of MHC class I alleles on licensing of Ly49A+ NK cells. *J Immunol* **184**:3424-3432.
77. **Kamogawa-Schifter, Y., J. Ohkawa, S. Namiki, N. Arai, K. Arai, and Y. Liu.** 2005. Ly49Q defines 2 pDC subsets in mice. *Blood* **105**:2787-2792.
78. **Karlhofer, F. M., R. K. Ribaudo, and W. M. Yokoyama.** 1992. MHC class I alloantigen specificity of Ly-49+ IL-2-activated natural killer cells. *Nature* **358**:66-70.
79. **Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling.** 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* **319**:675-678.
80. **Khakoo, S. I., C. L. Thio, M. P. Martin, C. R. Brooks, X. Gao, J. Astemborski, J. Cheng, J. J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A. M. Little, G. J. Alexander, M. E. Cramp, S. J. O'Brien, W. M. Rosenberg, D. L. Thomas, and M. Carrington.** 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science* **305**:872-874.
81. **Kielczewska, A., M. Pyzik, T. Sun, A. Krmpotic, M. B. Lodoen, M. W. Munks, M. Babic, A. B. Hill, U. H. Koszinowski, S. Jonjic, L. L. Lanier, and S. M. Vidal.** 2009. Ly49P recognition of cytomegalovirus-infected cells expressing H2-Dk and CMV-encoded m04 correlates with the NK cell antiviral response. *J Exp Med* **206**:515-523.
82. **Kiessling, R., E. Klein, H. Pross, and H. Wigzell.** 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* **5**:117-121.
83. **Kiessling, R., E. Klein, and H. Wigzell.** 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol* **5**:112-117.
84. **Kim, D. K., J. Kabat, F. Borrego, T. B. Sanni, C. H. You, and J. E. Coligan.** 2004. Human NKG2F is expressed and can associate with DAP12. *Mol Immunol* **41**:53-62.
85. **Kim, S., K. Iizuka, H. S. Kang, A. Dokun, A. R. French, S. Greco, and W. M. Yokoyama.** 2002. In vivo developmental stages in murine natural killer cell maturation. *Nat Immunol* **3**:523-528.

86. **Kim, S., J. Poursine-Laurent, S. M. Truscott, L. Lybarger, Y. J. Song, L. Yang, A. R. French, J. B. Sunwoo, S. Lemieux, T. H. Hansen, and W. M. Yokoyama.** 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**:709-713.
87. **Koka, R., P. Burkett, M. Chien, S. Chai, D. L. Boone, and A. Ma.** 2004. Cutting edge: murine dendritic cells require IL-15R alpha to prime NK cells. *J Immunol* **173**:3594-3598.
88. **Kumanovics, A., A. Madan, S. Qin, L. Rowen, L. Hood, and K. Fischer Lindahl.** 2002. QUOD ERAT FACIENDUM: sequence analysis of the H2-D and H2-Q regions of 129/SvJ mice. *Immunogenetics* **54**:479-489.
89. **Lanier, L. L.** 1998. Activating and inhibitory NK cell receptors. *Adv Exp Med Biol* **452**:13-18.
90. **Lanier, L. L.** 1998. Follow the leader: NK cell receptors for classical and nonclassical MHC class I. *Cell* **92**:705-707.
91. **Lanier, L. L.** 2005. NK cell recognition. *Annu Rev Immunol* **23**:225-274.
92. **Lanier, L. L.** 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* **9**:495-502.
93. **Lanier, L. L., B. C. Corliss, J. Wu, C. Leong, and J. H. Phillips.** 1998. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature* **391**:703-707.
94. **Leibson, P. J.** 1997. Signal transduction during natural killer cell activation: inside the mind of a killer. *Immunity* **6**:655-661.
95. **Letourneur, O., I. C. Kennedy, A. T. Brini, J. R. Ortaldo, J. J. O'Shea, and J. P. Kinet.** 1991. Characterization of the family of dimers associated with Fc receptors (Fc epsilon RI and Fc gamma RIII). *J Immunol* **147**:2652-2656.
