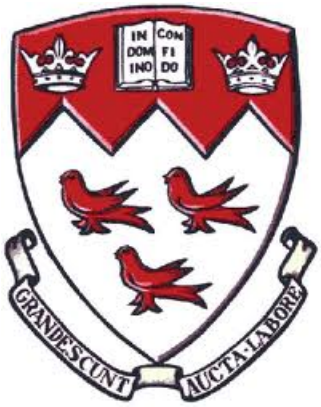

The role of natural killer cell receptors in the control of natural killer cell functions

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ABSTRACT

Natural killer (NK) cells possess the ability to destroy abnormal cells such as cancerous and virally-infected cells. A common feature of these cells is the downregulation of class I MHC molecules in an attempt to evade recognition by $CD8^+$ T lymphocytes. "Missing-self" recognition allows NK cells to identify and destroy cells with decreased MHC-I expression. MHC-I receptors are expressed by NK cells, therefore allowing "missing-self" recognition. The major MHC-I receptors on human NK cells are KIR while mouse NK cells express Ly49 receptors. In addition to the recognition of MHC-I on target cells, Ly49 receptors are also involved in the education of NK cells, a process in which developing NK cells acquire effector functions.

In human, *KIR* haplotypes vary greatly between individuals and the same phenomenon is observed in mice; *Ly49* haplotypes differ in various mouse strains. Sequencing of the non-obese diabetic (NOD) *Ly49* haplotype revealed that this strain encodes for the largest *Ly49* haplotype known to date. The NOD *Ly49* cluster is characterized by the largest number of genes coding for activating Ly49 receptors. The presence of a large number of Ly49 activating receptors may affect NK cell activation status and influence the development of diabetes in NOD mice.

To gain insight in the *in vivo* role of Ly49 receptors in the control of NK cell activity, a NKC-knockdown mouse was generated. NK cells from these mice show greatly decreased expression of NKC-encoded receptors such as Ly49. NKC-knockdown NK cells have reduced lysis of MHC-I-negative target cells *in vitro* and *in vivo*. The ability of NK cells to destroy MHC-I-negative cells is

restored by the re-introduction of a single Ly49 receptor. These results provide the first direct evidence that Ly49 receptors are involved in the *in vivo* surveillance of MHC-I molecules. Interestingly, the absence of Ly49 receptors results in defective "missing-self" recognition but activation of NK cells by MHC-I-independent mechanisms is not impaired.

NKR-P1 receptors, which bind to Clr proteins, are members of another receptor family expressed by NK cells. Interestingly, the genes coding for NKR-P1 and Clr ligands are located in the same cluster and are therefore inherited together. Previous analyses demonstrated that the *Nkrp1-Clr* gene cluster is conserved in B6 and BALB/c mice. Mapping of the 129-strain cluster confirmed the high degree of conservation of the *Nkrp1-Clr* gene order in 129-strain mice. Gene sequencing revealed conservation of 129 and BALB/c *Nkrp1* and *Clr* genes and divergence from the B6 genes, suggesting that the 129 cluster is more closely related to the BALB/c cluster than to the B6 cluster.

Taken together, the Ly49 receptor family varies greatly in contrast with the highly conserved NKR-P1 receptors. Loss of Ly49 receptor expression reveals that Ly49 receptors are required for the acquisition of "missing-self" responses by NK cells.

RESUME

Les cellules 'natural killer' (NK) possèdent l'habileté de détruire des cellules anormales telles que des cellules cancéreuses ou infectées par des virus. Un trait distinctif de ces cellules est la perte d'expression de molécules CMH de type I dans une tentative d'éviter la reconnaissance par les lymphocytes T $CD8^+$. La reconnaissance de la 'perte de soi' permet aux cellules NK d'identifier et de détruire les cellules ayant une diminution de l'expression des molécules CMH-I. Des récepteurs pour les CMH-I sont exprimés par les cellules NK permettant la reconnaissance de la 'perte de soi'. Les principaux récepteurs de CMH-I pour les cellules NK humaines sont les KIR tandis que les cellules NK de souris expriment les récepteurs Ly49. En plus de la reconnaissance de CMH-I à la surface de cellules cibles, les Ly49 sont également impliqués dans l'éducation des cellules NK, un procédé par lequel les cellules NK en développement font l'acquisition de leurs fonctions.

Chez l'humain, les haplotypes de gènes *KIR* varient grandement entre individus et le même phénomène est observé chez les souris; les haplotypes de gènes *Ly49* diffèrent entre plusieurs souches de souris. Le séquençage de l'haplotype *Ly49* des souris non-obèses diabétiques (NOD) a révélé que cette souche possède le plus grand haplotype de gènes *Ly49* connu à ce jour. L'haplotype *Ly49* des souris NOD est caractérisé par le plus grand nombre de gènes codant pour des récepteurs Ly49 activateurs. La présence d'un grand nombre de récepteurs Ly49 activateurs pourrait influencer le niveau d'activation des cellules NK et le développement du diabète chez les souris NOD.

Afin de déduire le rôle *in vivo* des récepteurs Ly49 dans le contrôle de l'activité des cellules NK, une souris 'NKC-knockdown' a été créée. Les cellules NK de ces souris ont une expression grandement diminuée des récepteurs encodés dans le NKC dont les Ly49. Les cellules NK 'NKC-knockdown' possèdent une lyse réduite des cellules négative pour les CMH-I autant *in vitro* que *in vivo*. La capacité des cellules NK à détruire des cellules négatives pour les CMH-I est restaurée par la réintroduction d'un seul récepteur Ly49. Ces résultats fournissent la première preuve directe que les récepteurs Ly49 sont impliqués dans la surveillance des molécules CMH-I *in vivo*. Étonnamment, l'absence des récepteurs Ly49 entraine une reconnaissance de la 'perte de soi' défectueuse mais l'activation des cellules NK par des mécanismes n'impliquant pas des molécules CMH-I n'est pas diminuée.

Les récepteurs NKR-P1, qui se lient aux protéines Clr, sont membres d'une autre famille de récepteurs exprimés par les cellules NK. Il est intéressant de noter que les gènes codant pour les NKR-P1 et leurs ligands Clr sont regroupés et sont donc hérités ensemble. Des analyses précédentes ont démontré que le groupe de gènes *Nkrp1-Clr* est conservé chez les souris B6 et BALB/c. La cartographie du groupe des souris de souche 129 a confirmé le haut degré de conservation de l'ordre des gènes dans le groupe *Nkrp1-Clr* des souris de souche 129. Le séquençage des gènes a démontré une conservation des gènes *Nkrp* et *Clr* chez les souris 129 et BALB/c tandis que ces gènes ont divergé chez les souris B6, suggérant que le groupe *Nkrp1-Clr* des souris de souche 129 est plus apparenté au groupe BALB/c qu'au groupe B6.

En résumé, la famille de récepteurs Ly49 varie grandement contrairement au faible degré de diversité observé chez les récepteurs NKR-P1. La perte d'expression des récepteurs Ly49 révèle qu'ils sont requis pour l'acquisition de la réponse à la 'perte de soi' par les cellules NK.

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I would like to take the time to thank everyone who helped me during my years as a Ph.D. student. First, I need to acknowledge my supervisor Dr. Andrew Makrigiannis who decide to first hire me as a summer student between my second and third year of my undergraduate degree. Working there for one summer made me choose his lab for my graduate studies and I am grateful he allowed me to work in his laboratory as a graduate student. His presence made this project possible and without his guidance, feedback and ideas, this research project would not have been successful. Even though he decided to move his laboratory to the University of Ottawa during my degree, this move turned out to be a fulfilling experience which I do not regret.

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TABLE OF CONTENTS

CONTRIBUTION TO ORIGINAL KNOWLEDGE

The following original results are presented in this thesis:

Analysis of the NOD* Ly49 *gene cluster.

The expression of Ly49 receptors on NOD splenic NK cells was analyzed by flow cytometry. This analysis revealed a unique Ly49 expression pattern. The *Ly49* cluster of NOD mice was sequenced and *Ly49* genes were cloned. The genomic sequence was analyzed for the presence of repetitive elements and for homology with other *Ly49* clusters. Finally, the functions of NOD NK cells were tested by *in vitro* cytotoxicity assays and by testing the resistance of NOD mice to viral infection. Sequence analysis revealed a unique *Ly49* cluster, the largest known to date. This novel *Ly49* cluster possesses interesting features such as a high proportion of putative activating receptors which mirrors the tendency of an elevated frequency of genes coding for activating KIR in diabetic patients. The *Ly49* cluster of NOD mice resembles that of B6 mice but also contains a large-scale duplication closely related to a region of the 129S6 *Ly49* cluster. The *in vitro* cytotoxic activity of NOD NK cells was lower than B6 NK cells but higher than 129S1 NK cells. Despite the presence of the Ly49H receptor involved in resistance to MCMV infection, NOD mice cannot control MCMV infection similar to the susceptible 129S1 mice.

Characterization of the functions of NKC-knockdown NK cells

Using a novel NKC-knockdown mouse model in which the expression of Ly49 and other NKC receptors is significantly decreased, we show for the first time the *in vivo* involvement of Ly49 receptors in the surveillance of MHC-I-

deficient cells. More specifically, NKC-knockdown NK cells display defective killing of MHC-I-deficient target cells *in vitro* and *in vivo* (defective "missing-self" response). This defect can be rescued by the expression of a self-Ly49 receptor on NK cells. Furthermore, MHC-I-deficient target cells expressing ligands for an NK cell activating receptor are efficiently killed by NKC-knockdown NK cells. This phenotype mirrors the defective "missing-self" recognition observed in $\beta 2m^{-/-}$ mice and the normal activities of $\beta 2m^{-/-}$ NK cells after activation by certain activating receptors.

Mapping of the 129-strain* Nkrp1-Clr *gene cluster

BAC mapping of the 129-strain *Nkrp1-Clr* gene cluster revealed that the genetic organization of this cluster is well conserved between B6, BALB/c and 129-strain mice. Cloning and sequencing of *Nkrp1* and *Clr* cDNA permitted comparison of these sequences with those from B6 and BALB/c. Sequence analysis demonstrated that the majority of *Nkrp1-Clr* genes are highly conserved between the BALB/c and 129 mice while most B6 genes show little divergence. Unlike the majority of *Nkrp1-Clr* genes, three genes (*Clr-c*, *Nkrp1b* and *Nkrp1c*) show a great degree of variation. Sequencing of the *Nkrp1-Clr* genes permitted the analysis of newly discovered interactions between NKR-P1 receptors and Clr ligands.

CONTRIBUTION OF AUTHORS

Chapter 1 is a review of the literature to provide the reader information for the evaluation of the thesis. Manuscript-based chapters include Chapters 2 and 3. Two sections not present in the manuscripts were added to Chapters 2 and 3. These sections include an extended discussion of the results and opportunities for future research. The results presented in Chapter 4 were published in a paper for which Simon Bélanger shares first authorship. Therefore, only the experiments performed by Simon Bélanger published in this article are presented in the thesis with a discussion of the results and opportunities for future research. Dr. Andrew P. Makrigiannis conceived and supervised the studies, prepared and edited the manuscripts. Simon Bélanger performed all the experiments presented in Chapter 2, the majority of the experiments presented in Chapter 3 and all experiments presented in Chapter 4. Detailed contributions are listed below.

Chapter 2:

Bélanger, S., Tai, L.-H., Anderson, S. K., and Makrigiannis A. P. 2008. *Ly49* cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome. *Genes and Immunity.* 9:509-521.

Simon Bélanger performed all laboratory experiments and sequence analysis; analyzed the results; prepared and edited the manuscript.

Dr. Lee-Hwa Tai co-wrote the introduction with Simon Bélanger.

Dr. Stephen K. Anderson performed initial sequence annotation and promoter sequence analysis (Figure 7). Dr. Stephen K. Anderson also participated in the preparation of the manuscript.

Dr. Andrew P. Makrigiannis conceived the experiments, analyzed the results, prepared the manuscript and supervised the project.

Simon Bélanger performed the experiment for the Additional Figure 1 and prepared Additional Figure 2. These two additional figures were not included in the manuscript and were added to supplement the extended discussion.

Chapter 3:

Bélanger, S., Patel, R., Tai, L.-H., Troke A. D., Wilhelm, B. T., Landry, J.-R., and Makrigiannis A. P. Ly49 Gene-Family Silencing Results in Loss of 'Missing-Self' Responses by Natural Killer Cells to MHC-I-Deficiency: A Dominant Role for NKG2D-Mediated Tumor Cell Killing. (Manuscript in preparation).

Simon Bélanger performed all the laboratory experiments but Figure 1C; analyzed the results; conceived the experiments; prepared and edited the manuscript.

Dr. Brian T. Wilhelm and Dr. Josette-Renée Landry performed the experiment and analyzed the results for Figure 1C.

Rajen Patel and Dr. Lee-Hwa Tai provided the WT mouse (B6.Ly49^{129}) used in the experiments of Figures 3, 5, 6 and Supplemental Figure 3.

Dr. Andrew P. Makrigiannis generated the NKC-knockdown mouse with the help of Angela D. Troke. Dr. Andrew P. Makrigiannis conceived the experiments, analyzed the results, prepared the manuscript and supervised the project.
Simon Bélanger performed the experiments for the two Additional Figures. These two additional figures were not included in the manuscript and were added to supplement the extended discussion.

Chapter 4:

The analysis of the *Nkrp1-Clr* gene cluster presented in this thesis has been published in the following article:

***Chen, P., *Bélanger, S., Aguilar, O. A., Zhang, Q., St-Laurent, A., Rahim, M. M., Makrigiannis, A. P., and Carlyle, J. R. 2011. Analysis of the mouse 129-strain Nkrp1-Clr gene cluster reveals conservation of genomic organization and functional receptor-ligand interactions despite significant allelic polymorphism. *Immunogenetics*. 63:627-640.**

*Peter Chen and Simon Bélanger contributed equally to this work.
Only the work realized by Simon Bélanger is presented in this thesis (Figures 1, 3 and 4).

Here are listed other manuscripts for which I contributed during my doctoral program, but that are not included in this thesis.

Patel R., **Bélanger S.**, Tai L.-H., Troke A. D. and Makrigiannis A. P. 2010. Effect of Ly49 haplotype variance on NK cell function and education. *The Journal of Immunology.* 185:4783-4792.

Tai L.-H., Goulet M.-L., **Bélanger S.**, Toyama-Sorimachi N., Fodil-Cornu N., Vidal S. M., Troke A. D., McVicar D. W. and Makrigiannis A. P. 2008. Positive Regulation of Plasmacytoid Dendritic Cell Function via Ly49Q recognition of class I MHC. *Journal of Experimental Medicine.* 205:3187-3199.

Fodil-Cornu N., Lee S. H., **Bélanger S.**, Makrigiannis A. P., Biron C. A., Buller R. M. and Vidal S. M. 2008. Ly49h-Deficient C57Bl/6 Mice: A New Mouse Cytomegalovirus-Susceptible Model Remains Resistant to Unrelated Pathogens Controlled by the NK Complex. *The Journal of Immunology.* 181:6394-6405.

Carlyle J. R., Mesci A., Fine J. H., Chen P., **Bélanger S.**, Tai L.-H., Makrigiannis A. P. 2008. Evolution of the Ly49 and Nkrp1 recognition systems. *Seminars in Immunology*. 20:321-30.

Tai L.-H., Goulet M.-L., **Bélanger S.**, Troke A. D., St-Laurent A. G., Mesci A., Toyama-Sorimachi N., Carlyle J. R. and Makrigiannis A. P. 2007. Recognition of H-2K(b) by Ly49Q Suggests a Role for Class Ia MHC Regulation of Plasmacytoid Dendritic Cell Function. *Molecular Immunology.* 44:2638-2646.

Carlyle J. R., Mesci A., Ljutic B., **Bélanger S.,** Tai L.-H., Rouselle E., Troke A. D., Proteau M.-F., Makrigiannis A. P. 2006. Molecular and Genetic Basis for Strain-Dependent NK1.1 Alloreactivity of Mouse NK Cells. *The Journal of Immunology,* 176:7511-7524.

ABBREVIATIONS

aCGH: Array-comparative genomic hybridization
ADCC: Antibody-dependent cellular cytotoxicity
ALAK: Adherent lymphokine activated killer, same as LAK
APC: Allophycocyanin
β2m: beta-2-microglobulin
B6: C57Bl/6
BAC: Bacterial articificial chromosome
BM: Bone marrow
BSA: Bovine serum albumin
CD: Cluster of differentiation
CFSE: Carboxyfluorescein succinimidyl ester
CHO: Chinese hamster ovary
CLP: Common lymphoid progenitor
Clr: C-type lectin related
CMV: Cytomegalovirus promoter
ConA: Concanavalin A
CRACC: CD2-like receptor activating cytotoxic cells
CTL: Cytotoxic T lymphocytes
DAP10/12: DNAX-activating protein of 10/12kDa
DC: Dendritic cell
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMBA: Dimethylbenz[a]anthracene
DN: Double-negative ($CD27^-CD11b^-$)
DNAM-1: DNAX-accessory molecule-1
DNMT: DNA methyltransferase
DP: Double-positive ($CD27^+CD11b^+$)
EBV: Epstein-Barr virus
ELISA: Enzyme-linked immunosorbent assay
ES: Embryonic stem
FBS: Fetal bovine serum
Fc: Fragment, cristallizable
FITC: Fluorescein isothiocyanate
Flt3L: Fms-like tyrosine kinase 3 ligand
G418: Geneticin
γ_c: Gamma common chain, encoded by *Ilr2g*
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GPI: Glycosylphosphatidylinositol

HA: Haemagglutinin
HBSS: Hank's balanced salt solution
HCMV: Human cytomegalovirus
HLA: Human leukocyte antigen
HSC: Hematopoietic stem cell
Hsp60: Heat-shock protein of 60kDa
ICAM: Intracellular adhesion molecule
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IRF: Interferon-regulatory factor
ITAM: Immunoreceptor tyrosine-based activating motif
ITIM: Immunoreceptor tyrosine-based inhibitory motif
KD: Knockdown
KIR: Killer immunoglobulin-like receptors
LAK: Lymphokine activated killer
LCR: Locus control region
LFA-1: Lymphocyte function-associated antigen-1
LINE1: Long interspersed nuclear element 1
LLT1: Lectin-like transcript 1
LPS: Lypopolysaccharide
LRC: Leukocyte receptor complex
LTR: Long terminal repeats
mAb: Monoclonal antibody
MCA: 3-Methylcholanthrene
MCMV: Murine cytomegalovirus
MEF: Mouse embryonic fibroblast
MHC-I: Major Histocompatibility Complex Class I
MICA/B: MHC class I polypeptide-related sequence A/B
MIP: Macrophage-inflammatory protein
MULT1: Murine UL16-binding protein-like transcript 1
Neo: Neomycin resistance gene
NK cell: Natural killer cell
NKC: Natural killer complex
NKC^{KD}: Natural killer complex knockdown
NKD: Natural killer domain
NKP: Natural killer precursor
NOD: Non-obese diabetic
PBS: Phosphate buffered saline
pDC: Plasmacytoid dendritic cell

PE: R-phycoerythrin
PFU: Plaque-forming unit
PGK: Phosphoglycerate kinase
PolyI:C: Polyinosinic:polycytidylic acid
Rae-1: Retinoic acid early inducible-1
RCMV: Rat cytomegalovirus
RCTL: Rat cytomegalovirus C-type lectin
RFLP: Restriction fragment length polymorphisms
RT-PCR: Reverse transcription polymerase chain reaction
S1P: Sphingosine-1-phosphate
SCF: Stem cell factor
SD: Standard deviation
SHIP: SH2-containing inositol polyphosphate 5-phosphatase
SHP-1/2: SH2-containing protein tyrosine phosphatase-1/2
SINE: Short interspersed element
SLAM: Signalling lymphocyte activation molecule
SNP: Single nucleotide polymorphism
T1D: Type 1, insulin-dependent, diabetes
TAP: Transporter associated with antigen processing
TCR: T cell receptor
Tg: Transgene
Th1: T helper type 1
TLR: Toll-like receptor
TNF: Tumour necrosis factor
TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand
TRAMP: Transgenic adenocarcinoma of the mouse prostate
ULBP: UL-16 binding protein
VZV: Varicella Zoster virus
WT: Wild-type

INTRODUCTION

1. Natural Killer cells

1.1. History

Until the 1970's the main dogma in immunology was that lymphocytes from healthy individuals are non-responsive against tumour cells without prior immunization. Lymphocytes were known to be antigen-specific since normal cells from cancer patients were not killed by lymphocytes that were able to kill tumour cells (1, 2). This view was challenged with the discovery that cells from normal, non-immunized patients also possess the ability to destroy tumour cells (3).

Studies in non-immunized mice have also shown the presence of naturally-occurring lymphocytes possessing lytic activity against syngeneic and allogeneic tumours (4, 5). This lytic activity was still present in the spleens of athymic mice, in splenocytes depleted of T and B lymphocytes and in several organs such as lymph nodes, bone marrow and blood (4, 6). These cells have the size and shape of small lymphocytes (6) but were confirmed to lack markers of T and B lymphocytes (7).

This newly discovered phenomenon was termed «natural-killer» activity due to the absence of the requirement for pre-immunization and the cells mediating this activity were named natural killer (NK) cells. Target cell lysis by NK cells is a rapid event since *in vitro* NK cell activity can be detected over the course of 4 hours. The reactivity was influenced by the age of the mouse as «natural-killer» activity was highest in mice aged between 1 and 3 months (4, 6).

The lytic activity of NK cells was found to be increased by treatment of mice with interferon or with inducers of type I interferons (8, 9).

In the first few years after their discovery, several groups attempted to find cellular targets for NK cells. Thymocytes, embryonic carcinoma cells and cells that are at an early stage in differentiation were all found to be targets for NK cells (10-12).

1.2. Missing-self hypothesis

Natural killer cells are able to destroy tumour cells without any prior sensitization but several observations concerning such target cells were quite puzzling. Thymocytes sensitive to T lymphocytes are resistant to lysis by NK cells suggesting that the recognition of target cells by NK cells differs from that of T lymphocytes (10). Further lines of evidence came from a study showing that as NK-sensitive cells differentiate and acquire MHC expression, the sensitivity to NK cells is lost (11). Interferons increase NK cell activity but treatment of target cells with interferon had opposite effects. Thymocytes isolated from virus-infected mice (virus infections are known inducers of interferon) became more resistant to lysis by NK cells (9). Similarly, *in vitro* treatment of NK-sensitive thymocytes or tumour cells with interferon decreased sensitivity to NK cell-mediated lytic activity (9, 13). A key observation was that the resistance of cells to NK cell lysis positively correlates with MHC expression levels after interferon treatment (9).

Discoveries in the field of bone marrow (BM) transplantation were quite surprising in the sense that they did not follow the rules governing the rejection of

skin grafts. Irradiated mice are able to reject allogeneic BM grafts while syngeneic grafts survived. For example, mice carrying H-2^d MHC-I were able to reject BM grafts of H-2^b origin but not grafts of H-2^d origin (14). This phenomenon was independent of thymus-derived cells as thymectomized mice are still able to reject allogeneic BM grafts (14). Another peculiarity of BM grafts is the hybrid resistance phenomenon in which H-$2^{b/d}$ offsprings reject parental H-2^b and H-2^d BM grafts while the H-2^b and H-2^d parents cannot reject F_1 H-$2^{b/d}$ grafts (15). Interestingly, the ability of mice to reject allogeneic grafts or to mediate hybrid resistance appears when mice are 3 weeks old, mirroring the appearance of NK cell lytic activity against tumour cells (14, 15). Mice devoid of T and B lymphocytes are able to reject allogeneic BM grafts further suggesting that NK cells may be involved in this process (16).

All the findings listed above pointed towards the use of MHC-I by NK cells to kill target cells. A seminal study by Kärre et *al.* solved this issue. Two cell lines were derived from the RBL-5 lymphoma tumour cell line by mutagenesis. One subline was selected against MHC-I expression to generate RMA-S cells. The second cell line did not undergo MHC-I selection and was named RMA. The MHC-I-positive RMA cells survived when injected in a syngeneic host while the MHC-I-negative RMA-S did not. RMA-S cells were found to be efficiently killed by NK cells *in vitro* while RMA were not, suggesting that NK cells recognize cells that have lost MHC-I expression (17). Furthermore, depletion of NK cells in mice allowed survival of injected RMA-S (18). The "missing-self" hypothesis was postulated to explain this phenomenon (Fig. 1A-B). The "missing-self"

hypothesis states that NK cells recognize and destroy cells lacking expression of MHC-I molecules (19). Expression of MHC-I but not MHC-II molecules in NK-sensitive targets inhibited NK cell activity further suggesting that MHC-I are recognized by NK cells (20).

The "missing-self" hypothesis could explain the hybrid resistance phenomenon. F_1 hybrid pups express MHC-I molecules from both parents while parental cells express one of the two MHC-I molecules present in the F_1 hybrid. When NK cells from the F_1 hybrids encounter BM cells from the parents, they recognize missing MHC-I molecules and destroy these cells. On the other hand, NK cells from the parent will always see cells from the F_1 hybrid as self due to the expression of parental MHC-I molecules.

Further experiments supporting the "missing-self" hypothesis came with the use of MHC-I-deficient mice as donors in BM engraftment experiments. Sub-lethally irradiated mice, in which BM cells are destroyed but with intact peripheral NK cells, are able to reject BM cells from syngeneic $\beta 2m^{-/-}$ donor mice (21). *In vitro* studies also revealed that cells isolated from $\beta 2m^{-/-}$ mice were killed by NK cells (22, 23).

Similarly, human NK cells attack cells with decreased expression of MHC-I molecules (24, 25) suggesting that the "missing-self" hypothesis also holds true for human NK cells. Furthermore, transfection of MHC-I molecules in NK-sensitive target endows resistance of these cells to human NK cells (26). The "missing-self" hypothesis has important implications to diseases as virus-infected cells and cancerous cells often downregulate the expression of MHC-I molecules

(27, 28) presumably to evade the cytotoxic response of $CD8^+$ T lymphocytes. The decrease in MHC-I expression would then be recognized by NK cells and these infected or transformed cells become sensitive to NK cell-mediated lysis.

1.3. Definition of an NK cell

Mature NK cells can be found in several lymphoid and non-lymphoid organs including the spleen, bone marrow, liver, lymph node, lung, intestine and placenta (29). Human NK cells are $CD3^-$ lymphocytes characterized by the expression of CD56 (a cell adhesion molecule part of the immunoglobulin superfamily). They can also express the IgG receptor FcγRIII (CD16) (30, 31). Two major subsets of human blood NK cells exist: $CD56^{dim}CD16^+$ and $CD56^{bright}CD16^{dim/negative}$ and these two markers are routinely used to detect human NK cells. $CD56^{dim}CD16^+$ NK cells represent around 90% of blood NK cells and the remaining NK cells are $CD56^{bright}CD16^{dim/negative}$ (32). $CD56^{bright}CD16^{dim/negative}$ NK cells are characterized by the high expression of IL-7R and c-kit while $CD56^{dim}CD16^+$ NK cells have low or no expression of these markers (33). Human NK cells possess several receptors such as NKG2D, KIR (killer Ig-like receptors), NKp46 and CD94-NKG2A allowing recognition of unhealthy cells.

Murine natural killer cells were initially characterized by the expression of the NK1.1 antigen (34, 35). NK1.1 expression is found in C57Bl/6 (B6) mice but not in 129S1 or BALB/c (34). The glycolipid asialo-GM1 is also a marker of mature NK cells (36). DX5, also known as CD49b, is a marker expressed by NK cells from all tested mice strains (37, 38). It is important to note that both NK1.1

and DX5 are not NK-specific markers as NK-T lymphocytes (loosely defined as lymphocytes that express a TCR and NK cell markers) also express NK1.1 and DX5 (39). Recently, NKp46 was defined as a true NK-specific marker as no other immune cells (even NK-T lymphocytes) express this marker (40). Thus murine NK cells can be defined by the expression of NKp46, DX5 (NK1.1 in certain strains) and by the absence of T lymphocyte markers such as CD3 and the T cell receptor (TCR). Mouse NK cells also express a wide variety of receptors that control their functions such as Ly49, NKG2D, 2B4 and CD94-NKG2.

1.4. Effector molecules of NK cells

It is thought that the major functions of NK cells are to recognize and destroy abnormal cells such as virally-infected or transformed cells. Upon recognition of these abnormal cells, NK cells will release effector molecules that will induce target cell death. The second major function of NK cells is to secrete cytokines and chemokines such as IFN-γ, TNF-α, MIP-1α, MIP-1β and IL-10 (41).

Natural killer cells require the expression of several molecules for proper lysis of target cells. NK cells isolated from mice lacking perforin cannot lyse tumour cells (42) and these mice cannot reject the NK-sensitive cell line RMA-S (43). Although perforin-deficient NK cells have a decreased cytotoxic activity, their ability to induce apoptosis is not completely abolished (44). In cooperation with perforin, granzyme molecules are used by NK cells to destroy target cells. Granzyme B induces lysis and DNA fragmentation of tumour target cells. Other granzymes such as granzyme A are also used by NK cells as shown by modest

DNA fragmentation detected when granzyme B-deficient NK cells are incubated with target cells for longer periods of time (45).

The mechanism of action of perforin is not clear. Initially, it was though that perforin would create large pores in the membrane of the cell targeted for destruction to allow entry of granzymes into the target cell (46). It was later shown that granzymes can be internalized in the absence of perforin and the pores formed by perforin at physiological concentration may be too small to allow entry of granzymes. Although the role of perforin is not clear, it clearly disrupts the plasma membrane of the target cell (46). On the other hand, the mechanism of action of granzymes is known. Both granzyme A and B induce DNA and membrane damage while only granzyme B can activate caspase 3 and disturb mitochondria leading to release of cytochrome c and activation of caspase 9, an initiator of apoptosis (46).

Granzyme B and granzyme A require cathepsin B for activation prior to release by the NK cell. NK cells from mice deficient for cathepsin B or from patients with loss-of-function mutations in the gene coding for this protein cannot generate mature granzymes and thus lack cytotoxic activity (47, 48). Transcription of the *Gzmb* and *Prf1* genes in resting NK cells allows rapid production of granzyme B and perforin proteins after activation while granzyme A protein is present in resting NK cells (49). The transcription of the *Ifng* gene in resting NK cells generates a pool of *Ifng* transcripts allowing rapid secretion of IFN-γ by NK cells upon activation (50). Lastly, NK cells also use FasL to trigger target cell death by binding to Fas on the surface of target cells as Fas^{+} tumour

cells are more sensitive to lysis by NK cells (51). Furthermore, TNF-related apoptosis-inducing ligand (TRAIL) is expressed on cytokine-activated NK cells and can trigger target cell death of some tumour cell lines (52).

1.5. Development of NK cells

Early *in vitro* experiments demonstrated that murine multipotent hematopoietic stem cells (HSC) isolated from BM can generate NK cells (53-55). Multipotent HSC are characterized by the absence of Lineage markers (B220, Gr-1, Mac-1, CD3, CD4, CD8 and Ter119) and the expression of Sca2 and c-kit (56). Culture of HSC in the presence of IL-7, Stem Cell Factor (SCF) and Fms-like tyrosine kinase 3 ligand (Flt3L) generates cells that become responsive to IL-15 (54). The differentiated HSC have now become NK cells precursors (NKP) which lack the potential to differentiate into T or B lymphocytes and express IL-15Rβ (CD122) (57). Addition of IL-15 to the culture of differentiated HSC results in the generation of NK cells. Alternatively, *in vitro* culture of sorted NKP in the presence of IL-2, IL-7, SCF and Flt3L also gives rise to NK cells (57). However, *in vitro* cultures require the addition of a bone marrow-derived stromal cell line for NK cells to acquire the expression of Ly49 receptors (54).

NK cell development in the mature mouse takes primarily place in the bone marrow. Accordingly, destruction of BM cells abrogates the generation of NK cells in the mouse (35). Early studies reported that injection of common lymphoid progenitors (CLP) into irradiated mice allows reconstitution of NK cells in the periphery (56). Five stages of NK cell development were identified in murine bone marrow (58). Stage I is composed of NKP ($CD122^{+}NK1.1^{-}DX5^{-}$). NKP

enter Stage II and begin to express high levels of NK1.1, CD94 and NKG2A, which are markers of mature NK cells. Stage II cells also express low levels of DX5, Mac-1 and CD43. Stage III is characterized by the upregulation of Ly49 receptors and c-kit. This stage is followed by extensive cell division (Stage IV). Lastly, mature NK cells (Stage V) lose expression of c-kit and acquire effector functions: cytotoxicity and cytokine production (58).

IL-15 is the critical cytokine required for proper development of NK cells. Mice deficient in IL-15 lack most peripheral NK cells (59). Similarly, mice lacking IL-15Rβ or IL-15Rα also lack peripheral NK cells (60, 61). IL-15R is made of a third component, the γ_c chain. Absence of the γ_c chain leads to a defective IL-15R and thus no NK cells develop in γ_c-deficient mice (62). These mice have normal numbers of NKP in their bone marrow but no further development is observed. Flt3L-deficient mice have highly reduced numbers of peripheral NK cells supporting *in vitro* data (63).

Several transcription factors have also been implicated in the normal development of murine NK cells. Mice deficient for the PU.1, Id2 and Ikaros transcription factors have severe defects in their peripheral NK cell population (64-66). The defect in NK cell development in *PU.1*$^{-/-}$ mice has been linked to reduced numbers of NKP in the BM (64). Similarly, *E4bp4*$^{-/-}$ mice lack peripheral NK cells but unlike *PU.1*$^{-/-}$ mice, NKP are present in the bone marrow (67). Id2 acts at a later stage than E4BP4 because *E4bp4*$^{-/-}$ HSC transduced with Id2 can generate NK cells (67). Another nuclear protein, the DNA-binding factor TOX, is also involved in the proper development of NK cells. *Tox* mRNA is expressed at

high levels in immature and mature NK cells in the bone marrow. Accordingly, there is impaired development of NK cells in *Tox*$^{-/-}$ mice (68). Expression of *Id2* is decreased in *Tox*$^{-/-}$ NK cells suggesting that TOX is involved in the induction of Id2. Interestingly, expression of Id2 in *Tox*$^{-/-}$ bone marrow progenitors does not allow the development of NK cells suggesting that Id2 is not the only target of TOX (68).

Ets-1 is another transcription factor required for development of NK cells as *Ets-1*$^{-/-}$ mice have highly reduced numbers of splenic NK cells (37). The IFN regulatory factor-2 (IRF-2) is involved in the final maturation stages of NK cells. *IRF-2*$^{-/-}$ mice show highly reduced numbers of mature NK cells in the spleen and bone marrow while the numbers of immature NK cells are normal (69). Similar to IRF-2, IRF-1 also plays a role in the proper development of NK cells since *IRF-1*$^{-/-}$ mice have reduced numbers of peripheral NK cells (70). Peripheral NK cells can develop in irradiated WT mice receiving *IRF-1*$^{-/-}$ BM while WT BM cannot generate NK cells when injected in irradiated *IRF-1*$^{-/-}$ mice indicating that the bone marrow environment of *IRF-1*$^{-/-}$ mice does not support NK cell development. IL-15 is a transcriptional target of IRF-1 and in the absence of IRF-1, there was no IL-15 transcription, thus possibly explaining the defective generation of NK cells in *IRF-1*$^{-/-}$ mice (70).

1.6. Peripheral NK cells

The frequency of NK cells in various organs varies between 1 and 10% of total lymphocytes. For example, NK cells represent roughly 3% of splenic lymphocytes and 6% of liver lymphocytes. NK cells are found in a decreasing

proportion in the following organs: lung, liver, blood, spleen, bone marrow, lymph nodes and finally thymus. Due to the size of the lymphocyte population, splenic NK cells represent the biggest population of NK cells in absolute numbers (29). The mechanisms governing NK cell migration from the bone marrow to different organs are largely unknown, but it was recently reported that the receptor $S1P_5$ allows NK cells to migrate in response to the lysophospholipid sphingosine 1-phosphate (S1P). S1P binds to plasma proteins such as albumin and a gradient of S1P is maintained *in vivo* with high concentrations of S1P in extracellular fluids and low concentrations in organs (71). In $S1P_5$-deficient mice, NK accumulate in the bone marrow and lymph nodes and lower numbers of NK cells are observed in the spleen, blood and lung (72). S1P may be one signal used by NK cells to leave the bone marrow after differentiation.

NK cells home to the red pulp of the spleen and some NK cells can be found in the marginal zone (29). Most of the splenic NK cells can be found in the blood sinuses and in close contact with $CD11b^{high}$ and $CD11c^{high}$ cells that are most likely macrophages and dendritic cells. The mechanism used by NK cells to home to different organs is not clear but it seems that CD62L (L-selectin) is used by NK cells to enter lymph nodes (73). NK cells express CCR2, CCR5, CXCR3, CX3CR1 which are all receptors for inflammatory chemokines (29). The expression of these chemokine receptors may favour recruitment of NK cells to sites of infection. S1P may also be implicated in the recruitment of NK cells to inflamed organs since $S1P_5$-deficient NK cells have defective migration from the spleen to the liver during inflammation (72).

NK cell survival in the periphery depends on IL-15. The average survival of NK cells transferred into *Il15*$^{-/-}$ mice is less than 5 days while NK cells can be found 60 days after transfer into WT recipients (74, 75). This survival was dependent on the action of Bcl-2 as Bcl-2^{tg} NK cells survived after transfer into *Il15*$^{-/-}$ mice (74). IL-15 also affects other proteins involved in the apoptosis pathway. The pro-apoptotic protein Bim is upregulated in NK cells deprived of IL-15 while its presence induces the expression of the Bcl-2 family member Mcl-1 (76). Although NK cells require IL-15 for survival, IL-15Rα-deficient NK cells survive in normal hosts while normal NK cells die when transferred in IL-15Rα-deficient recipients (77). Recent evidence have shown that IL-15 needs to be presented to NK cells by IL-15Rα (a phenomenon called transpresentation) for NK cell homeostasis and development thus explaining the finding that expression of IL-15Rα by NK cells is not required for their survival (78).

NK cells were initially described as short lived cells since a rapid loss of "natural cytotoxicity" was observed when cells are killed *in vivo* by injections of a cell cycle-specific cytotoxic drug (79). It was later shown that the half-life of splenic mouse NK cells is roughly 17 days (80). In young mice, NK cells proliferate rapidly and this mirrors an increase in total NK cell number as mice age. As the number of NK cells peaks, the proliferation of NK cells decreases to a minimum (80). While peripheral NK cells are not proliferating, NK cells in the bone marrow are actively dividing, consistent with the proliferation of NK cell precursors in Stage IV of NK cell development (58, 80).

1.7. NK cell subsets

Mature NK cells in the mouse can be divided in four subsets based on the expression of CD11b and CD27: double-negative (DN) $CD27^{-}CD11b^{-}$, single-positive $CD27^{+}CD11b^{-}$ and $CD27^{-}CD11b^{+}$, and double-positive (DP) $CD27^{+}CD11b^{+}$ (81, 82). These subsets are not equally distributed in peripheral organs. Bone marrow tend to have a high proportion of $CD27^{+}CD11b^{-}$ NK cells while this subset is almost absent from the blood and the lung. On the other hand, $CD27^{-}CD11b^{+}$ NK cells are mostly found in the spleen, liver, blood and lung while only a few are present in the bone marrow (81). DN and $CD27^{+}CD11b^{-}$ NK cells have a lower proportion of Ly49-positive cells than the other two subsets suggesting that the DN and $CD27^{+}CD11b^{-}$ NK cells are less mature than the DP and $CD27^{-}CD11b^{+}$ NK cells (81, 82). The lower percentage of DN and $CD27^{+}CD11b^{-}$ NK cells positive for the mature NK markers CD43 and CD62L also favours this hypothesis. Following NK cell depletion, the first subset of NK cells to reappear is the DN, followed by the $CD27^{+}CD11b^{-}$, then the DP and lastly the $CD27^{-}CD11b^{+}$ (82) suggesting that NK cells mature in the periphery following the order listed above. Functional differences exist between these subsets: DP $CD27^{+}CD11b^{+}$ NK cells produce more IFN-γ and have a higher cytotoxic potential than $CD27^{-}CD11b^{+}$ NK cells (81). Based on these findings, it was proposed that $CD27^{-}CD11b^{+}$ NK cells are long-lived and senescent cells (81).

Similar to murine NK cells, human NK cells can also be divided in two subsets: $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim/negative}$ cells (32). $CD16^{+}$ NK cells have higher cytotoxic potential than the $CD16^{dim/negative}$ NK cells while the latter

subset produces higher levels of cytokines such as IFN-γ, TNF-α, IL-10, IL-13 and GM-CSF (83, 84). Analysis of the transcriptional profiles of the two subsets confirmed the observed functional differences. $CD56^{dim}CD16^{+}$ NK cells express high levels of perforin and granzymes A, B and M transcripts correlating with their high cytotoxic potential. On the other hand, $CD56^{bright}CD16^{dim/negative}$ NK cells express transcripts for adhesion molecules such CD44 and CD62L, suggesting that this subset preferentially homes to lymphoid organs (85, 86). Several lines of evidence suggest that $CD56^{bright}CD16^{dim/negative}$ NK cells are precursors of $CD56^{dim}CD16^{+}$ cells. Upon activation with cytokines, $CD56^{bright}CD16^{dim/negative}$ NK cells acquire CD16 while CD56 expression decreases (33). Furthermore, $CD16^{-}$ NK cells are more prevalent in blood early after HSC transplantation while the proportion of $CD16^{+}$ NK cells in the blood increases with time (87). $CD16^{-}$ NK cells also have longer telomeres than $CD16^{+}$ NK cells indicating that they have undergone a smaller number of cell divisions (33). Lastly, transfer of $CD56^{bright}CD16^{-}$ NK cells in humanized mice allows the generation of $CD16^{+}$ NK cells (78).

1.8. Natural killer cells and diseases

1.8.1. Cancer

Since their discovery, NK cells have been implicated in immune responses directed against tumour cells (4, 6, 17, 88). Beige mice display defective NK cell activity and have an increased mortality rate compared to WT mice due to appearance of spontaneous tumours (89). Similarly, human NK cells have been implicated in the surveillance of tumour cells. Patients with Hodgkin's disease

have defective NK cell responses (90). Infiltration of tumours by NK cells seems to correlate with better prognosis in various carcinomas (91). The ability of NK cells to destroy tumour cells has been the focus of several therapeutic approaches. NK cells expanded and activated *in vitro* with IL-2 (also known as Lymphokine Activated Killers: LAK) have been used in the treatment of cancer patients (92). Unfortunately, injection of LAK cells into cancer patients has proved unsuccessful (93). Injections of IL-2 to activate NK cells *in vivo* was also used as a therapeutic approach to treat cancer but proved to be inefficient (94). Although injections of IL-2 are known to increase the number of tumour-infiltrating NK cells, no tumour regression was observed probably because patients with large tumour burdens were enrolled in almost all trials (91). Furthermore, injections of IL-2 favour the maintenance of regulatory T cells that suppress $CD8^+$ T lymphocytes (95).

1.8.2. Viruses

NK cells have been strongly implicated in the surveillance of viral infections. A patient devoid of NK cells could not control normally innocuous viral infections such as cytomegalovirus and chicken pox (96). Another case study revealed that patients lacking NK cells suffer from recurrent severe varicella infections (48). Other studies have shown that patients with mutations affecting NK cell functions also suffer from viral infections. A patient with normal NK cell lytic activity but bearing a mutation in FcγRIII (CD16) suffered from recurrent respiratory tract infections. This patient had severe clinical problems due to infection with Epstein-Barr virus (EBV) and Varicella Zoster virus (VZV) (48,

97). Another report demonstrated that NK cells from a patient with chronic EBV infection had decreased cytolytic activity and diminished secretion of IFN-γ (98). It is not known if the decreased NK cell activities in this patient are caused by the chronic EBV infection or if defective NK cells allowed for the chronic EBV infection. Similar to human NK cells, murine NK cells have also been implicated in the control of viral infections. The murine model of cytomegalovirus infection, murine cytomegalovirus (MCMV), is controlled by NK cells as their depletion leads to an increased viral burden and death in MCMV-infected mice (99). NK cells are also essential for the resistance of mice to infections with ectromelia virus, the causative agent of mousepox (100).