96. **Liao, N. S., M. Bix, M. Zijlstra, R. Jaenisch, and D. Raulet.** 1991. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science* **253**:199-202.
97. **Lieberman, J.** 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* **3**:361-370.
98. **Ljunggren, H. G., and K. Karre.** 1990. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol Today* **11**:237-244.
99. **Llano, M., N. Lee, F. Navarro, P. Garcia, J. P. Albar, D. E. Geraghty, and M. Lopez-Botet.** 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur J Immunol* **28**:2854-2863.

100. **Lodolce, J. P., D. L. Boone, S. Chai, R. E. Swain, T. Dassopoulos, S. Trettin, and A. Ma.** 1998. IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9**:669-676.
101. **Lopez-Botet, M., M. Llano, F. Navarro, and T. Bellon.** 2000. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol* **12**:109-119.
102. **Lucas, M., W. Schachterle, K. Oberle, P. Aichele, and A. Diefenbach.** 2007. Dendritic cells prime natural killer cells by trans-presenting interleukin 15. *Immunity* **26**:503-517.
103. **Makrigiannis, A. P., P. Gosselin, L. H. Mason, L. S. Taylor, D. W. McVicar, J. R. Ortaldo, and S. K. Anderson.** 1999. Cloning and characterization of a novel activating Ly49 closely related to Ly49A. *J Immunol* **163**:4931-4938.
104. **Makrigiannis, A. P., D. Patel, M. L. Goulet, K. Dewar, and S. K. Anderson.** 2005. Direct sequence comparison of two divergent class I MHC natural killer cell receptor haplotypes. *Genes Immun* **6**:71-83.
105. **Makrigiannis, A. P., A. T. Pau, A. Saleh, R. Winkler-Pickett, J. R. Ortaldo, and S. K. Anderson.** 2001. Class I MHC-binding characteristics of the 129/J Ly49 repertoire. *J Immunol* **166**:5034-5043.
106. **Makrigiannis, A. P., E. Rousselle, and S. K. Anderson.** 2004. Independent control of Ly49g alleles: implications for NK cell repertoire selection and tumor cell killing. *J Immunol* **172**:1414-1425.
107. **Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T. I. Arnon, Y. Bushkin, D. M. Davis, J. L. Strominger, J. W. Yewdell, and A. Porgador.** 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409**:1055-1060.
108. **Martin, A. M., J. K. Kulski, C. Witt, P. Pontarotti, and F. T. Christiansen.** 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol* **23**:81-88.
109. **Martin, M. P., X. Gao, J. H. Lee, G. W. Nelson, R. Detels, J. J. Goedert, S. Buchbinder, K. Hoots, D. Vlahov, J. Trowsdale, M. Wilson, S. J. O'Brien, and M. Carrington.** 2002. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* **31**:429-434.
110. **Martin, M. P., G. Nelson, J. H. Lee, F. Pellett, X. Gao, J. Wade, M. J. Wilson, J. Trowsdale, D. Gladman, and M. Carrington.** 2002. Cutting edge: susceptibility to psoriatic arthritis: influence of activating killer Ig-like receptor genes in the absence of specific HLA-C alleles. *J Immunol* **169**:2818-2822.

111. **Mason, L. H.** 2000. Recognition of CHO cells by inhibitory and activating Ly-49 receptors. *J Leukoc Biol* **68**:583-586.
112. **Mason, L. H., J. Willette-Brown, S. K. Anderson, P. Gosselin, E. W. Shores, P. E. Love, J. R. Ortaldo, and D. W. McVicar.** 1998. Characterization of an associated 16-kDa tyrosine phosphoprotein required for Ly-49D signal transduction. *J Immunol* **160**:4148-4152.
113. **Matos, M. E., G. S. Schnier, M. S. Beecher, L. K. Ashman, D. E. William, and M. A. Caligiuri.** 1993. Expression of a functional c-kit receptor on a subset of natural killer cells. *J Exp Med* **178**:1079-1084.
114. **McVicar, D. W., L. S. Taylor, P. Gosselin, J. Willette-Brown, A. I. Mikhael, R. L. Geahlen, M. C. Nakamura, P. Linnemeyer, W. E. Seaman, S. K. Anderson, J. R. Ortaldo, and L. H. Mason.** 1998. DAP12-mediated signal transduction in natural killer cells. A dominant role for the Syk protein-tyrosine kinase. *J Biol Chem* **273**:32934-32942.
115. **McVicar, D. W., R. Winkler-Pickett, L. S. Taylor, A. Makrigiannis, M. Bennett, S. K. Anderson, and J. R. Ortaldo.** 2002. Aberrant DAP12 signaling in the 129 strain of mice: implications for the analysis of gene-targeted mice. *J Immunol* **169**:1721-1728.
116. **Mehta, I. K., J. Wang, J. Roland, D. H. Margulies, and W. M. Yokoyama.** 2001. Ly49A allelic variation and MHC class I specificity. *Immunogenetics* **53**:572-583.
117. **Mesci, A., B. Ljutic, A. P. Makrigiannis, and J. R. Carlyle.** 2006. NKR-P1 biology: from prototype to missing self. *Immunol Res* **35**:13-26.
118. **Moretta, A., R. Biassoni, C. Bottino, M. C. Mingari, and L. Moretta.** 2000. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytotoxicity. *Immunol Today* **21**:228-234.
119. **Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta.** 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytotoxicity. *Annu Rev Immunol* **19**:197-223.
120. **Moretta, A., M. Vitale, C. Bottino, A. M. Orengo, L. Morelli, R. Augugliaro, M. Barbaresi, E. Ciccone, and L. Moretta.** 1993. P58 molecules as putative receptors for major histocompatibility complex (MHC) class I molecules in human natural killer (NK) cells. Anti-p58 antibodies reconstitute lysis of MHC class I-protected cells in NK clones displaying different specificities. *J Exp Med* **178**:597-604.
121. **Nagasawa, R., J. Gross, O. Kanagawa, K. Townsend, L. L. Lanier, J. Chiller, and J. P. Allison.** 1987. Identification of a novel T cell surface disulfide-bonded dimer distinct from the alpha/beta antigen receptor. *J Immunol* **138**:815-824.

122. **Nagler, A., L. L. Lanier, and J. H. Phillips.** 1990. Constitutive expression of high affinity interleukin 2 receptors on human CD16-natural killer cells in vivo. *J Exp Med* **171**:1527-1533.
123. **Nakamura, M. C., E. C. Niemi, M. J. Fisher, L. D. Shultz, W. E. Seaman, and J. C. Ryan.** 1997. Mouse Ly-49A interrupts early signaling events in natural killer cell cytotoxicity and functionally associates with the SHP-1 tyrosine phosphatase. *J Exp Med* **185**:673-684.
124. **Nice, T. J., L. Coscoy, and D. H. Raulet.** 2009. Posttranslational regulation of the NKG2D ligand MUlt1 in response to cell stress. *J Exp Med* **206**:287-298.
125. **Ohlen, C., J. Bastin, H. G. Ljunggren, L. Foster, E. Wolpert, G. Klein, A. R. Townsend, and K. Karre.** 1990. Resistance to H-2-restricted but not to allo-H2-specific graft and cytotoxic T lymphocyte responses in lymphoma mutant. *J Immunol* **145**:52-58.
126. **Ohlen, C., G. Kling, P. Hoglund, M. Hansson, G. Scangos, C. Bieberich, G. Jay, and K. Karre.** 1989. Prevention of allogeneic bone marrow graft rejection by H-2 transgene in donor mice. *Science* **246**:666-668.
127. **Olcese, L., P. Lang, F. Vely, A. Cambiaggi, D. Marguet, M. Blery, K. L. Hippen, R. Biassoni, A. Moretta, L. Moretta, J. C. Cambier, and E. Vivier.** 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J Immunol* **156**:4531-4534.
128. **Orr, M. T., W. J. Murphy, and L. L. Lanier.** 2010. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nat Immunol* **11**:321-327.