1.8.3. Other pathogens

NK cells not only participate in the immune response directed towards cancerous and virally-infected cells but they have also been implicated in the control of other pathogens such as intracellular bacteria and protozoan parasites. Human NK cells can kill monocytes and alveolar macrophages infected with *Mycobaterium tuberculosis* (101). Depletion of NK cells leads to increased bacterial burden in murine models of *Legionella pneumophila* and *Shigella flexneri* (102, 103). NK cells also produce IFN-γ in response to infection by these two bacteria. The production of IFN-γ by NK cells is also induced by infection with the protozoan *Toxoplasma gondii* (104) while depletion of NK cells reduces survival of mice infected with *Trypanosoma cruzi* (105). *Plasmodium yoelii* is used to model human malaria. This parasite induces recruitment of NK cells to the liver of mice infected with sporozoites, a stage of its life cycle that allows

infection of hepatocytes. While NK cells can control the growth of *P. yoelii* when it infects hepatocytes, this parasite cannot be controlled by NK cells when it infects red blood cells (106).

1.8.4. Influence of NK cells on adaptive immune responses

NK cells not only directly control pathogens and tumour cells but they can also influence adaptive immune responses. In mice, NK cells are able to prime Th1 responses. This priming ability requires activation of the NK cells by dendritic cells (DC), recruitment of NK cells to lymph nodes and the production of IFN-γ (107). Resistance of mice to infection with *Leishmania major* depends on Th1 immune response and on the production of the protective cytokine IFN-γ. In mice depleted of NK cells, production of IFN-γ was impaired leading to an increased parasite burden, indicating that NK cells are required for the initiation of a Th1 response after infection with *L. major* (108).

Human NK cells can also affect subsequent immune responses by activating DC. NK cells can prime DC which can in turn induce T cell proliferation and IFN-γ secretion by T cells (109, 110). It is not clear if the activation of DC by NK cells requires cellular interaction as some studies have shown that DC separated from NK cells by a porous membrane are not activated and that cytokines produced by NK cells cannot activate DC (109, 110). Others have suggested that both IFN-γ and TNF-α produced by NK cells are required for optimal activation of DC by NK cells (111). Interestingly, the ratio between NK cells and DC has very important consequences on the outcome of the interaction between these cells. Low NK:DC ratios favour the priming of DC while high

NK:DC will result in lysis of DC by the NK cells (110). NK cells can possibly modulate the outcome of immune responses by preferentially killing immature DC (112).

1.9. Activation of NK cells by dendritic cells

Although NK cells can kill tumour cells without any prior sensitization, early studies have shown that NK cell activity can be enhanced by injecting mice with inducers of type I interferon (8). Incubation of NK cells with IL-12 and IL-18 or IL-12 in combination with IL-15 induces cytokine production by NK cells suggesting a role for these three cytokines in the *in vivo* activation of NK cells (41). It is now known that DC play a critical role in the activation of NK cells. Both immature and mature DC possess the ability to trigger NK cell cytotoxicity and production of IFN-γ (112). The production of IL-12 by DC is required for activation of NK cells. Interestingly, the secretion of IL-12 is limited to the site of contact between DC and NK, otherwise known as the immunological synapse (113). Disruption of the immunological synapse hinders the ability of DC to prime NK cells. Formation of the synapse is mediated by interactions between DC-SIGN present on the surface of DC and its ligand ICAM-3 on NK cells (113). LFA-1 on the surface of NK cells is also recruited to the immunological synapse and is required for NK cell activation following interaction with DC (113, 114). The IRF-3-dependent NK-activating molecule (INAM) expressed by both DC and NK cells is also involved in the activation of NK cells by DC presumably by facilitating the formation of immunological synapses between NK cells and DC (115).

IL-15 plays a critical role in the activation of NK cells. DC lacking IL-15Rα cannot activate NK cells (116). Incubation of NK cells with a soluble form of IL-15Rα associated with IL-15 permits NK cell activation suggesting that similar to development and NK homeostasis, transpresentation of IL-15 plays a role in priming NK cells (116). To efficiently produce IL-15, DC require the expression of IL-15Rα and only coordinate expression of both IL-15 and IL-15Rα by DC can lead to activation of NK cell (117). Although IL-15Rα-IL-15 complexes can be secreted, only the membrane-bound complexes support *in vivo* NK cell activation (117) further supporting the notion that transpresentation of IL-15 by IL-15Rα by DC is required for NK cell activation.

NK cells are not activated by injections of interferon inducers in DC-depleted mice suggesting an *in vivo* role for DC in the priming of NK cells (118). DC first need to be activated by TLR agonists such as poly I:C (TLR-3 ligand) or LPS (TLR-4 ligand). This activation will induce production of type I IFN by DC. Type I IFN is required for the production of IL-15 by DC which is then transpresented by IL-15Rα to NK cells *in vivo* (118). Although IL-15 is a critical factor involved in the activation of DC, the few NK cells present in IL-15-deficient mice can be activated after infection with MCMV and this activation is driven by IL-12 (119) suggesting that IL-15 is not the only cytokine able to activate NK cells *in vivo*.

After activation, NK cells will upregulate the early activation marker CD69 and lose the expression of DX5 (38). Activated NK cells upregulate the inhibitory receptor KLRG1 (120, 121). $KLRG1^{+}$ NK cells have a reduced ability to secrete

IFN-γ and have a slower proliferation rate (120, 121). It is thought that the expression of KLRG1 serves to dampen NK cell responses. Interestingly, $CD27^-$ $CD11b^+$ NK cells express higher levels of KLRG1 than the other NK cell subsets (81) further suggesting that these NK cells are senescent.

2. NK cell receptors

2.1. General properties of NK cell receptors

NK cell receptors fall into two broad categories: activating and inhibitory receptors. The majority of inhibitory receptors expressed by NK cells possess a signalling motif in their cytoplasmic tail allowing the delivery of negative signals. This signalling motif is called an immunoreceptor tyrosine-based inhibitory motif (ITIM) and is defined by the following consensus sequence: Ile/Val/Leu/Ser-x-Tyr-x-x-Leu/Val (122). Upon ligand binding, the tyrosine residue present in the ITIM becomes phosphorylated and serves as a docking site for the recruitment of tyrosine-specific phosphatases such as SHP-1 (SH2-containing protein tyrosine phosphatase-1), SHP-2 and SHIP (SH2-containing inositol polyphosphate 5-phosphatase). Recruitment of these phosphatases to phosphorylated ITIMs will result in a decrease in the phosphorylation levels of several signalling proteins ultimately leading to dampened NK cell activities (122).

Unlike inhibitory receptors, most activating receptors expressed by NK cells lack a signalling motif in their cytoplasmic tail and need to associate with adaptor molecules to transmit their signals. These adaptor molecules are characterized by the presence of an immunoreceptor tyrosine-based activation motif (ITAM) defined by the following amino acid sequence: Asp/Glu-x-x-Tyr-x-

x-Leu/Ile-x_{6-8}-Tyr-x-x-Leu/Ile (122). NK cells express three ITAM-containing adaptor molecules: DAP12, FcεRIγ and CD3ζ. Upon ligand recognition by the activating receptors, the tyrosine residues in the ITAM become phosphorylated and recruit Syk and Zap70 tyrosine kinases that then initiate a signalling cascade leading to activation of NK cell functions (122). Another adaptor molecule expressed by NK cells and used by activating receptors to convey positive signals is DAP10 which is characterized by the presence of a Tyr-x-x-Met motif that initiates a signalling cascade independent of Syk-family protein tyrosine kinases (123).

The balance of signals delivered to the NK cell will determine its activation status. If activating signals are stronger, the NK cell will become activated and will release contents from its granules to initiate cytotoxicity and to produce cytokines. On the other hand, if inhibitory signals predominate, the NK cell will not be activated and the target cell will be spared (122).

2.2. NK signalling pathways

ITAMs located on adaptor molecules are initially phosphorylated by protein tyrosine kinases of the Src family (Lck and Fyn). The phosphorylated tyrosines on the ITAM will then recruit Syk and Zap70 followed by recruitment of adaptors such as LAT (Linker of activated T cells) and SLP-76 (SH2 domain-containing leukocyte phosphoprotein of 76 kD) (123). Initial signals delivered through DAP10 will induce the recruitment of PI3K (phosphatidylinositol 3-kinase). Signalling pathways initiated by ITAM-containing adaptors or by DAP10 ultimately converge on a common pathway leading to activation of NK cell

cytotoxicity. This pathway involves the activation of Rac which will activate PAK1 which in turn activates MEK leading to ERK activation (123). While signals initiated by ITAM-containing adaptor lead to cytotoxicity and cytokine secretion, DAP10 can only induce NK cell cytotoxicity. ITAM-containing adaptors but not DAP10 activate PLC-γ1 (phospholipase C-γ1) resulting in the initiation of Ca^{2+} flux (123). The inability of DAP10 to activate PLC-γ1 may explain why it cannot trigger IFN-γ production by NK cells.

2.3. NK cell receptors gene clusters

In humans, the receptors that belong to the Ig superfamily are encoded in the Leukocyte receptor complex (LRC), a gene cluster located on chromosome 19 (124). Genes present in the human LRC include *NKp46*, *KIR*, *ILT* (Ig-like transcripts), *LAIR* (Leukocyte-associated inhibitory receptors), *SIGLEC* (Sialic-acid binding Ig-like lectins), *CD66*, *DAP12* and *DAP10* (Fig. 2). The LCR is also present in mice on chromosome 7 with some highly conserved regions while other regions present in the human LCR are absent from its mouse counterpart (Fig. 2). *Nkp46* is present in the mouse LCR and so are *PIR* (Paired Ig-like receptors) which are thought to be orthologous to human *ILT* (124).

C-type lectin receptors are encoded by genes present in a cluster located on human chromosome 12 and mouse chromosome 6 called the Natural killer complex (NKC). The human NKC contains the following genes: *NKR-P1A*, *LLT1* (Lectin-like transcript 1), *NKG2D*, *CD94*, *NKG2A*, *NKG2C*, *NKG2E* and *LY49L* (Fig. 2) (124). The mouse NKC is similar to the human NKC and it contains the

following genes: *Nkrp1*, *Clr*, *Nkg2d*, *Cd94*, *Nkg2a*, *Nkg2c*, *Nkg2e* and *Ly49* (Fig. 2) (124-126).

2.4. NKG2D

The gene coding for NKG2D is located in the NKC (Fig. 2) and it encodes for an activating receptor involved in the recognition of MHC-like molecules. NKG2D is a type II transmembrane glycoprotein expressed as a homodimer, unlike other NKG2 members which heterodimerize with CD94. NKG2D is present on NK cells, activated $CD8^+$ T cells, subsets of γδ T cells and subsets of NK-T cells (127, 128). While activation through NKG2D is sufficient to trigger NK cell functions, it acts as a costimulatory molecule on $CD8^+$ T cells (128). The mouse *Nkg2d* gene can generate two isoforms: a long form called NKG2D-L and a short form named NKG2D-S (129). While NKG2D-L uses DAP10 as an adaptor molecule and is expressed in resting and activated NK cells, NKG2D-S associates with both DAP10 and DAP12 and is present in $CD8^+$ T cells but only on activated NK cells (129, 130). Unlike murine NKG2D, human NKG2D selectively associates with DAP10 (131, 132).

The ligands for human NKG2D are known to include MICA, MICB, ULBP1, ULBP2, ULBP3 and ULBP4 (122, 127, 133) and those for mouse NKG2D are Rae-1α, Rae-1β, Rae-1γ, Rae-1δ, Rae-1ε, H60 and Mult1 (122, 134, 135). These molecules are all distantly related to MHC-I molecules. Indeed ULPBs show ~20% homology with the human MHC-I molecule HLA-A2 in the α1 and α2 domains. Unlike MHC-I molecules that possess an α3 domain for membrane attachment, ULBPs possess a glycosylphosphatidylinositol (GPI)

linkage to the membrane (133). Similarly, mouse Rae-1 and H60 have a ~30% homology in the α1 and α2 domains with mouse MHC-I molecules and are also GPI-linked surface proteins (134, 135). Crystal structure of MICA and Rae-1β revealed that these molecules have a distorted MHC-I structure. Both MICA and Rae-1β possess a remnant of a peptide-binding groove formed by α1 and α2 domains but it is likely that these domains do not allow for binding of peptides (136, 137).

The ligands for NKG2D are preferentially expressed on various tumour cells such as YAC-1, RAW and DC2.4 (135). Expression of ligands for NKG2D on tumour cells triggers cytotoxicity and IFN-γ production by NK cells (134, 135) and cells expressing NKG2D ligands have decreased survival in mice compared to their ligand-negative counterpart (130). Tumour cells isolated from NKG2D-deficient mice display high expression of NKG2D ligands compared to tumour cells isolated from wild-type mice (138). This finding suggests that tumour cells will undergo an immunoediting process to evade surveillance by NKG2D. Another mechanism used by tumour cells to evade NKG2D surveillance is shedding of ligands. The serum of some cancer patients with MICA-positive tumours contains solube MICA, which induces the downregulation of NKG2D on $CD8^+$ T cells (139).

Ligands for NKG2D have been shown to be expressed in response to several stress conditions including DNA-damaging agents and DNA synthesis inhibitors (140). Infection of cells with human cytomegalovirus (HCMV) or murine cytomegalovirus (MCMV) also induces expression of NKG2D ligands

(141, 142). NKG2D has also been implicated in the resistance of mice to infection with ectromelia (143). Blocking of NKG2D *in vivo* led to an increase in viral titer and it was shown that ectromelia infection induces the expression of NKG2D ligands (143). In summary, NKG2D allows NK cells to survey the host for stressed cells (damaged cells, transformed cells or infected cells).

2.5. NKG2-CD94

Both humans and mice possess genes encoding the CD94-NKG2 family of receptors (122). Similar to *NKG2D*, these genes are located in the NKC (Fig. 2). Human possess four additional *NKG2* genes (*NKG2A*, -*C*, -*E* and –*F*) (122, 144) while mice have three (*Nkg2a*, -*c* and -*e*) and both species have one *CD94* gene (145, 146). CD94 and NKG2 are type II transmembrane proteins of the C-type lectin family. NKG2 family member proteins need to form heterodimers with CD94 for surface expression (147, 148). NKG2A possesses two ITIM motifs in its cytoplasmic tail and can deliver inhibitory signals (148). On the other hand, NKG2C and NKG2E lack ITIMs and need to associate with DAP12 for proper expression and initiation of signals (146, 149). This association depends on the presence of a lysine residue in the transmembrane domain of NKG2C and on an aspartic acid residue in DAP12 (149). Roughly half of NK cells express NKG2-CD94 receptors as well as γδ T cell and some memory $CD8^+$ T cells (122).

Human CD94-NKG2A and CD94-NKG2C bind to the MHC-Ib molecule HLA-E (150, 151). Mouse CD94-NKG2A, CD94-NKG2C and CD94-NKG2E recognize a non-classical MHC-I molecule, Qa-1^b (146, 152). Unlike MHC-I molecules which bind to a wide variety of peptides, HLA-E and Qa-1^b molecules

bind to peptides derived from the leader sequences of certain MHC-I molecules (153, 154). Qa-1^b can present the leader peptides of H-2L and H-2D (153) while leader peptides from HLA-A, -B, -C and –G can be loaded onto HLA-E (150, 154). Being an inhibitory receptor, human CD94-NKG2A can prevent lysis of target cells expressing MHC-I molecules whose leader peptide can be loaded onto HLA-E (150). Similarly, cells expressing Qa-1^b that present the leader peptide from H-2D are protected from lysis by CD94-NKG2A^+ NK cells (152). Although the activating CD94-NKG2C and CD94-NKG2E bind to HLA-E, their affinity for HLA-E is much lower than the affinity of CD94-NKG2A for HLA-E (155). Furthermore, *Nkg2a* transcripts are 20 times more abundant than *Nkg2c* or *Nkg2e* transcripts in NK cells (146). In most situations, it is possible that the interaction between CD94-NKG2C/E and HLA-E may not activate NK cells.

The leader peptide of MHC-I molecules is not the only peptide bound by HLA-E. The signal peptide from Hsp60 can also be bound by HLA-E (156). Cellular stress leads to upregulation of HLA-E molecules loaded with the signal peptide from Hsp60. This HLA-E/Hsp60 signal peptide complex is not recognized by CD94-NKG2A resulting in increased NK-mediated cytotoxicity (156). CD94-NKG2C receptor is also not able to bind to HLA-E/Hsp60 signal peptide suggesting that the increase in cytotoxicity is due to lack of inhibitory signals normally delivered by CD94-NKG2A (156). UL40, a protein encoded by HCMV, can also be loaded onto HLA-E. The presence of UL40 leads to an upregulation of the surface levels of HLA-E and unlike HLA-E/Hsp60, HLA-E/UL40 expression results in decreased cytotoxicity by NK cells (157, 158). The

expression of HLA-E/UL40 complexes by HCMV-infected cells may serve to dampen NK cell activation driven by the decreased expression of MHC-I on HCMV-infected cells (157).

The CD94-NKG2 receptors are also involved in the control of viral infections in mice. CD94-deficient mice cannot mount an immune response against ectromelia and show elevated viral titers and die rapidly after infection (159). Infection of mice with ectromelia leads to increased NKG2E expression on NK cells and high levels of Qa-1^b (159), and recognition of Qa-1^b by CD94-NKG2E complexes is required for proper control of ectromelia virus (159).

2.6. NKp46

The gene coding for the activating receptor NKp46 is located in the LRC (Fig. 2). NKp46 is a member of the Ig superfamily and possesses no signaling motif in its cytoplasmic tail and needs to associate with the adaptor molecule CD3ζ to initiate activating signals (160, 161). NKp46 is expressed in NK cells in both human and mice where it can trigger lysis of tumour cells and production of IFN-γ (40, 162). Mouse NKp46 is also expressed on NK-like cells located in gut lamina propria (163, 164). Human $NKp46^+$ NK cell clones have a higher cytotoxic activity directed towards transformed cells than $NKp46^-$ NK cell clones (165) and it was found that NK cells from patients with acute myeloid leukemia have decreased expression of NKp46 (166). Furthermore, NKp46 is involved in the destruction of neuroblastoma and myeloma cells by NK cells (167, 168). The presence of NKp46 on NK cells induces tumour immunoediting as shown by the

elevated expression of NKp46 ligands on tumours isolated from *Nkp46*$^{-/-}$ mice (169).

Human NKp46 binds to haemagglutinin (HA) on influenza-infected cells and triggers NK cell cytotoxicity against the infected cells (170). The binding of NKp46 to HA requires the presence of sialic acid moieties on NKp46, which is consistent with the fact that HA recognizes sialic acid (170). Similarly, murine NKp46 preferentially binds to influenza-infected cells and *Nkp46*$^{-/-}$ mice succumb more rapidly influenza infection than WT mice (171).

2.7. DNAM-1

DNAM-1 (DNAX accessory molecule-1) is a member of the Ig superfamily expressed on NK cells, T cells and on a subset of B cells. DNAM-1 is able to activate the cytotoxic activity of NK cells (172). NK cells isolated from patients suffering of leukocyte adhesion deficiency (LAD), a disease characterized by a lack of CD18, are not able to be activated by crosslinking DNAM-1, suggesting that DNAM-1 needs to be associated with the integrin LFA-1 (CD11a/CD18) to transmit activating signals (173). DNAM-1 binds to the poliovirus receptor (CD155) and to Nectin-2 (CD112). Interaction between DNAM-1 on the NK cell and its ligands on a target cell induces lysis of the target cell (174, 175). Simultaneous activation of NK cells through DNAM-1 and LFA-1 leads to increased cytokine production indicating cooperation between both receptors (175). The association between DNAM-1 and LFA-1 may also explain why ligation of LFA-1 with its ligand ICAM-1 is sufficient to trigger NK cell cytotoxicity (176). The ligands for DNAM-1 are expressed on endothelial cells

and on hematopoietic cells (177, 178). DNAM-1 ligands are also expressed on a wide variety of tumour cells: gastric and colon cancer tissues, ovarian carcinoma, neuroblastoma and myeloma (179). *DNAM-1*$^{-/-}$ mice have increased tumour burden and mortality after transplantation of CD155-expressing fibrosarcoma cells (179). Tumours developing in DNAM-1-deficient mice after injections of chemical carcinogens expressed higher levels of CD155 than tumour cells in wild-type mice (179). These results indicate that DNAM-1 is involved in the *in vivo* surveillance of tumour cells.

2.8. SLAM receptors

This family of receptors is encoded on human and mouse chromosome 1 and encodes for several receptors expressed on NK cells such as 2B4, Ly9, Ly108, CRACC and CD84 (180). These receptors are part of the CD2 Ig superfamily and are characterized by the presence of several tyrosine-based motifs in their cytoplasmic tail (181). These motifs are named immunoreceptor tyrosine-based switch motif (ITSM) and are defined by the following sequence: Thr-x-Tyr-x-x-Val/Ile (180).

2B4 (CD244) is expressed on activated CD8$^+$ T cells, NK cells and mature DC (181). 2B4 can activate cytotoxicity and production of IFN-γ by human NK cells (180). Its ligand, CD48, is expressed on hematopoietic cells (182, 183). The presence of CD48 on target cells increases susceptibility to 2B4-mediated NK cell lysis (184). Unlike human 2B4, mouse 2B4 inhibits NK cell function since CD48$^+$ cells are protected from lysis by mouse NK cells (185). Furthermore, 2B4-deficient NK cells display a higher cytotoxic activity against CD48$^+$ targets (185).

Expression of CD48 on cells provides an MHC-I-independent self-recognition system used by mouse NK cells since MHC-I-deficient target cells are protected from NK cell lysis when they express CD48 (186). Interestingly, a separate study revealed that 2B4 can activate the lytic activity of mouse NK cells against $CD48^+$ cells (187). These discrepancies could be explained by the different strains of mice and NK cell preparation used. The use of LAKs from B6-background mice resulted in inhibition of NK cell activity by 2B4 (185) while activation of NK cells by 2B4 was detected using freshly isolated NK cells from 129S1-derived mice (187).

Ly108 (NTB-A) is expressed on NK cells, T cells, B cells and eosinophils (180, 188). Ly108 is able to activate NK cells through homophilic interactions (189). Ly9 (CD229) is expressed on resting and activated T cells, B cells, macrophages and NK cells (181). Ly9 binds to itself and $Ly9^+$ cells are killed preferentially by NK cells over cells lacking this molecule (187, 190). CD84 is expressed on T cells, B cells, mature DC, macrophages and on mouse but not human NK cells (180, 181). CD84 is also a self-ligand and its presence on target cells induces NK cell cytotoxicity (187, 191).

CRACC (CD2-like receptor activating cytotoxic cells) is another member of the SLAM family of receptors that is also expressed by NK cells. T cells, B cells, DC and macrophages also express CRACC (192, 193). Similarly to Ly9, Ly108 and CD84, CRACC is a self-ligand and its ligation induces activation of NK cells against a wide variety of targets (192-194). As expected, NK cells from

CRACC-deficient mice display defective lytic activity but the production of IFN-γ is unaffected by the absence of this receptor (193).

2.9. NKR-P1 receptors

A single NKR-P1 receptor was initially cloned in rat and then in mouse (126, 195). The NKR-P1 receptor was found to be a type II transmembrane protein belonging to the C-type lectin superfamily expressed as a disulphide-linked homodimer (Fig. 3). The gene coding for this receptor is located in the NKC and RFLP analysis revealed variation at this locus in different mice strains (126). Other NKR-P1 receptors were later cloned and 6 NKR-P1 receptors are now known: NKR-P1A, -P1B/D (alleles of the same gene), -P1C, -P1E, -P1F and -P1G (196, 197). The NK1.1 antigen, recognized by the PK136 monoclonal antibody, which defines NK cells is encoded by the *Nkrp1c* gene in B6 mice while *Nkrp1b* encodes for another NK1.1 antigen in strains such as Swiss.NIH (198, 199). Cross-linking of NKR-P1C leads to activation of NK cells (200) and this activation requires the association of NKR-P1C with the adaptor FcεRIγ (Fig. 3) (201).

NKR-P1B is an inhibitory receptor characterized by the presence of an ITIM motif in its cytoplasmic tail (Fig. 3). Antibody-mediated cross-linking of NKR-P1B on NK cells dampens activation signals by recruiting the phosphatase SHP-1 (199). The absence of an ITIM and the presence of a charged residue in the transmembrane domain of NKR-P1A and -P1F suggest that these receptors are activating while NKR-P1G has an ITIM and is presumed to be inhibitory (122). A single NKR-P1 gene, *NKRP1A*, is present in human. This gene is located in the

NKC and encodes for a protein that can inhibit NK cell functions despite not containing a recognizable ITIM in its cytoplasmic tail (202). Mouse NKR-P1 receptors are expressed by NK cells (203) and NKR-P1C is also found on NK-T lymphocytes. Human NKR-P1A is present on a subset of NK cells, on peripheral T cells and γδ T cells (122, 202).

The ligands of two NKR-P1 receptors have been identified (204, 205). NKR-P1B/D binds to Clr-b while NKR-P1F recognizes Clr-g. Clr are C-type lectin molecules encoded by the *Clr* genes located in the NKC (Fig. 2). The *Clr* genes are intertwined with the *Nkrp1* genes meaning that the receptors and their ligands are inherited together (206). Similar to *Nkrp1*, there are multiple *Clr* genes: *Clr-a*, *-b*, *-c*, *-d*, *-e*, *-f*, *-g* and *-h* (197, 206). Transcripts for *Clr-b* were found in a wide variety of organs and cells: thymus, lung, spleen, bone marrow, ovary, ileum, liver, kidney and NK cells. *Clr-f* and *Clr-g* have a more restricted expression pattern: *Clr-f* transcripts are found in the ileum, liver, kidney and NK cells while only NK cells were found to express *Clr-g* (206). The expression patterns of the remaining *Clr* have not been reported. Clr-b was confirmed to be expressed on the surface of T cells, NK cells, DC, B cells and on thymocytes by flow cytometry (204). Expression of Clr-b on a target cell can inhibit the activation of NK cells through interaction with NKR-P1B/D (204, 205). Interestingly, the expression of Clr-b is often downregulated on various tumour cells (204). Both genotoxic stress and cellular stress can induce downregulation of Clr-b from the surface of cells leading to a loss of NKR-P1B-mediated inhibitory signals and to an increased sensitivity to NK cell lysis (207). Even target cells

with decreased MHC-I expression such as RMA-S can be protected from NK cells lysis if Clr-b is present on their surface (205). Thus the NKR-P1/Clr axis provides NK cells with an MHC-I-independent self-recognition system.

The gene order from the centromeric end to the telomeric end of the mouse *Nkrp1-Clr* gene cluster is *Nkrp1a*, *Clr-h*, *Clr-f*, *Nkrp1g*, *Nkrp1c*, *Nkrp1b/d*, *Clr-g*, *Clr-d*, *Clr-e*, *Clr-c*, *Nkrp1f*, *Clr-a*, *Nkrp1e* and *Clr-b* (Fig. 2) (197). The gene order of the cluster is conserved in B6 and BALB/c mice (208). *Nkrp1a*, *Nkrp1f* and *Nkrp1g* have more than 99% identity between B6 and BALB/c mice while *Nkrp1b/d* and *Nkrp1c* show significantly more divergence. *Nkrp1d* was first found to be present in B6 mice but an obvious homologue could not be found in other strains such as BALB/c. *Nkrp1b* was cloned in BALB/c and Swiss mice (199, 208) and mapping studies indicated that it could be an allele of *Nkrp1d* (208). Indeed, $Nkrp1d^{B6}$ is 95% identical to $Nkrp1b^{BALB/c}$ demonstrating that these two genes are actually alleles (208). Despite the allelic nature of NKR-P1D^{B6} and NKR-P1B$^{BALB/c}$, both receptors interact with Clr-b (208).

Similar to mouse NKR-P1, human NKR-P1A recognizes a lectin-like protein: LLT1 (209, 210) whose gene is located on chromosome 12 in close proximity to the *NKRP1A* gene (Fig. 2) (197, 211). Interaction between LLT1 on a target cell and NKR-P1A on NK cells leads to inhibition of NK cell cytotoxic activity and IFN-γ production (209, 210). LLT1 is expressed on TLR-9-activated B cells, TLR-activated DC and on TLR-activated pDCs (212).

Rat NKR-P1 receptors also bind to Clr and similar to mice and humans, *Nkrp1* and *Clr* genes are encoded together allowing co-inheritance of the

receptors and their ligands (197). An example of the importance of this system for NK cell regulation was the finding that rat cytomegalovirus (RCMV) encodes for a C-type lectin gene product named RCTL (RCMV C-type lectin) possessing high homology to rat Clr-b (213). Cells infected with RCMV rapidly lose expression of Clr-b but this does not lead to increased lysis by NK cells. RCMV-infected cells upregulate RCTL which then interacts with the inhibitory receptor NKR-P1B preventing lysis of RCMV-infected cells (213). Thus, the NKR-P1/Clr axis can be subverted by viruses to evade NK cell response.

2.10. KIRs (Killer Ig-like receptors)

KIRs are receptors for MHC-Ia on human NK cells and are the functional orthologues of Ly49 in mice. *KIR* genes encode for type I transmembrane proteins of the Ig superfamily. They are characterized by the presence of two Ig-like domains (KIR2D) or three Ig-like domains (KIR3D) in their extracellular region (Fig. 4) (122, 214). The cytoplasmic tail of KIR varies in length: it can either be long (KIR2/3DL) with one or two ITIM sequences or short (KIR2/3DS) with no signalling motif (Fig. 4) (122). KIR2/3DS possess a lysine residue in their transmembrane domain allowing for interaction with the adaptor protein DAP12 (215). *KIR2DL4* is a distinct member of this receptor family. Similar to KIR2/3DL, an ITIM is found in its cytoplasmic tail (216) but a charged arginine residue is also present in its transmembrane domain (Fig. 4) (217). Unlike activating KIR, KIR2DL4 associates with the FcεRIγ adaptor molecule (122). KIR2DL4 is able to activate NK cells and if the interaction between KIR2DL4 and FcεRIγ is prevented, KIR2DL4 can deliver inhibitory signals (216).

NK cells, γδ T cells and memory/effector αβ T cells express *KIR* genes (122). *KIR* genes are expressed in a variegated fashion on NK cells (218). All NK cells express KIR2DL4 further differentiating this receptor from the other KIR (219). The variegated expression of *KIR* genes can be explained by the properties of their promoters. *KIR* genes possess two promoters: one located close to the initiation site (proximal) and a second promoter located 1kb upstream of the start codon (distal) (220). The proximal promoter is a probabilistic promoter and can initiate transcription in both forward and reverse orientations (220). Reverse transcripts originating from the proximal promoter are detected in KIR^- NK cells while proximal forward transcripts are present in KIR^+ NK cells (220). The model of *KIR* expression proposes that reverse proximal transcripts will inhibit gene transcription. In immature NK cells, reverse proximal transcripts will anneal to transcripts originating from the distal promoter and dsRNA-mediated silencing will trigger methylation of the *KIR* gene. If the proximal promoter initiates transcription in the forward direction, no silencing occurs and the gene will be expressed (218).

The ligands for KIR are the MHC-I molecules HLA-A, -B and -C which are encoded on chromosome 6 (122, 124). Members of the KIR2DL group bind to HLA-C proteins, KIR3DL1 recognizes HLA-B and HLA-A proteins positive for the Bw4 epitope and lastly, KIR3DL2 binds to HLA-A molecules (122). Despite high similarity between inhibitory and activating KIR, only a few weak ligands have been found for activating KIR receptors. KIR2DL1 and KIR2DS1 are highly related and form a pair of inhibitory and activating receptors that likely arose by

gene duplication. KIR2DL1 binds with high affinity to HLA-C*0401 but KIR2DS1 binds weakly to this MHC-I molecule (122).

The *KIR* gene family, present in human but not in mice, encodes for fifteen genes and two pseudogenes located in the LRC (122). *KIR* genes show great variability between individuals as the number of genes differs in different individuals and each gene shows extensive allelic polymorphisms (221). Two haplotypes, A and B, have been identified based on the number of genes present (221). Interestingly, four framework genes are present in all haplotypes identified so far: *KIR3DL3*, the pseudogene *KIR3DP1*, *KIR2DL4* and *KIR3DL2* (122). The *KIR* genes located between framework genes greatly vary in both haplotypes. Haplotype A can encode for six inhibitory and one activating receptor while the B haplotype contains up to four activating and five inhibitory receptors (222). The high sequence similarity between each *KIR* gene is hypothesized to have facilitated the rapid evolution of different *KIR* haplotypes by unequal crossing over (223).

The presence of some *KIR* genes and their MHC-I ligands in certain individuals has been linked with susceptibility or resistance to certain viral infection, cancer and some autoimmune diseases (224). Patients carrying the activating *KIR2DS1* and *KIR2DS2* genes are susceptible to the development of psoriatic arthritis but only if the ligands (HLA-C) for their inhibitory counterparts KIR2DL1 and KIR2DL2/3 are absent (225). The presence of KIR B haplotypes has been linked to a faster loss of $CD4^+$ T cells in HIV-infected individuals and progression to AIDS (226). With respect to cancer, genotypes that predict high

NK cell inhibition (*KIR3DL1* and *KIR2DL1* in combination with their ligands) provide protection against cervical neoplasia while genotypes with high activation potential (absence of the ligands for *KIR3DL1* and *KIR2DL1* with the presence of the activating *KIR3DS1*) are linked to increased susceptibility against cervical neoplasia (227). This finding can be explained by the knowledge that inhibitory receptors are involved in the acquisition of full functions by NK cells (228) and continuous signalling by an activating receptor can induce NK cell anergy (229).

3. Ly49 receptors

3.1. Structure

Ly49 genes encode for type II transmembrane proteins that are members of the C-type lectin family. Ly49 proteins are expressed as disulphide-linked homodimers. They are composed of a short cytoplasmic tail, a transmembrane domain, a long flexible stalk and a ligand-binding domain, the natural killer domain (NKD) (Fig. 5). Similar to NKR-P1 and KIR, Ly49 can be either activating or inhibitory (Fig. 5). Inhibitory Ly49 receptors, which possess an ITIM (230), bind to MHC-I, thereby preventing NK cell activation upon encountering cells expressing normal levels of MHC-I. Activating receptors need to associate with the DAP12 adaptor for proper expression and function (Fig. 5) (231). The presence of a positively charged arginine residue in the transmembrane domain of activating Ly49 receptors allows association with DAP12 by interacting with the negatively charged aspartic acid residue located in the transmembrane domain of DAP12. Recent reports have shown that activating Ly49 can be expressed on the surface of NK cells in the absence of DAP12,

although at levels significantly reduced compared to WT and that DAP10 allowed for this residual expression by forming complexes with activating Ly49 (232, 233). It is unclear whether DAP10 is an adaptor for activating Ly49 since one group reported that activating Ly49 require both DAP12 and DAP10 for optimal function (232), while a second demonstrated that activating Ly49 receptors do not require DAP10 for their functions (233). Similar to inhibitory Ly49, activating Ly49 receptors also recognize MHC-I molecules. They also bind to virus-encoded MHC-I mimics and to MHC-I of foreign origin such as rat and hamster MHC.

3.2. Expression

Ly49 proteins are expressed mainly on NK cells but other leukocytes have also been shown to express these receptors. Inhibitory Ly49 are expressed on memory $CD8^+$ T cells where they can inhibit TCR-mediated activation (234). Inhibitory Ly49 receptors are also present on NK-T cells and on $CD3^+CD8^+$ intestinal epithelial lymphocytes (IEL) (235, 236). Unlike other Ly49 receptors, Ly49B, E and Q have distinct expression patterns. Ly49B is expressed on $CD11b^+Gr1^{low/int}$ macrophages, neutrophils and eosinophils (237) while Ly49Q is present on plasmacytoid dendritic cells (pDC), neutrophils and osteoclasts (238). Ly49E is expressed on fetal NK cells and expression is lost early after birth (239). A fraction of adult thymic NK-T cells and epidermal γδ T cells also express Ly49E (240).

NK cells acquire Ly49 expression during their development in the bone marrow. Ly49 are expressed on NK cells that have reached Stage III of the developmental program after the acquisition of CD94-NKG2 receptor in Stage II

and before the expansion phase (Stage IV) (58). Few NK cells express Ly49 receptors at birth, but as mice age, NK cells acquire more Ly49 on their surface and the adult levels are reached around the age of 6-8 weeks. The acquisition of Ly49 expression correlates with the appearance of NK cell activity as defined by the ability of mice to reject MHC-I-deficient BM grafts (241). Individual NK cells express up to six different Ly49 receptors and due to random expression, an abundance of patterns of receptor expression are observed (242). In addition to this random expression, not all Ly49 are equally expressed (243).

The stochastic and unequal expression of Ly49 proteins can be explained by the properties of their three distinct promoters. Pro1, inactive in mature NK cells, is located between 4 and 10kb upstream of Pro2 which is found at the 5' end of the first exon (244). A third promoter, Pro3, can initiate transcription of *Ly49* a few nucleotides upstream of the start codon present in the second exon (245). Pro1 has a bidirectional transcriptional activity as it can initiate transcription either in forward or reverse direction. The strength of specific *Ly49* Pro1 promoters in the forward orientation correlates with the percentage of NK cells expressing that particular Ly49. *Ly49* genes under the control of a Pro1 that have a strong reverse activity are not frequently expressed (246). If Pro1 is active in the forward orientation in immature NK cells, transcripts can dislodge suppressor complexes located on Pro2 leading to the expression of the *Ly49* gene in mature NK cells. Pro1 promoters that initiate transcription in the reverse orientation will not activate Pro2 resulting in no transcription of the *Ly49* gene (246).

Several transcription factors are involved in the expression of Ly49 on NK cells such as NF-κB p50 (247). The transcription factor TCF-1 is required for the expression of Ly49A and D. Ly49A and Ly49D are expressed on 20% and 55% of WT B6 NK cells, respectively, and this proportion decreases to 1 and 18% on *TCF-1*$^{-/-}$ NK cells (248). Conversely, the expression of Ly49G, which is present on 50% of WT NK cells, is increased to 70% on *TCF-1*$^{-/-}$ NK cells (248).

3.3. Ly49 receptors ligands

The discovery of MHC-I molecules as the ligands of inhibitory Ly49 receptors provided a molecular explanation for the "missing-self" hypothesis (230). The ligation of inhibitory Ly49 with MHC-I on target cells prevents NK cell activation. In the absence of MHC-I expression, the inhibition is relieved and NK cells are activated. Some inhibitory Ly49 can bind to a wide variety of MHC-I as exemplified by the broad reactivity of Ly49V which binds to MHC-I of the H-2^{b}, H-2^{d} and H-2^{k} haplotypes (249), while others are very specific, like Ly49G which can bind only to H-2L^{d} and H-2D^{d} (249). Interestingly, activating Ly49 can also bind to MHC-I molecules albeit much more weakly than inhibitory Ly49. Ly49D recognizes MHC-I molecules from the H-2^{d}, H-2^{r} and H-2^{sp2} haplotypes and Ly49P can bind to H-2D^{d} (249). Ly49E has been suggested to have a unique ligand: urokinase plasminogen activator (uPA) which is secreted by epithelial and hematopoietic cells and is upregulated during tissue remodelling (250).

The activating Ly49D binds to MHC molecules (Hm1-C4) expressed by a Chinese hamster cell line and to rat MHC-I molecules (251, 252), although this is thought to be a coincidence based on structural similarity between mouse and

hamster MHC-I molecules with no real physiological relevance. The activating Ly49H binds to m157 (253), an MHC-I like mimic encoded by MCMV, and the presence of the *Ly49h* gene is associated with resistance of mice to infection with MCMV (254). Interestingly, the m157 viral product is also recognized by the inhibitory $Ly49I^{129S1}$ (253). It is possible that the initial role of m157 was to bind to $Ly49I^{129S1}$ to prevent lysis of MCMV-infected cells by NK cells. *Ly49h* could have originated later by joining the exons of an *Ly49d*-like gene that code for the cytoplasmic tail and transmembrane domain of an activating receptor and the exons of an *Ly49i*-like gene coding for the NKD (255). Ly49P is also involved in the resistance to infection with MCMV by binding to $H\text{-}2D^{k}$ complexed with the viral protein m04 (256). This viral protein associates with MHC-I, travels with these molecules to the cell surface, thereby preventing their downregulation. This interaction was also detected for $Ly49L^{BALB/c}$, $Ly49D_2^{PWK/Pas}$ and $Ly49P_1^{NOD/LtJ}$ (257).

Interestingly, the expression of Ly49 is influenced by the presence of its ligand. The percentage of Ly49-expressing NK cells rises in MHC-I-deficient mice ($\beta 2m^{-/-}$ or $Tap^{-/-}$) and the amount of Ly49 present on individual NK cells is also increased in the absence of MHC-I ligands (258). Similarly, Ly49 expression is decreased when NK cells are transferred from a ligand-negative donor mouse to a ligand-positive recipient (259). Such calibration of Ly49 receptors when ligands are present may insure that NK cells receive the proper amount of negative signals in the presence of MHC-I molecules.

Similar to the TCR, the recognition of MHC-I molecules by Ly49 requires the presence of a peptide in the peptide-binding groove of the MHC-I molecules (260). But unlike TCR, Ly49 lack peptide specificity. Expression of H-2D^d in the TAP-deficient cell line RMA-S does not confer protection against lysis by Ly49A$^+$ NK cells implying that Ly49A cannot bind to empty MHC-I molecules (260, 261). These cells were protected when H-2D^d molecules were loaded with any D^d-binding peptide. Protection from lysis correlated with stabilization of MHC-I molecules on the surface of the target cells. Similar to Ly49A, Ly49C binds to H-2K^d loaded with a wide array of peptides but Ly49I recognizes H-2K^d loaded with specific peptides (262).

The crystal structure of Ly49A and Ly49C bound to their respective ligands brought further evidence that Ly49 lack peptide specificity. The NKD of Ly49 binds to a region of MHC-I located under the peptide-binding groove. The Ly49-MHC interaction site is composed of the α2 and α3 domains of the MHC-I molecule as well as the β2m subunit (263, 264). The presence of a peptide in the peptide-binding groove of the MHC-I molecule could affect the interface recognized by Ly49 allowing recognition. An empty peptide-binding groove would disrupt the general structure of the MHC-I molecule; the interface recognized by Ly49 would be changed and Ly49 binding prevented.

3.4. Trans and cis interactions

The classical view of MHC recognition states that Ly49 receptors bind to MHC-I in *trans* meaning that Ly49 on the surface of NK cells bind to MHC-I molecules present on the surface of a target cell. It is now known that Ly49A can

bind to H-2D^d expressed on the surface of the NK cell (*cis* interactions) and this possibly provides an explanation for the increased Ly49 expression observed in MHC-I-deficient mice (265). Ly49 receptors binding to MHC-I in *cis* are most likely not efficiently recognized by monoclonal antibodies due to steric hindrance. It was proposed that *cis* interaction between Ly49A and its MHC-I ligand on the surface of NK cells lowers the threshold of activation of NK cells allowing for a more sensitive monitoring of abnormal cells (265). The binding of Ly49A to H-2D^d in *cis* reduces the number of Ly49A receptors available for recognition of H-2D^d on the surface of the target cell resulting in decreased inhibitory signals delivered to the NK cell by *trans* interactions. Therefore, weaker activating signals can activate NK cells in the presence of *cis* interactions. Ly49C, Ly49I and Ly49Q have also been shown to bind to their respective ligands in *cis* (266).

When the crystal structures of Ly49A and Ly49C bound to their ligands were solved, two different NKD conformations were observed (Fig. 6) (263, 264). It was demonstrated that one conformation was used for binding to MHC-I in *trans* while the second NKD conformation was used in *cis* interactions (267). When Ly49 contacts MHC-I on the surface a target cell (*trans*), the NKDs are backfolded on the extended stalk and the Ly49 homodimer contacts two MHC-I molecules (Fig. 6). On the other hand, *cis* interaction requires a loop domain located between the stalk and the NKD to be extended. In this conformation, the NKD is dissociated from the stalk and contacts a single MHC-I molecule (Fig. 6) (267).

3.5. Genomic organization

Cloning of the first *Ly49* gene revealed its location in the NKC and that it is part of a gene family (Fig. 2) (125). Like *KIR*, the number of *Ly49* genes varies between different inbred mouse strains and several genes show a high degree of polymorphism. Analysis of *Ly49* gene clusters from three different mice strains has revealed extensive variations in gene number (Fig. 7) (255, 268, 269). The *Ly49* gene cluster of B6 mice contains fifteen genes: two activating (*Ly49d*, *h*), eight inhibitory (*Ly49a*, *c*, *j*, *g*, *i*, *f*, *e*, *q*) and five pseudogenes (255). BALB/c mice encode the smallest cluster known to date with eight *Ly49* genes: one activating (*Ly49m*), six inhibitory (*Ly49a*, *c*, *g*, *i*, *e*, *q*) and one pseudogene (270). Lastly, nine-teen *Ly49* genes are present in the cluster of 129S6 mice: three activating (*Ly49p*, *u*, *r*), nine inhibitory (*Ly49o*, *g*, i_1, *t*, *s*, ec_2, *v*, *e*, q_1) and seven pseudogenes (271). The high sequence homology between different *Ly49* genes suggests that novel *Ly49* genes were created by duplication events and exon shuffling, ultimately leading to the generation of different *Ly49* haplotypes (255, 270). Exon shuffling is thought to be responsible for the generation of hybrid *Ly49* genes (255). For example, *Ly49p* is thought to have arisen by joining the exons coding for the intracellular portion of an *Ly49d*-like gene and those coding for the extracellular domain of an *Ly49a*-like gene (272).

Roughly 50% of the *Ly49* gene clusters are composed of various repetitive elements, which is strikingly higher than the 39% for the rest of the mouse genome (270, 271). LINE1 elements make up 36% of the total sequence of the different *Ly49* clusters and are found at a higher frequency in intergenic regions

(197). The high frequency of repetitive sequences in this region of the genome is thought to have favoured the rapid evolution of this gene family. The repetitive elements may promote unequal crossing over events leading to duplication or deletion of regions (271).

Despite the high diversity in the gene content of the *Ly49* gene cluster, a major conservative feature is the presence of three pairs of framework genes that are present in all sequenced clusters. On the centromeric end of the cluster, the first pair consists of *Ly49q* and *Ly49e*. In the middle of the cluster, *Ly49i* and *Ly49g* form a second pair of framework genes and the last pair, *Ly49c* and *Ly49a*, is located at the telomeric end of the cluster (Fig. 7) (271). *Ly49o* and *Ly49i*$_2$ are the alleles for *Ly49a* and *Ly49c* in the 129S6 *Ly49* cluster that make up the telomeric pair of framework genes (271). *Ly49b* is an additional *Ly49* gene encoded on mouse chromosome 6 but it is located at least 0.8 Mb away from the telomeric end of the *Ly49* cluster (Fig. 2) (273).

Human possess a single *Ly49* gene, *Ly49L*, located in the human NKC on chromosome 12. *Ly49L* is a pseudogene and is transcribed at low levels (122). Other species such as primates, cow, pig, dog and cat also encode a single *Ly49* gene while six *Ly49* genes have been identified in horse (122).

4. Education of NK cells

4.1. NK cell functions in MHC-I-deficient mice

The ability of NK cells to recognize MHC-I-deficient cells depends on the environment in which they develop. NK cells from MHC-I-deficient mice such as *β2m*$^{-/-}$ or *Tap*$^{-/-}$ mice cannot destroy MHC-I-deficient cells (22, 23, 274). Because

of this phenotype, NK cells from MHC-I-deficient mice are widely considered as hyporesponsive or non-functional but interestingly these NK cells efficiently lyse the tumour cell line YAC-1 (23, 274). NK cells from MHC-I-deficient mice develop in the absence of MHC-I molecules and therefore it is not surprising that MHC-I-deficient NK cells have a defective "missing-self" response. Similarly, NK cells from TAP2-deficient patients cannot destroy autologous MHC-I-deficient cells (275).

Cells from hematopoietic and non-hematopoietic origin both control the acquisition of NK cell reactivity towards loss of MHC-I molecules (276, 277). Rejection of $\beta 2m^{-/-}$ cells was assessed in bone marrow chimeric mice. WT mice receiving $\beta 2m^{-/-}$ grafts ($\beta 2m^{-/-} \rightarrow$WT chimera) cannot reject MHC-I-deficient cells (276, 277). Similarly, WT$\rightarrow \beta 2m^{-/-}$ chimaeras are not able to reject MHC-I-deficient cells (276, 277). Therefore, the presence of MHC-I-deficient cells in the host can induce NK cell tolerance.

4.2. Importance of the MHC-I environment for NK cell specificity

The introduction of a single MHC-I molecule as a transgene has major impacts on the specificity of NK cells and their ability to reject cells of different origins. B6 mice do not reject RMA cells since they originate from B6 mice (17). B6 transgenic for H-2D^d (a non-B6 MHC-Ia molecule) show NK-mediated rejection of RMA cells and other MHC-I$^+$ tumour cells of B6 origin (278). The same mice are also able to reject B6 bone marrow cells indicating that the H-2D^d transgene induces a change in the specificity of NK cells (279). NK cells from B6 mice expressing H-2D^d are educated to recognize H-2D^d in addition to the

presence of MHC from B6 mice (H-2D^b and H-2K^b). Cells from B6 mice are thus considered by NK cells from H-2D^d transgenic mice as having lost MHC-I expression due to the lack of H-2D^d expression.

H-2D^d mosaic transgenic mice in which the transgene is only expressed by a fraction of cells have a different phenotype. These mice cannot reject cells of B6 origin indicating that NK cells from these mice are educated to see both non-transgenic and transgenic cells as normal (280). Interestingly this tolerance can be reverted by *in vitro* culture. H-2D^d-positive and H-2D^d-negative NK cells sorted from the mosaic mice and cultured separately were tested for their ability to destroy B6 cells. Sorted H-2D^d-negative NK cells could not kill B6 cells while H-2D^d-positive NK cells had acquired the ability to destroy B6 cells (280). Culture of sorted H-2D^d-positive and H-2D^d-negative NK cells allowed the H-2D^d-positive cells to reset their specificity leading to acquisition of cytotoxicity towards H-2D^d-negative cells. This important finding suggests that tolerance of NK cells towards a specific MHC-I environment is not fixed and that it can be changed in certain conditions such as *in vitro* culture in a new MHC environment.

4.3. Role of self-receptors in education of NK cells

Due to stochastic expression of Ly49 receptors, not all NK cells express the same repertoire of Ly49 (242). With the diverse MHC-I specificities of Ly49 receptors, it is therefore not surprising that some NK cells only express Ly49 that do not bind to MHC-I expressed by the host mouse (non-self receptors). Approximately 10% of NK cells in B6 mice only express non-self receptors while the rest of NK cells express the self receptors Ly49C,I and NKG2A which

directly (Ly49C and I) or indirectly (NKG2A, through Qa-1^b) recognize H-2^b MHC-I molecules (281). Ly49C/I/NKG2A^+ NK cells secrete more IFN-γ and have better cytotoxic responses than Ly49C/I/NKG2A^- NK cells (281). The differential activities between these two subsets is absent in mice expressing H-2^f MHC indicating that Ly49C/I and NKG2A require their H-2^b ligands to educate NK cells (281). In B6 mice, Ly49A does not bind to H-2^b MHC and is considered as a non-self receptor explaining why Ly49C^+ NK cells from B6 mice respond more vigorously than Ly49A^+ NK cells (282). Ly49A^+ NK cells acquire robust responses when they develop in mice that carry the strong ligand for Ly49A, H-$2D^d$ (282). These results collectively demonstrate that NK cells expressing receptors for self-MHC are better responders than NK cells lacking such receptors and this process has been called education of NK cells.

Similar to murine NK cells, human NK cells that express KIR receptors for self-MHC-I molecules secrete more IFN-γ than NK cells lacking these self-receptors (228). Interestingly, educated and non-educated human NK cells do not show any difference in the expression of various surface receptors (228). Educated mouse NK cells are nearly indistinguishable from non-educated NK cells except for the reduced expression of KLRG1 by non-educated NK cells (281). KLRG1 is known to be expressed on activated NK cells (120, 121) and the low percentage of non-educated NK cells expressing KLRG1 may be a consequence of a reduced activation status in these NK cells.

The presence or absence of self-receptors is not the only factor influencing NK cell education; the number of self-receptors on individual NK cells correlates

with increased functions. Introduction of H-2D^{d} MHC-I as a transgene in B6 mice increases the number of self-receptors available for NK cell education and indeed, NK cells from these mice have better responses than B6 NK cells (283). Conversely, decreasing the number of MHC-I available (*H-2K*$^{b-/-}$ or *H-2D*$^{b-/-}$ mice) decreases the responses of NK cells (283). Furthermore, increasing numbers of self-receptors on individual NK cells correlates with stronger responses (283, 284). NK cells expressing three self-receptors produce more IFN-γ than NK cells expressing two self-receptors which, in turn, have better responses than NK cells with one self-receptor (284). B6 mice congenic for the *Ly49* gene cluster of 129S1 mice carry more self-receptors than B6 mice and these mice show an increased ability to reject MHC-I-deficient cells (285). These results are explained by the rheostat model which proposes that the amount of negative signals received by individual NK cells during development determines the strength of their functions.

Not all MHC-I molecules have the same impact on education of NK cells. The presence of H-2D^{b} in B6 mice leads to weaker NK cells responses than that of H-2K^{b} (283, 284, 286). This may be explained by the number of self-receptors for each of these two MHC molecules: H-2D^{b} is indirectly recognized by NKG2A while Ly49C and Ly49I bind to H-2K^{b}. Furthermore, the strength of the interaction between an inhibitory Ly49 and its MHC-I ligand positively influences the outcome NK cells education. In mice where there is a strong ligand for Ly49A (H-2D^{d}), Ly49A^{+} NK cells respond vigorously while in H-2^{q} mice, Ly49A^{+} NK cells have a weaker activity (287). Interestingly, the strength of *cis* interaction

between Ly49A and its various ligands was found to be a good indicator of the educating potential of MHC-I molecules (287). Indeed, it was later reported that *cis* interactions allow for education of NK cells while *trans* interaction are dispensable for this process (288).

4.4. NK cell education in MHC-I-deficient mice

The education hypothesis of NK cells explains why NK cells from MHC-I-negative mice have a hyporesponsive phenotype compared to WT mice and can be considered as non-educated. The presence of MHC-I and Ly49 recognizing these MHC-I molecules is required for the proper acquisition of NK cell effector functions. The effect of education imparted by Ly49C, I and NKG2A in B6 is lost in $\beta 2m^{-/-}$ mice and all NK cells, regardless of Ly49C, I and NKG2A expression, show a reduced function in response to NK-sensitive target cells compared to NK cells from WT mice (281, 282).

However, NK cells from $\beta 2m^{-/-}$ mice are not completely hyporesponsive since $\beta 2m^{-/-}$ mice are not susceptible to infection with the NK-sensitive MCMV (289). Furthermore, lysis of YAC-1 target cells and Ly49D-mediated activity are almost normal in $\beta 2m^{-/-}$ NK cells (23, 251). The hyporesponsiveness is not fixed as it can be reverted when $\beta 2m^{-/-}$ cells are transferred to WT mice. $\beta 2m^{-/-}$ NK cells transferred to WT mice produce more IFN-γ than $\beta 2m^{-/-}$ NK cells and this production is similar to that of WT NK cells (277, 290) indicating that NK cells can tune their responsiveness according to the levels of MHC-I expression. Similarly, WT NK cells transferred to $\beta 2m^{-/-}$ mice tune down their functions and

secrete IFN-γ at levels comparable to $\beta 2m^{-/-}$ NK cells (277). The percentage of KLRG1^{+} NK cells changes after the transfer indicative of a change in the activation status of transferred NK cells. $\beta 2m^{-/-}$ NK cells transferred to a WT host have increased expression of KLRG1, while WT NK cells transferred to $\beta 2m^{-/-}$ hosts show decreased expression of KLRG1 (277). These findings agree with the rheostat model which proposes that NK cell responsiveness is affected by the amount of inhibitory signals delivered to the NK cells. NK cells adapt to changes in expression of MHC-I in their surroundings and change their activation threshold to keep it at a level that allows both sufficient inhibition by self MHC-I and optimal activation.

Similar to NK cells from $\beta 2m^{-/-}$ mice, uneducated NK cells (Ly49C/I/NKG2A^{-}) from B6 mice are not fully hyporesponsive. These uneducated NK cells respond to infection with *Listeria monocytogenes* by producing IFN-γ at levels comparable to educated NK cells suggesting that uneducated NK cells can play a role in response to infection with pathogens (281). A recent report revealed that uneducated Ly49H^{+} NK cells in B6 mice are essential for the control of MCMV infection (291). MCMV expresses the m04 protein that sequesters MHC-I on the surface of MCMV-infected cells presumably to prevent activation of NK cells via the "missing-self" response (292). Due to expression of self-receptors, educated Ly49H^{+} NK cells are inhibited by MHC-I retained on the surface of MCMV-infected cells while the uneducated Ly49H^{+} NK cells which lack self-receptors are not inhibited by MHC-I and can respond vigorously to MCMV infection (291). Therefore the

activity of uneducated NK cells is not completely turned off and that these NK cells can become activated given the proper conditions.

5. Figures

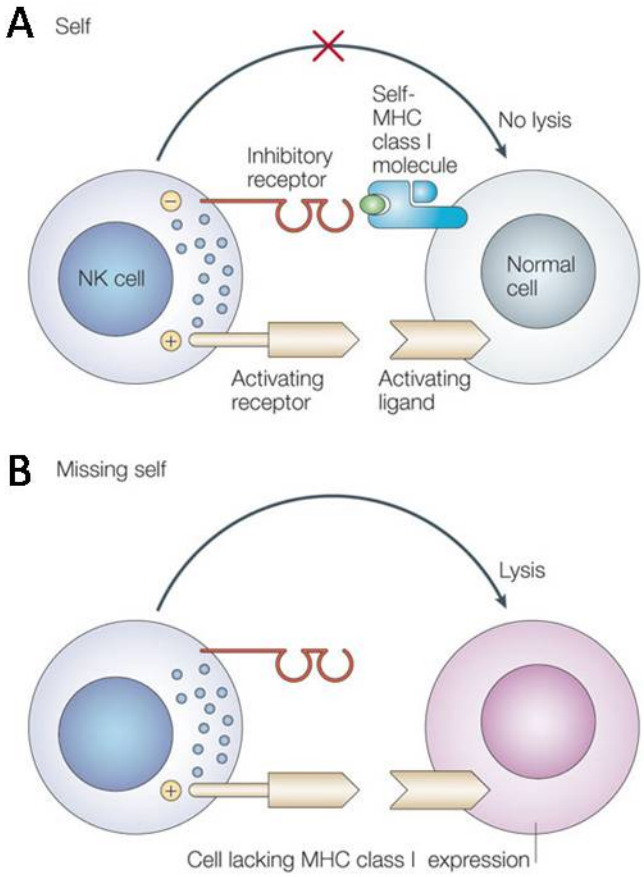

Figure 1: "Missing-self" hypothesis. Simplified representation of the "missing-self" hypothesis. (A) The interaction between a NK cell and a MHC-I-expressing target cell results in inhibition of NK cell activity. The presence of inhibitory MHC-I receptors prevents NK cell activation. (B) NK cells are activated when a target cell lacking MHC-I expression is encountered. The lack of MHC-I on the target cells relieves the inhibition delivered by inhibitory MHC-I receptors resulting in NK cell activation.

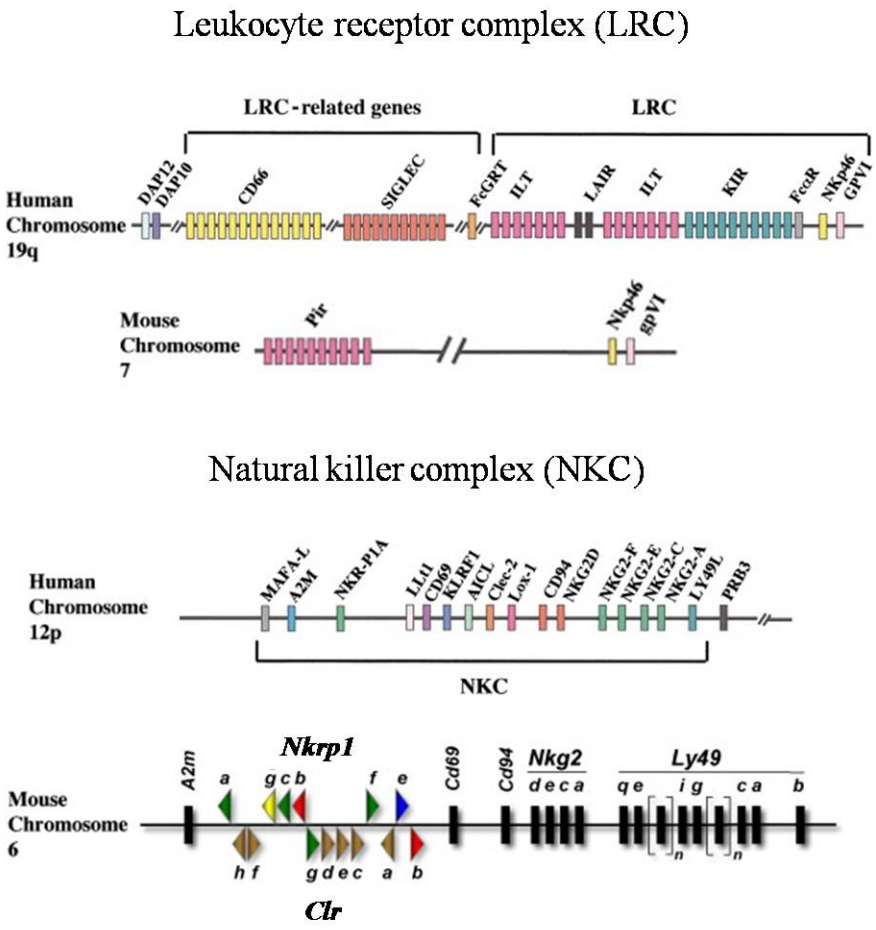

Figure 2: Maps of the human and mouse Leukocyte receptor complex (LRC) and Natural killer complex (NKC). Maps are not to scale and do not reflect the actual number of genes in the *KIR* and *Ly49* clusters. The centromeric end of the mouse NKC is on the left hand side.

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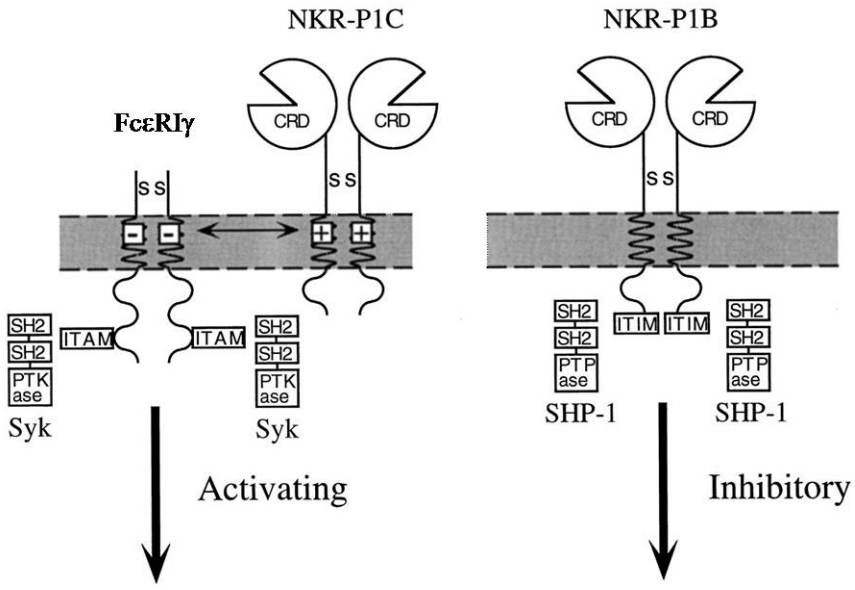

Figure 3: Schematic representation of the NKR-P1 receptors. On the left is depicted NKR-P1C, an activating NKR-P1 receptor, that associates with the ITAM-containing adaptor molecule FcεRIγ to transmit activating signals. On the right, the inhibitory NKR-P1B is drawn. The presence of ITIM in its cytoplasmic tail allows this receptor to transmit negative signals. Note that both receptor types are expressed as disulphide-linked homodimers.

Carlyle J., et al. (1999). *The Journal of Immunology.* 162:5917-23. *Copyright 1999. The American Association of Immunologists, Inc.*

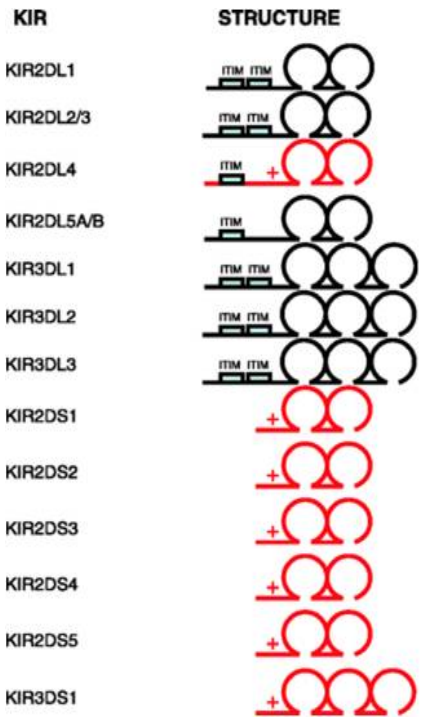

Figure 4: Schematic representation of KIR receptors. KIR receptors are depicted. Inhibitory KIR possess a long cytoplasmic tail with one or two ITIM while the activating KIR have a short cytoplasmic tail and a positively charged lysine or arginine in their transmembrane domains. Non-closed circles represent the Ig domains of the extracellular region of KIR. KIR either have two (KIR2) or three (KIR3) Ig domains.

(Williams A. P., Bateman A. R. and Khakoo S. I. 2005. Hanging in the Balance: KIR and Their Role in Disease. *Molecular Interventions* 5:226-240).

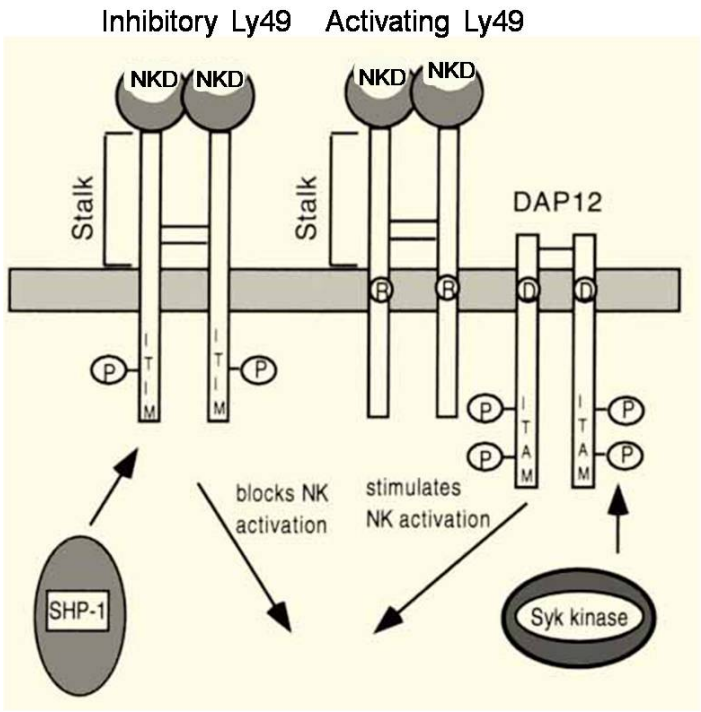

Figure 5: Representation of the structure of Ly49 receptors. Ly49 receptors are type II transmembrane proteins of the C-type lectin family of receptors expressed as disulphide-linked homodimers. They are composed of the following domains: a short cytoplasmic tail, a transmembrance domain, a long-flexible stalk and the ligand-binding domain: the natural killer domain (NKD). Inhibitory Ly49 possess an ITIM in their cytoplasmic tail allowing them to prevent NK cell activation after recognition of their MHC-I ligand. Activating Ly49 receptors associate with the ITAM-containing DAP12 in order to transmit stimulatory signals. The positively charged arginine residue in the Ly49 transmembrane domain interacts with the negatively charged aspartic acid residue in the transmembrane domain of DAP12 allowing non-covalent association between Ly49 and DAP12.

(Adapted from Kane K., et al. (2001). *Immunological Reviews.* 181:104-14).

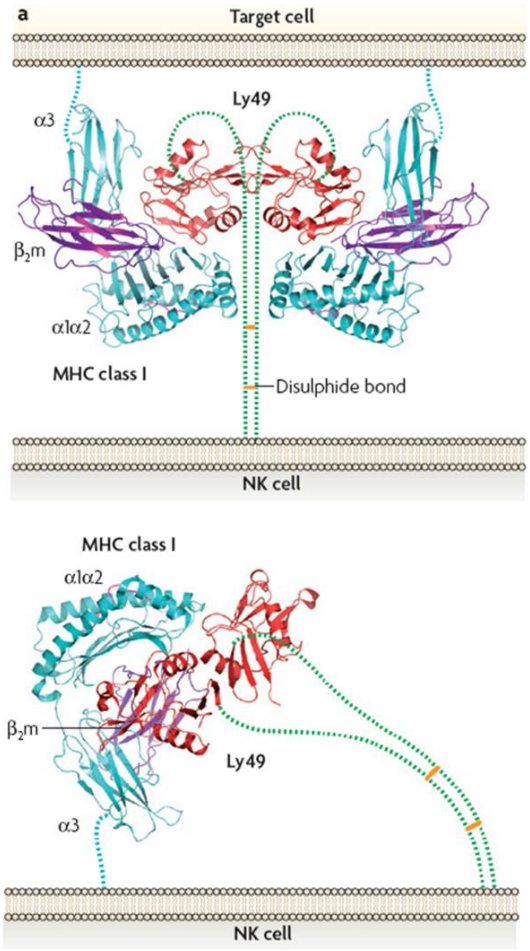

Figure 6: *Trans* and *cis* interaction between Ly49 and their MHC-I ligands. Top: *Trans* interaction between Ly49 (red ribbon and green dotted line) on an NK cell and MHC-I (blue and purple ribbons) on the surface of a target cell. The NKD (red ribbon) of the Ly49 is backfolded on the stalk (green dotted line) allowing contact with two MHC-I molecules. Bottom: *Cis* interaction between an Ly49 receptor and MHC-I on the surface of a single NK cell. The stalk is extended and the NKD is dissociated from the stalk allowing contact with one MHC-I molecule located on the same plasma membrane.

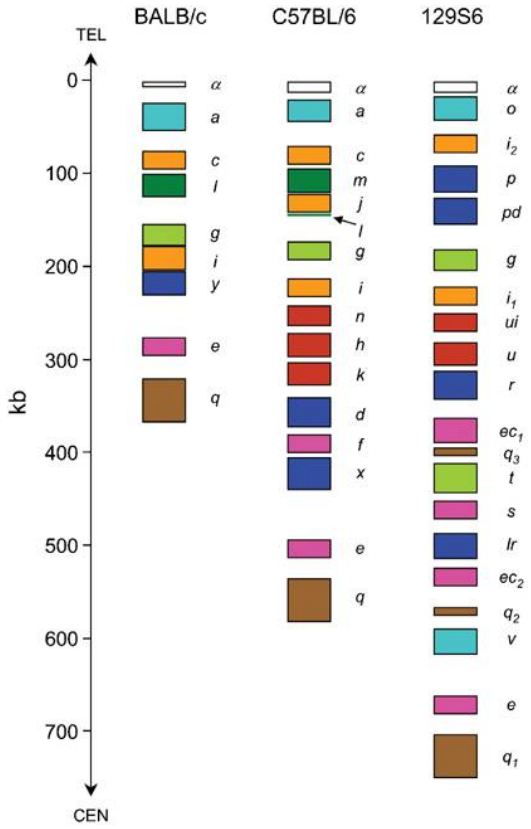

Figure 7: Gene content of three different *Ly49* haplotypes. The *Ly49* genes of BALB/c, C57Bl/6 and 129S6 mice are graphically represented. The figure is drawn to scale with each rectangle representing the size of each gene from the first to the last exon. The scale in kilobases (kb) is shown on the left. The colors of each gene represent *Ly49* subgroups after phylogenetic analysis.

RESEARCH OBJECTIVES AND HYPOTHESES

Prior to our study, little was known about Ly49 receptors in NOD mice. Two activating receptors were cloned from NOD mice (293, 294). The first is an allele of *Ly49p*^{129} and the second, *Ly49w*, is a novel *Ly49* gene absent from the three previously elucidated clusters (270). The extracellular portion of Ly49W closely resembles that of Ly49G but unlike Ly49G, Ly49W is an activating receptor (294). A diabetes susceptibility locus (*idd 6*) was mapped close to the *Ly49* cluster in the NOD genome (295) suggesting that some NK cell receptors may be implicated in the appearance of diabetes. The discovery of a novel activating Ly49 receptor and the possibility that these receptors may be involved in the development of diabetes prompted us to analyze the *Ly49* cluster from NOD mice.

Since their discovery, Ly49 receptors are known to play a critical role in the control of NK cell function by contributing to the "missing-self" response with their ability to recognize MHC-I molecules. Although several mice expressing Ly49 receptors as transgene have been described (262, 296), no Ly49-deficient mouse model has been created. An NKC-knockdown mouse in which the expression of Ly49 is greatly reduced would be a great tool for studying the role played by Ly49 receptors in the control of NK cell functions. We propose two possible outcomes concerning NK cell functions in NKC-knockdown mice: first, the lack of inhibitory signals delivered by Ly49 results in increased NK cell functions or secondly, NKC-knockdown NK cells are hyporesponsive due to the lack of MHC-I-mediated NK cell education.

In contrast to the highly diverse *Ly49* gene cluster, the *Nkrp1-Clr* gene cluster shows minimal differences between B6 and BALB/c mice (208). The NKR-P1 receptors bind to Clr ligands and the expression of Clr-b on target cells prevents NK cell activation through recognition by the inhibitory NKR-P1D (204, 205). Even though BALB/c mice do not encode *Nkrp1d*, they possess its allele *Nkrp1b* which also binds to Clr-b. We sought to map the *Nkrp1-Clr* gene cluster from 129S1 mice and to clone *Nkrp1* and *Clr* genes to confirm the conservation of this cluster. This data was used to analyse newly discovered interactions between NKR-P1 receptors and Clr ligands (203, 297, 298).

The specific objectives of this research project are as follows:

1) Characterize the sequence of the NOD *Ly49* cluster.
2) Characterize the phenotype and development of NK cells from NKC-knockdown mice.
3) Study the "missing-self" response of NKC-knockdown NK cells.
4) Map the *Nkrp1-Clr* gene cluster in 129S1 mice.

PREFACE TO CHAPTER 2

Ly49 clusters from three different mouse strains have been previously sequenced (255, 270, 271). *Ly49* clusters from B6, BALB/c and 129S6 mice are all different but have some commonalities. One shared feature of the *Ly49* clusters is the presence of three pairs of framework genes present in all three clusters. Another striking feature of the *Ly49* clusters is the high density of repetitive elements. It was proposed that the high density of repetitive sequences in *Ly49* clusters favoured the rapid evolution of the clusters. Despite these commonalities, the three *Ly49* clusters vary greatly in gene number. The BALB/c cluster is the smallest with nine *Ly49* genes while the 129S6 cluster is the largest with twenty genes. The B6 cluster has an intermediate size with its sixteen genes. Despite the cloning of two genes in NOD mice coding for activating Ly49 receptors (Ly49P and W) (293, 294), little is known about the *Ly49* genes from NOD mice. NOD mice carrying the B6 NKC including the *Ly49* cluster have a delayed onset of diabetes (299). Elucidating the *Ly49* cluster from NOD mice may give indications on the role played by NK cells, more specifically by Ly49 receptors, in the development of diabetes.

Based on current knowledge of *Ly49* clusters, we hypothesize that the *Ly49* cluster from NOD mice will contain three pairs of framework genes: *Ly49a* and *c*, *Ly49i* and *g* and lastly *Ly49e* and *q*. We also expect that the *Ly49* cluster from NOD mice will be different than the three other sequenced clusters since Ly49W is a unique Ly49 receptor only found on NOD NK cells (294).

CHAPTER 2

Ly49 cluster sequence analysis in a mouse model of diabetes: an expanded repertoire of activating receptors in the NOD genome

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1. Abstract

The mouse *Ly49* and human killer cell immunoglobulin-like receptors (*KIR)* gene clusters encode activating and inhibitory class I MHC receptors on natural killer (NK) cells. A direct correlation between the presence of multiple activating KIR and various human autoimmune diseases including diabetes has been shown. Previous studies have implicated NK cell receptors in the development of diabetes in the non-obese diabetic (NOD) inbred mouse strain. To assess the contribution of Ly49 to NOD disease acceleration the *Ly49* gene cluster of these mice was sequenced. Remarkably, the NOD *Ly49* haplotype encodes the largest haplotype and the most functional activating Ly49 of any known mouse strain. These activating *Ly49* include three *Ly49p*-related and two *Ly49h*-related genes. The NOD cluster contains large regions highly homologous to both B6 and 129 haplotypes, suggesting unequal crossing over as a mechanism of *Ly49* haplotype evolution. Interestingly, the 129-like region has duplicated in the NOD genome. Thus, the NOD *Ly49* cluster is a unique mix of elements seen in previously characterized *Ly49* haplotypes resulting in a disproportionately large number of functional activating *Ly49* genes. Finally, the functionality of activating Ly49 in NOD mice was confirmed in cytotoxicity assays.

Keywords:

Ly49, diabetes, natural killer cells, comparative immunology/evolution, cell surface molecules

2. Introduction

Type 1, insulin-dependent, diabetes (T1D) is an autoimmune disease characterized by targeted destruction of insulin-producing β-cell islets of the pancreas by infiltrating lymphocytes (300). Much of our understanding of T1D has come from the study of disease in the non-obese diabetic (NOD) mouse (301, 302). In addition to being a model of spontaneous T1D, NOD mice spontaneously develop other tissue-related autoimmune responses (301, 303). Several genetic loci have been associated with susceptibility and development of diabetes in both humans and NOD mice (304). Multiple candidate genes have been identified that contribute to the genetic susceptibility of NOD mice to autoimmune diseases, including MHC class II, *Il2* and *Ctla4* (301, 302).

Natural killer (NK) cells are large granular lymphocytes that are able to lyse virally-infected and transformed cells (122). Interestingly, several NK anomalies have been linked with diabetes. An early study demonstrated that diabetic patients have lower NK activity compared to healthy controls (305), whereas a second group found that NK cells isolated from diabetic patients showed an activated phenotype compared to NK cells from healthy controls (306). Several recent studies have reported abnormal NOD NK cell functions and may be indicative of a possible association between diabetes development and NK cell dysfunction (299, 307, 308). Cytotoxicity of NK cells from NOD mice against NK-sensitive tumour cell targets is lower than the killing activity from NK cells of non-diabetic mice (309). In particular, Johansson *et al.* (307) showed defective killing triggered by FcR and Ly49D activating receptors in NOD mice.

Similarly, impaired NKG2D modulation in NOD mice has recently been demonstrated. Ogasawara *et al.* (308) suggested that NKG2D, an activating receptor, may become desensitized to its ligands as they are overexpressed in autoimmune, inflammatory environments resulting in decreased NKG2D expression and defective cytotoxicity and cytokine secretion by NOD NK cells. Collectively, these studies suggest that multiple NK cell activation pathways may be affected in NOD mice.

Cytokine production and cytotoxicity in mouse NK cells is regulated to a large extent by Ly49 family members through their binding to MHC class I and related molecules (230). Ly49 proteins are disulfide-linked homodimeric type II transmembrane receptors belonging to the C-type lectin receptor superfamily (122). *Ly49* genes are grouped in a gene cluster as part of the natural killer gene complex on chromosome 6. There are large differences in the gene content of the *Ly49* haplotypes found in different mouse strains. To date, the 129S6 *Ly49* gene cluster is the largest known with 20 genes and BALB/c is the smallest with 9 genes (270, 271). The Ly49 receptor family is composed of two major groups: (1^{st} group) inhibitory Ly49 receptors possess an immunoreceptor tyrosine-based inhibitory motif (ITIM; I/VxYxxL/V) in the intracellular domain that recruits SHP-1 upon phosphorylation (310, 311), and (2^{nd} group) activating Ly49 molecules lack an intact ITIM sequence but possess an arginine in their transmembrane domain for association with the signal-transducing protein DAP12 (231, 312). The binding of inhibitory Ly49 receptors to MHC class I ligands results in an inhibition of cytotoxicity (230, 313). In contrast, NK killing can be

triggered by activating Ly49 recognition of MHC class I expressed on target cells, suggesting a possible mechanism of NK cell-induced autoimmunity (293, 294). Furthermore, cross-linking of activating Ly49 molecules by specific monoclonal antibody (mAb) or MHC ligand results in cytokine production and intracellular calcium ion mobilization (272, 293, 314).

Killer cell immunoglobulin-like receptors (KIRs) are the human functional equivalents of murine Ly49 molecules (122). Diabetic patients tend to have KIR haplotypes that differ from those of healthy controls. These individuals generally have an increased number of activating KIR in their genome compared to healthy controls or are more likely to possess KIR haplotypes containing specific activating KIR (306, 315). Mapping studies in the NOD mouse model have shown that a diabetes susceptibility locus (*idd 6*) is present on chromosome 6 near the *Ly49* gene cluster (295). In agreement with this finding, NOD mice congenic for B6 (B6) NK1.1, a marker near the *Ly49* cluster, manifested reduced disease incidence and improved NK and NKT cell performance as compared to wild-type NOD mice (299).

To better understand the role activating Ly49 receptors and NK cells may play in the development of diabetes, we analyzed the *Ly49* gene cluster of the NOD/ShiLtJ (NOD) inbred mouse. Initial sequencing of *Ly49* cDNAs revealed the presence of closely related duplicated genes. Subsequent sequence analysis of the NOD *Ly49* gene cluster revealed a significantly increased number of activating *Ly49* genes compared to other known *Ly49* haplotypes. This is due, in part, to a large-scale duplication from a 129-related *Ly49* region. A separate

region of the NOD *Ly49* cluster also shows great similarity to a part of the B6 haplotype. Furthermore, the identification of novel *Ly49* genes in the NOD mouse is confirmed through flow cytometry. Elucidation of the MHC receptors regulating NOD NK cells will facilitate linking potential NK cell dysfunction to the induction of autoimmunity in the NOD mouse.

3. Materials and methods

Mice, cells and viruses

B6, 129S1 and NOD/ShiLtJ mice were purchased from the Jackson Laboratory (Bar Harbour, ME, USA) and then bred and maintained at the IRCM animal care facility in accordance with institutional guidelines. Animal studies were reviewed and approved by the IRCM Animal Ethics Committee. ALAK preparation and cytotoxicity assays were performed as described previously (316). Spleens were first treated with collagenase for flow cytometry of pDC. MCMV plaque assay was performed as described previously (317). BALB/c MEF used for plaque assays were a kind gift of Dr. Silvia Vidal (McGill University, Montréal, QC, Canada).

cDNA cloning

Total RNA was extracted from ALAK cultures using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada). cDNA was synthesized using the Superscript First Strand cDNA synthesis kit (Invitrogen). Full length *Ly49g*$_1$ and *Ly49g*$_2$ coding sequences were amplified with the following primers: forward 5′-CTTCATACATCATTCCCAAG-3′ and reverse 5′-ATTTTACACTCGTTGGAGAG-3′. PCR products were cloned with the pCR2.1–TOPO cloning kit (Invitrogen) and sequenced.

Flow cytometry

Splenocytes were isolated from mice and stained for various cell surface markers with the following mAb: APC-CD49b (DX5), PE-TCRβ (H57-597) (eBioscience, San Diego, CA, USA), FITC-Ly49D (4E5), (BD Biosciences,

Toronto, Ontario, Canada), APC-mPDCA-1/BST2 (JF05-1C2.4.1) (Miltenyi Biotech, Auburn, CA, USA), FITC-Ly49Q (2E6) (MBL), and purified SiglecH (440c) (HyCult, Uden, The Netherlands). MAb to Ly49A/D (12A8), Ly49G (4D11), Ly49H (3D10) and SiglecH were biotinylated using a kit (Roche, Laval, Quebec, Canada) and detected using FITC/PE-Streptavidin (BD Biosciences). Fc receptors were blocked with rat serum (Sigma, Oakville, Ontario, Canada) and dead cells were excluded with propidium iodide (BD Biosciences). Flow cytometry and analysis was performed using a FACsCalibur (BD Biosciences) with CellQuest software (BD Biosciences).

NOD Ly49 BAC sequencing

Funds were made available to The Wellcome Trust Sanger Institute (Cambridge, UK) by The National Institute of Allergy and Infectious Diseases (NIAID), the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and the Juvenile Diabetes Research Foundation International (JDRF) for sequencing *Idd* regions in the NOD mouse. The Wellcome Trust Sanger Institute generated initial sequences from the ends of BACs from a NOD BAC library (CHORI-29; Pieter de Jong, Research Genetics), which was developed from the NOD/ShiLtJ mouse strain. An application was made to the Sanger Institute by APM and Christophe Benoist (Harvard Medical School, Boston, MA, USA) for the sequencing of the *Ly49* cluster (IDD6.3). CHORI-29 BACs with end-sequence homologous to the *Ly49* region of the B6 genome were shotgun subcloned and sequenced. Finished *Ly49*-containing BAC sequence is publicly available online (http://www.sanger.ac.uk/Projects/M_musculus-NOD/) and

through GenBank with the following accession numbers: CH29-584N12 (CU442703), CH29-511G10 (CU467659), CH29-575J15 (CU463286), CH29-171N14 (CU424478), CH29-540A18 (CU570803), CH29-4H7 (CU469450) and CH29-493D4 (CU207332).

Sequence analysis

The NCBI BLAST program was used to identify the location of *Ly49* exons with known *Ly49* cDNAs as queries on individual BAC sequences. This was followed by Lasergene DNASTAR program analysis to map and annotate the exons on the sequence. Putative cDNA sequences were constructed manually and putative amino-acid sequences were determined with Sequencher 4.6 software. cDNA and amino-acid sequences were compared using the BLAST 2 sequence analysis tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Putative cDNA and amino-acid sequences were aligned using ClustalX version 1.83. Dotplot comparison of different sequences was performed using Dotter (available from http://www.cgb.ki.se/groups/sonnhammer/Dotter.html). Repetitive elements were identified with Repeatmasker version 3.1.8 (AFA Smit and P Green unpublished; available at http://www.repeatmasker.org). A PIP of the repeat-masked NOD/ShiLtJ *Ly49* sequence vs itself was constructed using Advanced Pipmaker with a setting of single coverage (available at http://pipmaker.bx.psu.edu/pipmaker). Bootstrap analysis of 1000 replicates was performed on aligned cDNA sequences using PHYLIP (Phylogeny Inference Package) version 3.5c (available at http://evolution.genetics.washington.edu/phylip.html). Phylograms were

constructed with TreeExplorer version 2.12 (K Tamura, unpublished; available from http://evolgen.biol.metrou/ac.jp/TE/TE_man.html).