129. **Orr, M. T., J. C. Sun, D. G. Hesselein, H. Arase, J. H. Phillips, T. Takai, and L. L. Lanier.** 2009. Ly49H signaling through DAP10 is essential for optimal natural killer cell responses to mouse cytomegalovirus infection. *J Exp Med* **206**:807-817.
130. **Ortaldo, J. R., E. W. Bere, D. Hodge, and H. A. Young.** 2001. Activating Ly-49 NK receptors: central role in cytokine and chemokine production. *J Immunol* **166**:4994-4999.
131. **Ortaldo, J. R., L. H. Mason, T. A. Gregorio, J. Stoll, and R. T. Winkler-Pickett.** 1997. The Ly-49 family: regulation of cytokine production in murine NK cells. *J Leukoc Biol* **62**:381-388.
132. **Ortaldo, J. R., R. Winkler-Pickett, J. Willette-Brown, R. L. Wange, S. K. Anderson, G. J. Palumbo, L. H. Mason, and D. W. McVicar.** 1999. Structure/function relationship of activating Ly-49D and inhibitory Ly-49G2 NK receptors. *J Immunol* **163**:5269-5277.

133. **Paolini, R., R. Molfetta, M. Piccoli, L. Frati, and A. Santoni.** 2001. Ubiquitination and degradation of Syk and ZAP-70 protein tyrosine kinases in human NK cells upon CD16 engagement. *Proc Natl Acad Sci U S A* **98**:9611-9616.
134. **Parham, P.** 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol* **5**:201-214.
135. **Pereira, R. A., A. Scalzo, and A. Simmons.** 2001. Cutting edge: a NK complex-linked locus governs acute versus latent herpes simplex virus infection of neurons. *J Immunol* **166**:5869-5873.
136. **Perussia, B., M. M. Tutt, W. Q. Qiu, W. A. Kuziel, P. W. Tucker, G. Trinchieri, M. Bennett, J. V. Ravetch, and V. Kumar.** 1989. Murine natural killer cells express functional Fc gamma receptor II encoded by the Fc gamma R alpha gene. *J Exp Med* **170**:73-86.
137. **Proteau, M. F., E. Rousselle, and A. P. Makrigiannis.** 2004. Mapping of the BALB/c Ly49 cluster defines a minimal natural killer cell receptor gene repertoire. *Genomics* **84**:669-677.
138. **Radaev, S., and P. D. Sun.** 2003. Structure and function of natural killer cell surface receptors. *Annu Rev Biophys Biomol Struct* **32**:93-114.
139. **Ranson, T., C. A. Vosshenrich, E. Corcuff, O. Richard, W. Muller, and J. P. Di Santo.** 2003. IL-15 is an essential mediator of peripheral NK-cell homeostasis. *Blood* **101**:4887-4893.
140. **Raulet, D. H., R. E. Vance, and C. W. McMahon.** 2001. Regulation of the natural killer cell receptor repertoire. *Annu Rev Immunol* **19**:291-330.
141. **Ravetch, J. V., and L. L. Lanier.** 2000. Immune inhibitory receptors. *Science* **290**:84-89.
142. **Rawlinson, W. D., H. E. Farrell, and B. G. Barrell.** 1996. Analysis of the complete DNA sequence of murine cytomegalovirus. *J Virol* **70**:8833-8849.
143. **Renedo, M., I. Arce, A. Rodriguez, M. Carretero, L. L. Lanier, M. Lopez-Botet, and E. Fernandez-Ruiz.** 1997. The human natural killer gene complex is located on chromosome 12p12-p13. *Immunogenetics* **46**:307-311.
144. **Robertson, M. J., and J. Ritz.** 1990. Biology and clinical relevance of human natural killer cells. *Blood* **76**:2421-2438.
145. **Robertson, M. J., R. J. Soiffer, S. F. Wolf, T. J. Manley, C. Donahue, D. Young, S. H. Herrmann, and J. Ritz.** 1992. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J Exp Med* **175**:779-788.

146. **Rohrschneider, L. R., J. F. Fuller, I. Wolf, Y. Liu, and D. M. Lucas.** 2000. Structure, function, and biology of SHIP proteins. *Genes Dev* **14**:505-520.
147. **Rosmaraki, E. E., I. Douagi, C. Roth, F. Colucci, A. Cumano, and J. P. Di Santo.** 2001. Identification of committed NK cell progenitors in adult murine bone marrow. *Eur J Immunol* **31**:1900-1909.
148. **Roth, C., J. R. Carlyle, H. Takizawa, and D. H. Raulet.** 2000. Clonal acquisition of inhibitory Ly49 receptors on developing NK cells is successively restricted and regulated by stromal class I MHC. *Immunity* **13**:143-153.
149. **Russell, J. H., and T. J. Ley.** 2002. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol* **20**:323-370.
150. **Ryan, J. C., J. Turck, E. C. Niemi, W. M. Yokoyama, and W. E. Seaman.** 1992. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. *J Immunol* **149**:1631-1635.
151. **Salazar-Mather, T. P., T. A. Hamilton, and C. A. Biron.** 2000. A chemokine-to-cytokine-to-chemokine cascade critical in antiviral defense. *J Clin Invest* **105**:985-993.
152. **Salcedo, M., M. Andersson, S. Lemieux, L. Van Kaer, B. J. Chambers, and H. G. Ljunggren.** 1998. Fine tuning of natural killer cell specificity and maintenance of self tolerance in MHC class I-deficient mice. *Eur J Immunol* **28**:1315-1321.
153. **Sasawatari, S., M. Yoshizaki, C. Taya, A. Tazawa, K. Furuyama-Tanaka, H. Yonekawa, T. Dohi, A. P. Makrigiannis, T. Sasazuki, K. Inaba, and N. Toyama-Sorimachi.** 2010. The Ly49Q receptor plays a crucial role in neutrophil polarization and migration by regulating raft trafficking. *Immunity* **32**:200-213.
154. **Scalzo, A. A., M. G. Brown, D. T. Chu, J. W. Heusel, W. M. Yokoyama, and C. A. Forbes.** 1999. Development of intra-natural killer complex (NKC) recombinant and congenic mouse strains for mapping and functional analysis of NK cell regulatory loci. *Immunogenetics* **49**:238-241.
155. **Scalzo, A. A., N. A. Fitzgerald, A. Simmons, A. B. La Vista, and G. R. Shellam.** 1990. *Cmv-1*, a genetic locus that controls murine cytomegalovirus replication in the spleen. *J Exp Med* **171**:1469-1483.
156. **Scalzo, A. A., N. A. Fitzgerald, C. R. Wallace, A. E. Gibbons, Y. C. Smart, R. C. Burton, and G. R. Shellam.** 1992. The effect of the *Cmv-1* resistance gene, which is linked to the natural killer cell gene complex, is mediated by natural killer cells. *J Immunol* **149**:581-589.

157. **Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume.** 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**:163-189.
158. **Shibuya, A., K. Nagayoshi, K. Nakamura, and H. Nakauchi.** 1995. Lymphokine requirement for the generation of natural killer cells from CD34+ hematopoietic progenitor cells. *Blood* **85**:3538-3546.
159. **Sivakumar, P. V., A. Gunturi, M. Salcedo, J. D. Schatzle, W. C. Lai, Z. Kurepa, L. Pitcher, M. S. Seaman, F. A. Lemonnier, M. Bennett, J. Forman, and V. Kumar.** 1999. Cutting edge: expression of functional CD94/NKG2A inhibitory receptors on fetal NK1.1+Ly-49- cells: a possible mechanism of tolerance during NK cell development. *J Immunol* **162**:6976-6980.
160. **Sivori, S., M. Falco, M. Della Chiesa, S. Carlomagno, M. Vitale, L. Moretta, and A. Moretta.** 2004. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. *Proc Natl Acad Sci U S A* **101**:10116-10121.