4. Results

New and unusual Ly49 genes detected in NOD mice

Previous in-depth studies of the Ly49 expressed by NOD mice by the Kane laboratory reported allelic variations of Ly49 known to exist in multiple mouse strains (Ly49A, G, D and E), in 129 mice (Ly49P), in B6 mice (Ly49M) and the NOD-specific Ly49W (293, 294). As the above combination of *Ly49* genes is unique, it appears that NOD inbred mice possess a haplotype different from the three sequenced mouse haplotypes (B6, 129 and BALB/c). To better understand the Ly49 expressed by NOD mice, flow cytometry was performed with anti-Ly49-specific mAb on NK cells present in fresh splenocyte isolations. Fresh *ex vivo* spleen NK cells from NOD mice showed reactivity with 12A8, 4E5, 4D11 and 3D10 mAbs (Fig. 1A), which in B6 mice indicate the presence of Ly49A, D, G and H/I (313, 318, 319). However, no binding with Cwy-3 ($Ly49G^{B6}$), AT8 ($Ly49G^{BALB/129}$), 5E6 ($Ly49C/I^{B6}$) or 14B11 ($Ly49C/I/F/H^{B6}$) was detected (data not shown), possibly due to differences in allele epitopes or missing genes. Interestingly, the activating Ly49D receptor appears to be expressed on a majority of NOD NK cells as evidenced by mAb 4E5 staining of NK cells. However, it is possible that these mAbs bind to additional NOD Ly49 as seen for 129 NK cells (320).

Ly49 expression by splenic NKT cells of NOD mice was also evaluated. Relative to B6 NKT cells, very few NOD NKT cells expressed Ly49. Ly49 expression on NOD NKT was detected using 12A8, 4D11 and 3D10 mAb. The small but significant percentage of 3D10-specific NKT cells suggests that either

Ly49H is expressed on NKT cells or, more likely, that a closely related inhibitory receptor, such as Ly49I, cross-reacts with this mAb in NOD mice. Similarly, the 12A8 binding to NOD NKT cells is likely due to Ly49A expression. NOD plasmacytoid dendritic cells (pDC) bound to the 2E6 mAb specific for Ly49Q, but the mean fluorescence intensity was significantly lower compared to B6-derived pDC (Fig. 1B). In summary, mAb staining suggests that Ly49A, D, G, H, I and Q-like proteins are encoded by genes present in the NOD genome.

Reverse transcription (RT)-PCR on NOD ALAK cDNA using Ly49-specific primers was then employed to identify novel Ly49 expressed by NOD NK cells. A cDNA closely matching the already described *Ly49g*^{NOD} was isolated (98.5% identity). Interestingly, the novel *Ly49g*-related cDNA appears to be a pseudogene as it contains an early in-frame stop codon in exon 4 (Fig. 2A). In addition, a cDNA corresponding to the *Ly49g*^{NOD} allele (now termed *Ly49g*$_2^{NOD}$ due to its position in the cluster) previously reported by Kane was also isolated (294). Thus, the *Ly49* cluster of NOD mice is unique in that there appears to be a duplication of the *Ly49g* gene. To completely elucidate the complement of *Ly49* in the NOD mouse, a request was put forward and approved by the NOD Genome Sequencing Project at The Wellcome Trust Sanger Institute to completely sequence the NOD *Ly49* region.

NOD Ly49 cluster sequence

NOD BACs containing *Ly49* genes were identified by end-sequencing and comparison to the relevant region of the B6 genome. A total of seven BACs covering the entire *Ly49* cluster from the NOD genomic library (CHORI-29) were

fully sequenced using the shotgun approach. BAC and gene order was assured by significant overlap (2 kb minimum) between neighbouring BACs. The *Ly49* cluster of NOD mice contains 22 genes as deduced by BLAST analysis of the genomic sequence using known *Ly49* cDNAs for similarity searches. The new NOD genes were named based on their best BLAST matches (Table 1), and the allele relationships implied there are supported by phylogenetic analysis (Fig. 3). All new *Ly49* genes fit into the *Ly49* subfamilies defined in previous sequence alignment/grouping analyses. The NOD *Ly49* gene order is: *Ly49a*, *c*, *h*, *m*, *w*, p_1, pd_1, g_1, i_1, p_2, i_2, p_3, pd_2, g_2, i_3, *u*, *d*, *f*, *x*, *e* and *q*. The framework *Ly49* genes (*a*, *c*, *g*, *i*, *e* and *q*) previously identified in B6, BALB/c, and 129 mice are maintained in this haplotype (269). The relative spacing and size of each gene along with location of exons and repetitive elements is shown in the PIP diagram of Fig. 4A. The NOD *Ly49* cluster is approximately 850 kb in length with a single gap between BACs CH29-511G10 and CH29-171N14 (Fig. 4B). This gap is between the *Ly49m* and $Ly49p_1$ genes. The previously reported (294), but missing, *Ly49w* gene has been tentatively placed here for the following reasons: (1) the entire cluster is covered by overlapping BACs except for this gap, (2) there is sufficient additional BAC sequence upstream and downstream of the cluster to know that the *Ly49w* gene cannot be immediately present outside the main cluster and (3) the placement beside *Ly49m* would be logical as these genes are closely related and most likely arose as a tandem duplication similar to *Ly49p* and *pd* in 129 mice and *Ly49n*, *h* and *k* in B6 mice.

Evidence for intra-Ly49 cluster recombination in the NOD genome

The NOD *Ly49* cluster is composed of a unique combination of six regions (Fig. 5). The cluster begins with *Ly49a* and *c*, which is common to all *Ly49* haplotypes. Next, the unique combination of *Ly49h*, *m* and *w* genes are present. As mentioned above, *Ly49w* and *Ly49m* are likely the result of a tandem duplication event. The residual *Ly49l* exon 7 in the B6 cluster may be the remains of a *Ly49w* gene that was destroyed by the creation of *Ly49j*, which itself is presumably a duplication of a *Ly49c*/*i*-like gene. Remarkably, $Ly49h^{NOD}$ which is closely related to the MCMV-m157 binding receptor encoded by $Ly49h^{B6}$ and the 129 allele *Ly49u*, has been transposed from its normal position 5′ of *Ly49d* and *r* in the B6 and 129 clusters, respectively. This may be the result of a duplication event, since a similar gene ($Ly49u^{NOD}$) is found in the typical location, but it is more likely that the unusual location of $Ly49h^{NOD}$ may be a result of an unequal crossing-over event. This finding highlights the great plasticity in *Ly49* cluster evolution. Following *Ly49h*, *m* and *w* is a cluster of genes first identified in 129 mice: $Ly49p_1$, pd_1, g_1 and i_1 (271). This region is in turn followed by duplications of *Ly49p* and *i*-related genes: $Ly49p_2$ and i_2. Following these two genes a large duplication of the $Ly49p_1$-i_1 *r*egion is present: $Ly49p_3$, pd_2, g_2 and i_3. This large-scale gene duplication explains the initial identification of two *Ly49g*-related cDNAs from NOD NK cDNA (Fig. 2A). It should be noted that the *Ly49p* and *Ly49g* cDNAs first identified by Kane in NOD mice correspond to $Ly49p_3$ and $Ly49g_2$, respectively, in the present study.

Finally, a region closely resembling the centromeric end of the B6 *Ly49* cluster is found: *Ly49u*, *d*, *f*, *x*, *e* and *q*.

The DOTTER analysis was employed to better assess the similarity of the various regions of the NOD *Ly49* cluster to that of B6 and 129 mice. DOTTER genomic sequence comparison displays identity between two sequences as an uninterrupted diagonal line. Comparison of the NOD and the 129 *Ly49* clusters shows that the NOD *Ly49p$_1$-i$_1$* and *Ly49p$_3$-i$_3$* regions are closely related to the 129 *Ly49p-i* region (Fig. 6A). However, in both cases there are breaks in the diagonal indicative of insertions/deletions as a result of divergence over time. DOTTER analysis of the NOD *Ly49p$_1$-i$_1$* and *Ly49p$_3$-i$_3$* regions reveals that they are more closely related to each other than to the homologous region in the 129 cluster, suggesting their likely origin as a large duplication (Fig. 6B). Sequence comparison between the NOD and B6 *Ly49* clusters reveals that the 3′ ends stretching from *Ly49h/u* to *q*, although not identical, are closely related (Fig. 6C). Otherwise, comparison of the total NOD cluster to that of either 129 or B6 only reveals homology to the beginning and end of the clusters, due to *Ly49*α, *a*, *c* and *Ly49e*, *q* regions being highly conserved in all haplotypes, thus far sequenced (data not shown). Thus, DOTTER analysis strongly supports the possibility that the NOD *Ly49* cluster was, in large part, the result of an unequal cross-over between 129-like and B6-like haplotypes present in an ancestral mouse heterozygous for these *Ly49* haplotypes. However, whether the duplication of the NOD Ly49*p-i* region occurred before, during, or after the B6/129 cross-over is not clear.

NOD Ly49 cluster open reading frames

Putative NOD *Ly49* cDNAs were constructed after artificially splicing together the predicted exons of the new genes. The NOD haplotype has seven *Ly49* genes that are predicted to produce functional activating receptors: *Ly49h*, *m*, *w*, p_1, p_3, *u* and *d*. The *Ly49h*, p_1 and *u* genes are newly identified in the current study and their surface expression needs to be confirmed by the production of specific monoclonal antibodies. Like Ly49H, Ly49P has been implicated in the resistance of MA/My mice to murine cytomegalovirus (MCMV) (317), thus, the finding of three *Ly49p*-related genes in one haplotype is especially intriguing. The close identity between the functional $Ly49p_1$, p_3, and the pseudogene $Ly49p_2$ cDNAs (and previously identified *Ly49p* alleles) is shown in Fig. 2B.

Similar to 129 and B6, but not BALB/c inbred mice, the NOD *Ly49* cluster contains a large number of pseudogenes with *Ly49*α, pd_1, g_1, p_2, i_2, pd_2, i_3, *f* and *x* predicted to be non-functional for various reasons including missing exons, early in-frame stop codons, and single or multiple insertions leading to frame-shift stop codons (Table 1). As with their 129 or B6 counterparts, the *Ly49*α, pd_1, pd_2 and *x* genes are non-functional in NOD mice. The NOD *Ly49c* allele, unlike its B6 counterpart, has an early stop in the last exon. However, $Ly49c^{NOD}$ is not necessarily a pseudogene, as the majority of the protein including the carbohydrate recognition domain should be properly translated and folded. There are four members of the *Ly49c/i* subfamily in NOD mice (*Ly49c*, i_1, i_2 and i_3), the most known in any one haplotype. The $Ly49i_1$ gene

may be the only functional member of this group in NOD; *Ly49i*$_2$ and *i*$_3$ are definitely pseudogenes and the status of *Ly49c* is unclear. Similarly, *Ly49g*$_1$ and *g*$_2$ is the second known instance of *Ly49g* duplicating within a haplotype; *Ly49t* is closely related to *Ly49g* in 129 mice. However, in NOD mice the *Ly49g* duplication is more recent, but one gene is non-functional due to an early in-frame stop codon in exon 4 of *Ly49g*$_1$. Repetitive element-related open reading frames are the only additional open reading frames found in the NOD *Ly49* cluster. The incidence of *SINE*, *LINE*, *LTR* and other simple repeats in the NOD *Ly49* region was similar to that seen for 129, B6 and BALB/c *Ly49* regions (data not shown).

NOD Pro1 promoter polymorphisms

Previous studies have indicated that the *Ly49* Pro1 promoter is active in immature NK cells and plays an important role in *Ly49* gene activation and the control of variegated expression (244, 246). Fig. 7 shows a comparison of the Pro1 regions of the activating and inhibitory NOD *Ly49* genes. The activating genes have promoters that are nearly identical to the previously characterized Pro1 promoters of the B6, 129 and BALB/c *Ly49* genes. The *Ly49p*$_2$ gene has a deletion that selectively removes the TATA element associated with Pro1 antisense transcription, similar to the 1640 bp deletion observed for the BALB/c *Ly49y* gene (270), and it was suggested that this deletion may allow expression of the gene as *in vitro* studies have shown that the forward Pro1 promoter is active in reporter constructs lacking the reverse TATA box (246). Although the NOD *Ly49p*$_2$ gene contains a premature stop codon in exon 6,

it is transcribed, as a NOD *Ly49p*-related cDNA reported in GenBank (AK172530) is identical to the predicted *Ly49p*$_2$ transcript. The *Ly49p*$_3$ gene has a typical Pro1 element, and the predicted *Ly49p*$_3$ transcript corresponds to the NOD Ly49P-coding cDNAs previously identified (GenBank AF074458 and AF218080). No sequences identical to the predicted *Ly49p*$_1$ transcript were found in GenBank; however, the sequence of the *Ly49p*$_1$ Pro1 element is not available due to a gap in the sequence assembly.

The forward Pro1 TATA elements of the inhibitory *Ly49a* and *Ly49g*$_2$ genes are distinct from the Pro1 promoters observed for these genes in the B6, 129 and BALB/c *Ly49* gene clusters. The *Ly49g* Pro1 forward TATA element is 'TATAAAT' in the three previously characterized strains; however, it has the sequence 'TGTAAAT' in NOD. Conversely, the *Ly49a* Pro1 TATA element is 'TATAAAT' in NOD, whereas it is 'TGTAAAT' in the other three strains. Characterization of the functional activity of the B6 and 129 Pro1 promoters demonstrated that *Ly49g* Pro1 had significantly higher forward transcriptional activity than the *Ly49a* Pro1 promoter (246), correlating with a higher frequency of Ly49G expression than Ly49A in these mouse strains. These observations predict that the frequency of Ly49A expression should be higher than Ly49G expression in NOD NK cells if Pro1 is the major element controlling the degree to which each gene is expressed. In agreement with this prediction previous studies have shown that anti-Ly49A-specific mAbs YE1/48 and A1 stain the majority of NOD NK cells in contrast to

anti-Ly49G mAb (Fig. 1) (293). It should be noted that Ly49P-related proteins may have also been detected on NOD NK cells with these mAb.

Activating Ly49 function in NOD mice

The presence of multiple and specific activating class I MHC receptors in humans has been correlated with various autoimmune diseases including diabetes (225, 315). However, activating Ly49 do not signal normally in certain mouse strains such as 129X1 (321). To further explore the possibility that the activating Ly49 in NOD NK cells contribute to the onset of diabetes, their functional activity in Chinese hamster ovary (CHO) cell cytotoxicity and MCMV infection assays was assessed. CHO cell killing was tested as NK cells from B6 mice preferentially lyse these tumour cells due to a fortuitous affinity of the activating Ly49D receptor for the hamster MHC Hm1-C4 expressed by CHO cells (251). To determine if Ly49D present on NOD NK cells is functional, ALAK from B6, NOD and 129S1 mice were used as effectors in cytotoxicity assays with CHO as target cells. B6 ALAK displayed high killing ability in agreement with previous reports (252). Also in agreement with previous publications, 129S1 ALAK displayed low cytotoxicity towards CHO cells (321), which may be due to defects in the DAP12 signalling pathway of 129-background mice (Fig. 8A). NOD NK cells from individual mice behaved similarly to B6 mouse NK cells, consistently showing significant cytotoxicity towards CHO cells. Note that this killing was typically lower than that displayed by B6 NK cells; the reasons for this are unclear. To determine if the lower cytotoxicity displayed by NOD NK cells is due to a defect in DAP12 signal transduction similar to that seen in 129 NK cells, the

YB2/0 target cell that is killed by an Ly49D-independent interaction was tested. Interestingly, the pattern of NK cell cytotoxicity observed for the three mouse strain was similar to the CHO killing: B6>NOD>129S1 (Fig. 8B). This suggests that the lower CHO killing displayed by NOD NK cells is due to non-Ly49D-related factors and is in agreement with previous studies of NOD NK cell cytotoxic function (307, 322).

The ability of B6 mice to resist MCMV infection is due to Ly49H, which has been shown to bind to the MHC-like m157 protein on the surface of infected cells, thus triggering cytotoxicity and cytokine production by $Ly49H^+$ NK cells (253). Similarly, Ly49P provides resistance to MCMV infection in MA/My mice although the mechanism is distinct from Ly49H (317). The current study reveals that NOD mice may have up to four functional anti-MCMV activating Ly49 (*Ly49h*, *u*, p_1 and p_3). Thus, NOD, like B6 and MA/My, may be resistant to MCMV. To test this possibility, NOD mice along with 129S1 and B6 controls were infected with MCMV and after 3 days spleen viral titer was assessed. B6 and 129S1 mice had low and high viral titers, respectively, as previously reported. Interestingly, NOD mice had high splenic MCMV levels similar to 129S1 mice (Fig. 8C). Thus, NOD, like 129S1, is an MCMV-susceptible mouse strain despite the four *Ly49h* and *p*-related genes in its genome.

5. Discussion

The NOD mouse strain has been used extensively in studies identifying genes that contribute to diabetes development and severity. There is strong evidence that NK cells or NK cell receptors play an important role in the induction of autoimmune disease in this mouse strain (323-325). NK cell function is dependent on the types of class I MHC receptors that are expressed on their surface. It has long been realized that *in vitro* NK cell function determined by cytotoxicity assays against specific tumour cell targets and *in vivo* responses such as bone marrow or skin graft rejection is highly dependent on mouse strain background. This is not only due to differing MHC haplotypes, but equally due to variable receptors for MHC (such as Ly49 and KIR) expressed by NK cells.

The *Ly49* repertoire is highly variable among mouse strains. The number of genes can vary from 9 to 20, including six framework genes that are always present (269). All of the framework genes are inhibitory in nature. However, each of the mouse *Ly49* haplotypes previously characterized also contain at least one functional activating Ly49, with a maximum of three in the 129 mouse. The NOD mouse now is known to have the largest known mouse *Ly49* haplotype, with 22 genes. This includes up to seven genes coding for activating Ly49, but the functional status of these proteins is uncertain. The role of activating Ly49 has been the subject of much debate. Various ligands have been found for activating Ly49 and all are MHC or MHC-like. Ly49H has been shown to bind the MCMV-m157 gene product on infected target cells (253, 326). Other activating Ly49 have been found to bind normal MHC class I from mouse and other rodent species. For

example, Ly49D^{+} NK cells preferentially lyse H-2D^{d} target cells, especially when the NK cells are also negative for inhibitory receptors of H-2D^{d} such as Ly49G (327, 328). Ly49D has also been shown to recognize other types of rodent class I MHC including hamster Hm1-C4 and ligands encoded by the rat MHC (251, 252).

Similarly, the activating Ly49P and Ly49W of NOD mice were shown to specifically activate NK cell cytotoxicity toward target cells expressing H-2D^{d} and H-2D^{k} class I MHC alleles (293, 294). The outcome of an activating NK cell receptor having affinity for a ubiquitous self-MHC molecule could be either autoimmunity or anergy. Inhibitory Ly49 guard against NK cell autoimmunity and, thus, provide tolerance by binding to self-MHC on normal target cells. However, the physiological significance and functional role for activating NK cell receptors binding to self-MHC still needs to be addressed. At least two activating Ly49 (Ly49P and H) have been shown to be necessary for resistance to MCMV infection; however, the two mechanisms are very different. In contrast to Ly49H, Ly49P recognizes MCMV-infected cells in the context of H-2D^{k} (317). Thus, it is not surprising that Ly49P has some affinity for MHC of non-infected cells. As the Ly49D subfamily is closely related to Ly49P (Fig. 3), it is logical that Ly49D also has affinity for normal MHC. Perhaps, Ly49D has an *in vivo* function similar to Ly49P. The role of the Ly49W group is unknown, but it is more similar to Ly49P than to Ly49H, both in terms of evidence for MHC binding and in sequence identity.

Thus, the protective effect of an anti-viral activating Ly49 may come at the price of an increased chance for autoimmunity. This may be the reason why most murine Ly49 haplotypes have relatively few activating Ly49. Such a hypothesis can be tested directly in the NOD mouse. The present study has found that NOD mice may express up to a maximum of seven activating Ly49. The analogy to observations seen in human diabetic patients is intriguing. Human diabetics have on average more activating KIR (the analogues of activating Ly49 on human NK cells) than non-diabetic individuals (315). The presence of specific activating KIR in combination with certain HLA alleles is also positively correlated with other types of autoimmune disease incidence such as psoriatic arthritis and rheumatoid arthritis (225, 329). However, activating KIR also appear to have positive protective effects against viruses similar to the observations with activating Ly49 in mice. Individuals with HIV or HCV that express KIR3DS1 and HLA-B Bw4-80Ile are protected from disease progression (330, 331).

Although the high number of activating Ly49 in NOD mice is consistent with the hypothesis that activating class I MHC receptors contribute to autoimmune disease incidence, the assumed strong anti-viral protective effect of many activating Ly49 is not found when NOD mice are challenged with MCMV (Fig. 8C). This is despite the presence of two *Ly49h*- and two *Ly49p*-related genes. The reason for the lack of function of these genes is not clear, but there are several possibilities. First, it is unknown if Ly49P_1 and P_3 are expressed on the surface of NOD NK cells due to a lack of specific mAb. Second, Ly49P resistance in MA/My mice is dependent on the presence of H-$2D^k$ (317) and NOD mice

instead have the non-protective H-2D^{b} allele. Third, the NOD *Ly49h* and *u* cDNAs are not identical to B6 *Ly49h* and the amino-acid differences may result in loss of binding to m157, similar to Ly49U of 129 mice (253). This is supported by a recent report that Y146 and G151 of Ly49H^{B6} are critical for functional recognition of m157 (332). In contrast to G151 of Ly49H^{B6}, Ly49U^{NOD} possesses S151 and partially explains the MCMV-susceptibility of NOD mice. Ly49H^{NOD} has both necessary residues, but it is unclear whether the 3D10 staining of Fig. 1 reveals surface expression of Ly49H^{NOD}, Ly49U^{NOD} or both. Interestingly, the inhibitory Ly49I$_1$^{NOD} like Ly49I$_1$^{129}, also share the m157 binding residues making it possible that this subset of NOD NK cells are inhibited by MCMV-infected target cells as speculated for Ly49I$_1$$^{+}$ 129 NK cells (253).

Although the MCMV infection assays are not informative, the CHO killing results suggest that the activating Ly49D is functional in NOD NK cells although NOD ALAK cytotoxicity was lower than that seen for B6 ALAKs (Fig. 8A and B). The reasons for the lower cytotoxicity displayed by NOD NK cells are likely multifactorial, but previous studies of NK cells from human T1D patients show significantly lower cytotoxicity and decreased expression of activating receptors (306, 333). The observed cytotoxicity against CHO targets and the unusually large number of activating Ly49 in NOD mice, is especially intriguing in light of recent reported correlations between diabetes incidence and the number of activating KIR expressed by an individual's NK cells (306, 315). Supporting evidence for this hypothesis is found in the observation that disease incidence is reduced in NOD mice congenic for the B6 *NKC* (299), which encompasses the

B6 *Ly49* region and contains only two functional activating Ly49 in contrast to the seven predicted for NOD.

In conclusion, this study reveals the full panel of MHC class I receptors that may be expressed by NOD NK cells through analysis of the genomic sequence of the NOD *Ly49* region. In addition to finding that the NOD mouse has the largest known haplotype in terms of total genes and total length, NOD mice also possess the most activating Ly49 receptors. Furthermore, these activating Ly49 are functional as determined by cytotoxicity assays. Thus, the NOD inbred mouse is a suitable model to study the contribution of activating MHC class I receptors expressed by NK cells to diabetes induction.

6. Acknowledgements

We thank Christophe Benoist for his essential aid in initiating the sequencing of the NOD *Ly49* region. We also thank Dr Silvia Vidal for her help with MCMV plaque assays and reagents. We gratefully acknowledge the genome sequencing efforts of The Wellcome Trust Sanger Institute. This work was supported by an Operating Grant from the Canadian Institutes of Health Research (CIHR MOP 62841). This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research. This project has also been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-12400. SB is supported by a scholarship from the Fonds de la recherche en santé du Québec. L-HT is supported by a CIHR Cancer Training Program scholarship. APM is supported by a New Investigator Award from the CIHR.

Animal care was provided in accordance with the procedures approved by the IRCM Animal Care Committee.

Note: The manuscript ends here.

7. Extended discussion (not included in the manuscript)

Initial flow cytometric analysis of NOD splenic NK cells revealed that Cwy-3 ($Ly49G^{B6}$), AT8 ($Ly49G^{BALB/129}$), 5E6 ($Ly49C/I^{B6}$) or 14B11 ($Ly49C/I/F/H^{B6}$) antibodies do not stain NOD NK cells (Add. Fig. 1). Although Cwy-3 and AT8 did not stain NOD NK cells, these cells were positive for 4D11 ($Ly49G^{B6}$ and $Ly49G/T^{129}$) suggesting that the $Ly49G^{NOD}$ expressed is different from $Ly49G^{B6}$ or $Ly49^{129}$. $Ly49W^{NOD}$ is bound by the 4D11 and Cwy-3 antibodies (294), but no Cwy-3 staining was detected on NOD NK cells even though roughly 20% of these cells were positive for 4D11. Taken together, this suggests that Ly49W may not be expressed at high levels by NOD NK cells and that 4D11 detects Ly49G on NOD NK cells.

Although no 5E6 ($Ly49C/I^{B6}$) and 14B11 ($Ly49C/I/F/H^{B6}$ and $Ly49I_1^{129}$) staining was detected on NK cells (Add. Fig. 1), NOD mice possess the *Ly49c* and *Ly49i1* genes that encode for putative functional receptors. *Ly49i1* likely encodes for a functional Ly49 receptor while the status of *Ly49c* is not clear due to the presence of an early stop codon located in the last exon predicting a truncated NKD. Analysis of the promoter sequences of both genes suggests that they should be expressed. Both possess a mutated reverse TATA element (GATAAAT) and a normal forward TATA element (Fig. 7). These promoter sequences predict transcription of the genes in the forward orientation suggesting possible surface expression (246). If we assume that these genes are expressed normally in NOD NK cells, allelic polymorphisms in the *Ly49c* and *Ly49i1* genes can explain the lack of 5E6 and 14B11 staining seen in NOD mice.

The lack of 14B11 staining on NOD NK cells suggests that Ly49F and Ly49H are not expressed (Add. Fig. 1). On the other hand, NOD NK cells were stained with 3D10 ($Ly49H^{B6}$) and it is possible that $Ly49H^{NOD}$ is not bound by 14B11 but by 3D10. Furthermore, $Ly49F^{NOD}$ may not be recognized by the 14B11 antibody. Alternatively, $Ly49U^{NOD}$, which is closely related to $Ly49H^{NOD}$ (Fig. 3), could be recognized by 3D10.

The sequencing of the *Ly49* cluster of NOD mice resulted in the discovery of a fourth *Ly49* haplotype. Previous analysis of the *Ly49* cluster of B6, 129S6 and BALB/c mice revealed the presence of six framework genes present in all clusters (270, 271). Similar to the previously sequenced clusters, the NOD *Ly49* cluster possesses all six framework genes: *Ly49a*, *c*, *g*, *i*, *e* and *q*. Due to the duplication of the *p-i* region in the NOD cluster, it is not known whether g_1 and i_1 or g_2 and i_3 form the pair of central framework genes. The homology line seen in the DOTTER plot in Fig. 6A reveals higher sequence similarity between g_1^{NOD}, i_1^{NOD} and g^{129}, i_1^{129} than between g_2^{NOD}, i_3^{NOD} and g^{129}, i_1^{129}. It can be speculated that g_1^{NOD}, i_1^{NOD} were acquired by NOD from 129S1 mice before the *p-i* duplication event that generated the second pair of *Ly49g* and *i* genes.

A recent genomic analysis of the NKC from different mice strains revealed that *Ly49* gene clusters can fall into five major groups, each one characterized by a different RFLP pattern (334). As predicted by previous sequence analyses, this study demonstrated that *Ly49* clusters from BALB/c, 129S1 and B6 fall into separate groups. This analysis revealed that the NOD *Ly49* gene cluster belongs to a fourth group, confirming its divergence (334).

Evolution of *Ly49* clusters cannot be easily predicted, but it is tempting to put forward a model explaining the formation of the NOD *Ly49* cluster. The starting point would be a putative ancestral B6 cluster slightly modified from the actual cluster due to the presence of an intact *Ly49l* gene (255) (Add. Fig. 2). The first step would involve the deletion of *Ly49j* followed by a duplication (or possibly an unequal crossing-over event) of *Ly49h* and its relocation between *Ly49c* and *m* (ancestral B6 and cluster #1). This cluster would undergo an unequal crossing over event with an ancestral 129S6 *Ly49* cluster to acquire the *Ly49p-i* region (cluster #2) followed by duplications of this region (clusters #3 and #4) and deletion of the central *Ly49pd* and *g* genes and of *Ly49n* and *k* (cluster #5) to create the NOD *Ly49* cluster. This scenario uses an ancestral B6 as a starting point due to the high similarity between the centromeric and telomeric ends of the B6 and NOD *Ly49* clusters. It cannot be ruled out that the B6 and NOD *Ly49* evolved in parallel or that they have diverged from a common ancestral cluster.

NOD mice are susceptible to MCMV infection despite the presence of *Ly49h* that allows B6 mice to be resistant to MCMV (254). As stated in the discussion section of the manuscript, $Ly49H^{NOD}$ possesses the residues involved in the recognition of the MCMV-encoded protein m157 (332) but it is not certain if $Ly49H^{NOD}$ is expressed on NOD NK cells. The presence of the amino acid residues allowing recognition of m157 by $Ly49I_1{}^{NOD}$ suggests that these two proteins interact which should result in NK cell inhibition. The uncertainty concerning the expression of $Ly49H^{NOD}$ and the possible binding of $Ly49I_1{}^{NOD}$ to m157 could explain the susceptibility of NOD mice to MCMV infection. It is also

possible that Ly49H^{NOD} recognizes a m157 variant from a strain other than the commonly studied Smith strain. A recent study revealed that although Ly49H^{B6} binds to m157^{Smith}, it could not bind to the majority of m157 variants tested (335). The Ly49H residues involved in binding to m157 are known but it is remains to be tested if they are also involved in binding to non-Smith m157 variants.

Recently, a second mechanism of resistance to MCMV involving Ly49 receptors has been uncovered (256, 317). Ly49P$^{MA/My}$ recognizes MCMV-infected cells by binding to H-2D^{k} complexed with the MCMV-encoded protein m04 (256). More recently, Ly49L^{BALB} and Ly49P$_1$^{NOD} were also found to recognize MCMV-infected cells in the context of H-2^{k} and H-2^{a}, but not H-2^{b} or H-2^{g7} MHC-I molecules and this recognition required the MCMV protein m04 (257). Since NOD mice possess H-2^{g7} MHC-I molecules that include H-2K^{b} and H-2D^{b}, Ly49P$_1$^{NOD} cannot confer resistance to MCMV in NOD mice. It is therefore tempting to speculate that NOD mice congenic for H-2^{k} MHC-I molecules would be less susceptible to MCMV infection than NOD mice. In NOD mice with H-2^{k} MHC-I molecules, Ly49P$_1$^{NOD} should recognize MCMV-infected cell through recognition of H-2D^{k}-m04 complexes and trigger NK cell-mediated lysis.

As shown in Fig. 8A-B, NOD NK cells have reduced lysis of CHO and YB2/0 cells correlating with previous findings showing that NOD NK cells have decreased lytic activity against RMA-S, YAC-1 and *β2m*$^{-/-}$ cells (307, 308). Several groups have suggested that NOD NK cells are anergic, similar to *β2m*$^{-/-}$ NK cells. It is now clear that NOD NK cells possess defective NK cell activity

compared to B6 NK cells but this NK cell activity is definitely higher than that of 129S1 NK cells (Fig. 8A-B) arguing against anergic NOD NK cells. The anergy of *β2m*$^{-/-}$ NK cells can generally be explained by a lack of MHC-I education. NOD mice carry H-2D^{b} and H-2K^{b} MHC-I molecules; therefore NKG2A, Ly49C and Ly49I$_1$ should act as the self-receptors on NOD NK cells similar to B6 NK cells (281). NKG2A is expressed by NOD NK cells (336) while the expression of Ly49C and I$_1$ has yet to be confirmed. The other putative inhibitory receptors expressed by adult NOD NK cells are Ly49A and G$_2$. Ly49A binds to H-2D^{d} and H-2D^{k} while Ly49G recognizes H-2D^{d} and H-2L^{d} (249) and are probably not self-receptors in NOD mice. The defective lytic activities of NOD NK cells cannot be explained by a lack of MHC-I education since self-receptors should be expressed, although this hypothesis needs to be verified. Furthermore, the decreased activity of *β2m*$^{-/-}$ NOD NK cells supports the notion that NOD NK cells can be educated by MHC-I molecules (307).

The defective lytic activity of NOD NK cells directed against YAC-1 cells can be explained in part by the decreased expression of the activating receptor NKG2D on the surface of NOD NK cells (308). YAC-1 expresses ligands for NKG2D (135) and the lysis of YAC-1 is initiated by the activation of NK cells through NKG2D. The decreased expression of NKG2D is most likely caused by the expression of its ligand Rae-1 in the pancreas of NOD mice (323). This is reminiscent of mice transgenic for *Rae-1*, which have defective NKG2D-dependent functions (337, 338). Thus, the defective lytic activities of NOD NK cells may be caused by the presence of Rae-1 in the pancreas. These ligands

would induce continuous signalling through NKG2D leading to downregulation of NKG2D from the surface of NK cells and defective NKG2D-dependent responses (339). Continuous signalling through NKG2D also affects NKG2D-independent NK cell functions since MHC-I-negative cells are not efficiently rejected by *Rae-1* transgenic mice (338). *In vitro* culture of NK cells with Rae-1-expressing target cells also induces tolerization of NKG2D-independent pathways such as lysis of RMA-S (339). The NKG2D-induced tolerization of the "missing-self" response requires signals initiated by both DAP12 and DAP10 (339). Interestingly, the downregulation of NKG2D on NOD NK cells requires a functional DAP10 (308) and this could lead to tolerization of NKG2D-independent pathways. Collectively, this suggests that the presence of Rae-1 in the pancreas of NOD mice is recognized by NKG2D on NK cells. The continuous NKG2D signalling induces downregulation of NKG2D and thus NKG2D-dependent activities are impaired. This signalling also affects NKG2D-independent pathways such as "missing-self" response. Thus, loss of NKG2D from the surface of NOD NK cells may explain their hyporesponsive phenotype.

As the downregulation of NKG2D during *in vitro* culture of NK cells with cells expressing NKG2D ligands is accompanied by a loss of DAP10 and DAP12 proteins (340), it is possible that NOD NK cells have decreased levels of these two adaptor proteins. Therefore, activating receptors requiring DAP12 for initiation of their signals may be unable to activate NOD NK cells. This could explain why Ly49H^{NOD} cannot confer resistance to MCMV despite its predicted ability to bind to m157. Similarly, the decreased lysis of CHO cells by NOD NK

cells may be due to an inability of Ly49D to properly initiate activating signals after binding to the hamster MHC-I molecule Hm1-C4. *Dap12*$^{-/-}$ NK cells have slightly decreased expression of activating Ly49, but no defects in cytotoxicity are observed while the role of DAP10 in Ly49 expression and function is unclear (232, 233). Until the expression levels of DAP12, DAP10, Ly49D and Ly49H in NOD NK cells are directly verified, no conclusions can be drawn concerning their role in the defective lytic activity of NOD NK cells.

It was previously suggested that a majority of NOD NK cells express Ly49A because of the strong staining of YE1/48 that also recognizes the activating receptor Ly49P$_3^{NOD}$ (293). Moreover, Ly49A does not bind to MHC-I expressed in NOD mice (249). This implies that a large proportion of NOD NK cells express a non-self receptor. The expression of a Ly49 receptor in the absence of its ligand has a negative impact on NK cell activity (288). Ly49C is a self-receptor in B6 mice and the presence of Ly49A on Ly49C$^+$ NK cells (Ly49C$^+$A$^+$) reduces the production of IFN-γ by these NK cells compared to Ly49C$^+$A$^-$ NK cells. Importantly, this is not observed in B6 mice transgenic for the Ly49A ligand H-2D^d (288). Collectively, this indicates that unengaged inhibitory Ly49 receptors can dampen the functions of NK cells. This finding has important implications for the analysis of the activity of NOD NK cells as a majority of these cells presumably express Ly49A in the absence of its ligand. It is therefore tempting to speculate that unengaged Ly49A receptors on NOD NK cells dampen their activity. The downregulation of NKG2D and the high expression of

unengaged Ly49A, and possibly Ly49G, could both contribute towards the hyporesponsive phenotype of NOD NK cells.

The correlation between NOD NK cell responses and the development of diabetes is unclear. Interestingly, NOD mice congenic for the B6 NK1.1 marker (NOD.NK1.1) have a decreased incidence of diabetes compared with NOD mice (299). This study also demonstrated that the lytic activity of NOD.NK1.1 NK cells is higher than that of NOD NK cells. This increase in NK cell activity was not reproduced in a separate study (308). Due to these conflicting results, it is difficult to correlate NOD NK cell lytic activity with the development of diabetes.

Studies of diabetic patients demonstrated that activating KIRs are found more frequently in diabetic patients than in control subjects (306, 315). Furthermore, the presence of activating KIRs and their ligands is associated with an increased risk of developing diabetes (315). Our study of the NOD *Ly49* complex revealed that NOD mice possess the largest *Ly49* known to date. Furthermore, this novel cluster contains seven putative activating receptors, more than any other known *Ly49* cluster (197). Indeed, the BALB/c *Ly49* cluster encodes one activating receptor, the B6 cluster encodes two and the 129S6 cluster three. The high number of activating Ly49 may have an impact on NK cell activities in NOD mice. Recognition of MHC-I by activating Ly49 may promote unwanted NK cell activation in NOD mice favouring NK-mediated cellular damage in the pancreas or leading to NK cell exhaustion. In support of this hypothesis, Ly49D, $Ly49P_3^{NOD}$ and Ly49W are known to bind to MHC-I

molecules (249, 293, 294) and all three receptors could contribute to this unwanted NK cell activation.

NOD.NK1.1 mice carry the B6 NKC and thus have the B6 *Ly49* cluster that encodes for a smaller number of activating receptors. This smaller number of activating receptors may contribute towards the decreased incidence of diabetes observed in NOD.NK1.1 mice. It can be speculated that the smaller number of activating receptors in NOD.NK1.1 mice reduces unwanted NK cell activation. The decreased NK cell activation in NOD.NK1.1 mice would result in decreased damage to the pancreatic β-cells. This hypothesis suggests that activating Ly49 receptors contribute to hyperactivation of NOD NK cells but this does not seem to agree with the decreased NOD NK cell functions. NK cells can be rendered hyporesponsive by continuous signalling through an activating receptor (338). It can then be hypothesized that NOD NK cells become activated in the NOD pancreatic environment, participate in the damage to the pancreas and then become hyporesponsive due to constant activation. The pancreatic damage would then favour the development of diabetes. On the other hand, NOD.NK1.1 NK cells would receive less activating signals due to decreased number of activating Ly49 receptors. In the absence of unwanted NK cell activation, damage done to the pancreas would be reduced favouring a delayed onset of diabetes. It can also be speculated that while NK cells are activated in the pre-diabetic pancreatic environment, they secrete cytokines that activate T lymphocytes thereby promoting the development of diabetes.

Not only the number of activating Ly49 receptors in the Ly49 cluster of NOD mice may have an impact on the development of diabetes but the expression levels of inhibitory receptors as well. As stated earlier, it is suggested that a majority of NOD NK cells express the inhibitory Ly49A and that the absence of its ligand in NOD mice may have negative effects on the function of NOD NK cells. NK cells from NOD.NK1.1 express Ly49A at levels comparable to B6 NK cells (341). A decrease in the number of NK cells expressing unengaged inhibitory Ly49 receptors may favour "normal" NK cell functions if we consider B6 NK cells as having "normal" functions. NK cells from diabetic patients are thought to have abnormal functions (306), thus NK cell responses closer to "normal" in NOD.NK1.1 mice may contribute to the delay in the development of diabetes.

A recent study shed light on the differences between splenic and pancreatic NK cells in NOD mice (336). NK cells can be detected in the pancreas of NOD mice and as the mice age, these NK cells are found to infiltrate the islets where the insulin-producing β-cells are found. Interestingly, pancreatic NK cells show signs of prior activation due to high KLRG1 and CD69 expression. Pancreatic NK cells also have decreased expression of NKG2D (336) probably caused by the expression of Rae-1 in the pancreas of NOD mice (323). Interestingly, NK cells are also found in the pancreas of non-diabetic mice such as B6 and BALB/c, but pancreatic NK cells from these mice do not show signs of activation. This suggests that the pancreas of NOD mice possess unique properties able to influence the activation status of NK cells such as the expression of Rae-1

(323). Indeed, NOD splenic NK cells transferred to NOD mice can be found in the pancreas and these transferred NK cells upregulate KLRG1 and CD69 (336). Previous studies, including ours, were focusing on splenic NK cells from NOD mice but the focus should be perhaps changed to pancreatic NK cells. These cells are likely to be directly involved in the pathogenesis of diabetes as they were found in association with pancreatic islet β-cells and their presence in islets appears to predate that of T cells (336). Due to the differences between splenic and pancreatic NK cells, great care must be taken when trying to link diabetes with the functions of splenic NK cells. Our results suggest that Ly49 receptors are potentially involved in favouring diabetes development through the control of NK cell functions.

8. Research opportunities

Directly testing the education hypothesis in NOD mice would give reliable information concerning the activation status of NOD NK cells. Although interesting, it may be difficult to perform due to the lack of antibodies specific for NOD Ly49 proteins. The currently accepted experiment used to assess the education potential of different Ly49 receptors is to determine production of IFN-γ in different $Ly49^+$ NK cell subsets after activation by cross-linking an activating receptor such as NK1.1 or NKG2D. Due to lack of NK1.1 staining and decreased NKG2D expression, these two activating receptors should not be used in these experiments. NKp46 is expressed on NK cells from a wide variety of strains and cross-linking NKp46 induces production of IFN-γ by NK cells (40). If NKp46 is normally expressed on NOD NK cells, it can be used as an activating stimulus to test the education status of NOD NK cells.

As stated earlier, it is hypothesized that NOD mice congenic for $H\text{-}2^k$ MHC-I would be resistant to MCMV infection. Testing this hypothesis would not only confirm in a third mouse model the interplay between Ly49, MHC-I and MCMV proteins and the role of these interactions in immune responses. It would also give a good indication of the functional properties of activating Ly49 molecules in NOD mice. Due to the possible defects in DAP12 and DAP10 signalling in NOD mice, this congenic model may not give a good indication of the functional properties of NOD Ly49 receptors.

Despite the presence of predicted binding of $Ly49H^{NOD}$ to m157, NOD mice are susceptible to MCMV infection. The unknown expression status of

Ly49H on NOD NK cells was proposed to explain this phenomenon. To directly prove that Ly49H^{NOD} binds to m157, binding assays should be conducted. One method would be to assess the binding of m157-Fc variants from several MCMV strains to cell lines transfected with Ly49H^{NOD}. Alternatively, binding could be tested using a BWZ reporter cell line expressing the extracellular domain of Ly49H^{NOD} with stimulator cells expressing m157 variants.

Another method that can be used to verify if NOD Ly49 receptors are functional is to generate Ly49 congenic mice. The creation of a B6 mouse congenic for the *Ly49* cluster of 129S1 mice revealed that Ly49 receptors in 129S1 mice are functional (285). Similarly, creating a B6 mouse congenic for NOD *Ly49* genes could permit the testing of the role of Ly49H^{NOD} and Ly49I$_1$^{NOD} in controlling NK cell responses to MCMV. Alternatively, further studies using the NOD.NK1.1 mouse model could be performed to test the education hypothesis. NOD.NK1.1 mice could easily be used to test the ability of the NOD NK cell signalling environment to allow education by Ly49 molecules. It is currently known that NOD.NK1.1 NK cells produce less IFN-γ than B6 NK cells (341), but the responses of the Ly49C$^+$, Ly49I$^+$ and NKG2A$^+$ subsets in these mice have not been studied.

NOD NK cells are likely educated by MHC-I present in NOD mice as shown by the defective "missing-self" response seen in NOD.*β2m*$^{-/-}$ mice (307). Studying the incidence and severity of diabetes in these mice could unveil a potential link between diabetes in the education status of NK cells. Due to recent discoveries concerning pancreatic NK cells in NOD mice, a careful analysis of

pancreatic NK cells in NOD.$\beta 2m^{-/-}$ mice should also be performed if any differences in the onset of diabetes are observed in NOD.$\beta 2m^{-/-}$ mice.

9. *Figures*

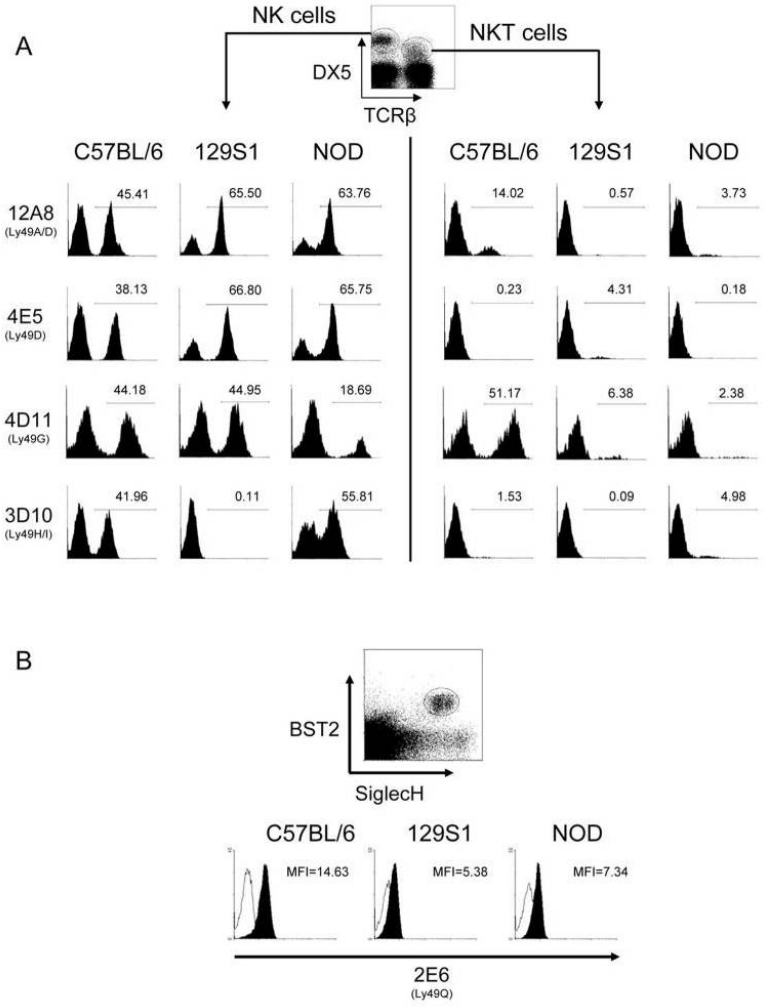
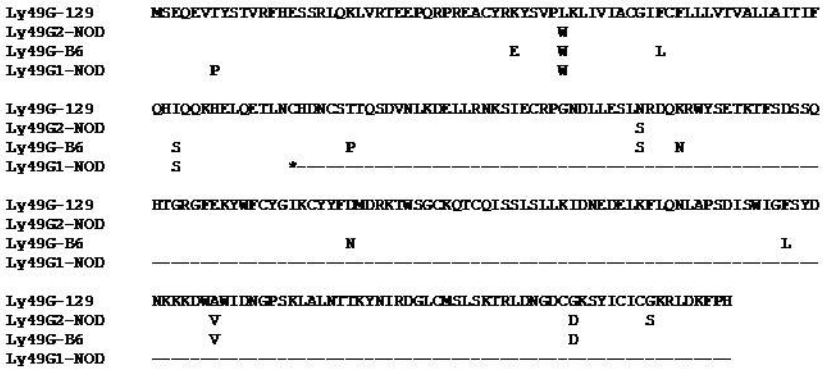


Figure 1: Ly49-specific mAb reactivity of NOD, 129S1 and B6 NK and NKT cells. (A) Splenocyte suspensions of the indicated mouse strains were stained with anti-TCRβ, DX5 and the indicated Ly49-specific mAb. The three left and three right columns show Ly49 surface expression of gated NK ($DX5^+$ $TCR\beta^-$) and NK-T ($DX5^+$ $TCR\beta^+$) cells, respectively. The B6 specificities for each of the mAb used are indicated in parentheses. (B) Collagenase-liberated splenocytes were stained for BST2, SiglecH and Ly49Q. pDC were analyzed by gating on $BST2^+SiglecH^+$ events. The empty and filled histograms indicate pDC binding of isotype control and anti-Ly49Q mAb, respectively.

A



B

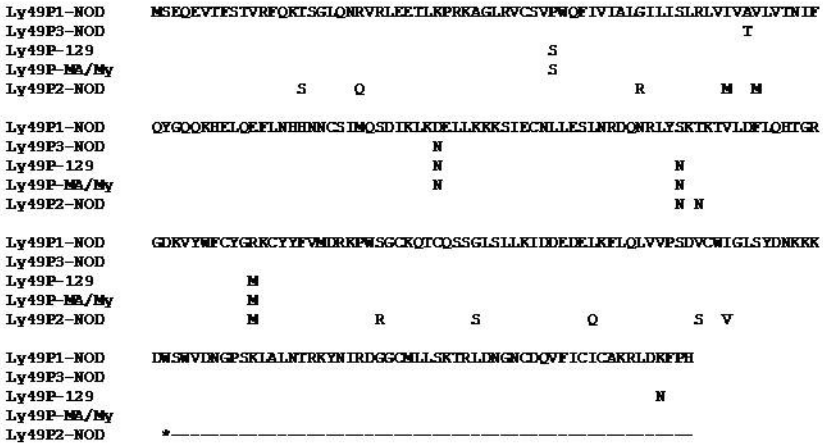


Figure 2: NOD mice have two *Ly49g* and three *Ly49p* genes. Alignment of the putative amino-acid translation of the novel NOD (A) Ly49G and (B) Ly49P alleles is shown along with their 129, B6 and MA/My counterparts. NOD $Ly49g_1$ cDNA was isolated by RT-PCR of NOD ALAK mRNA. NOD $Ly49p_1$ cDNA was deduced from genomic sequence, whereas $Ly49p_2$ cDNA was previously submitted to GenBank (AK172530). The NOD $Ly49g_2$ and $Ly49p_3$ sequences were previously reported (293, 294), but renumbered here due to their location in the genome. Dashes indicate non-existing amino acids due to an early stop.

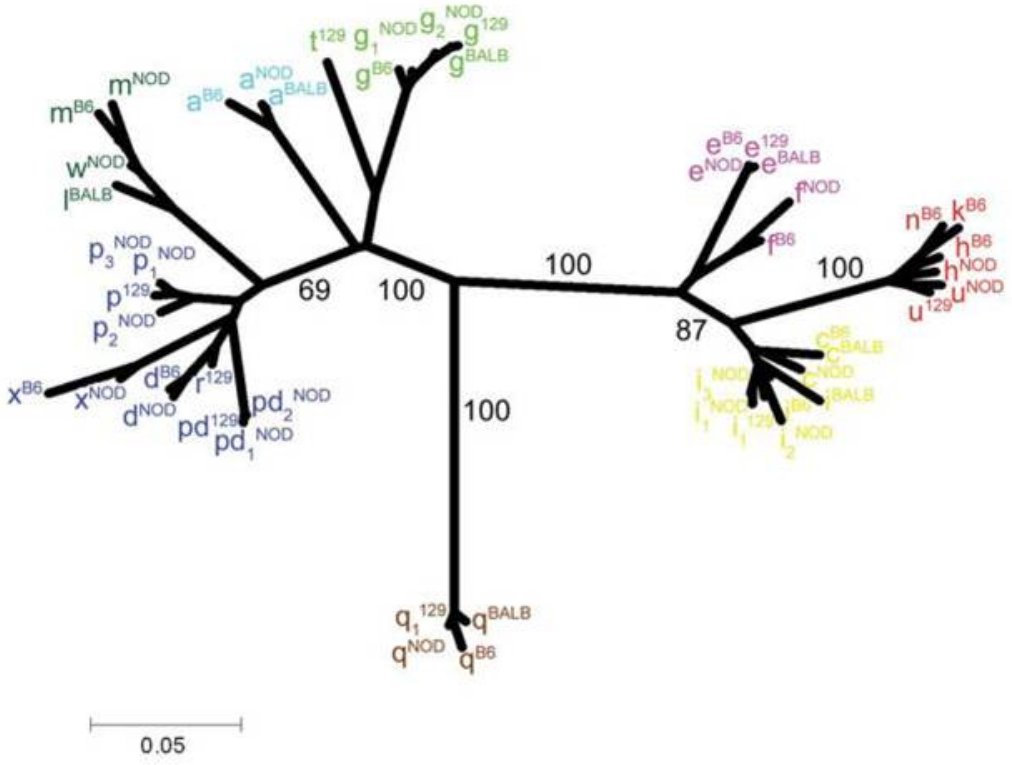


Figure 3: Phylogenetic analysis of all NOD *Ly49*. *Ly49* cDNA sequences from NOD, B6, BALB/c and 129S6 mice, including artificially spliced cDNAs from non-transcribed pseudogenes, were aligned using ClustalX. Bootstrap analysis was performed on 1000 data sets with PHYLIP and the final consensus phylogram was visualized with TreeExplorer. The percentage bootstrap values are given for major branchpoints. The *Ly49* family of genes can be subdivided into the D, L, A, G, Q, E, H and C-related groups. The scale bar indicates the percentage of divergence between cDNAs.

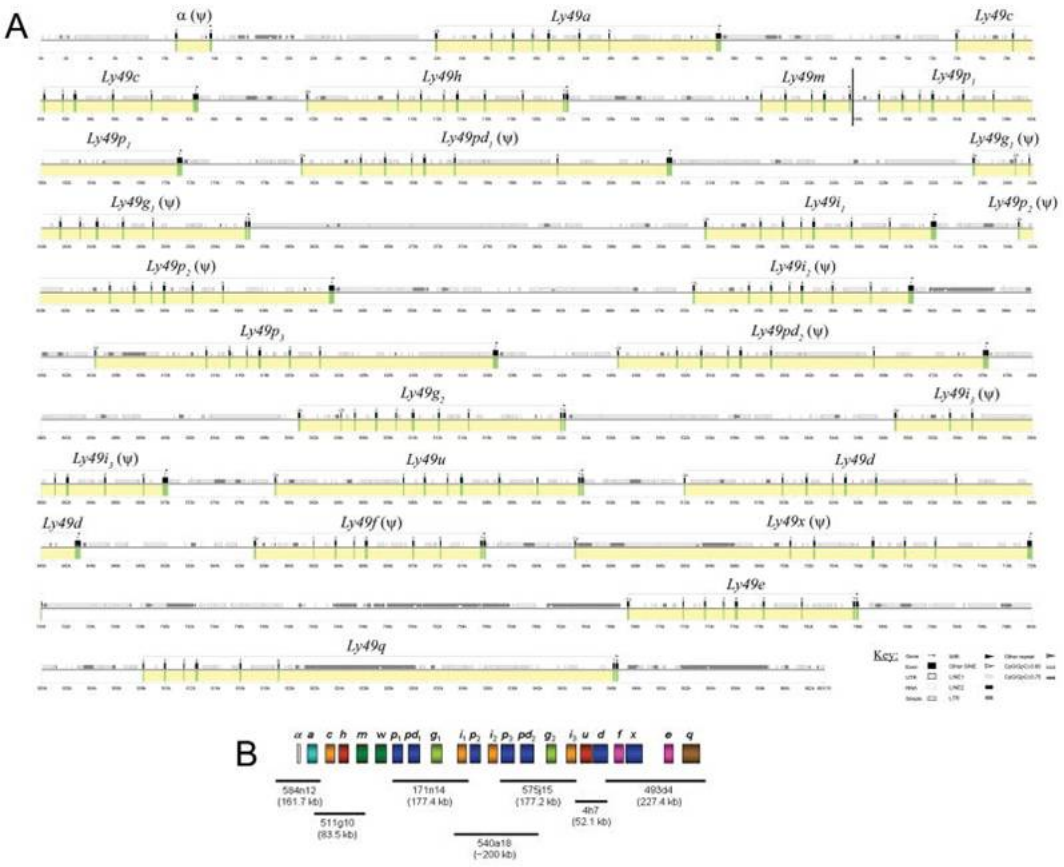


Figure 4: Organization of the NOD *Ly49* gene cluster. (A) A 862 kb sequence of the NOD *Ly49* cluster is represented in graphical form using Pipmaker. The sequence is demarcated below the plot in kilobases. Regions that contain genes are marked in yellow and exons in green. The name of the gene is given above the yellow area and predicted pseudogenes are denoted with a (ψ). A vertical bar between the *Ly49m* and p_1 genes indicates the gap believed to contain *Ly49w*. See key for symbols of different types of repeats and other sequence elements identified using Repeatmasker. (B) An inset (bottom) displaying the location, name, and size of the BACs used for sequencing the cluster.

Table 1: Characteristics of NOD/ShiLtJ *Ly49* genes.

Ly49	*Arg/ITIM* [a]	*Observed sequence anomalies*	*Best cDNA match (B6 or 129)*	*Accession numbers*
a	NA[b]	Exons 2 and 4 only	NA	
a	ITIM	None	98.23% B6 *Ly49a*	AF218077
c	ITIM	Extra 97 nt in exon 7 (early stop)	97.08% B6 *Ly49c*	
h	Arg	None	96.12% B6 *Ly49h*	
m	Arg	None	98.75% B6 *Ly49m*	AF283252
w	Arg	None	96.65% B6 *Ly49m*	AF283250
p 1	Arg	None	99.42% 129 *Ly49p*	
pd 1	NA	Early stop in exon 2	99.62% 129 *Ly49pd*	
g 1	NA	Early stop in exon 4	98.51% B6 *Ly49g*	
i 1	ITIM	None	97.75% B6 *Ly49i*	
p 2	NA	Early stop in exon 6	96.59% 129 *Ly49p*	AF172530
i 2	NA	Early stop in exon 4	97.25% B6 *Ly49i*	
p 3	Arg	None	99.24% 129 *Ly49p*	AF218080
pd 2	NA	Early stop in exon 2	99.62% 129 *Ly49pd*	
g 2	ITIM	None	99.00% 129 *Ly49g*	AF283249
i 3	NA	Early stops in exons 2 and 5	97.63% B6 *Ly49i*	
u	Arg	None	98.13% 129 *Ly49u*	
d	Arg	None	98.48% B6 *Ly49d*	AF218078
f	NA	No splice exon 6, no exon 7	98.32% B6 *Ly49f*	
x	NA	Early stop in exon 4, no exon 3	97.35% B6 *Ly49x*	
e	ITIM	None	100% B6 *Ly49e*	
q	ITIM	None	99.15% B6 *Ly49q*	

[a] The predicted presence of an arginine (Arg) in the transmembrane region or an ITIM in the cytoplasmic domain is indicated. Complementary DNA for new genes were deduced from the genomic sequence.

[b] Not applicable.

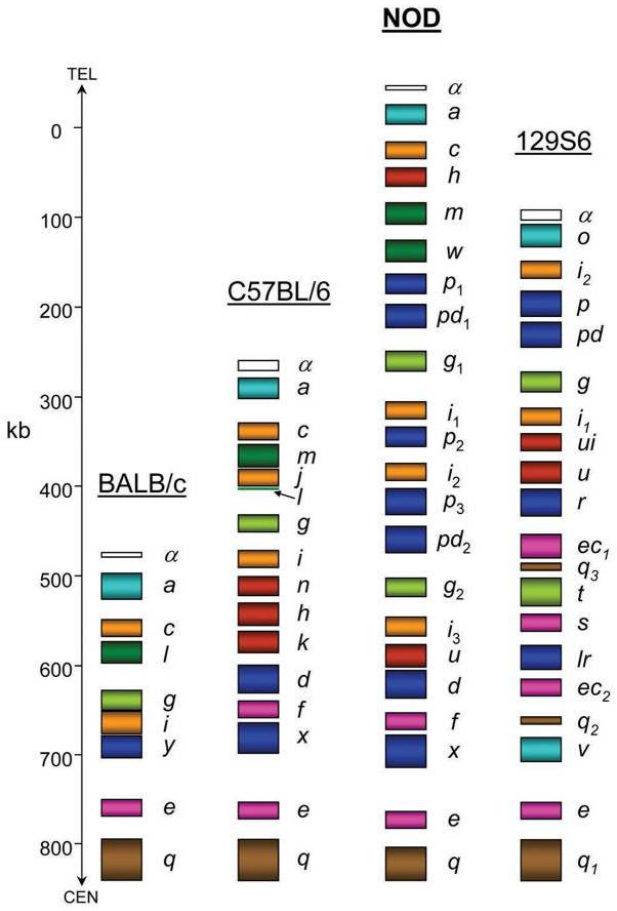


Figure 5: Comparison of the NOD *Ly49* cluster to known murine *Ly49* haplotypes. The location and number of *Ly49* genes of BALB/c, B6, NOD and 129 are compared graphically. The figure is drawn to scale and the scale in kilobases is shown on the left along with the relative locations of the chromosome 6 telomere (TEL) and centromere (CEN). The colors of the various *Ly49* genes follow the scheme of Figure 3. For each gene, the rectangle covers the first known exon to the last. The location of the last exon of $Ly49m^{NOD}$ is inferred from the B6 haplotype. The length of *Ly49w* is unknown and has been tentatively placed in the only gap between *Ly49m* and p_1.

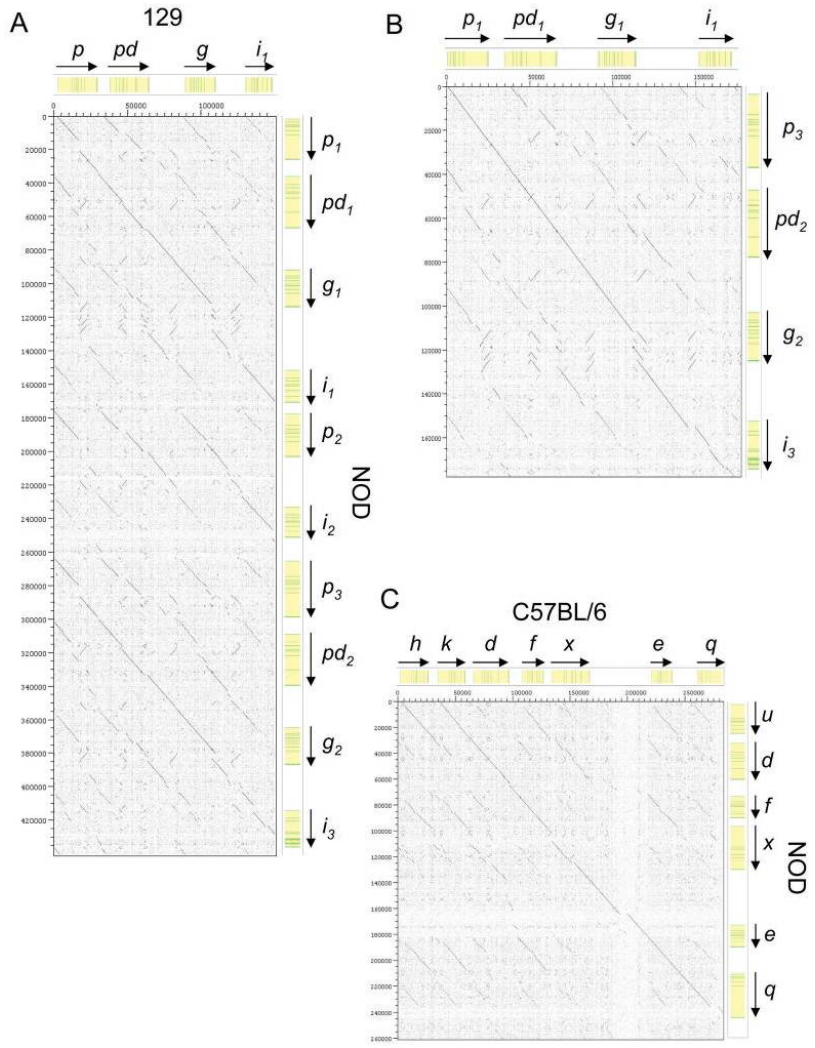


Figure 6: Direct sequence comparison between the B6, 129S6 and NOD *Ly49* gene clusters. Specific regions of B6, 129S1 and NOD *Ly49* gene clusters were directly compared at the nucleotide level using the Dotter program. Sequence comparisons are shown for (A) the *Ly49p-i* regions of 129 vs NOD, (B) the NOD *Ly49p$_1$-i$_1$* and *Ly49p$_3$-i$_3$* regions and (C) the centromeric portion of the NOD vs B6 haplotypes. Diagonal lines represent regions of sequence homology. The location of genes and exons is shown above and on the right side of the plot. The scale on both axes is in base pairs. Breaks in continuity of the homology lines indicate the locations of haplotype-specific deletions/insertions such as repetitive elements

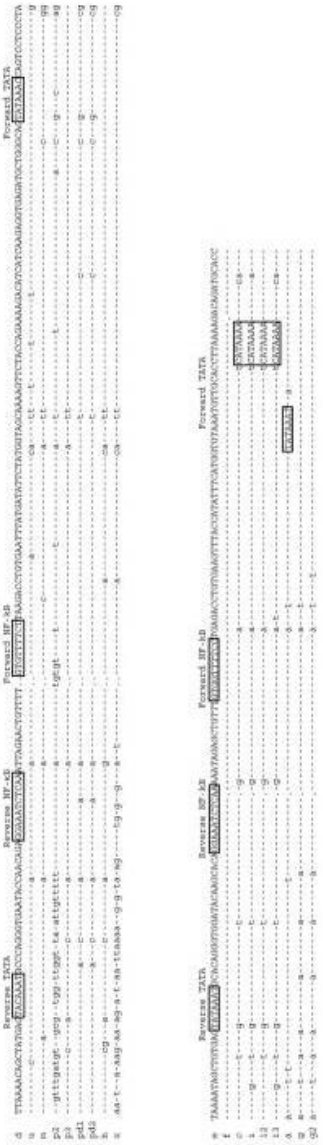


Figure 7: Promoter region analysis of NOD *Ly49* genes. The Pro1 regions of the NOD *Ly49* genes were aligned and separated into activating or inhibitory subgroups. The important transcription factor binding sites are boxed. A dash (−) indicates identity and a period (.) indicates absent nucleotides.

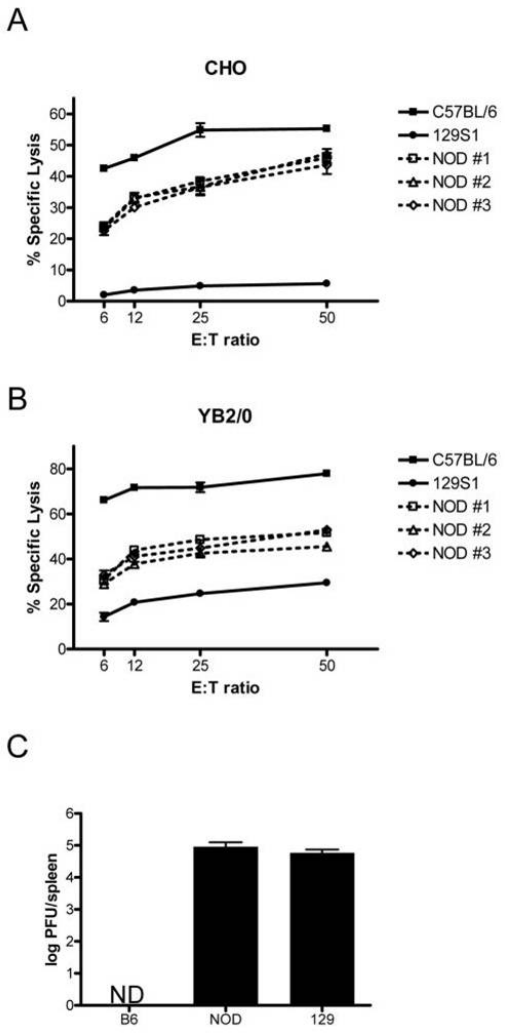
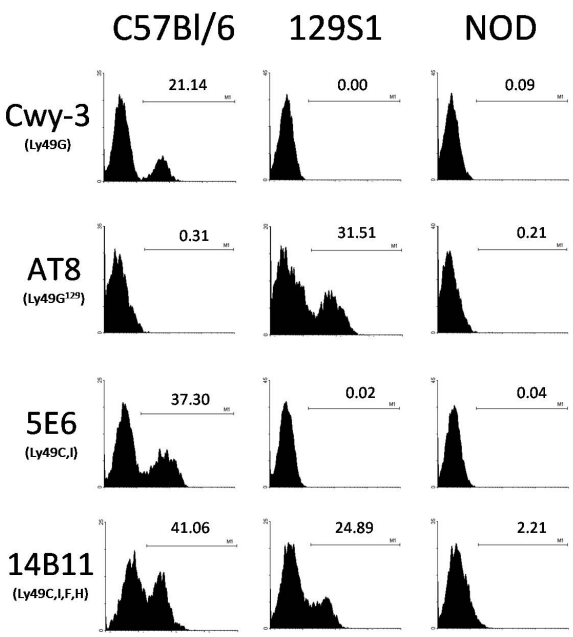
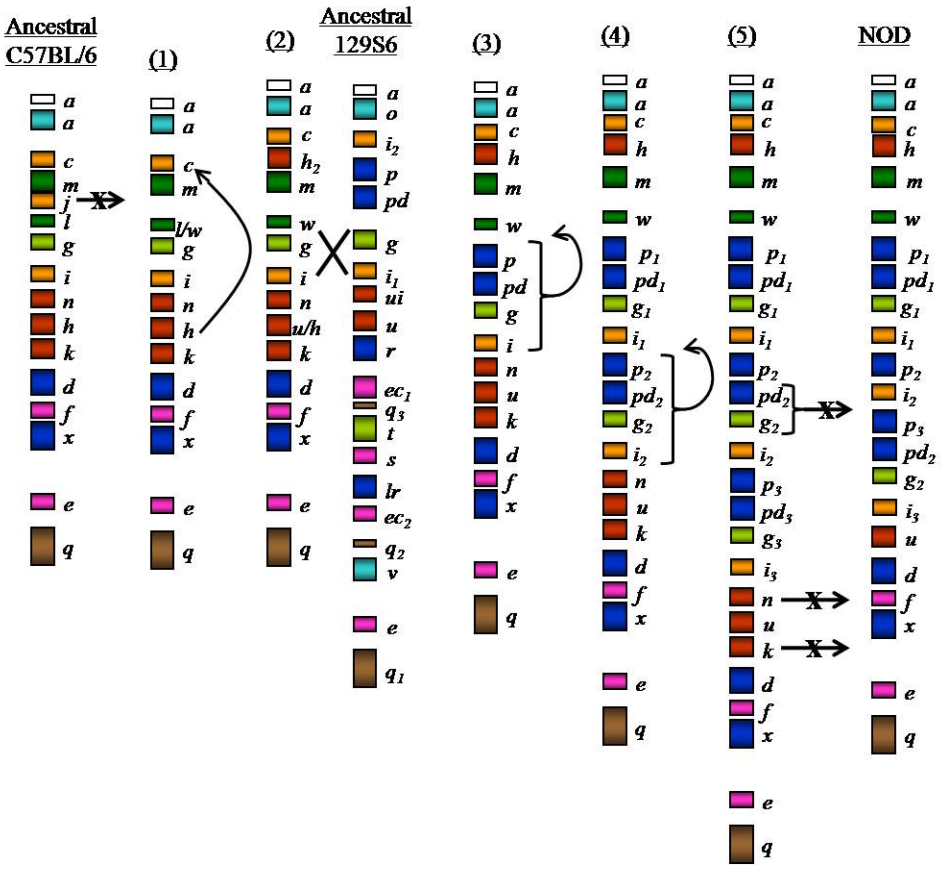


Figure 8: NOD activating Ly49 function. Splenic NK cells cultured in the presence of IL-2 from B6, 129S1 and NOD mice were prepared and used in chromium release assays against (A) CHO, and (B) YB2/0 target cells. Killing of CHO cells is largely dependent on competent signaling through the activating Ly49D receptor. Each line on the cytotoxicity graph indicates the killing achieved from ALAK cultures of different individual mice. (C) Three mice each of the indicated strains were injected i.p. with 5000 PFU MCMV and after 3 days spleen homogenates were prepared and used to infect BALB/c MEF monolayers to assess plaque forming potential. Data are shown in log scale with standard deviation. ND, not detectable



Additional Figure 1: Ly49-specific mAb reactivity of NOD, 129S1 and B6 NK cells. Splenocyte suspensions of the indicated mouse strains were stained with anti-TCRβ, DX5 and the indicated Ly49-specific mAb. The B6 specificities for each of the mAb used are indicated in parentheses. The three columns show Ly49 surface expression of gated NK ($DX5^{+}TCR\beta^{-}$).



Additional Figure 2: Possible scenario for the generation of the NOD *Ly49* gene cluster. This scenario uses an ancestral C57Bl/6 *Ly49* gene cluster as a starting point.

PREFACE TO CHAPTER 3

The discovery of Ly49 receptors and their recognition of MHC-I molecules provided an explanation for the ability of NK cells to destroy MHC-I-deficient cells (230). Antibody-mediated depletion of different Ly49-expressing subsets was attempted to address the role played by different Ly49 receptors in the rejection of MHC-I-mismatched bone marrow cells (342). Generating mice lacking a single *Ly49* gene could be used as another method for assessing the *in vivo* role of Ly49 receptors but sequencing of *Ly49* gene clusters revealed the complexity of *Ly49* haplotypes (197) and therefore deleting a single *Ly49* gene may not give a good insight in the control of NK cell activities by Ly49 receptors.

More recently, Ly49 receptors binding to MHC-I molecules expressed in the host (self-receptors) have been implicated in the acquisition of NK cell effector functions. NK cells expressing self-receptors show more robust responses than self-receptor-negative NK cells (281, 282). The number of such self-receptors expressed on individual NK cells is now known to correlate with the strength of the effector functions of NK cells (283, 284). The generation of a mouse model lacking Ly49 expression provides an opportunity to study the role played by Ly49 receptors in the *in vivo* control of NK cell functions including rejection of MHC-I-deficient cells, viral infections, cancer or autoimmune diseases such as diabetes.

CHAPTER 3

Ly49 Gene-Family Silencing Results in Loss of 'Missing-Self' Responses by Natural Killer Cells to MHC-I-Deficiency: A Dominant Role for NKG2D-Mediated Tumor Cell Killing

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1. Abstract

Natural killer (NK) cell recognition of MHC-I molecules on host cells via Ly49 receptors is considered to be vital for NK cell regulation and education, but studies with animal models in which these receptors are deficient are lacking. Here, we describe "NKC knockdown" (NKC^{KD}) mice that display silencing of Ly49 receptors on ~80% of NK cells, thus providing a novel model for testing the role of self-MHC-I receptors in NK cell education and function. NKC^{KD} NK cells exhibit defective natural killing of MHC-I-deficient targets resulting in diminished *in vivo* rejection of $\beta 2m^{-/-}$ and $H\text{-}2K^{b-/-}H\text{-}2D^{b-/-}$, but not NKG2D-ligand^{+} cells. The role of Ly49 is confirmed via transgenic restoration of these "missing-self" responses in NKC^{KD} mice. These results provide direct genetic evidence that Ly49 expression is necessary for NK cell education to self-MHC-I molecules and that the absence of these receptors leads to loss of MHC-I-dependent "missing-self" immunosurveillance by NK cells.

2. Introduction

Natural killer (NK) cells are a lymphocyte subset that is a unique and integral part of the innate immune system. Immunosurveillance of the host by NK cells for malignant and virus-infected cells results in direct cytotoxicity and the production of cytokines and chemokines to enhance the immune response. Individuals without NK cells or lacking normal NK cell activity suffer from persistent and life-threatening infections of normally innocuous viruses (48, 96). NK cells are able to distinguish normal cells from unhealthy cells by monitoring surface expression of a variety of molecules. The most well-characterized self-recognition system involves surveillance of host class I MHC (MHC-I) molecules, a process initially described by the "missing-self hypothesis" (17). This hypothesis states that target cells lacking normal expression of self MHC-I molecules are specifically recognized and lysed by NK cells.

Several surface receptors are known to activate or inhibit the function of NK cells. The net balance or integration of all activating and inhibitory signals delivered by engaged surface receptors on an individual NK cell dictates whether or not it will respond to a given target cell (122). Numerous NK cell receptors such as the NKG2D, CD94/NKG2, NKR-P1, and Ly49 families of C-type lectin-like transmembrane proteins are encoded in a region on murine chromosome 6 termed the Natural Killer gene Complex (NKC). Among these, NKG2D is an activating receptor capable of recognizing MHC-related stress-inducible ligands such as Rae-1, H60, and Mult1 (134, 135, 140). The CD94/NKG2A heterodimeric receptor indirectly monitors MHC-I expression by recognizing the MHC-Ib

molecule, Qa-1^b (152), which in turn presents peptide derived from the signal sequence of the MHC-Ia molecule, H-$2D^b$. The NKR-P1 family of receptors and their Clr ligands are encoded together in the NKC. The existence of this recognition system suggests that NK cell functions can also be regulated by non-MHC ligands. On the other hand, the most well-characterized MHC-I-specific receptors on NK cells are the *Ly49* gene products, which represent the murine functional equivalents of the human killer-cell Ig-like receptor (KIR) family (122). Unlike the *Nkrp1-Clr* family of genes, the *Ly49* family of receptors is highly polymorphic, with significant variation in gene content between mouse strains. The multiple *Ly49* haplotypes found in different mouse strains parallels the diversity of KIR haplotypes in humans (122).

Ly49 receptors are divided into two major groups: activating and inhibitory receptors. Activating Ly49 receptors have been implicated in direct recognition of virally-encoded MHC-I-like molecules on infected target cells (253). Activating Ly49 associate with the immunoreceptor tyrosine-based activating motif (ITAM)-containing DAP12 adaptor molecule, which is required for proper surface expression and function. Upon ligand binding of activating Ly49 receptors, the associated DAP12 ITAMs become phosphorylated by tyrosine kinases and a signalling cascade is initiated, leading to Ca^{2+} influx, cytokine secretion, degranulation and cytotoxicity (122). In contrast, inhibitory Ly49s each possess an immunoreceptor tyrosine-based inhibition motif (ITIM) in their cytoplasmic domain, allowing these receptors to dampen positive signals initiated by activating receptors through the recruitment of the phosphatases SHP-

1, SHP-2, or SHIP (122). Inhibitory Ly49 receptors recognize specific MHC-I molecules and alleles, resulting in some Ly49 that can bind "self" MHC-I and some that cannot.

During development, NK cells sequentially express Ly49 receptors and subsequently acquire the ability to recognize and kill cells with reduced MHC-I expression. The MHC-I environment in which NK cells develop thus has a profound effect on the functional activity of mature NK cells. For example, in an environment lacking ligands for Ly49 receptors, such as in $\beta 2m^{-/-}$ or $Tap1/2^{-/-}$ mice, mature NK cells possess a significant defect in their ability to kill MHC-I-deficient cells (22, 274). Conversely, introduction of an H-2D^{d} transgene (a "non-self" MHC) into the B6 (H-2^{b}) background also has an effect on NK cell specificity by "educating" these transgenic mice to reject donor cells from non-transgenic B6 mice (279). Interestingly, mosaic expression of H-2D^{d} transgene (on only some "self" cells) prevents this functional change in "missing-self" NK cell specificity (280). Moreover, some MHC-I molecules have a greater impact on NK cell education than others (286).

NK cells have been directly implicated in MHC-I surveillance in the periphery, as revealed by bone-marrow transplantation studies using MHC-I-deficient mice in conjunction with depletion of NK cells using mAb (21), as well as studies of transgenic mice with NK-cell deficiency (343). However, the specific role of Ly49 molecules in the surveillance of MHC-I expression *in vivo* has yet to be rigorously tested. We have generated a mutant mouse strain via ES cell gene-targeting in which the expression of all Ly49 and several neighbouring

NKC genes is highly reduced, allowing for the direct testing of the hypothesis that Ly49 are required for NK cell discrimination between MHC-I^+ and MHC-I^- target cells. In this study, we assess the development and the function of NK cells in Ly49-deficient mice, and provide the first genetic evidence that Ly49 receptors are directly responsible for NK cell immunosurveillance of self-MHC-I *in vivo*.

3. Materials and methods

Mice.

B6, 129S1 and *β2m*$^{-/-}$ mice were purchased from The Jackson Laboratory. *H-2K*$^{b-/-}$, *H-2D*$^{b-/-}$ and *H-2K*$^{b-/-}$*D*$^{b-/-}$ mice were purchased from Taconic Farms. Ly49A^{tg} and Ly49G^{tg} mice were a kind gift from Dr. David H. Raulet (University of California, Berkeley, CA), Rae1ε^{tg} mice were provided by Dr. Lewis L. Lanier (University of California, San Francisco, CA) and Ly49I^{tg} mice were a kindly provided by Dr. Kenneth S. Tung (University of Virginia, VA). The generation of B6 mice congenic for the *Ly49*^{129S1} gene cluster (B6.Ly49^{129}) has been described before (285). NKC^{KD} mice were generated by the targeting of the *Ly49o* gene promoter region with a floxed neomycin cassette that was electroporated into Ly49Q$^{lox/wt}$ R1 ES cells (129-strain background). Neomycin-resistant ES cells were electroporated with CMV-Cre plasmid and recombinant ES cells were selected by PCR using the following primers for: 5'-GGC TTG AAG ACT CAG GGT TTT GCT C-3' and rev: 5'-TCT TGA CCC TTG ATT GTC CTC AGG C-3'. Chimeric mice were produced with *Ly49o*-targeted ES cells and heterozygous mice were produced by breeding with 129S1 females. Homozygous *Ly49o*-targeted pups were determined using the above PCR strategy plus a PCR for the absence of the Ly49Q^{WT} allele using the following primers: for: 5'-CCT AAA AGT AAT TGC TGT GAC TAT T-3' and rev: 5'-CTT TCT AAC TAG CTA ACA ACA G-3'. B6.NKC^{KD} mice were produced by backcrossing NKC^{KD} mice to the B6 background for 10 generations and selecting for 129-specific *Ly49v* gene by genomic PCR at each generation as previously described (285). To

determine B6 vs. 129S1 genome content, SNP analysis was performed using an Illumina Beadstation 500G mouse medium density linkage panel (The Center for Applied Genomics-Sick Kids Hospital, Toronto, ON). The whole genome of B6.NKC^{KD} mice is of B6 origin except for a region containing the NKC on chromosome 6 spanning nucleotides 127,954,449-138,203,431 deduced from SNP markers rs3681620 and rs13479071, respectively. All breeding and manipulations performed on animals were in accordance with university guidelines and approved by the University of Ottawa animal ethics committee.

Cells and viruses.

RMA (H-2^b thymoma) and RMA-S (MHC-I-deficient variant of RMA) were a kind gift from Dr. André Veillette (IRCM, Montréal, Québec). YAC-1 was purchased from the ATCC. RMA-S cells were transfected with a Rae-1β-pEF6 expression construct using Lipofectamine 2000 (Invitrogen). Stable transfectants were selected in 5 μg/mL blasticidin and screened for the surface expression of Rae-1β with a pan-Rae-1 antibody (R&D Systems). These cell lines were grown in complete RPMI medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/mL streptomycin). Adherent lymphokine-activated killers (LAK) cells were generated as previously described (285).

Semi-quantitative RT-PCR.

RNA was isolated from IL-2 activated NK cells using TRIzol reagent (Invitrogen). cDNA was synthesized using the Superscript First Strand cDNA synthesis kit (Invitrogen). Semi-quantitative RT-PCR was performed on serial 5-

fold dilutions of the stock cDNA using primers specific for individual *Ly49* genes and for *Gapdh* as previously described (268, 271). *Nkrp1b*, *Clr-b*, and *Clr-g* were amplified as previously described (208). *Klri* was amplified as previously described (344).

Antibodies and flow cytometry.

Anti-CD49b (DX5), anti-TCRβ (H57-597), anti-CD16 (93), anti-LFA-1 (M17/4), anti-CD62L (MEL-14), anti-CD2 (RM2-5), anti-CD11b (M1/70), anti-CD122 (TM-beta1), anti-NKp46 (29A1.4), anti-CD43 (eBioR2/60), anti-CD27 (LG.7F9), anti-CD69 (H1.2F3), anti-Thy1.2 (30-H12), anti-NKG2D (CX5), anti-CD94 (18d3), anti-Ly49A/D (12A8), anti-Ly49C/I/F/H (14B11), anti-granzyme B (16G6), anti-KLRG1 (2F1), were purchased from eBiosciences. Anti-NKG2A/C/E (20d5), anti-Ly49D (4E5), and anti-Ly49G (4D11) were purchased from BD Biosciences. Anti-B220 (RA3-6B2) was purchased from Miltenyi Biotech. Anti-granzyme A (3G8.5) was purchased from Santa Cruz. Isotype controls were purchased from eBioscience and BD Biosciences. Anti-CRACC was a kind gift from Dr. André Veillette and anti-CD44 was provided by Dr. Woong-Kyung Suh (IRCM, Montréal, Québec). Fc receptors were blocked using rat serum (Sigma) and dead cells were excluded with propidium iodide (BD Biosciences). Flow cytometry was performed on FACScan, FACsCalibur (BD Biosciences) using the CellQuestPro software (BD Biosciences) and on a CyAN-ADP using Summit software (Beckman Coulter). Data was analyzed with CellQuestPro, FlowJo and Kaluza softwares.

In vitro *NK cell assays*.

Cytotoxicity experiments were performed using LAK cells as previously described (345). ConA blast target cells were prepared by culturing $2.5x10^6$ cells/mL of splenocytes from B6, MHC-I-deficient or Rae-1ε^{tg} strains for 2 days in NK medium containing 5 μg/mL ConA (Sigma).

In vivo *NK cell assays.*

The splenocyte rejection assay and calculation was performed as previously described (285). Briefly, splenocytes from MHC-deficient mice and B6 mice were isolated and labelled with 5 μM and 0.5 μM CFSE (Invitrogen), respectively. A mixture of $5x10^6$ splenocytes of each population were injected in the tail vein of 129-background recipient mice treated with poly I:C (150 μg, 24 hours earlier). B6-background recipient mice were left untreated prior to the injection of the cell mixture. After 16 hours, spleens from recipient mice were analyzed for the presence of CFSE-labelled donor cells by flow cytometry. In order to control for the role of NK cells in splenocytes rejection, NK cells were depleted by i.p. injection of 40 μL anti-asialo GM1 antiserum (Wako Chemicals) 2 days prior to poly I:C injection.

Southern blots.

RFLP analysis was performed on DNA isolated from the thymus and digested with EcoRI, KpnI or BamHI. Blots were probed with a mixture of *Ly49g* and *Ly49e* cDNA. The presence of the concatemer was discovered by probing with a probe fragment located in the *Ly49o-Neo*^{r} targeting construct.

Genome Analysis.

Oligos for tiling microarrays were designed using a custom Perl script on the Repeatmasked (Smit, AFA, Hubley, R & Green, P. RepeatMasker Open-3.0. 1996-2010 <http://www.repeatmasker.org>) 129 sequence spanning the Ly49 cluster and flanking genes (693,835bp). In addition, a 5 Mbp Repeatmasked region, centered on the Ly49 cluster (NCBI 37:chr6:127300000-132300000), based on the published B6 genome was also used to design tiling array probes. Regions containing repetitive elements were excluded from the analysis and appear as an absence of signal. All 60 bp probes were tiled at approximately every 15 bp and the custom arrays were subsequently generated by NimbleGen. Hybridizations were performed by the functional genomic platform at IRIC (Montréal, Québec). Genomic DNA from WT and KD were labelled with Cy5- and Cy3-labeled 9mers (Trilink technologies) using 3'-5' exo- Klenow fragment (NEB) and a dye swap was performed. Two-colour hybridizations were carried out using 6 μg of Cy5-labeled and 6 μg of Cy3-labeled gDNA using the NimbleGen hybridization kit as recommended by the manufacturer. Arrays were scanned at 5 μm resolution using a GenePix4000B scanner (Molecular Devices). Data from scanned images were extracted and analysed using NimbleScan 2.5 extraction software (NimbleGen Systems, Inc.)