161. **Smith, H. R., J. W. Heusel, I. K. Mehta, S. Kim, B. G. Dorner, O. V. Naidenko, K. Iizuka, H. Furukawa, D. L. Beckman, J. T. Pingel, A. A. Scalzo, D. H. Fremont, and W. M. Yokoyama.** 2002. Recognition of a virus-encoded ligand by a natural killer cell activation receptor. *Proc Natl Acad Sci U S A* **99**:8826-8831.
162. **Smith, K. M., J. Wu, A. B. Bakker, J. H. Phillips, and L. L. Lanier.** 1998. Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors. *J Immunol* **161**:7-10.
163. **Stetson, D. B., M. Mohrs, R. L. Reinhardt, J. L. Baron, Z. E. Wang, L. Gapin, M. Kronenberg, and R. M. Locksley.** 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* **198**:1069-1076.
164. **Sun, P. D.** 2003. Structure and function of natural-killer-cell receptors. *Immunol Res* **27**:539-548.
165. **Tai, L. H., M. L. Goulet, S. Belanger, N. Toyama-Sorimachi, N. Fodil-Cornu, S. M. Vidal, A. D. Troke, D. W. McVicar, and A. P. Makrigiannis.** 2008. Positive regulation of plasmacytoid dendritic cell function via Ly49Q recognition of class I MHC. *J Exp Med* **205**:3187-3199.
166. **Tai, L. H., M. L. Goulet, S. Belanger, A. D. Troke, A. G. St-Laurent, A. Mesci, N. Toyama-Sorimachi, J. R. Carlyle, and A. P. Makrigiannis.** 2007. Recognition of H-2K(b) by Ly49Q suggests a role for class Ia MHC regulation of plasmacytoid dendritic cell function. *Mol Immunol* **44**:2638-2646.

167. **Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch.** 1994. FcR gamma chain deletion results in pleiotropic effector cell defects. *Cell* **76**:519-529.
168. **Tamir, I., J. M. Dal Porto, and J. C. Cambier.** 2000. Cytoplasmic protein tyrosine phosphatases SHP-1 and SHP-2: regulators of B cell signal transduction. *Curr Opin Immunol* **12**:307-315.
169. **Tassi, I., G. Le Friec, S. Gilfillan, T. Takai, W. M. Yokoyama, and M. Colonna.** 2009. DAP10 associates with Ly49 receptors but contributes minimally to their expression and function in vivo. *Eur J Immunol* **39**:1129-1135.
170. **Titus, J. A., P. Perez, A. Kaubisch, M. A. Garrido, and D. M. Segal.** 1987. Human K/natural killer cells targeted with hetero-cross-linked antibodies specifically lyse tumor cells in vitro and prevent tumor growth in vivo. *J Immunol* **139**:3153-3158.
171. **Tomasello, E., L. Olcese, F. Vely, C. Geourgeon, M. Blery, A. Moqrich, D. Gautheret, M. Djabali, M. G. Mattei, and E. Vivier.** 1998. Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12. *J Biol Chem* **273**:34115-34119.
172. **Tormo, J., K. Natarajan, D. H. Margulies, and R. A. Mariuzza.** 1999. Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. *Nature* **402**:623-631.
173. **Toyama-Sorimachi, N., Y. Tsujimura, M. Maruya, A. Onoda, T. Kubota, S. Koyasu, K. Inaba, and H. Karasuyama.** 2004. Ly49Q, a member of the Ly49 family that is selectively expressed on myeloid lineage cells and involved in regulation of cytoskeletal architecture. *Proc Natl Acad Sci U S A* **101**:1016-1021.
174. **Trapani, J. A., and M. J. Smyth.** 1993. Killing by cytotoxic T cells and natural killer cells: multiple granule serine proteases as initiators of DNA fragmentation. *Immunol Cell Biol* **71 (Pt 3)**:201-208.
175. **Trinchieri, G.** 1989. Biology of natural killer cells. *Adv Immunol* **47**:187-376.
176. **Trinchieri, G., and N. Valiante.** 1993. Receptors for the Fc fragment of IgG on natural killer cells. *Nat Immun* **12**:218-234.
177. **Uhrberg, M., N. M. Valiante, B. P. Shum, H. G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L. L. Lanier, and P. Parham.** 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity* **7**:753-763.