Statistical analysis.

Statistical significance was determined by student t test with a cutoff P value of 0.05. Data is presented as +/- SD.

Results

Generation of mutant mice containing a concatemer upstream of the Ly49 *complex*

In order to create a mouse model genetically deficient in Ly49 expression a strategy was devised to delete the entire *Ly49* locus via Cre-mediated recombination of flanking LoxP sites. The *Ly49o* gene promoter region was targeted with a floxed PGK-Neo selection cassette in 129-background R1 ES cells containing an $Ly49q^{lox}$ allele previously used to make Ly49Q-deficient mice (346) (Fig. 1A). After generating mice from targeted ES cells, it was discovered that the long-range deletion was unsuccessful due to non-homologous recombination of the *Ly49o*-targeting construct resulting in a total gain of DNA sequence. Specifically, we found that when the *Ly49o* targeting-construct was used as a probe on genomic DNA from WT and mutant mice, a 10-12 kb DNA fragment hybridized very strongly and specifically to mutant mouse DNA (Fig. 1B). In order to identify the source of the additional DNA, we performed array-comparative genomic hybridization (aCGH) analysis using custom microarrays that spanned the *Ly49* locus and flanking genes. Our results confirmed that the gained sequence originated from the *Ly49o* targeting-construct as the mutant genomic DNA preferentially bound to oligonucleotide probes spanning the promoter region of *Ly49o* and other *Ly49* from the same subfamily (Fig. 1C). A blow-up of the amplified region shows the increase in copy number in more detail (Fig. 1C, lower panel). These data suggest the targeting construct integrated as a concatemer. The location of the concatemer was verified to be at the intended

recombination site of the *Ly49o* promoter region by semiquantitative PCR analysis of genomic DNA using primers either within or outside the concatemerized targeting construct. Only when both primers were inside the *Ly49o* promoter region was there a significantly increased amplification signal; when one primer was located outside the *Ly49o* promoter region, the amplification levels were normal (Fig. 1D).

Silenced Ly49*,* Nkg2*,* Cd94*, and* Klri *gene expression in* NKC^{KD} *mice*

While the total deletion attempt was unsuccessful, we sought to determine whether or not *Ly49o* gene expression was affected due to promoter disruption in mice generated using *Ly49o*-targeted ES cells. Initial analysis of Ly49O surface expression by flow cytometry showed significant loss of positively staining NK cells from homozygous mutant mice relative to WT littermate controls (Fig. 2A). Unexpectedly, additional staining of NK cells from *Ly49o*-targeted mice with mAb specific for Ly49V, R, G, T, and I, individually or as a cocktail, showed that these subsets were also decreased by 70-80% (Fig. 2A, B). These results were surprising, as no other *Ly49* genes were intentionally targeted during ES cell manipulation. Interestingly, heterozygous mice have near normal $Ly49^+$ NK cells subsets, although a completely stochastic model of Ly49 acquisition would predict intermediate numbers (Fig. 2B). *Ly49* gene downregulation was detectable at the level of mRNA as deduced by semi-quantitative RT-PCR experiments using primers specific for *Ly49r, u, v, s,* and *g* (Fig. 2C). Thus, the mechanism of Ly49 downregulation may involve multi-locus transcriptional silencing.

Ly49 are co-expressed on average at 2-3 receptors per NK cell, although 0-6 receptors is the range of variegated expression (242). The residual Ly49^{+} NK cells from *Ly49o*-targeted mice lost on average ~50% of Ly49 receptor co-expression, as shown by multi-parameter flow cytometry (Fig. 2D). Therefore, NK cells from mutant mice were mostly Ly49-negative and the remaining Ly49-positive cells had a decreased probability of expressing multiple Ly49. Southern blot analysis of WT and mutant mouse genomic DNA with a mixture of *Ly49* cDNAs as probes resulted in highly similar patterns using three different restriction enzymes (Fig. 2E). These data suggest that Ly49 downregulation is not due to gene deletion or reorganization within the gene cluster. The downregulation was not limited to *Ly49* genes; neighbouring genes in the NKC, including the *Nkg2a/c/d/e, Cd94*, and *Klri* loci, were also affected, albeit to a degree dependent on the proximity to the *Ly49* locus, as assessed by semi-quantitative RT-PCR or flow cytometry (Fig. 2C, F). Due to the overall NK receptor expression phenotype of these mice, they are referred to as NKC-knockdown (NKC^{KD}) mice hereafter.

In summary, the attempted gene-targeting of *Ly49o*, the most telomeric *Ly49* in the 129-strain *Ly49* gene cluster, resulted in an insertion of the *Ly49o* targeting-construct as a concatemer upstream of the *Ly49* gene cluster. This insertion leads to a regional transcriptional silencing and the subsequent loss of the majority of Ly49 receptor expression on NKC^{KD} NK cells, including additional downregulation of neighbouring *Cd94/Nkg2* and *Klri* gene families.

NKC^{KD} NK cells display normal development and maturation markers

The phenotype of NK cells from NKC^{KD} mice suggests that these mice, although generated inadvertently, are potentially useful for studying NK development and education. To determine whether development, localization, or differentiation marker expression (other than Ly49/CD94/NKG2/KLRI) was affected on NKC^{KD} NK cells, flow cytometry was performed on single-cell suspensions from a variety of tissues and organs using a panel of mAb for known NK cell markers.

NK cell numbers and percentages in the spleen, lungs, liver, and blood were similar in WT and NKC^{KD} mice (Supplementary Fig. 1A). Therefore, Ly49/CD94/NKG2/KLRI receptors are not absolutely required for NK cell maturation or seeding of peripheral organs. Twelve markers expressed by NK cells (CD16, LFA-1, CD62L, CD2, CRACC, CD44, CD11b, CD122, NKp46, CD43, CD27, and B220) were further tested by flow cytometry and were found to be expressed at normal levels and proportions of NK cells (Supplementary Fig. 1B). Additionally, the NKC-resident *Nkrp1b, Clr-b,* and *Clr-g* genes were unaffected, as expression levels were normal when analyzed by RT-PCR (Supplementary Fig. 2A). As a control, *Ly49i$_1$* was amplified from the same cDNA aliquot and found to be significantly downregulated similar to the other *Ly49* genes in NKC^{KD} NK cells.

Several markers on NK cells are only detectable after activation by cytokines or ligation of stimulatory surface receptors. NKC^{KD} NK cells cultured in IL-2 for 3 days displayed normal intracellular levels of granzymes A and B,

two granule-associated proteins necessary for target cell killing via granule exocytosis. Similarly, upregulation of surface CD69, a marker of T and NK cell activation, was normal, but the number of NKC^{KD} NK cells expressing the KLRG1 activation marker was reduced by approximately a third relative to NK cells from WT littermates (Supplementary Fig. 2B). This reduction was found to be statistically significant after analyzing multiple mice of each genotype (Supplementary Fig. 2C). *Klrg1* is located in the NKC, but at the opposite end relative to the *Ly49* and beyond the *Nkrp1/Clr* gene clusters. As the *Nkrp1/Clr* genes appear to be normally expressed, it is likely that the decreased KLRG1 expression is a result of altered activation or terminal differentiation of NKC^{KD} NK cells, rather than a long-range silencing effect. In support of this, B6 strain Ly49C/I/NKG2A-negative NK cells (uneducated) also possess significantly reduced KLRG1 levels similar to NKC^{KD} NK cells (281). In summary, the non-NKC NK cell markers analyzed (over 15) on NKC^{KD} NK cells appear to be expressed at normal levels. Furthermore, the development and migration of NK cells to peripheral tissues in naive NKC^{KD} mice is normal. Thus, the deficiency of NKC^{KD} mice appears to be restricted to only NKC-encoded MHC-I-specific receptor expression on mature NK cells.

NKC^{KD} mice exhibit defective natural killing of MHC-I-deficient hematopoietic cells

NK cells have the ability to rapidly reject transplanted hematopoeitic cells from mice genetically deficient for β2m or TAP1/2 molecules, which are both necessary for normal MHC-I surface expression (21, 274). Since the known

ligands for most inhibitory Ly49 are MHC-Ia molecules, the *in vivo* rejection of MHC-I-deficient cells by NK cells is thought to be due to a loss of MHC-I-specific inhibition mediated by inhibitory Ly49, otherwise known as the "missing-self" hypothesis. NKC^{KD} mice provide a genetic model to directly test this hypothesis without using mAb-dependent cell blocking or depletion techniques, which may produce artifacts.

The cytotoxicity assays were performed using B6-background mice as initial experiments with 129-background NK cells yielded low *in vitro* killing, as previously observed for this strain, making data interpretation difficult (285). In the following experiments, the WT control consisted of the previously described B6.Ly49^{129} congenic mouse, which carries a 129-derived *Ly49* cluster (and NKC) on the B6 genetic background (285), while the NKC^{KD} NK cells were isolated from B6.NKC^{KD} mice, which were obtained by backcrossing NKC^{KD} mice with B6 mice for 10 generations and selecting for the 129-strain NKC at each generation before producing homozygous B6.NKC^{KD} mice. Ly49 molecule expression is downregulated to a similar degree in B6.NKC^{KD} mice (Supplementary Fig. 3).

ConA blasts were prepared from various MHC-I-sufficient and -insufficient mouse strains and used in cytototoxicity assays as targets for LAK prepared from WT or NKC^{KD} mice. Both WT and NKC^{KD} LAK were unable to kill WT ConA blasts (Fig. 3A). In contrast, ConA blasts prepared from *β2m*$^{-/-}$ mice, which are missing both MHC-Ia and MHC-Ib β2m-dependent molecules, were lysed efficiently by WT LAK (Fig. 3B). However, NKC^{KD} LAK killing of

β2m$^{-/-}$ blasts was considerably lower (Fig. 3B). Similarly, the killing of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ ConA blasts was high for WT LAK and approximately 50% lower for NKC^{KD} LAK (Fig. 3C). Notably, WT LAK killing of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ blasts was less than that seen for *β2m*$^{-/-}$ blasts, suggesting that β2m-dependent MHC-Ib molecules can also inhibit NK cell killing, in line with previous reports (347-350). *H-2K*$^{b-/-}$ ConA blasts were efficiently killed by WT LAK, but poorly lysed by NKC^{KD} LAK (Fig. 3D). In contrast, very low killing of *H-2D*$^{b-/-}$ ConA blasts was exhibited by either WT or NKC^{KD} LAK (Fig. 3E). This is in agreement with reports suggesting that H-2K^{b} is the main self-educating MHC-I molecule for NK cells in B6 mice (286). To control for cytotoxic potential independent of MHC-I, ConA blasts were prepared from Rae1ε-transgenic mice and used as target cells. These were efficiently lysed by both WT and NKC^{KD} LAK (Fig. 3F), showing that the decreased killing of MHC-I-deficient blasts by NKC^{KD} LAK is not due to lower overall killing ability.

To determine if NKC^{KD} NK cells effectively monitor the expression of MHC-I on peripheral cells *in vivo*, splenocytes were isolated from WT and MHC-I-deficient mice, differentially labelled with CFSE, mixed in a 1:1 ratio and injected into the tail vein of 129-background WT or NKC^{KD} littermates. The next day, the relative levels of CFSE^{+} WT vs. MHC-I-deficient cells in the spleens of recipient mice were analyzed by flow cytometry. Approximately 80% of *β2m*$^{-/-}$ splenocytes are rejected by WT mice in this assay, however, NKC^{KD} mice displayed only half as much rejection (Fig. 4A). The weak rejection displayed by NKC^{KD} mice is likely due to residual Ly49 expression, resulting in low numbers

of MHC-I-educated NK cells. The rejection of *β2m*$^{-/-}$ splenocytes mediated by both WT and NKC^{KD} mice was due to NK cells as shown by pre-treatment of mice with NK cell-depleting anti-asialo GM1 serum.

Next, the rejection by NKC^{KD} mice of splenocytes specifically deficient in MHC-Ia molecules was tested. Rejection of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ splenocytes by WT mice was ~20% lower than rejection of *β2m*$^{-/-}$ splenocytes, but still twice as high as that mediated by NKC^{KD} mice (Fig. 4B). The higher overall rejection of *β2m*$^{-/-}$ cells compared to *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ cells by both types of mice suggests that β2m-dependent MHC-Ib molecules are being recognized by NK cell receptors and are modulating cytotoxic responses. The rejection of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ cells was also shown to be due to NK cells by serum-mediated NK cell depletion (Fig. 4B).

The ability of NKC^{KD} mice to destroy cells missing single MHC-Ia molecules was determined next. NK cells can be educated by single MHC-Ia molecules as shown using MHC-Ia-transgenic mice backcrossed onto a MHC-I-deficient background (286). The rejection of *H-2K*$^{b-/-}$ splenocytes by WT mice was less than that observed for *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ splenocytes, but NKC^{KD} mice rejected *H-2K*$^{b-/-}$ splenocytes significantly less well than WT mice (Fig. 4C). The rejection of *H-2D*$^{b-/-}$ splenocytes by both WT and NKC^{KD} were very low, but not statistically different (Fig. 4D). The low rejection of *H-2D*$^{b-/-}$ cells compared to *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ and *H-2K*$^{b-/-}$ is consistent with studies showing that H-2D^{b} is not a strong educator of self for NK cells compared to H-2K^{b}, but the synergistic effect of H-2D^{b} co-expression is evident in the higher rejection of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ versus *H-2K*$^{b-/-}$ splenocytes (286). In summary, these data provide the first genetic

evidence that Ly49 receptors are directly responsible for the ability of educated NK cells to specifically reject MHC-I-deficient hematopoietic cells *in vivo*.

An Ly49 *transgene restores MHC-I immunosurveillance in NKC^{KD} mice*

In addition to *Ly49, Cd94, Nkg2,* and *Klri* expression is reduced in NKC^{KD} mice. CD94/NKG2A plays an indirect role in MHC-I immunosurveillance via binding to Qa-1^{b} and although the ligand of the KLRI/E heterodimer is not yet reported, it is a strong possibility that it will also bind to MHC-I-related structures. To determine the direct contribution of Ly49 to the deficient "missing-self" responses in NKC^{KD} mice, three different Ly49 transgenic mice (Ly49A^{tg}, Ly49G^{tg}, and Ly49I^{tg}) were individually backcrossed to NKC^{KD} mice on the B6 background. B6 background NKC^{KD} mice show the same Ly49 downregulation as seen on the 129S1 background (Supplementary Fig. 3) compared to control B6.Ly49^{129} congenic mice, which bear a 129S1 *Ly49* cluster on a B6 background (285). As reported for WT NK cells, Ly49A, G, and I transgenes were expressed on the majority of NK cells from NKC^{KD} mice (Fig. 5A). Ly49A and Ly49G transgenes, which do not bind to H-2K^{b} or H-2D^{b}, did not rescue the ability of NKC^{KD} mice to reject MHC-deficient cells (Fig. 5B, C). However transgenic expression of Ly49I, which binds to H-2K^{b} (262, 266), was able to restore the majority of missing self responses (Fig. 5D). Thus, Ly49 silencing is responsible for the majority of deficient "missing-self" responses in NKC^{KD} mice.

Defective killing of MHC-I-deficient tumour cells by NKC^{KD} NK cells is masked by activation through NKG2D

The ability of NKC^{KD} NK cells to kill tumour cells was determined next. Tumour cells are more complex target cells than non-transformed cells from MHC-I-deficient mice, having undergone immune selection resulting in the up or downregulation of many surface receptors, including MHC-I and other ligands modulating NK cell activation. WT and NKC^{KD} LAK killing of MHC-I-sufficient RMA tumour cells was very low (Fig. 6A), showing that the diminished expression of MHC-I-specific receptors does not result in a loss of MHC-I-mediated inhibition in agreement with the low killing of WT ConA blast targets. In contrast, the killing of the MHC-I-deficient variant RMA-S was higher; however, NKC^{KD} NK cells killed RMA-S only half as well as WT NK cells (Fig. 6B) in line with observations using MHC-I-deficient ConA blasts as targets, and again suggesting that the ability to respond to MHC-I-deficient cells requires MHC-I receptor expression during NK cell development. Unexpectedly, the killing of the prototypical mouse NK tumour target, YAC-1, was found to be almost equally high by both WT and NKC^{KD} NK cells (Fig. 6C), most likely due to the near normal levels of NKG2D in NKC^{KD} NK cells (Fig. 2F). NKG2D is an activating receptor for the Rae-1 family of stimulatory ligands expressed by YAC-1 and other tumour cells (135). The large and small decreases in RMA-S and YAC-1 killing, respectively, were not due to an inherently impaired cytotoxic ability of NKC^{KD} LAK as ADCC-mediated killing using RMA as targets cells was equally high by both types of effectors (Fig. 6D).

YAC-1 are MHC-low and express NKG2D ligands (135). Therefore, we hypothesized that in the absence of overriding NKG2D activation signals, YAC-1 should be differentially killed by WT vs. NKC^{KD} LAK similar to RMA-S. To test this hypothesis, the YAC-1 cytotoxicity assay was repeated in the presence of blocking anti-NKG2D mAb. WT LAK killing decreased marginally in the presence of anti-NKG2D mAb, but NKC^{KD} LAK killing was dramatically reduced similar to NKC^{KD} LAK killing of RMA-S (Fig. 6E). To further test this hypothesis, RMA-S was made more "YAC-1-like" by stable expression of Rae1β and used in cytotoxicity assays. Similar to YAC-1, both WT and NKC^{KD} NK cells exhibited similar high killing of RMA-S-Rae-1β target cells (Fig. 6F). In addition, anti-NKG2D blocking of WT LAK decreased killing of RMA-S-Rae1β only slightly, but killing by NKC^{KD} LAK was considerably lower (Fig. 6F). In sum, "missing-self" immunosurveillance against tumour cells is also deficient in NKC^{KD} mice, but this deficiency can be masked by activating signals such as NKG2D.

Finally, to test whether these results were unique to NK cells from NKC^{KD} mice or to MHC-I uneducated NK cells in general, YAC-1 killing by LAK from $\beta 2m^{-/-}$ mice in the presence or absence of anti-NKG2D mAb was tested. WT and $\beta 2m^{-/-}$ LAK killed YAC-1 almost equally well, but in the presence of anti-NKG2D mAb, only $\beta 2m^{-/-}$ LAK killing was significantly decreased revealing deficient "missing-self" responses (Fig. 7A). Similarly, the same pattern was observed when using RMA-S-Rae1β as target cells; high killing by both WT and $\beta 2m^{-/-}$ LAK, but significantly decreased killing by blocking NKG2D only with

$\beta 2m^{-/-}$ LAK (Fig. 7B). Collectively, these results reinforce that defective MHC-I education, whether through absence in receptors (NKC^{KD} mice) or absence in ligands ($\beta 2m^{-/-}$ mice), still allows NK cells to display cytotoxicity towards some canonical tumour cells (such as YAC-1), but that the "missing-self" cytotoxicity stimulated solely by the loss of MHC-I molecules (such as RMA-S versus RMA), requires MHC-I receptor expression during NK cell education and functional maturation.

4. Discussion

There is overwhelming evidence that NK cells are able to discriminate between MHC-I^{+} and MHC-I^{-} cells both in humans and mice. In turn, the discovery of MHC-I receptors on human (KIR) and mouse (Ly49) NK cells provides a molecular basis for MHC-I-dependent immunosurveillance. To directly establish that these receptors are responsible for *in vivo* MHC-I discrimination has proven to be more difficult. To date this has only been attempted with injection into mice of mAb specific for different Ly49. However, it is not always clear if the mAb is simply blocking the receptor-ligand interaction, causing depletion of the NK cell subset via complement/ADCC (and partial depletion of other Ly49 subsets due to co-expression), or even activating/inhibiting the NK cells by simulating ligand binding.

The unintended, but fortuitous, silencing of the whole *Ly49* gene cluster in *Ly49o*-targeted mice allows for the direct testing of the hypothesis that Ly49 are responsible for the ability of NK cells to survey for the presence of self-MHC-I on host cells. We show that NK-dependent *in vitro* killing and *in vivo* rejection of MHC-I-deficient cells is significantly lower in NKC^{KD} mice, and that this deficiency can be rescued by an Ly49 transgene. These results are consistent with the hypothesis that self-MHC-I-specific inhibitory Ly49 expression during NK cell development is necessary for NK cells to achieve optimal responsiveness towards MHC-I-deficient cells in the periphery. If the only function of Ly49 is inhibition in mature NK cells, then the loss of Ly49 should result in the increased killing of MHC-I^{+} cells. However, the lack of RMA tumour cell and WT ConA

blast killing by NKC^{KD} NK cells argues against this model. One explanation is that NKC^{KD} NK cells are simply "abnormal" and lack cytotoxic ability unrelated to their Ly49 downregulation, but their high YAC-1 and ADCC-mediated killing argues against this. The data best fit a model whereby self-MHC-I-specific inhibitory Ly49 receptor expression is required during NK cell development and education for the subsequent acquisition of cytotoxicity that can be specifically triggered by the loss of self-MHC-I on target cells.

We have shown that acquisition of cytotoxicity is not strictly dependent on Ly49 expression during development. NKC^{KD} NK cells, most of which are Ly49-negative, cells are not totally hyporesponsive: they clearly kill NKG2D-ligand positive cells as efficiently as WT NK cells, in agreement with the near normal levels of NKG2D and normal levels of effector molecules (granzyme A and B) of NKC^{KD} NK cells as assessed by flow cytometry. The high killing of YAC-1 and RMA-S-Rae1β by NKC^{KD} NK cells suggests that the MHC-I requirement for education is not necessary for the recognition of MHC-I-deficient cells that possess additional activating signals, such as NKG2D ligands (135). Our data agree with the recent findings showing that inhibitory Ly49-mediated NK cell education has little or no bearing on cytotoxicity triggered through activating receptors like Ly49H (291). Thus, with respect to cytotoxicity, self-MHC-I-uneducated NK cells are only hyporesponsive towards MHC-I-deficient cells, whereas cytotoxicity triggered through activating receptors is normal in Ly49-negative NK cells.

The downregulation of the whole *Ly49* gene cluster (and some neighbouring genes) after targeting of the most upstream/telomeric gene is reminiscent of the first granzyme B-targeted mouse, which unintentionally resulted in silencing of the other granzyme gene family members with possible magnification of the resulting NK and CTL deficiency phenotype (351). The multiple neomycin selection cassettes are thought to contribute to the silencing effect in granzyme B-targeted mice. However, southern blot analysis revealed that NKC^{KD} mice contain no neomycin genes due to Cre-mediated deletion at the ES cell stage, in agreement with the inability of NKC^{KD} NK cells to survive in neomycin-containing culture medium (data not shown).

The granzyme and globin gene families are hypothesized to be under the control of a locus control region (LCR) that governs expression temporally and with regard to tissue/cell type (351). There is currently no evidence that the *Ly49* gene cluster is under the control of an LCR. Instead, Ly49 expression appears to be a stochastic event governed by bidirectional promoters upstream of each gene, with expression being detectable if the forward promoter is activated rather than the reverse (246). The silencing of the whole gene cluster is possibly a result of the concatemer insertion interrupting an LCR-mediated opening of the cluster that subsequently allows for bidirectional promoter firing. Another possible silencing mechanism is long-range methylation caused by the repetitive elements in the original targeting construct turning on repetitive element-dependent DNA methylases whose function is to repress such regions of the genome (352). Efforts

are ongoing to try and understand the molecular mechanism behind the silencing of the telomeric end of the NKC in NKC^{KD} NK cells.

As three related, adjacent gene families (Ly49, CD94/NKG2, KLRI) are silenced in NKC^{KD} mice, any of the three, or combinations thereof, may be responsible for the loss of MHC-I immunosurveillance. In the specific case of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ and *H-2K*$^{b-/-}$ cells, the defective *in vitro* and *in vivo* killing by NKC^{KD} NK cells can be mostly attributed to the loss of Ly49 expression and altered education in NKC^{KD} mice. Ly49 molecules are known to be direct receptors for the H-2K^{b} and H-2D^{b} MHC-Ia molecules on NK cells. The absence of H-2D^{b} by itself, did not elicit significant cytotoxicity or rejection (Fig. 3E and 4D), in agreement with prior studies showing that H-2K^{b} is a stronger self-educating ligand than H-2D^{b} (286). However, the rejection response by WT mice was clearly increased when H-2K^{b} and H-2D^{b} were both absent compared to H-2K^{b}-deficiency alone (Fig. 4B, C), strongly suggesting that H-2D^{b} does play a partial role in the education of NK cells.

When both MHC-Ia and β2m-dependent MHC-Ib molecules are absent as in the case of β2m-deficient cells, the rejection and killing by WT NK cells is higher than that seen when only MHC-Ia molecules are absent (*H-2K*$^{b-/-}$*H-2D*$^{b-/-}$) (compare Fig. 4A, B). This suggests that β2m-dependent MHC-Ib molecules are also used by NK cells to monitor missing self-MHC expression. Expression of MHC-Ib molecules such as Qa-1^{b}, H-2^{Blastocyst}, CD1d, and Q9 have all been shown to inhibit NK cell cytotoxicity (347-350), implying that they may also play a role in NK cell education. However, only a receptor for Qa-1^{b} has been

identified (NKG2A) (152). The ligand for KLRI is unknown, but the structural and primary homology to the NKG2 family suggests that the ligands may also be MHC-Ib proteins. Regardless, transgenic expression of an Ly49 that can bind to $H\text{-}2^b$ molecules restored most of the "missing-self" responses suggesting that the loss of missing self-MHC-I immunosurveillance in NKC^{KD} mice can be attributed mostly to Ly49 downregulation. The data further suggest that inhibitory Ly49 receptors for self-MHC-Ia must be expressed at key moments in NK cell development in the bone marrow for the specific acquisition of cytotoxicity towards MHC-I-deficient cells.

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Abbreviations: MHC, major histocompatibility complex; NK, natural killer; NKC, natural killer gene complex; NKC^{KD}, NKC-knockdown

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6. Extented discussion (not included in the manuscript)

Ly49 *repertoire in NKC^{KD} NK cells.*

Individual NK cells can express up to six different Ly49 receptors (242) and this generates a diverse NK cell repertoire. As shown in Fig. 2D, WT NK cells display high levels of Ly49 co-expression. For example, ~80% of WT Ly49G,T-positive NK cells also express Ly49R. Similarly, WT NK cells positive for Ly49R, Ly49O,V,R or Ly49I$_1$ also express other Ly49 receptors in high proportions. Unlike WT NK cells, NKC^{KD} NK cells have a decreased co-expression of Ly49 receptors. The low co-expression of Ly49 receptors on NKC^{KD} NK cells, coupled with the highly decreased proportion of Ly49$^+$ NK cells, should result in a smaller number of different Ly49 receptors on each NKC^{KD} NK cell. Taken together, these results suggest a decreased diversity in the Ly49 repertoire on the surface of NKC^{KD} NK cells.

Ly49 receptor expression in heterozygous NK cells.

Both chromosomes contribute to Ly49 expression since (B6 x BALB)F$_1$ NK cells express Ly49G^{B6} and/or Ly49G^{BALB} (316). It is then surprising to observe only a small decrease in Ly49 expression on NK cells from heterozygous mice (Fig. 2B). The absence of one WT chromosome should result in approximately a 50% loss in the number of Ly49$^+$ NK cells. The proportion of Ly49$^+$ NK cells in heterozygous mice can be predicted by averaging the percentages of Ly49$^+$ WT and NKC^{KD} NK cells. Based on this prediction, it can be calculated that ~35% of heterozygous NK cells will express Ly49R. 35% is the average of the percentages of Ly49R$^+$ NK cells in WT mice (~60%) and NKC^{KD}

mice (~10%). Ly49R is expressed on more NK cells than what was predicted by these calculations since 42% of NK cells from heterozygous mice express Ly49R. The same phenomenon is observed for Ly49O,V,R (~35% predicted vs. 45% expressed), Ly49G,T (~20% vs. 26%), Ly49I_1 (~15% vs. 20%) and total Ly49 (~46% vs. 59%). During their development, it is possible that heterozygous NK cells express *Ly49* genes at a higher frequency than what is predicted in an attempt to obtain a normal proportion of Ly49$^+$ NK cells in order to maintain normal NK cell functions. This would suggest that heterozygous NK cells sense a reduction in the number of Ly49 receptors expressed on their surface which signals the developing NK cells to transcribe more *Ly49* genes. Alternatively, the high expression of *Ly49* genes by heterozygous NK cells could be caused by an increased binding of transcription factors to *Ly49* promoters located on the remaining wild-type *Ly49* gene cluster. If we assume that the levels of transcription factors driving *Ly49* gene expression are normal in NKC^{KD} NK cells, these transcription factors only have one wild-type *Ly49* cluster available for efficient binding in heterozygous NK cells since the knockdown *Ly49* cluster does not allow efficient transcription of *Ly49* genes (Fig. 2C). Therefore, more transcription factors are bound to *Ly49* promoters on the wild-type *Ly49* cluster and transcription of *Ly49* genes is favoured ultimately resulting in increased Ly49 expression by heterozygous NK cells.

NKC^{KD} NK cells ignore MHC-I expression

In vitro cytotoxicity results revealed that similar to WT NK cells, NKC^{KD} NK cells are inhibited by the presence of MHC-I molecules on the surface of

target cells (Fig. 3A and 6A). The low cytotoxic activity of WT NK cells against MHC-I-expressing target cells can be explained by the presence of inhibitory Ly49 and NKG2A-CD94 receptors recognizing the expression of MHC-I on target cells and preventing NK cell activation. Despite the absence of Ly49 receptors on the majority of NKC^{KD} NK cells, minimal lysis of MHC-I-expressing targets is observed. Ly49-mediated inhibition is expected to be functional for the small Ly49- and NKG2A-positive subset of NKC^{KD} NK cells. The majority of NKC^{KD} NK cells never bind MHC-I molecules during their development and are educated to ignore MHC-I molecules on the surface of host cells. It can then be concluded that the ignorance of MHC-I molecules results in tolerance to host cells ultimately leading to a low lytic activity against $MHC\text{-}I^{+}$ cells. Alternatively, it can be hypothesized that an unknown MHC-I-receptor renders $Ly49^{-}$ NK cells tolerant to MHC-I molecules. The effect of this receptor would be masked in WT mice but in the absence of Ly49 receptors, it would become the major MHC-I receptor. MHC-I-independent mechanisms could also prevent NK cell activation. The recognition of Clr-b by the inhibitory NKR-P1B provides (204, 205), and the recognition of CD48 by 2B4 can provide NK cells with MHC-I-independent inhibition (186).

Defective "missing-self" response of NKC^{KD} NK cells.

NKC^{KD} NK cells cannot efficiently recognize the loss of such molecules from the surface of target cells as shown by the defective *in vitro* lysis of $MHC\text{-}I^{-}$ cells (Fig. 3B-D and 6B). These results indicate that NKC^{KD} NK cells have a defective "missing-self" response. This resembles the phenotype of $\beta 2m^{-/-}$ NK

cells which are generally considered to be uneducated because they cannot destroy MHC-I^{-} cells. Furthermore, rare uneducated NK cells from B6 mice lacking self-receptors (Ly49C,I and NKG2A) have decreased functions compared to educated B6 NK cells that express self-receptors (281, 282). Therefore, it is not surprising that the lack of self-receptors on NKC^{KD} NK cells results in reduced lytic activity against MHC-I^{-} target cells. Thus, NKC^{KD} NK cells can be considered as uneducated.

Even though NKC^{KD} NK cells have a highly decreased ability to destroy RMA-S and *β2m*$^{-/-}$ blasts compared to WT NK cells, the lysis of these targets is significantly higher than that of their respective MHC-I^{+} variants (either RMA or WT blasts) (Fig. 3A-B and 6A-B). This suggests that NKC^{KD} NK cells can to some extent recognize loss of MHC-I molecules on the surface of target cells. The residual expression of Ly49 on a minority of NKC^{KD} NK cells most likely allows for this partial "missing-self" recognition. The rheostat model (283, 284) predicts that Ly49^{+} NKC^{KD} NK cells have weaker responses than the equivalent subset in WT mice because of a decreased co-expression of Ly49 receptors. It is then possible that Ly49^{+} NKC^{KD} NK cells are not responsible for all of the residual lysis against MHC-I^{-} cells. Ly49^{-} NK cells from NKC^{KD} mice could theoretically still be involved in the destruction of MHC-I^{-} cells. It is possible that NK cells are able to recognize loss of MHC-I molecules in an Ly49-independent manner. One of the receptors possibly involved in this process could be NKG2A but only a minority of NKC^{KD} NK cells express this receptor (Fig. 2F). As suggested earlier,

an unknown MHC-I receptor could allow the education of NK cells and a partial recognition of the loss of MHC-I molecules by NKC^{KD} NK cells.

The results obtained with *in vitro* experiments were corroborated by *in vivo* cytotoxicity assays in which the ability of mice to reject MHC-I^- cells was tested. Similar to the defective killing of RMA-S and $\beta 2m^{-/-}$ blasts, NKC^{KD} mice did not reject various MHC-I^- cells (either $\beta 2m^{-/-}$, $H\text{-}2K^{b-/-}H\text{-}2D^{b-/-}$ or $H\text{-}2K^{b-/-}$) as efficiently as WT mice (Fig. 4A-C) further suggesting that NKC^{KD} NK cells have a defective "missing-self" response. This provides evidence that Ly49 receptors are involved in the *in vivo* surveillance of cells that have lost MHC-I expression. Depletion of NK cells by injecting anti-asialo GM1 serum permitted the assessment of the role played by NK cells in the rejection of MHC-I^- cells. Rejection of both $\beta 2m^{-/-}$ and $H\text{-}2K^{b-/-}H\text{-}2D^{b-/-}$ cells by WT mice is almost completely abolished after NK cell depletion. Similarly, the residual rejection of $\beta 2m^{-/-}$ cells by NKC^{KD} mice is abolished in the absence of NK cells (Fig. 4A). This suggests that NKC^{KD} NK cells recognize loss of MHC-I in an *in vivo* setting. This is reminiscent of the inefficient but detectable *in vitro* lysis of MHC-I^- targets by NKC^{KD} NK cells. The residual rejection of $\beta 2m^{-/-}$ cells is most likely mediated by the few NKC^{KD} NK cells that still express Ly49 and NKG2A receptors. In conclusion, the ability of NK cells to recognize loss of MHC-I molecules is severely impaired in the absence of MHC-I receptors. The defective "missing-self" recognition may render NKC^{KD} mice susceptible to cancer or pathogens that can modulate MHC-I expression.

Taken together, these results suggest that NKC^{KD} NK cells are uneducated analogous to *β2m*$^{-/-}$ NK cells and to uneducated B6 NK cells (281, 282). Analysis of surface receptors expressed by educated and non-educated NK cells from B6 mice revealed no major difference except for KLRG1: a lower proportion of non-educated NK cells express this marker as compared to educated NK cells (281). *β2m*$^{-/-}$ NK cells also express lower levels of KLRG1 compared to B6 NK cells (353). KLRG1 could then be considered as a marker of successful NK cell education. Interestingly, KLRG1 expression was also found to be decreased on NKC^{KD} NK cells (Supplementary Fig. 2B-C), further suggesting that NKC^{KD} NK cells were not educated by MHC-I molecules.

Normal MHC-I-independent functions of NKC^{KD} NK cells.

NKC^{KD} NK cells are able to efficiently kill RMA cells coated with anti-Thy1.2 antibodies and YAC-1 cells (Fig. 6C and D). The lysis of antibody-coated target cells (antibody-dependent cellular cytotoxicity or ADCC) is triggered by the recognition of the Fc portion of antigen-bound antibodies by the activating receptor CD16. CD16 is expressed at comparable levels between NKC^{KD} and WT NK cells (Supplementary Fig. 1B) and this is reflected by normal ADCC responses. The equal ability of WT and NKC^{KD} NK cells to destroy antibody-coated RMA cells can be surprising. The activating signals delivered by CD16 override the inhibition conferred by the presence of MHC-I molecules on RMA resulting in NK cell activation and lysis of the antibody-coated target cell. NKC^{KD} NK cells could be expected to kill antibody-coated RMA cells more efficiently than WT NK cells due to the absence of inhibitory Ly49 receptors. In fact,

antibody-coated RMA are killed equally well by both WT and NKC^{KD} NK cells (Fig. 6D). As stated earlier, it is possible that NKC^{KD} NK cells could be inhibited by non-Ly49 MHC-I-receptors. It is also possible that the ligation of CD16 initiates a maximal NK cell response and that this maximum is still attained in the presence of signals delivered by inhibitory Ly49 receptors. In other words, maximal NK cell activation would be reached regardless of the strength of inhibition. This could explain the similar lysis of antibody-coated RMA by both WT and NKC^{KD} NK cells.

Unlike uneducated B6 NK cells that show diminished lysis of YAC-1 cells (281), NKC^{KD} NK cells lyse YAC-1 almost as efficiently as WT NK cells (Fig. 6C). YAC-1 express ligands for the activating receptor NKG2D (135) and lysis of YAC-1 is severely impaired in *Nkg2d*$^{-/-}$ mice (138). The expression of NKG2D on NKC^{KD} NK cells is slightly decreased (Fig. 2F) and it is possible that this minor decrease does not affect the lysis of YAC-1. Normal ADCC and YAC-1 lysis by NKC^{KD} NK cells indicates that a defective "missing-self" response does not affect MHC-I-independent functions mediated by activating receptors such as CD16 and NKG2D. While these receptors are able to activate NKC^{KD} NK cells, uneducated NK cells from B6 mice cannot respond to the cross-linking of these receptors. In contrast, *β2m*$^{-/-}$ NK cells show near normal lysis of YAC-1 (Fig. 7A) (22, 23). The hyporesponsive phenotype of both NKC^{KD} and *β2m*$^{-/-}$ NK cells can be reversed by triggering activating receptors and therefore, the phenotype of NKC^{KD} NK cells closely matches that of *β2m*$^{-/-}$ NK cells. It can be concluded that

the absence of MHC-I receptors on NK cells leads to a phenotype similar to the one created by the absence of MHC-I molecules.

Defective "missing-self" response can be reversed by the presence of NKG2D ligands.

The normal lysis of YAC-1 by NKC^{KD} NK cells could possibly be explained by the presence of a ligand for an activating receptor that would "mask" the reduced MHC-I expression on YAC-1 cells. The killing of YAC-1 was significantly reduced when NKG2D was blocked on NKC^{KD} NK cells compared to NKG2D-blocked WT NK cells (Fig. 6E). Interestingly, $\beta 2m^{-/-}$ NK cells, which killed YAC-1 almost as efficiently as WT (B6) NK cells, had highly reduced lysis of YAC-1 after NKG2D blockade while blockade of NKG2D on WT NK cells had a marginal effect on their ability to kill YAC-1 (Fig. 7A). These results suggest that NKC^{KD} and $\beta 2m^{-/-}$ NK cells can efficiently kill an MHC-I^- target cell if this cell also expresses NKG2D ligands. The lysis of YAC-1 by WT NK cells is only marginally decreased after NKG2D blockade and this is intriguing because NKG2D is thought to be the major activating receptor involved in the recognition of YAC-1 cells (138). Our results suggest that "missing-self" recognition of YAC-1 may then play a larger role than what was previously thought.

Expression of NKG2D ligands on RMA-S gave similar results. RMA-S, which is not efficiently lysed by NKC^{KD} and $\beta 2m^{-/-}$ NK cells, became highly susceptible to NK cell-mediated lysis when the NKG2D ligand Rae-1β is expressed on its surface (Fig. 6F and 7B). The ability of NKC^{KD} and $\beta 2m^{-/-}$ NK cells to kill RMA-S-Rae-1β was similar to that of WT NK cells. Altogether, these

results suggest that the presence of a ligand for an activating receptor on a MHC-I$^-$ target cell allows for normal lysis by NKC^{KD} NK cells. Similarly, the low lytic activity of *β2m*$^{-/-}$ NK cells against the MHC-I$^-$ RMA-S can be increased to the levels of WT NK cells when a ligand for NKG2D is expressed on RMA-S. These results further strengthen the resemblance between NKC^{KD} and *β2m*$^{-/-}$ NK cells since the defective "missing-self" response of these NK cells can be rescued by the presence of ligands for an activating receptor on MHC-I$^-$ cells.

These findings emphasize the reversibility of NK cell hyporesponsiveness. *β2m*$^{-/-}$ NK cells are generally considered hyporesponsive compared to B6 NK cells but our results indicate that *β2m*$^{-/-}$ NK cells can efficiently destroy specific target cells. The same phenomenon is observed for NKC^{KD} NK cells. These results agree with the finding that *β2m*$^{-/-}$ mice are resistant to MCMV infection (289). In addition, uneducated B6 NK cells produce IFN-γ at levels comparable to educated NK cells after infection with *L. monocytogenes* (281) and respond more efficiently to MCMV-infected cells than educated B6 NK cells (291). Altogether, the defective "missing-self" recognition of NKC^{KD} and *β2m*$^{-/-}$ NK cells does not result in globally impaired NK cell responses.

Transgenic expression of Ly49 on NKC^{KD} NK cells restores the "missing-self" response.

In an attempt to educate NKC^{KD} cells and to rescue their defective "missing-self" response, NKC^{KD} mice were crossed with three different Ly49 transgenic mouse strains to generate three separate NKC^{KD} mice expressing

Ly49A, G or I. Ly49G does not bind to H-2^{b} MHC-I molecules (249) and therefore its expression was expected to have no impact on the education of NKC^{KD} NK cells. Indeed NKC^{KD}-Ly49G^{tg} mice could not reject *β2m*$^{-/-}$ cells more efficiently than NKC^{KD} mice (Fig. 5C). There is some evidence that H-2D^{b} can educate Ly49A^{+} NK cells (283, 287) and it was predicted that the expression of Ly49A on all NKC^{KD} NK cells would lead to a modest increase in the rejection of *β2m*$^{-/-}$ cells. In fact, the ability of NKC^{KD} mice to reject MHC-I^{-} cells was unaffected by the expression of Ly49A (Fig. 5B). This suggests that Ly49A does not bind to H-2D^{b} or that this interaction is too weak to educate NKC^{KD} NK cells. Ly49I should positively influence the rejection of *β2m*$^{-/-}$ cells by NKC^{KD} mice since it binds to H-2K^{b} (262, 266) and this interaction can educate Ly49I^{+} NK cells (283, 284). Expression of Ly49I rescued the rejection of MHC-I^{-} cells by NKC^{KD} mice (Fig. 5D), suggesting that the defective "missing-self" recognition observed in NKC^{KD} mice is most likely caused by the loss of Ly49 receptors. Interestingly, the rejection of *β2m*$^{-/-}$ cells by NKC^{KD}-Ly49I^{tg} mice was lower than the rejection of these cells by WT mice. NK cells expressing only one self-receptor have weaker responses than NK cells expressing two or three self-receptors (283, 284). It is therefore possible that the presence of only one self-receptor on NKC^{KD} NK cells does not permit their full education. Consequently, more than one self-receptor should be required to allow "missing-self" recognition by NKC^{KD} NK cells.

Recognition of MHC-Ib molecules by NK cells does not require Ly49 receptors.

Our results demonstrate that NK cells monitor the periphery for the expression of MHC-Ia and MHC-Ib molecules. Both MHC-Ia and MHC-Ib molecules are absent in *β2m*$^{-/-}$ mice while only MHC-Ia molecules are absent in *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ mice. The absence of MHC-Ib leads to an increase in NK cell cytotoxicity (Fig. 4A and B) revealing recognition by NK cells. Interestingly, a similar difference in the killing of *β2m*$^{-/-}$ and *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ cells by NKC^{KD} NK cells was observed suggesting that Ly49 are not involved in the recognition of MHC-Ib molecules by NK cells. This confirms numerous studies showing that Ly49 receptors bind almost exclusively to MHC-Ia molecules (249). Part of the recognition of MHC-Ib molecules by WT NK cells could be mediated by NKG2A as it binds to Qa-1^{b} (152) and this could explain why *β2m*$^{-/-}$ cells are more susceptible to NK cell-mediated lysis than *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ cells. The expression of NKG2A is decreased on NKC^{KD} NK cells and as a result, the recognition of MHC-Ib molecules by NK cells can also be mediated by receptors other than NKG2A. It cannot be ruled out that the remaining NKG2A expression on NKC^{KD} NK cells is responsible for the NK cell-mediated recognition of MHC-Ib molecules.

It is also possible that MHC-Ib molecules other than Qa-1^{b} are recognized by NK cells. Such molecules include CD1d, H-2^{blastocyst} (MHC molecule expressed in the blastocyst and the placenta) or Q9. These molecules are all recognized by NK cells and their expression result in NK cell inhibition (347,

348, 350). CD1d and Q9 require β2m for proper expression and it is likely that their absence on *β2m*$^{-/-}$ cells increases their susceptibility to NK-mediated lysis. The receptors involved in the recognition of these molecules are unknown but there is some evidence showing that NKG2A recognizes H-2^{blastocyst} through Qa-1^{b} (350). Qa-1^{b} can bind to the leader peptide of H-2^{blastocyst} and this complex is recognized by NKG2A resulting in inhibition of NK cells (350). H-2^{blastocyst} can be predicted to play a minor role in the recognition of missing MHC-Ib molecules by NK cells due to its restricted expression in the embryo. Other uncharacterized MHC-Ib molecules are also probably able to inhibit NK cells by acting as ligands for unknown NK cell inhibitory receptors.

Lack of Ly49Q does not affect activation of NKC^{KD} NK cells.

One of the steps in the generation of the NKC^{KD} mouse was the insertion of a floxed-neomycin cassette in the *Ly49q$_1$* gene. Ly49Q is expressed by plasmacytoid dendritic cells (pDC), neutrophils and osteoclasts (238) and Ly49Q is predicted to be knocked-out in NKC^{KD} mice. Not surprisingly, pDC from NKC^{KD} mice lack Ly49Q expression (Additional Fig. 1). It is possible that the absence of Ly49Q may influence the activation of NK cells during *in vivo* experiments. Previous experiments revealed that *Ly49q$_1$*$^{-/-}$ mice reject MHC-I^{-} cells as efficiently as WT mice (285). Therefore, the absence of Ly49Q has no major influence on the priming of NK cells during the rejection of MHC-I^{-} cells by NKC^{KD} mice.

Silencing of NKC in NKC^{KD} mice is possibly induced by the presence of transposable elements.

The decreased expression of *Ly49* genes in the NKC^{KD} mouse is intriguing since the *Ly49* genes are present. The normal expression of Ly49 receptors in the *Ly49q$_1$*$^{-/-}$ mouse indicates that the targeting of *Ly49q$_1$* had no influence on the expression of NKC genes (346). Therefore, the homologous recombination involving the *Ly49o* targeting construct is the likely cause of the decreased expression of NKC-encoded genes. Indeed, the *Ly49o* targeting construct was integrated in the genome of NKC^{KD} mice as a concatemer (Fig. 1C and D). The *Ly49o* targeting construct contains a neomycin resistance gene under the control of the PGK promoter and it is possible that the presence of multiple neomycin cassettes near *Ly49o* reduces the expression of *Ly49* and other NKC genes. It has been reported that the insertion of a neomycin resistance gene near the *Gzmb* gene resulted in loss of expression of *Gzm* genes located in the *Gzmb* gene cluster (351). However, NKC^{KD} mice lack a neomycin gene as revealed by Southern blot analysis. Furthermore, culture of WT and NKC^{KD} NK cells in the presence of IL-2 and G418 resulted in death of ~99% of the cells. On the other hand, roughly 25% of NK cells from an unrelated gene-knockout mouse carrying the neomycin resistance gene survived this treatment. Altogether, this indicates that the downregulation of *Ly49* and other NKC genes in NKC^{KD} is not caused by the presence of a neomycin resistance gene.

The presence of repetitive sequences in the *Ly49o* targeting construct may affect the expression of nearby genes. Repetitive sequences, especially

transposable elements, can be deleterious for the genome if left unchecked. To prevent unwanted duplication of transposable elements, cells prevent their transcription by epigenetic modifications (354). Interestingly, the silencing of transposable elements can spread to neighbouring genes. Antisense transcripts originating from the reverse LTR region of LINE1 elements can form dsRNA complexes with forward LINE1 transcripts and this initiates dsRNA-mediated silencing of LINE1 that can propagate to nearby genes (354). Previous analysis of the 129 *Ly49* sequence revealed the presence of LINE1 elements close to the 5' end of the *Ly49o* gene (271) that were included in the *Ly49o* targeting construct. Multiple insertions of the *Ly49o* targeting construct as a concatemer then resulted in a local increase in the number of transposable elements and silencing of this region may have been initiated to prevent expression of LINE1 elements. Other transposable elements present in the *Ly49o* targeting construct such as SINE may also influence the expression of NKC genes.

Alternatively, silencing of the cluster could have been initiated by transcripts originating from *Ly49* promoters. The *Ly49o* targeting construct contains Pro1, Pro2 and possibly Pro3 of *Ly49o*. During development of NK cells, transcription in forward or reverse orientation will be initiated from Pro1 (246). This suggests than when these promoters are active, the concatemer region generates multiple copies of forward and reverse *Ly49o* transcripts. These transcripts could anneal to each other and form dsRNA that would in turn recruit the machinery required for gene silencing. The high sequence similarity of *Ly49* genes may have favoured the spreading of the dsRNA-mediated silencing to

nearby genes. On the other hand, the silencing of the *Nkg2-Cd94* genes cannot be explained by sequence similarity. It is also possible that the silencing spread in a non-specific manner to other NKC genes.

Several lines of evidence suggest that chromatin remodelling is used to silence transposable elements (354). Methylation of histone H3 at lysine 9 (H3K9) results in inactive chromatin and mutations in the methyltransferase responsible for H3K9 methylation results in an increase in the transcription of transposable elements in mouse ES cells (354). Another means used to silence transposable sequences is DNA methylation by DNMT1 and DNMT3 DNA methyltransferases. This methylation needs to be targeted at every cell division to maintain silencing (354). Interestingly, sorted $Ly49^{+}$ NKC^{KD} NK cells expanded in IL-2 lost Ly49 expression; after 6 days of culture, ~60-70% of NK cells have become $Ly49^{-}$ (Additional Fig. 2). This contrasts with the $Ly49^{+}$ subset of WT NK cells that retained Ly49 expression after *in vitro* culture with IL-2. Furthermore, $Ly49^{-}$ NK cells from WT and NKC^{KD} remained $Ly49^{-}$ after expansion in IL-2. This suggests that Ly49 expression in NKC^{KD} NK cells is not stable and that *Ly49* genes are silenced when these NK cells divide. This strongly implicates DNA methylation in the silencing of NKC genes in NKC^{KD} NK cells. Altogether, it is likely that the presence of transposable elements and *Ly49o* promoters in multiple copies near the *Ly49* cluster may recruit DNA methyltransferases and other chromatin remodelling proteins resulting in silencing of *Ly49* gene expression.

7. Research opportunities

$Ly49^+$ NKC^{KD} NK cells are thought to be responsible for the residual rejection of $\beta 2m^{-/-}$ cells by NKC^{KD} NK cells. Depletion of these NK cells by injection of anti-Ly49 antibodies before the injection of the MHC-I^- cells could give an indication of the role played by the $Ly49^+$ subset of NKC^{KD} NK cells in the *in vivo* "missing-self" response. If the rejection of $\beta 2m^{-/-}$ cells by Ly49-depleted NKC^{KD} mice is comparable to what is seen in NK-depleted NKC^{KD} mice, then this subset is responsible for the rejection observed in NKC^{KD} mice. If the rejection is partially abrogated, it would suggest the involvement of $Ly49^-$ NK cells or another NK cell receptor in the rejection of $\beta 2m^{-/-}$ cells. Due to the lack of antibodies specific for all Ly49 receptors expressed in 129-strain mice, it is likely that not all $Ly49^+$ NK cells would be depleted with the currently available antibodies.

Our experiments were performed using mice carrying H-2^b MHC-I molecules. Changing the MHC-I molecules in our mice would also change the self-receptors and this can be used as a method to study the role of $Ly49^+$ NK cells in the rejection of $\beta 2m^{-/-}$ cells by NKC^{KD} mice. In NKC^{KD} mice congenic for H-2^d MHC-I, Ly49G, I_1, O and V would be the Ly49 receptors binding to self-MHC-I (249, 320). Importantly, these mice would need to be compared to WT mice also made congenic for H-2^d. It could be predicted that H-2^d-congenic mice would reject MHC-I^- cells more efficiently than our current WT mice due to the expression of more self-receptors. Similarly, the residual "missing-self" response in the NKC^{KD}-H-2^d mice could be stronger than in NKC^{KD} mice. If this is

phenotype is observed, the Ly49^{+} subset of NKC^{KD} mice is most likely responsible for the residual rejection of MHC-I^{-} cells.

Another experimental approach that could be used to study the role of different NK cell subsets in the rejection of *β2m*$^{-/-}$ cells would be to transfer sorted NK cells into NK cell-deficient recipients (*Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice) (355). NK cells can be found 60 days after transfer into such NK-deficient hosts (355). A few weeks after transfer of sorted Ly49^{-} NKC^{KD} NK cells, recipient mice would be tested for their ability to reject *β2m*$^{-/-}$ cells. *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice receiving Ly49^{-} NKC^{KD} NK cells could be compared to *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice injected with unsorted NKC^{KD} NK cells. Unfortunately, this approach cannot be used to study the role of the Ly49^{+} subset of NKC^{KD} NK cells. After transfer into *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice, NK cells proliferate (355) and Ly49^{+} NKC^{KD} NK cells lose Ly49 expression after *in vitro* expansion. Similarly, analysis of Ly49 expression on NKC^{KD} NK cells transferred to *Rag2*$^{-/-}$ x *Il2rg*$^{-/-}$ mice would be expected to reveal a reduction in the percentage of Ly49^{+} NK cells.

The recognition of allogeneic cells by NK cells is similar to that of MHC-I^{-} cells in the sense that allogeneic cells lack the MHC-I molecules of the host. NK cells lacking inhibitory MHC-I receptors binding to the allogeneic MHC-I molecules will be activated while NK cells expressing receptors recognizing these MHC-I molecules will be inhibited. In addition to a defective "missing-self" response, *β2m*$^{-/-}$ mice cannot efficiently reject allogeneic cells (22). The ability of NKC^{KD} mice to reject cells of allogeneic origin such as from BALB/c mice (H-2^{d}) could be tested and it is expected that similar to their defective "missing-self"

response, these mice will display significantly reduced rejection of allogeneic cells compared to WT controls. It can also be predicted that NKC^{KD} mice will show residual rejection of allogeneic cells since NKC^{KD} NK cells have a weak response directed towards MHC-I^-cells.

It is assumed that the Ly49 receptors expressed on $Ly49^+$ NKC^{KD} NK cells are still functional. It is possible that cross-linking Ly49 receptors on $Ly49^+$ NKC^{KD} NK cells leads to reduced activation or inhibition of NK cell activity due to a decreased number of receptors on this subset. To address this issue, $Ly49G^+$ NK cells from NKC^{KD} mice could be sorted and tested for their response to antibody-mediated cross-linking of receptors. The activating receptor NKp46 can be used to activate NK cells since it is normally expressed on NKC^{KD} NK cells. The inhibitory Ly49G receptor would be simultaneously cross-linked to measure Ly49G-mediated inhibition that would be compared between $Ly49G^+$ NK cells from WT and NKC^{KD} mice. Similarly, the responses of $Ly49R^+$ NKC^{KD} NK cells to Ly49R cross-linking could be tested. However, the small numbers of $Ly49^+$ NKC^{KD} NK cells would make these studies difficult to undertake. Furthermore, this NK cell subset cannot be expanded because Ly49 expression is lost during culture.

Transgenic expression of Ly49A or Ly49G in NKC^{KD} did not result in enhanced rejection of MHC-I^- cells by these mice. The lack of rejection by these mice can be attributed to the inability of Ly49A and Ly49G to educate NK cells in an $H\text{-}2^b$ environment. Therefore, expressing the Ly49A or Ly49G transgene in an NKC^{KD} mouse congenic for $H\text{-}2^d$ should result in a partially rescued "missing-

self" response. The partial rescue in the "missing-self" response conferred by the Ly49I transgene was tentatively explained by the need of NK cells to express more than one self receptor. To address this issue, NKC^{KD}-$Ly49I^{tg}$ mice should be crossed to $Ly49C^{tg}$ and/or $NKG2A^{tg}$ mice. NKC^{KD}-Ly49I/C^{tg}, NKC^{KD}-Ly49I/$NKG2A^{tg}$ or even NKC^{KD}-Ly49C/I/$NKG2A^{tg}$ mice could be generated and each tested for their ability to reject $\beta 2m^{-/-}$ cells. The rheostat model of NK cell education predicts that NKC^{KD}-Ly49C/I/$NKG2A^{tg}$ mice would have the strongest rejection of $\beta 2m^{-/-}$ cells. Alternatively, NKC^{KD} mice could be crossed to $Ly49C^{tg}$ or $NKG2A^{tg}$ mice to assess the individual educational impact of these two self-receptors in NKC^{KD} mice.

Several lines of evidence suggest that *cis* interactions between Ly49 and their MHC-I ligands are the major determinant of NK cell education (287, 288). The generation of a mouse model in which Ly49 expression is severely impaired could be used to test the role of *cis* and *trans* interactions in NK cell education. The stalk of Ly49 receptors is flexible and this flexibility permits *cis* interactions between Ly49 and their MHC-I ligand (267). A chimeric Ly49A receptor in which the non-flexible stalk domain of CD72b (Ly49-72A) replaces the normal Ly49 stalk could be used to address this issue. This chimeric Ly49A-72A receptor cannot bind to its ligand in *cis* (288). This knowledge could be used to create a chimeric Ly49I receptor with the non-flexible stalk of CD72b (Ly49-72I), which in turn could be expressed as a transgene in NKC^{KD} mice. The "missing-self" response of NKC^{KD}-$Ly49\text{-}72I^{tg}$ mice would then be tested. Alternatively, we could make use of the already generated $Ly49\text{-}72A^{tg}$ mouse and breed this mouse

to a NKC^{KD} mouse congenic for $H\text{-}2^{d}$. If *cis* interactions are absolutely required for NK cell education, the expression of these chimeric receptors in NKC^{KD} mice should not result in an increase in the rejection of MHC-I^{-} cells.

NK cells patrol the periphery for the appearance of tumour cells. Several studies have revealed that various NK cell receptors are involved in the *in vivo* surveillance of tumour cells (138, 169, 179). The generation of a NKC^{KD} mouse provides the opportunity to directly study the role of Ly49 receptors in the surveillance of tumour cells. The tumour incidence in mice injected with the fibrosarcoma-inducing carcinogen 3-methylcholanthrene (MCA) could be assessed (138, 169, 179). Alternatively, papillomas could be induced by injections of dimethylbenz[a]anthracene (DMBA) (179). NKC^{KD} mice could also be bred to TRAMP (transgenic adenocarcinoma of the mouse prostate) mice and studied for signs of prostate carcinomas at the time of necropsy (138). Lastly, Eμ-myc transgenic mice that develop B cell lymphomas could be crossed with NKC^{KD} mice (138). Based on previous studies on mice deficient for NKp46 (169), NKG2D (138) or DNAM-1 (179), the absence of Ly49 receptors may not necessarily result in higher tumour burden or decreased mouse survival. Tumours developing in WT mice are expected to be different from those isolated in NKC^{KD} mice. The tumours developing in WT mice will be under selective pressure by Ly49 receptors to maintain expression of MHC-I molecules in an attempt to evade NK cell-mediated responses. On the other hand, in the absence of Ly49 receptors, tumours are expected to lose expression of MHC-I molecules to prevent

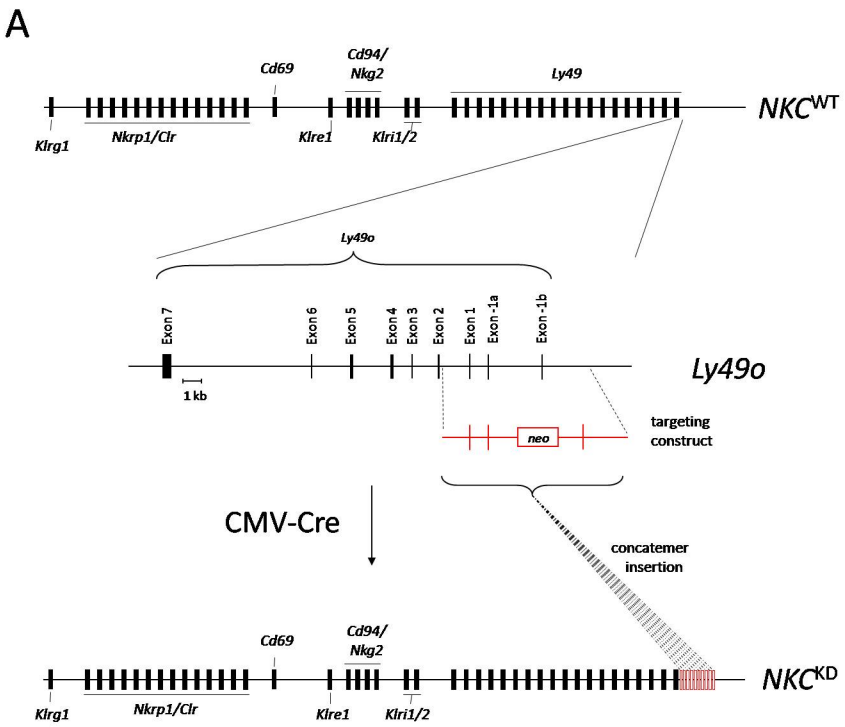
recognition by $CD8^+$ T lymphocytes. In order words, it is predicted that Ly49 receptors induce tumour immunoediting.

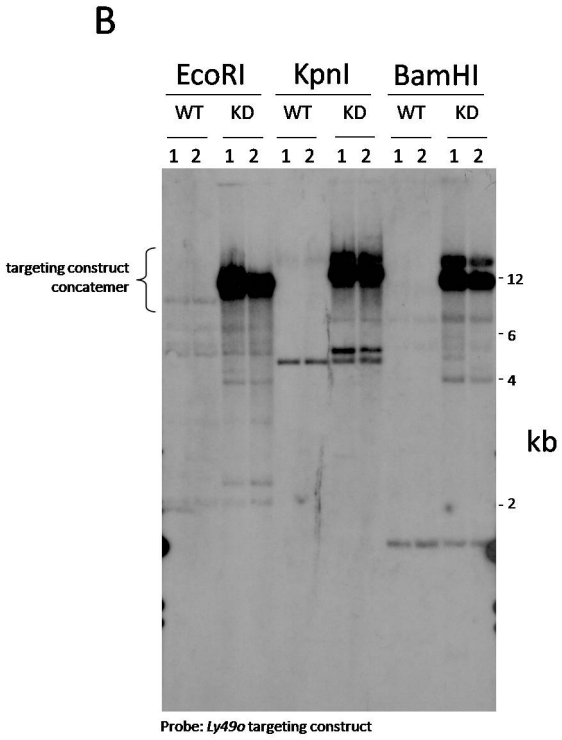
NK cells are required for the proper control of viral infections (96) and the NKC^{KD} mouse model is an ideal model for studying the role played by Ly49 receptors in the control of viral infections. A recent report revealed the importance of uneducated $Ly49H^+$ NK cells in the control of MCMV infection (291) and based on this result, it can be predicted that NKC^{KD} mice would be more resistant to viral infections that influence MHC-I expression. MCMV-infected cells are recognized by Ly49H (253, 326), which is absent from the *Ly49* cluster of 129 mice. Crossing the available $Ly49H^{tg}$ mouse (356) to NKC^{KD} mice would allow confirmation of the involvement of uneducated NK cells in the immune response directed against MCMV. NKC^{KD}-$Ly49H^{tg}$ mice should control MCMV infection better than WT-$Ly49H^{tg}$ mice because NKC^{KD} NK cells can be considered uneducated. WT-$Ly49H^{tg}$ NK cells will receive activating signals from Ly49H and inhibitory signals from self-MHC-I receptors and this will result in dampened NK cell responses while NKC^{KD}-$Ly49H^{tg}$ NK cells will only receive activating signals from Ly49H leading to strong NK cell activation and better control of MCMV. The presence of *Ly49h* confers strong resistance to MCMV infection (254) and it may be hard to observe a more efficient viral control by NKC^{KD}-$Ly49H^{tg}$ mice when assessing splenic viral titers after 3 days of infection. The amelioration of the viral control may be visible at earlier or later time points in the spleen or even in organs other than the spleen such as salivary glands (291). Alternatively, the activation status of NK cells (upregulation of CD69, production

of IFN-γ) after infection may be analyzed. Lastly, other viral models could be tested and if any differences in the survival of WT and NKC^{KD} mice are found, the relationship between the education status of NKC^{KD} NK cells and control of the viral infection should be investigated.

NK cell receptors are clearly involved in the development as shown by the reduced incidence of diabetes in NOD.NK1.1 mice (299). The NKC^{KD} mouse provides us with a tool to directly assess the role of Ly49 receptors in the development of diabetes. NKC^{KD} mice could be crossed to NOD mice to create $NOD.NKC^{KD}$ mice and the incidence of diabetes would be assessed in these mice using $NOD.Ly49^{129}$ as control. Specific KIR haplotypes have been linked with autoimmune disorders such as psoriatic arthritis (225). EAE is used to model multiple sclerosis in mice and could be used as a model to address the role of Ly49 receptors and NK cells in the development of autoimmune disorders.

8. *Figures*





Probe: *Ly49o* targeting construct

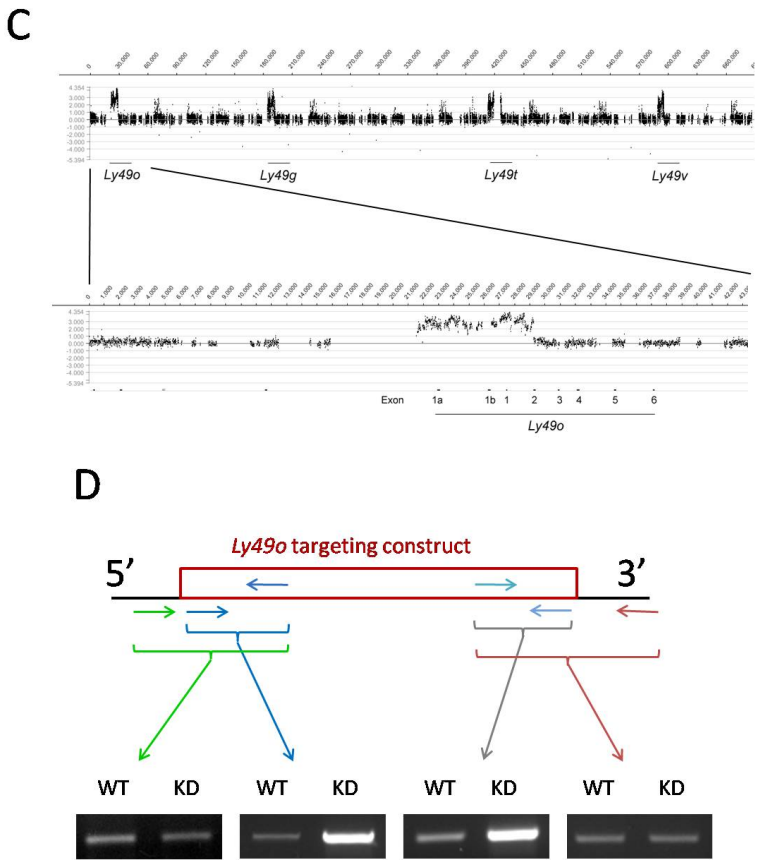
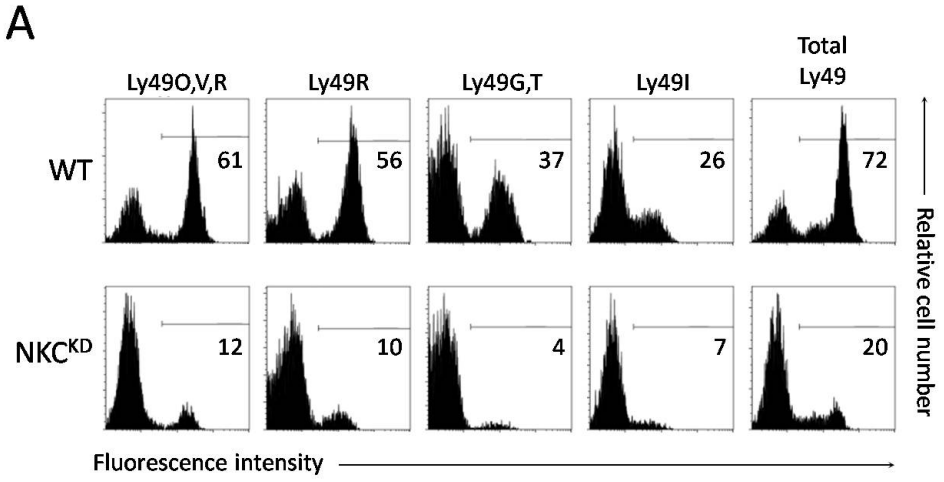


Figure 1: Generation of NKC^{KD} mice. (A) Strategy employed to target *Ly49o* in *Ly49q1*^{lox} R1 ES cells. (B) Southern analysis of WT and NKC^{KD} genomic DNA using a fragment of the *Ly49o* targeting construct as a probe. (C) Array CGH profile of the *Ly49* cluster in WT and NKC^{KD} mice. The x-axis represents the genomic region tiled on the microarrays while the y-axis shows differences in copy numbers between WT and NKC^{KD} ($\log_2$ ratio (NKC^{KD}/WT)). Positive values indicate regions showing copy number increases in the NKC^{KD} genome. (D) PCR analysis of the *Ly49o*-targeting construct. Forward and reverse primers were used to identify the copy level and integration site of the *Ly49o*-targeting construct. Data are representative of at least three similar experiments, except for array CGH which was performed twice.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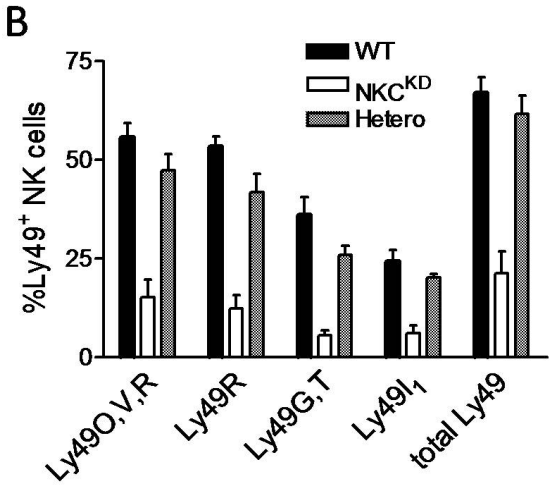
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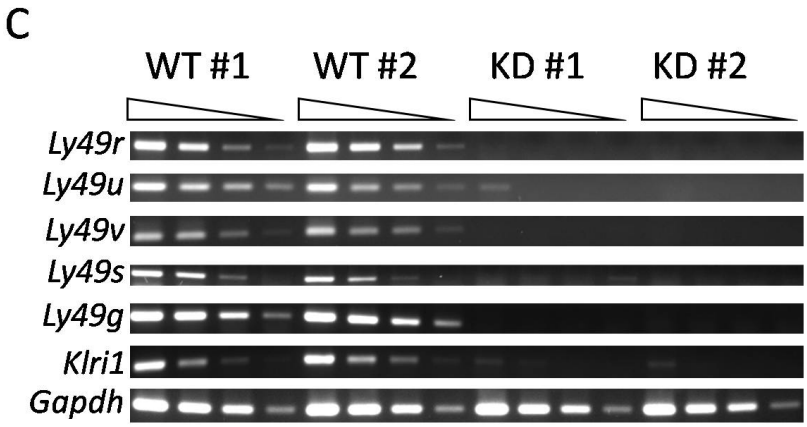
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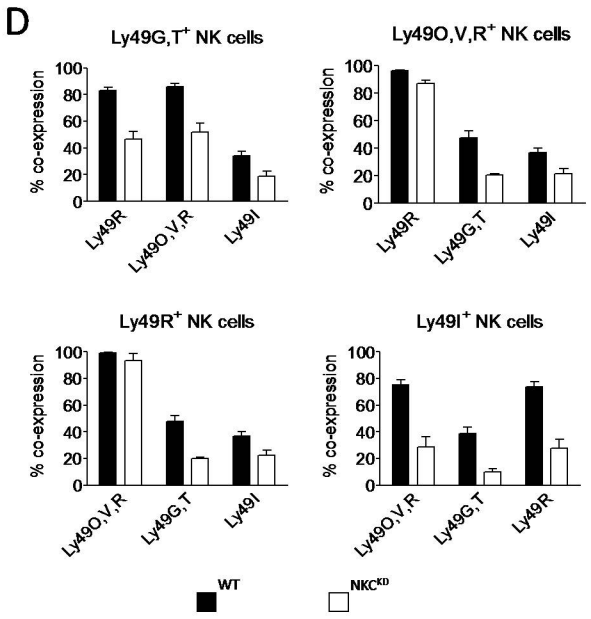
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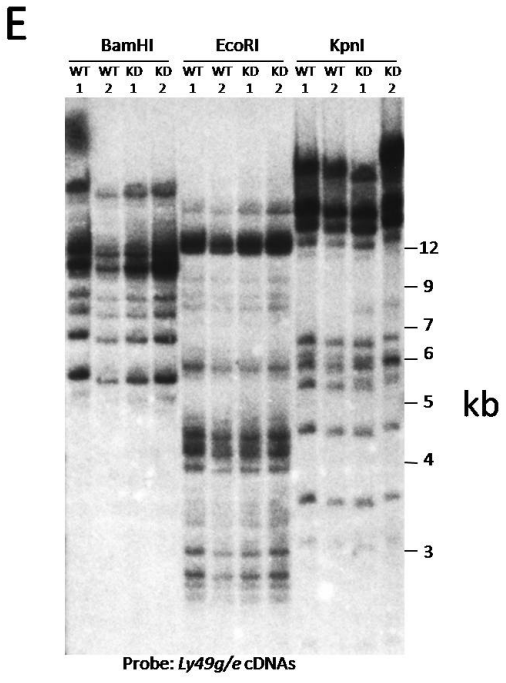
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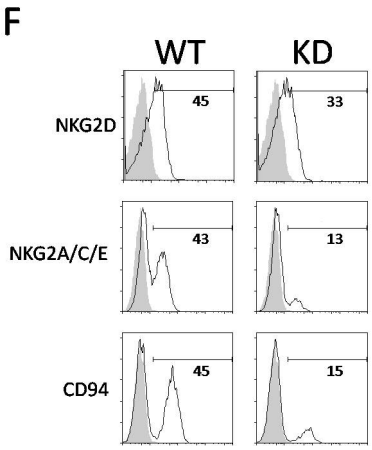
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NKG2A/C/E
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CD94
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Figure 2: Silenced *Ly49*, *Nkg2*, *Cd94* and *Klri* gene expression in NKC^{KD} mice
(A) NK cells (DX5^{+}TCRβ^{-}) from the spleens of mice with the indicated genotypes were analyzed by flow cytometry for Ly49 expression with mAb 4E5 (Ly49O/V/R), 12A8 (Ly49R), 4D11 (Ly49G/T), and 14B11 (Ly49I) individually or as a cocktail (Total Ly49). The percentage of NK cells positively stained is indicated. (B) Graph summarizing the expression of Ly49 receptors on NK cells from 8-12 mice of the indicated genotypes. Data are presented as the mean of Ly49^{+} NK cells ±SD. (C) Semi-quantitative RT-PCR for the indicated genes was performed on total RNA obtained from LAK cells prepared from two individual WT or NKC^{KD} mouse spleens. (D) Ly49 co-expression on NK cells from WT or NKC^{KD} mice was analyzed by multi-colour flow cytometry using the indicated Ly49-specific mAbs. The data are presented as the mean percentage of Ly49 co-expression when gated on a particular Ly49^{+} subset (n=6 mice for both genotypes) ±SD. (E) Southern analysis of *Ly49* gene content in NKC^{KD} mice. Restriction fragment length polymorphism analysis with *Ly49g* and *Ly49e* cDNAs. (F) The expression of NKG2/CD94 family of receptors on splenic NK cells from WT or NKC^{KD} littermates was analyzed by flow cytometry as described in (A). Data are representative of at least three similar experiments.

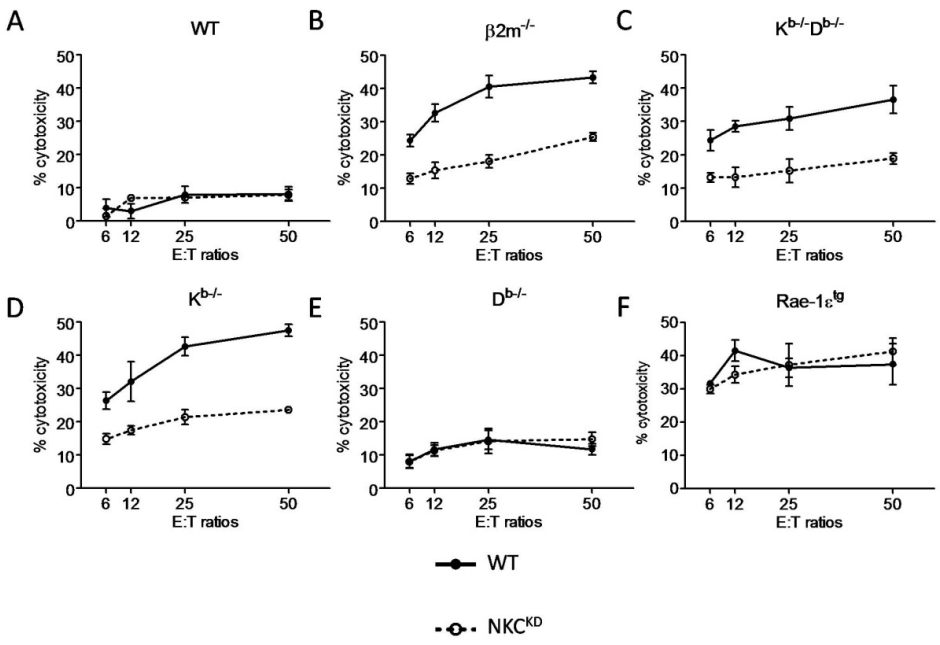


Figure 3: NKC^{KD} NK cells exhibit defective *in vitro* killing of MHC-I-deficient ConA blasts. ^{51}Cr-release assay testing the ability of LAK from B6-background WT and NKC^{KD} mice to kill ConA blasts from various mice. ConA blasts were prepared from (A) C57Bl/6, (B) *β2m*$^{-/-}$, (C) *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$, (D) *H-2K*$^{b-/-}$, (E) *H-2D*$^{b-/-}$ or (F) Rae-1ε^{tg}. The data are displayed as mean percent (±SD) of chromium release from triplicate wells for the indicated effector:target (E:T) ratios. Data are representative of three independent experiments.

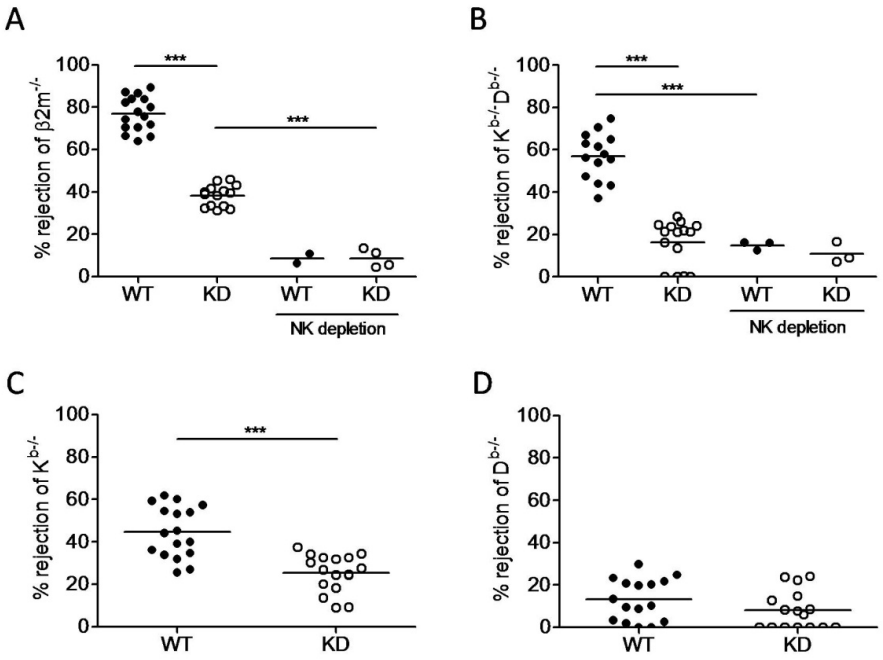


Figure 4: Reduced *in vivo* rejection of MHC-I-deficient cells by NKC^{KD} mice. Splenocytes from WT and various MHC-I^{-} mice were labelled with different concentrations of CFSE and injected in WT and NKC^{KD} mice on 129-background. (A) Rejection of *β2m*$^{-/-}$ splenocytes. (B) Rejection of *H-2K*$^{b-/-}$*H-2D*$^{b-/-}$ splenocytes. (C) Rejection of *H-2K*$^{b-/-}$ splenocytes. (D) Rejection of *H-2D*$^{b-/-}$ splenocytes. Each symbol represents an individual mouse and the small horizontal lines indicate the average. In (A) and (B) some mice were pretreated with anti-asialo GM1 Ab to deplete NK cells before injection of CFSE-labelled cells. Data are pooled from 3-5 independent experiments. ***P<0.0001.

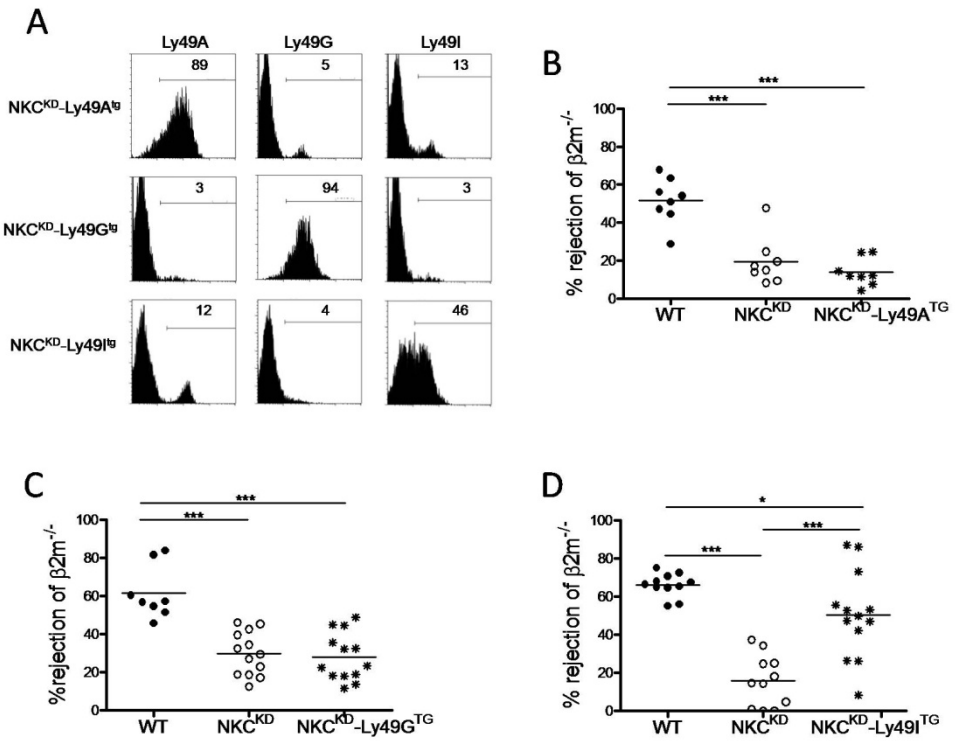


Figure 5: Ly49 silencing causes loss of MHC-I surveillance in NKC^{KD} mice. (A) Expression of Ly49 transgenes on NK cells (DX5^{+}TCRβ^{-}) from the respective NKC^{KD}-Ly49^{tg} mice on B6 background. The percentage of NK cells positively stained is indicated. Rejection of *β2m*$^{-/-}$ splenocytes by WT, NKC^{KD} and (B) NKC^{KD}-Ly49A^{tg}, (C) NKC^{KD}-Ly49G^{tg} or (D) NKC^{KD}-Ly49I^{tg} mice. *P<0.05 ***P≤0.0001.

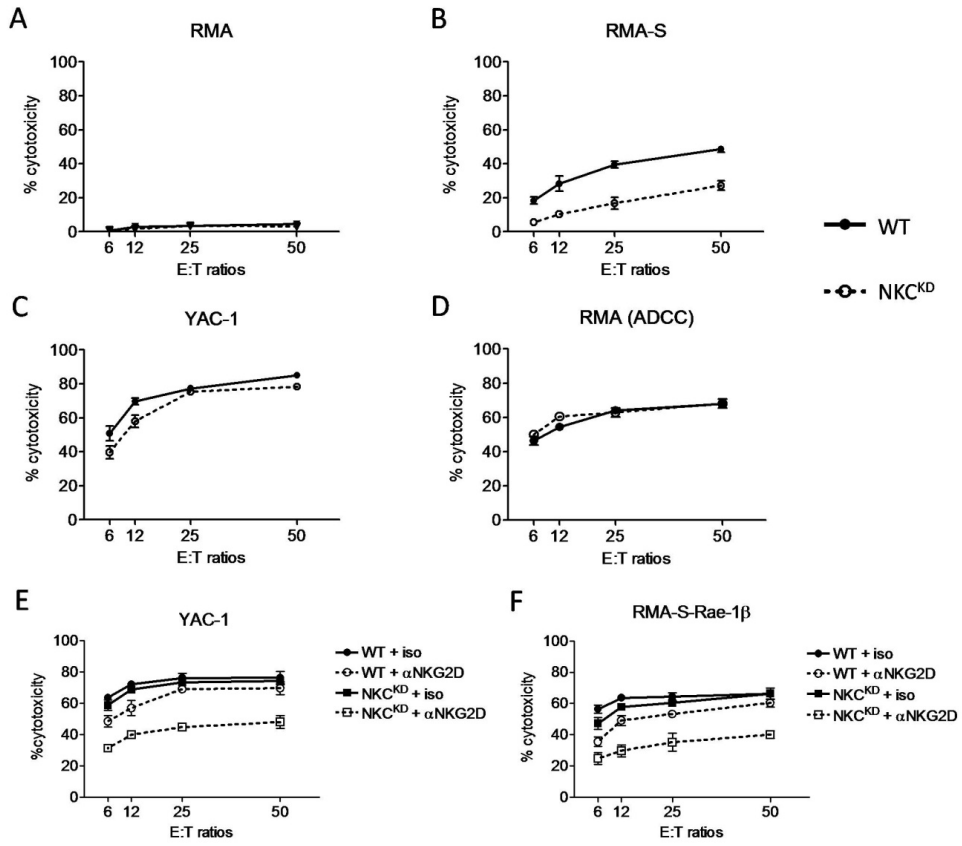


Figure 6: NKG2D can override the defective killing of MHC-I-deficient tumour cells by NKC^{KD} NK cells. ^{51}Cr-release assay testing the ability of LAK to kill target cells at different effector to target (E:T) ratios. LAK from B6-background WT and NKC^{KD} mice were used as effectors cells against (A) RMA, (B) RMA-S, (C) YAC-1 and (D) anti-Thy1.2-coated RMA tumour cells. (E) NKG2D was blocked on LAK cells and then tested for lysis against YAC-1. (F) Same as (E) but with RMA-S-Rae-1β tumour cells as targets. The mean percent (±SD) from triplicate wells is shown. Data are representative of at least three similar experiments.