178. **van der Slik, A. R., B. P. Koeleman, W. Verduijn, G. J. Bruining, B. O. Roep, and M. J. Giphart.** 2003. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. *Diabetes* **52**:2639-2642.
179. **Vance, R. E., J. R. Kraft, J. D. Altman, P. E. Jensen, and D. H. Raulet.** 1998. Mouse CD94/NKG2A is a natural killer cell receptor for the nonclassical major histocompatibility complex (MHC) class I molecule Qa-1(b). *J Exp Med* **188**:1841-1848.
180. **Veillette, A.** 2006. NK cell regulation by SLAM family receptors and SAP-related adapters. *Immunol Rev* **214**:22-34.
181. **Veillette, A., Z. Dong, and S. Latour.** 2007. Consequence of the SLAM-SAP signaling pathway in innate-like and conventional lymphocytes. *Immunity* **27**:698-710.
182. **Vivier, E., M. Ackerly, N. Rochet, and P. Anderson.** 1992. Structure and function of the CD16:zeta:gamma complex expressed on human natural-killer cells. *Int J Cancer Suppl* **7**:11-14.
183. **Voss, S. D., J. Daley, J. Ritz, and M. J. Robertson.** 1998. Participation of the CD94 receptor complex in costimulation of human natural killer cells. *J Immunol* **160**:1618-1626.
184. **Vosshenrich, C. A., M. E. Garcia-Ojeda, S. I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, E. Corcuff, D. Guy-Grand, B. Rocha, A. Cumano, L. Rogge, S. Ezine, and J. P. Di Santo.** 2006. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat Immunol* **7**:1217-1224.
185. **Wagtmann, N., S. Rojo, E. Eichler, H. Mohrenweiser, and E. O. Long.** 1997. A new human gene complex encoding the killer cell inhibitory receptors and related monocyte/macrophage receptors. *Curr Biol* **7**:615-618.
186. **Westgaard, I. H., S. F. Berg, S. Orstavik, S. Fossum, and E. Dissen.** 1998. Identification of a human member of the Ly-49 multigene family. *Eur J Immunol* **28**:1839-1846.
187. **Wilson, M. J., M. Torkar, A. Haude, S. Milne, T. Jones, D. Sheer, S. Beck, and J. Trowsdale.** 2000. Plasticity in the organization and sequences of human KIR/ILT gene families. *Proc Natl Acad Sci U S A* **97**:4778-4783.
188. **Wu, J., and L. L. Lanier.** 2003. Natural killer cells and cancer. *Adv Cancer Res* **90**:127-156.

189. **Wu, J., Y. Song, A. B. Bakker, S. Bauer, T. Spies, L. L. Lanier, and J. H. Phillips.** 1999. An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* **285**:730-732.
190. **Yen, J. H., B. E. Moore, T. Nakajima, D. Scholl, D. J. Schaid, C. M. Weyand, and J. J. Goronzy.** 2001. Major histocompatibility complex class I-recognizing receptors are disease risk genes in rheumatoid arthritis. *J Exp Med* **193**:1159-1167.
191. **Yokoyama, W. M., L. B. Jacobs, O. Kanagawa, E. M. Shevach, and D. I. Cohen.** 1989. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. *J Immunol* **143**:1379-1386.
192. **Yokoyama, W. M., P. J. Kehn, D. I. Cohen, and E. M. Shevach.** 1990. Chromosomal location of the Ly-49 (A1, YE1/48) multigene family. Genetic association with the NK 1.1 antigen. *J Immunol* **145**:2353-2358.
193. **Yu, H., T. A. Fehniger, P. Fuchshuber, K. S. Thiel, E. Vivier, W. E. Carson, and M. A. Caligiuri.** 1998. Flt3 ligand promotes the generation of a distinct CD34(+) human natural killer cell progenitor that responds to interleukin-15. *Blood* **92**:3647-3657.
194. **Yu, Y. Y., V. Kumar, and M. Bennett.** 1992. Murine natural killer cells and marrow graft rejection. *Annu Rev Immunol* **10**:189-213.