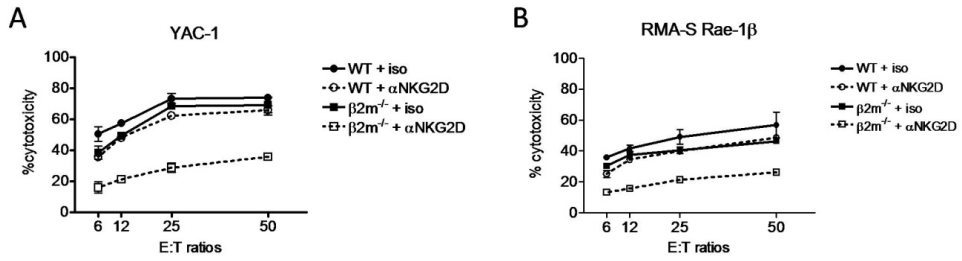
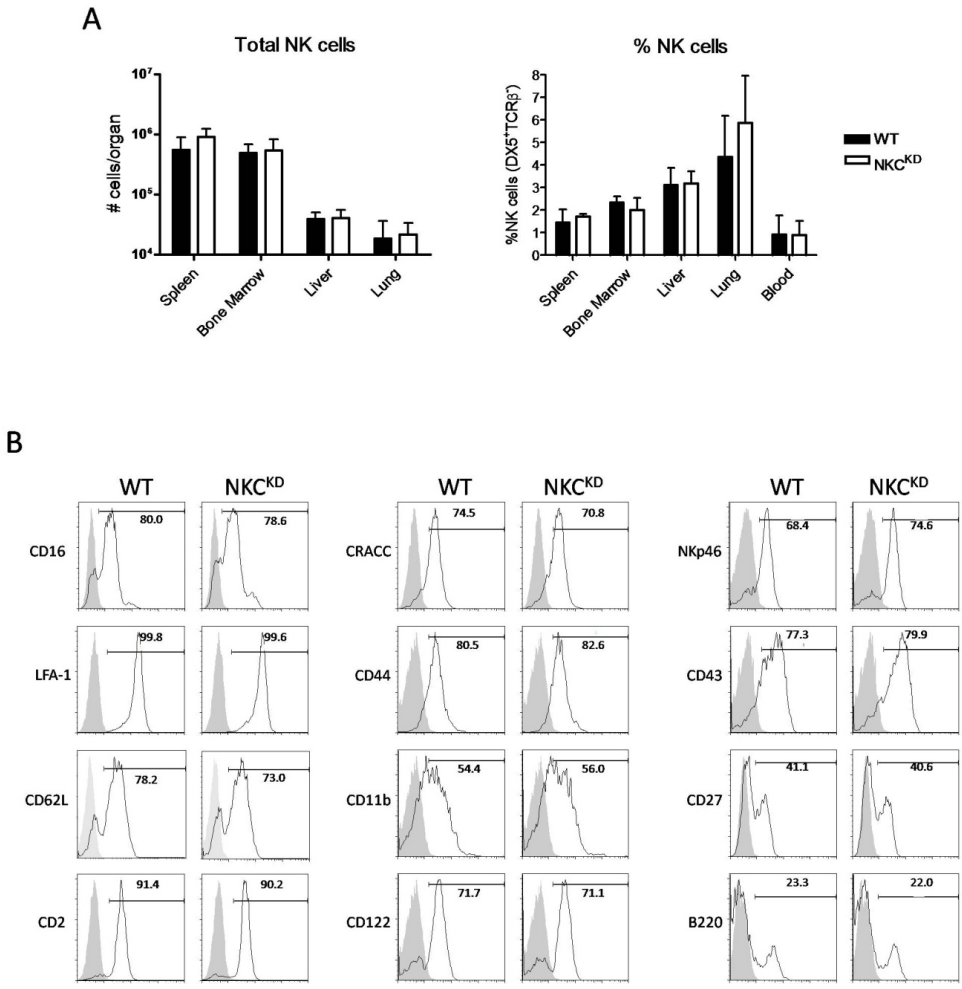
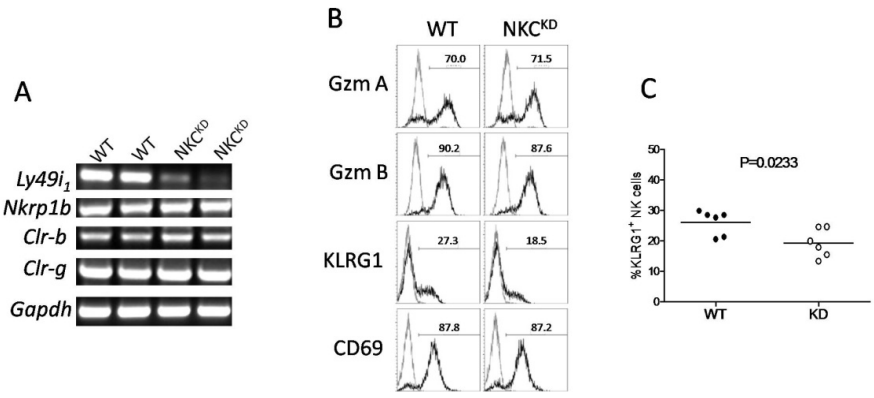


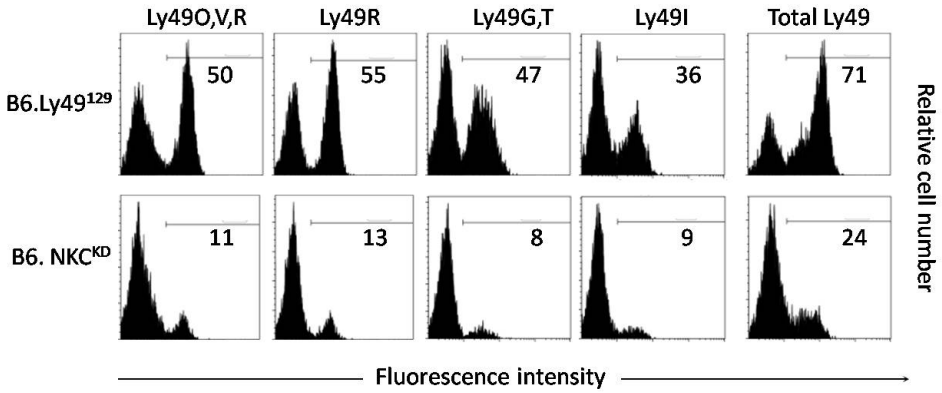
Figure 7: NKG2D can override the defective killing of MHC-I-deficient tumour cells by *β2m*$^{-/-}$ NK cells. ^{51}Cr-release assay testing the ability of LAK to kill target cells at different effector to target (E:T) ratios. NKG2D-blocked LAK cells from B6 and *β2m*$^{-/-}$ mice were used as effectors cells against (A) YAC-1 and (B) RMA-S Rae-1β. The mean percent (±SD) from triplicate wells is shown. Data are representative of at least three similar experiments.



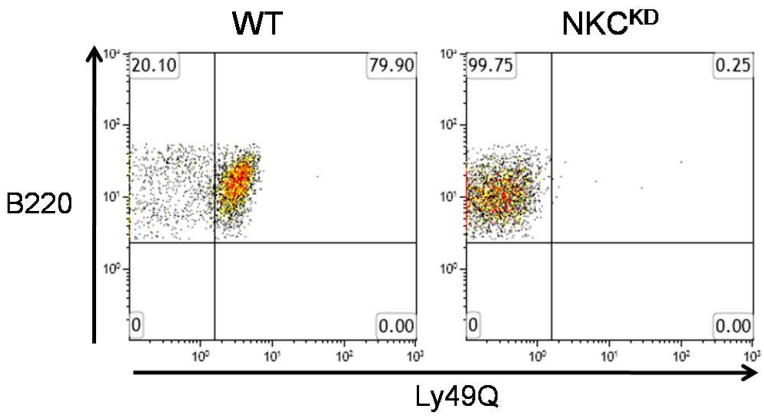
Supplemental Figure 1: Normal development of NKC^{KD} NK cells. (A) Lymphocytes from the indicated organs were isolated and stained for NK cells ($DX5^{+}TCR\beta^{-}$). The data is presented in graphs depicting the total number of NK cells and the proportions of NK cells in the indicated organs. Bars represent the mean ±SD (n=6 mice) (B) Flow cytometric analysis of the indicated cell surface markers (open peaks) was performed on splenocytes. The histograms represent fluorescence intensity from $DX5^{+}TCR\beta^{-}$-gated NK cells. The percentage of positively staining cells relative to the isotype control staining (grey peak) is indicated in the top right.



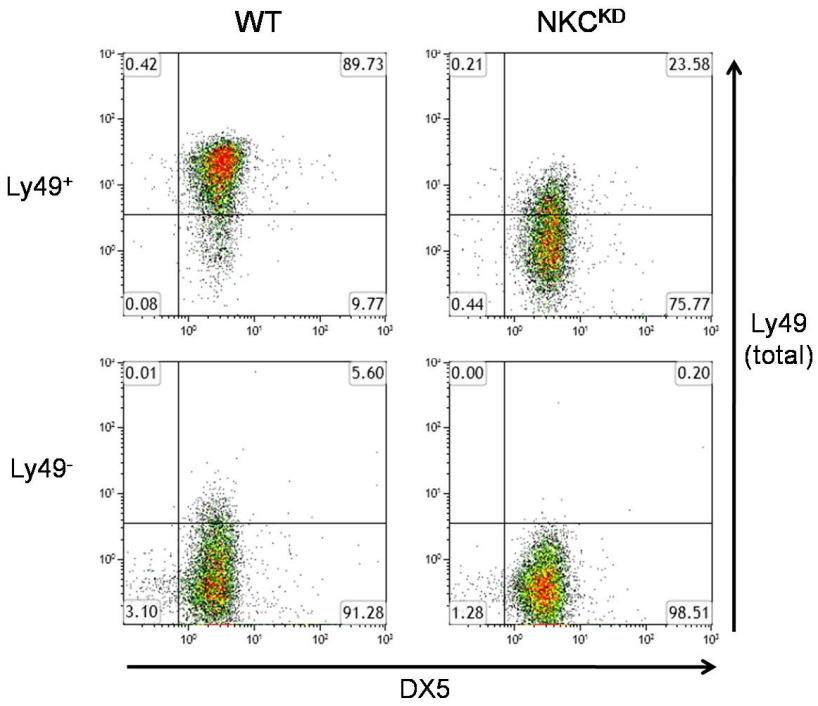
Supplemental Figure 2: Decreased KLRG1 expression on NKC^{KD} NK cells. (A) RT-PCR for the indicated NKC-resident genes and *Gapdh* was performed on total RNA isolated from WT and NKC^{KD} LAK cells. The RT-PCR result of two independent NK cell RNA isolations is shown for 35 cycles. (B) Intracellular or cell surface mAb staining followed by flow cytometry was performed on splenocytes cultured with IL-2 for 3 days to check for the expression of the indicated NK cell activation markers. Analysis was performed on DX5^{+}TCRβ^{-}-gated NK cells. The percent of positive staining for the indicated marker (dark line) is indicated in to top right of the histogram. The isotype control staining is shown as a light grey line. Data are representative of at least three similar experiments. (C) KLRG1 expression from multiple mice of each strain. The horizontal bar indicates the mean and each symbol represents a different mouse. The statistical significance is indicated. This experiment was performed three times with two individual WT and NKC^{KD} mice per experiment. Pooled data is shown.



Supplemental Figure 3: Ly49 silencing in NKC^{KD} NK cells on a B6 genetic background. NK cells (DX5^{+}TCRβ^{-}) from the spleens of mice with the indicated genotypes were analyzed by flow cytometry for Ly49 expression with mAb 4E5 (Ly49O/V/R), 12A8 (Ly49R), 4D11 (Ly49G/T), and 14B11 (Ly49I) individually or as a cocktail (Total Ly49). The percentage of NK cells positively stained by the mAb is indicated.



Additional Figure 1: Ly49Q expression is absent on NKC^{KD} pDC. Splenocytes were stained for B220, CD11c and Ly49Q. Dot plots show Ly49Q expression on gated B220^{+}CD11c^{+} pDC. The numbers in the quadrants indicate the percentage of total cells.



Additional Figure 2: Ly49-positive NKC^{KD} NK cells lose Ly49 expression after *in vitro* culture. WT and NKC^{KD} NK cells (DX5^{+}TCRβ^{-}) were sorted into Ly49^{+} and Ly49^{-}subsets with the 4E5, 12A8, 4D11 and 14B11 mAb and were cultured in NK medium supplemented with IL-2 for 7 days. NK cells were then harvested and stained for DX5, TCRβ and for total Ly49 (4E5, 12A8, 4D11 and 14B11). Dot plots show Ly49 expression on gated DX5^{+}TCRβ^{-} NK cells. The numbers in the quadrants indicate the percentage of total cells.

PREFACE TO CHAPTER 4

Two major families of NK cells receptors, the Ly49 and NKR-P1, are encoded in close proximity on murine chromosome 6 in a region called the Natural Killer Gene Complex (NKC). These evolutionary related families of lectin-like proteins share similarities and differences. One such similarity is that both receptor families possess activating and inhibitory receptors which regulate cellular function. In terms of differences, the ligands for NKR-P1 receptors are Clr molecules that are encoded by genes interspersed with the *Nrkp1* genes and, as a result, are inherited together (204, 205). This is unlike the Ly49 receptors which recognize MHC-I molecules that are encoded on a separate chromosome and are inherited separately. It has been suggested that this would limit the evolution of *Nkrp1* receptors and that the divergence between different clusters would be minimized. Indeed, unlike *Ly49* gene clusters which display great variability between different mouse strains (197), the comparison of B6 and BALB/c *Nkrp1-Clr* gene clusters has revealed minimal differences (208). While *Ly49* gene clusters have extensive allelic polymorphism and variation in gene number, the *Nkrp1-Clr* gene number and order was the same for both B6 and BALB/c mice (208).

NKR-P1D is known to bind Clr-b, while NKR-P1F binds to Clr-g (204, 205). Interestingly, BALB/c mice lack the *Nkrp1d* gene but instead, in its location *Nkrp1b* is found, suggesting that these two genes are in fact alleles. Indeed, NKR-P1B^{BALB} is also able to bind to Clr-b supporting the allelic nature of these two genes (208). Furthermore, the lack of NK1.1 reactivity in BALB/c mice cannot be

attributed to the lack of the two genes coding for NK1.1 antigens: *Nkrp1b* and *Nkrp1c*. Allelic polymorphisms in these two genes explain the lack of NK1.1 reactivity of BALB/c NK cells (208). In the following study, the mapping of the *Nkrp1-Clr* gene cluster in 129S6 mice confirmed that the genetic organization of the *Nkrp1-Clr* genes is conserved in this mouse strain as well. Sequence comparison of the coding regions of *Nkrp1* and *Clr* genes from the three strains was undertaken followed by a search for novel interactions between NKR-P1 and Clr.

CHAPTER 4

Analysis of the mouse 129-strain *Nkrp1-Clr* gene cluster reveals conservation of genomic organization and functional receptor–ligand interactions despite significant allelic polymorphism

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Note: Only the work performed by Simon Bélanger and published in this manuscript is presented in this chapter.

1. *Results*

Generation of a BAC map of the 129S6 Nkrp1-Clr *cluster*

129S6 BAC filters were probed with a mixture of B6 and BALB/c *Nkrp1* and *Clr* cDNA. BAC clones that were positive after hybridization were purchased, grown and BAC DNA was isolated for each clone. BAC DNA was screened for the presence of *Nkrp1* and *Clr* genes by PCR using primers that amplify the genomic region spanning intron 2 to intron 4 of each known *Nkrp1* and *Clr* gene (208). PCR products were cloned and sequenced to confirm the specificity of the PCR reaction. Using the sequenced-confirmed PCR products as probes, Southern blot analysis of BAC DNA confirmed the *Nkrp1-Clr* gene content of the individual BACs. All genes previously identified in the B6 and BALB/c *Nkrp1-Clr* gene clusters were also present in the 129S6 cluster: *Nkrp1a*, *Nkrp1b*, *Nkrp1c*, *Nkrp1e*, *Nkrp1f*, *Nkrp1g*, and *Clr-a*, *Clr-b*, *Clr-c*, *Clr-d*, *Clr-e*, *Clr-f*, *Clr-g*, *Clr-h*. A novel *Clr* gene fragment was identified from several BACs: *Clr-i*. So far, the only sequence available for *Clr-i* is limited to exons 3 and 4 and it is unknown if the gene is complete and if it codes for a functional protein. The available sequence was used to confirm the presence of similar exons in the B6 genome using the UCSC genome browser (http://genome.ucsc.edu/).

Several genes were present in multiple BACs and this allowed the construction of a map of the gene cluster (Fig. 1). Due to the random nature of BAC library generation, the order of the *Clr-h* and *Clr-f*, *Nkrp1f* and *Clr-a* and *Clr-b* and *Cd69* gene pairs could not be directly determined. These three gene pairs share common BACs and are not present on unique BACs, thereby not

allowing the elucidation of the gene order (Fig. 1). The gene order for the BALB/c *Nkrp1-Clr* gene cluster was previously determined by BAC mapping (208) and it was found that gene order was conserved in both clusters. Therefore, it is likely that the 129S6 cluster also possesses the same gene order. The gene order of *Clr-h* and *Clr-f*, *Nkrp1* and *Clr-a* and *Clr-b* and *Cd69* was based on the BALB/c and B6 *Nkrp1*-Clr clusters. Inversions in these pairs of genes are possible and cannot be formally ruled out, but are highly unlikely due to the conservation of gene order between the three mapped *Nkrp1-Clr* clusters.

The novel *Clr-i* gene was found to be located between *Clr-g* and *Clr-d* (Fig. 1). No BACs containing both *Clr-i* and *Clr-d* were found and the lack of such BACs created a gap in the middle of the map. It was therefore possible that the 129S6 cluster possessed unique genes located in the gap. To complete the map, one BAC positive for *Clr-d* (410a1) was end-sequenced and primers amplifying the 5' end of the BAC were designed and used to screen the *Clr-i*-containing BAC by PCR. It was shown that two of these BACs (519p18 and 429a7) contained the region found in the BAC 410a1 (data not shown). After sequencing, this PCR product was used as a probe in Southern blot analysis of BAC DNA confirming the PCR results. Thus, the BAC map was completed and no gaps are present (Fig. 1). Lastly, the sizes of all individual BAC were estimated by NotI digestion followed by pulsed-field gel electrophoresis (PFGE). The total size of the *Nkrp1-Clr* cluster (from *Nkrp1a* to *Clr-b*) of 129S6 mice was predicted to be approximately 600 kb, similar to the predicted size of the BALB/c cluster (208).

Cloning and sequence analysis of Nkrp1-Clr cDNA

To gain insight into the function and expression of *Nkrp1* and *Clr*, cloning and sequencing of full-length cDNAs was attempted. RNA was isolated from 129S1 ALAK, cDNA was synthesized and used in PCR reactions with primers designed to amplify the coding regions of the known *Nkrp1* and *Clr* genes. The complete cDNA coding regions from *Nkrp1a*, *Nkrp1b*, *Nkrp1c*, *Nkrp1f* and *Nkrp1g* were amplified. Coding region cDNA from *Clr-b*, *Clr-c*, *Clr-d*, *Clr-f* and *Clr-g* were also amplified (298). All amplified cDNA were cloned, sequenced and the putative amino acid sequences were compared to the sequences of the previously cloned B6 and BALB/c *Nkrp1* and *Clr* (Fig. 2).

It was previously reported that the NKR-P1 amino acid sequences are well conserved between B6 and BALB/c mice except for NKR-P1B/D and NKR-P1C (208). Despite the high divergence of these receptors, the 129S1 alleles of NKR-P1B and NKR-P1C are identical to the BALB/c alleles (Fig. 2A). The conservation of the NKR-P1B amino acid sequence suggests that similar to the B6 and BALB/c NKR-P1D/B receptors, NKR-P1B^{129} binds to Clr-b. Similarly, NKR-P1A^{129} is identical to the BALB/c allele and both differ from NKR-P1A^{B6} by only two amino acid substitutions located in the extracellular region (Fig. 2A). Likewise, NKR-P1G^{129} is identical to NKR-P1G^{BALB} and both differ from NKR-P1G^{B6} by only one amino acid substitution located in the extracellular domain. Unlike other NKR-P1 receptors, NKR-P1F^{129} differs from both NKR-P1F^{B6} and NKR-P1F^{BALB} (Fig. 2A) by two amino acid substitutions: one in the cytoplasmic domain and a second one in the extracellular domain. NKR-P1F^{B6} recognizes Clr-

g (205) and it can be assumed that NKR-P1F^{BALB} also binds Clr-g. Based on the conserved ligand of the divergent NKR-P1D^{B6} and NKR-P1B^{BALB} (357), it can be predicted that NKR-P1F^{129} also binds to Clr-g.

Similar to the NKR-P1 receptors, the Clr ligands are highly conserved among all three mouse strains with only one notable exception: Clr-c. Clr-c^{B6} and Clr-c^{BALB} are identical, but Clr-c^{129} is highly divergent with 38 amino acid differences including two missing amino acids in the predicted transmembrane domain (Fig. 2B). Interestingly, the NKR-P1B/D ligand, Clr-b is 100% identical between the three strains (Fig. 2B). Similar to most NKR-P1 receptors, Clr-d and Clr-f are identical between 129 and BALB/c and differ from the B6 proteins by seven and three amino acid substitutions, respectively. Lastly, Clr-g is nearly identical in the three strains. Clr-g^{BALB} possesses four extra residues in its cytoplasmic domain, probably due to alternative splicing.

In summary, the *Nkrp1* and *Clr* genes are highly conserved between the three strains except for the notable exceptions of *Nkrp1b*/*d*, *Nkrp1c* and *Clr-c*. The 129S1 NKR-P1 and Clr gene products are closely related to those of BALB/c mice due to their high sequence homology. The two exceptions are NKR-P1F and Clr-c which are conserved between B6 and BALB/c and are different in 129S1 mice. Thus, the BALB/c and 129 *Nkrp1-Clr* gene clusters seem to be closely related while the B6 cluster has diverged from the previous two.

Phylogenetic analysis of the Nkrp1 *and* Clr *nucleotide and amino acid sequences*

To establish evolutionary relationships, all known *Nkrp1* and *Clr* cDNA sequences were aligned followed by bootstrap analysis. The resulting phylogenetic trees are depicted in Fig. 3A and B. Initial phylogenetic analysis of the *Nkrp1d*, *Nkrp1f*, *Clr-b* and *Clr-g* coding region cDNA revealed that the *Nkrp1* and *Clr* cDNA sequences are divergent (205). To facilitate analysis, the *Nkrp1* and *Clr* sequences were compared separately (Fig. 3). The *Nkrp1* cDNA sequences can be divided into two clusters: *Nkrp1a*, *b*, *c* and *e* in the first cluster and *Nkrp1f* and *g* in the second (Fig. 3A). In the first cluster, *Nkrp1b/d* are in a separate subgroup while *Nkrp1a*, *c* and *e* are more closely related. *Nkrp1a* and *Nkrp1c* both encode for activating receptors, respectively, and may have risen by gene duplication. Moreover, these two genes share common ancestry with the inhibitory *Nkrp1b/d* genes and may have evolved by gene recombination with these two genes. In the second cluster, the activating *Nkrp1f* and the inhibitory *Nkrp1g* are clearly related and probably evolved by gene duplication and recombination possibly from a member of the *Nkrp1a*-containing cluster. Even though all *Nkrp1* genes share common ancestry, the two clusters were probably formed when only a few *Nkrp1* genes existed after which the clusters expanded by gene duplication. Phylogenetic analysis of the amino acid sequences of the NKR-P1 receptors supports this analysis since the proteins are found in the same clusters as their cDNA sequences (Fig. 3C).

Bootstrap analysis of the *Clr* cDNA sequences revealed that the relationship between these genes is more complicated than that of the *Nkrp1* genes (Fig. 3B). It is obvious that *Clr-a* and *f* are related as they form a separate cluster. Another obvious cluster is comprised of *Clr-b*, *d* and *g*. These three genes are clearly related and probably evolved by gene duplication. The relationship of *Clr-c* and *h* with the other *Clr* genes is not as obvious, but *Clr-h* tends to group closely with the *Clr-b*, *d* and *g* cluster while *Clr-c* does not seem to be closely related to any cluster. This is reminiscent of the phylogenetic analysis of *Ly49* genes in which *Ly49q* is the only gene present in its cluster (345). Similar to the *Nkrp1* genes and receptors, phylogenetic analysis of the amino acid sequences of the Clr receptors supports the analysis of the *Clr* cDNA sequences (Fig. 3D).

2. *Discussion*

BAC mapping of the 129-strain *Nkrp1-Clr* gene cluster revealed a genomic organization that was in agreement with the high degree of relatedness observed between the two sequenced B6 and BALB/c clusters (208). Furthermore all genes are organized in the same fashion, unlike the highly divergent *Ly49* clusters (197). The gene order of the 129-strain cluster is exactly the same as the B6 and BALB/c clusters and thus, it can be concluded that the genetic organization of *Nkrp1-Clr* clusters is highly conserved between different strains.

Analysis of the protein sequences of NKR-P1 receptors and Clr ligands revealed that the 129-strain and BALB/c gene products are highly related. Most of the genes in these two strains share the same sequences. On the other hand, genes from 129-strain and BALB/c clusters have diverged from the B6 genes. The notable exceptions are NKR-P1F and Clr-c which are conserved between B6 and BALB/c but have diverged in 129-strain mice. Regardless of allelic polymorphisms, most genes are highly conserved in 129-strain and B6 mice: Clr-b and Clr-g (conserved), NKR-P1G (one amino acid substitution), NKR-P1A and NKR-P1F (two substitutions), Clr-f (three substitutions) and Clr-d (seven substitutions). Three genes show significant divergence between the 129-strain and the B6 mice: NKR-P1B/D (23 substitutions), NKR-P1C (25 substitutions) and Clr-c (38 substitutions). Similarly, NKR-P1B/D and NKR-P1C are quite different between B6 and BALB/c while Clr-c is identical (208). It can be concluded that the BALB/c and 129-strain *Nkrp1-Clr* clusters are highly related whereas the B6 cluster shows signs of divergence. Our results are corroborated by

CGH and Southern blot analyses performed by Higuchi et al. These experiments demonstrated that the 129S1 and BALB/c *Nkrp1-Clr* clusters are highly related as they fall in the same subgroup as determined by the degree of relatedness of CGH patterns. On the other hand, the B6 cluster is different from the previous two and is part of another group (334).

BALB/c NKR-P1B and NKR-P1C lack the specific amino acids recognized by the NK1.1 antibody thus explaining why BALB/c NK cells are not stained by the NK1.1 antibody (208). Both NKR-P1B and NKR-P1C from 129-strain mice are identical to their respective BALB/c alleles (Fig. 2A) and this explains the lack of NK1.1 staining observed in 129-strain mice (34). Interestingly, the NK1.1 reactivity of NK cells from different mouse strains can be predicted by CGH analysis (334). Mice that possess a *Nkrp1-Clr* cluster similar to the B6 cluster are $NK1.1^{+}$ while those that possess a BALB/c or 129-like cluster are $NK1.1^{-}$ (334).

The discovery of a novel Clr ligand gene, *Clr-i*, adds to the complex NKR-P1 recognition system in which the genes for the ligands and the receptors are grouped together. Although it remains to be proven that *Clr-i* codes for a functional protein, its presence may be indicative of a gene that was deleted. Rat cytomegalovirus (RCMV) has acquired a Clr-like protein most likely from a rat *Nkrp1-Clr* cluster (213). This RCMV C-type lectin-like (RCTL) protein is bound by the inhibitory rat NKR-P1B and thereby favours the survival of RCMV-infected cells (213). If a similar Clr-like protein encoded by a murine virus exists, it becomes highly likely that the *Nkrp1-Clr* gene cluster is under high selective

pressure. It is then possible that *Clr-i* represents a gene fragment originating from a gene that has been subverted by a virus. Alternatively, *Clr-i* may have arisen during a homologous recombination event in which only a few exons were involved or was destroyed by a recombination event involving genes located nearby.

Initial studies revealed that NKR-P1D binds to Clr-b and that NKR-P1F recognizes Clr-g (204, 205). Despite significant divergence between NKR-P1D^{B6} and NKR-P1B^{BALB}, both recognize Clr-b (208). Novel NKR-P1/Clr interactions were recently discovered by several groups, including ours (in collaboration with the laboratory of Dr. James Carlyle at the University of Toronto) (203, 297, 298). NKR-P1F was found to have two other ligands: Clr-c and Clr-d while NKR-P1G recognizes Clr-d, Clr-f and Clr-g. Our study revealed that NKR-P1B^{129} also binds to Clr-b. Unlike the other studies which focused on the NKR-P1 and Clr proteins of B6 and BALB/c mice (203, 297), the ligand specificities of 129-strain receptors were investigated by our group (298). Interestingly, it was found that all interactions are conserved between B6 and 129-strain mice (298).

The conservation of the NKR-P1B/D and Clr-b interaction between B6, BALB/c and 129 strains is not surprising given the conservation of the ligand in all three strains (Fig. 2). If we consider the receptors, this interaction becomes quite puzzling: NKR-P1B^{129} and NKR-P1B^{BALB} are 100% identical and it is therefore not surprising that the NKR-P1B/Clr-b interaction is conserved in these two strains. NKR-P1D in B6 mice has significantly diverged from its NKR-P1B alleles but it still binds Clr-b, albeit significantly more weakly than all known

NKR-P1B alleles. Thus, the amino acids substitutions observed between these alleles do not affect the ligand specificity of the receptor, but they change the affinity of the interaction with the Clr-b ligand. These substitutions probably alter the ligand-binding interface in such a way that the interaction is conserved. Rat NKR-P1B binds to the viral RCTL protein (213) and it is therefore possible that mouse NKR-P1B/D binds to a yet unrecognized viral Clr-like ligand. The recognition of viral Clr-like protein by the inhibitory NKR-P1B/D would favour the acquisition of mutations so that the receptor loses its ability to bind to the viral protein while retaining its specificity towards Clr-b. The weak interaction between NKR-P1D and Clr-b could be indicative of a loss of interaction between NKR-P1D and this hypothetical viral Clr-like protein.

With 25 amino acid differences between the 129-strain and B6 alleles, the *Nkrp1c* gene is likely under high selective pressure. Unfortunately, no ligand for NKR-P1C has been found and it is difficult to interpret the divergence of the different NKR-P1C alleles. Similar to other NKR-P1 receptors, it is highly likely that the activating NKR-P1C binds to a Clr protein. This Clr protein may be of viral origin and the interaction would lead to NK cell activation. If this Clr-like ligand is also recognized by an inhibitory NKR-P1 receptor, the viral Clr-like ligand may rapidly evolve, thereby favouring amino acid modifications in NKR-P1C to retain the interaction with the viral Clr-like ligand. NKR-P1C could also bind to a Clr protein of mouse origin. The expression of this Clr ligand would need to be under strict control to prevent unwanted NK cell activation. The Clr ligand could be expressed in tumour cells or virally-infected cells. The observed

divergence in NKR-P1C could reflect attempts by some alleles to increase their affinity to the Clr ligand in an effort to increase NK cell activation after recognition of Clr-expressing diseased cells. Alternatively, it cannot be ruled out that NKR-P1C may have a non-Clr ligand and this non-Clr ligand diverges between different mouse strains thereby favouring accumulation of mutations in the receptor.

NKR-P1F^{B6} is known to recognize Clr-g (205) and this binding was confirmed for BALB/c (297) and 129-strain alleles (298). Similar to Clr-b, Clr-g is identical between 129-strain and B6 mice (Fig. 2B) so any difference in the NKR-P1F/Clr-g interaction would be caused by NKR-P1F. But unlike NKR-P1B/D which differs by 23 amino acids, NKR-P1F from B6 and 129-strain only differ by two amino acid substitutions (Fig. 2A), one of them located in the cytoplasmic domain and the second in the extracellular region. Therefore, these polymorphisms are not sufficient to change the specificity and the affinity of the interaction with Clr-g.

NKR-P1F can also bind to Clr-d and Clr-c (203, 297, 298). Clr-d from both 129-strain and B6 mice are both strongly bound by NKR-P1F (298). Similar to Clr-g, Clr-d is well conserved between these two strains (seven substitutions); therefore the conservation of the interaction is expected. On the other hand, Clr-c from B6 and 129-strain mice are quite different (38 amino acid differences), but both alleles act as ligands for NKR-P1F with no difference in affinity (298). Furthermore, NKR-P1F^{B6} does not show preferential binding to Clr-c^{B6} and the same applies for the 129-strain alleles. These results can be surprising at first but

if we consider the location of the polymorphisms, the conservation of the interaction makes more sense. Almost half of the differences between Clr-c^{B6} and Clr-c^{129} are located in the cytoplasmic region or in the transmembrane domain (Fig. 2B). These amino acid differences should not impact the recognition by NKR-P1F. The other substitutions are located in the extracellular domain, and most of them are found in close proximity to the transmembrane domain (Fig. 2B). It is therefore possible that the mutations do not affect the interface used by NKR-P1F for Clr-c recognition. This suggests that the *Clr-c* genes are under high selective pressure, but the polymorphisms may be limited to amino acids that do not affect the ability of Clr-c to be recognized by NKR-P1F. NKR-P1F is an activating receptor and recognition of Clr-c by NKR-P1F should lead to NK cell activation. It is possible that Clr-c expression is initiated during viral infection or cancer and this in turn triggers NK cell activity. Clr-c could be the target of viral proteins that alter its expression or function and it is tempting to speculate that the divergence of the Clr-c alleles may have occurred to prevent its downregulation or subversion during viral infection.

Interestingly, NKR-P1G shares two ligands with NKF-P1F: Clr-d and Clr-g. It also binds to Clr-f (297, 298). Similar to all NKR-P1/Clr interactions, the binding of NKR-P1G to its ligands is conserved in B6, BALB/c and 129-strain mice. This is not surprising since NKR-P1G from all three strains is highly conserved with only one amino acid substitution (Fig. 2A). Furthermore, Clr-d, Clr-f and Clr-g are relatively well conserved between B6 and 129-strain mice.

This high degree of relatedness of NKR-P1F and NKR-P1G (Fig 3A and 3C) can explain why these two receptors share Clr-d and Clr-g as ligands. The notion that these two receptors share some ligands reinforces the hypothesis that they evolved by gene duplication. NKR-P1F and NKR-P1G form a pair of activating and inhibitory receptors, respectively, and share ligand specificities. This is reminiscent of the shared ligand specificity of the inhibitory Ly49A and the activating Ly49P which both bind to H-2D^{d} (249). Interestingly, two of the ligands shared by NKR-P1F and NKR-P1G, Clr-d and Clr-g are also found in the same cluster after phylogenetic analysis (Fig. 3B and 3D) while the third ligand for each receptor, Clr-c or Clr-f is found in a separate cluster. We can speculate that an ancestral NKR-P1F/G recognized an ancestral Clr-d/g and that after separate or coordinate duplications of the receptor and the ligand, the interaction between the novel receptors and ligands were maintained. After the duplication each new receptor separately acquired the ability to bind to a new ligand.

NKR-P1F is expressed on the surface of NK cells (203) and *Nkrp1g* mRNA is detected in NK cells (297, 298). Even though mRNA can be detected, the expression status of NKR-P1G is unknown due to lack of antibodies (203). Due to the unknown expression status of NKR-P1G, it is not known which of the two receptors have a dominant effect on NK cell functions. Furthermore, *Clr-c*, *-d* and *-g* mRNA is present only in ALAK while the *Clr-f* gene is expressed in the ileum, liver, kidney and ALAKs (206, 298). There is some evidence that Clr-g is expressed in dendritic cells and macrophages generated by *in vitro* culture of bone marrow cells (205). It remains to be proven whether this expression pattern holds

true *in vivo*. Thus, it is possible that NKR-P1F does not encounter its ligands (Clr-c, -d and -g) in steady state. The ligands for NKR-P1F may only be expressed during viral infection or in cancerous cells and this could lead to NKR-P1F-mediated NK cell activation. In this model, NKR-P1G would serve to prevent overactivation of NK cells by recognizing and competing with NKR-P1F for the Clr-d and Clr-g ligands. This model would also predict that the upregulation of Clr-c after infection or transformation would lead to activation of NK cells since NKR-P1G does not recognize this ligand. It is therefore possible that the expression of Clr-c is under tight control to prevent unwanted NK cell activation.

3. Research opportunities

The *Nkrp1-Clr* cluster shows very little divergence when compared to the highly diverse *Ly49* gene cluster (197, 298). Even though this cluster is well conserved in different strains, CGH and Southern blot analysis revealed that the *Nkrp1-Clr* cluster of NOD mice is part of a subgroup separate from the two subgroups containing the B6, BALB/c and 129-strain clusters (334) suggesting a cluster different from the three currently mapped clusters. Mapping the NOD *Nkrp1-Clr* gene cluster would reveal the specific differences of this cluster. It can be speculated that the NOD genes coding for proteins bound by the NK1.1 antibody, *Nkrp1b* and *Nkrp1c*, differ from $Nkrp1c^{B6}$ and $Nkrp1b^{SJL}$ because NOD NK cells are $NK1.1^{-}$ (299, 334). Furthermore, the *Clr-c* gene displays high divergence between B6 and 129 mice and it is also possible that this locus also shows divergence in NOD mice. In addition, it is likely that another locus is highly divergent in NOD mice.

The presence of Clr-b on tumour cells prevents NK cell activity (204, 205). Despite similar binding of $NKR\text{-}P1D^{B6}$ and $NKR\text{-}P1B^{129}$ to Clr-b, the latter seems to bind to Clr-b with higher affinity (298). Because of the higher affinity of $NKR\text{-}P1B^{129}$ to Clr-b than $NKR\text{-}P1D^{B6}$, it is possible that 129-strain NK cells respond more vigorously to the loss of Clr-b from the surface of target cells. It has been demonstrated that B6 NK cells respond weakly to the loss of Clr-b while NK cells from CD-1 mice which possess NKR-P1B respond vigorously to the loss of Clr-b as assessed by *in vitro* cytotoxicity assays (204). Whether or not this translates into *in vivo* lysis or not requires further testing. First, the ability of 129

and B6 mice to reject Clr-b^{+} cells could be assessed. Due to the low lytic activity of 129-strain NK cells compared to B6 NK cells (321), a B6 mouse carrying the 129-strain NKC (B6.Ly49^{129}) should be used in these experiments (285). This mouse is currently available in our laboratory. In a separate experiment, mice could be injected with tumour cells treated with genotoxic agents that induce downregulation of Clr-b (207), and the rejection of these cells could be assessed in B6 and in B6.Ly49^{129} mice.

The activating NKR-P1F receptor recognizes several Clr proteins and it is unknown if the recognition of Clr-c, -d or -g by NKR-P1F results in NK cell activation. Transfection of Clr ligands into cell lines and testing these cells for susceptibility to NK cell lysis could answer this question. Due to the shared specificity of NKR-P1F and NKR-P1G for Clr-d and Clr-g, the focus of this study should be on Clr-c. A NKR-P1F-specific antibody unable to cross-link this activating receptor is available (203) and could be used to block the interaction between NKR-P1F and its ligands during cytotoxicity assays.

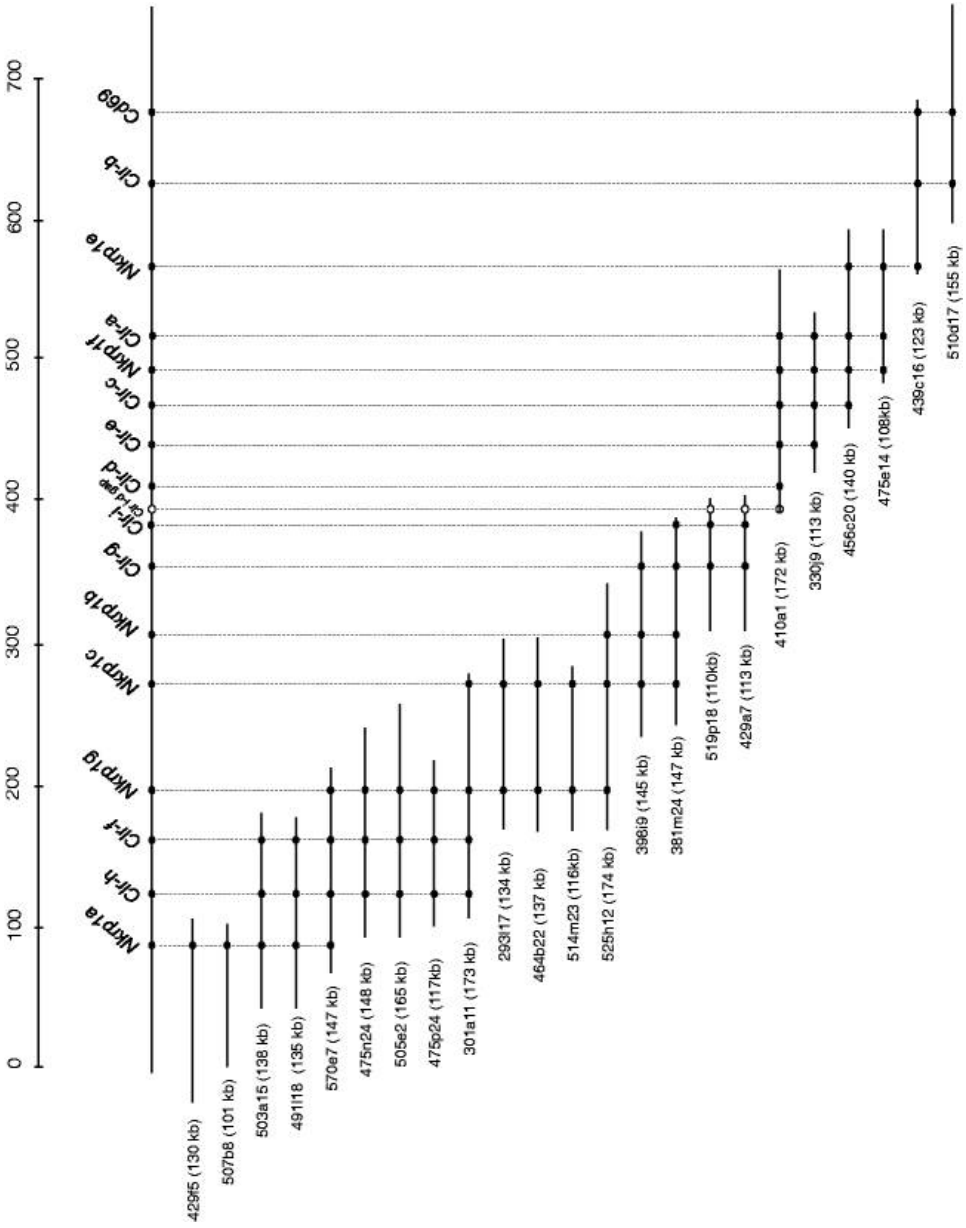
The lack of data concerning the expression of several Clr ligands precludes any functional study. The expression patterns of Clr-c, Clr-d and Clr-g seem to be confined to ALAKs while Clr-f is present in the ileum, liver and kidney as well as ALAKs (206). Testing the expression of these four Clr ligands in cell lines originating from different organs should be attempted. Furthermore, the expression of these ligands should be verified in the organs of mice infected with different viruses or in cell lines infected with the same viruses. Tumours isolated from mice treated with carcinogenic drugs could also be tested for the

expression of Clr ligands. Gaining knowledge of the expression of Clr proteins could help understanding the *in vivo* role played by NKR-P1 receptors on NK cells.

Clr-f mRNA is expressed in the ileum and NK cell subsets can be found in the lamina propria of the small intestine (163, 164). It is possible that Clr-f acts as a signal used by NK cells to respond to the gut microbiota. Testing the expression of *Clr-f* in the intestine of mice bred in germ-free conditions compared with mice bred in conventional facilities could answer this question. Alternatively, mice could be treated with antibiotics to deplete the gut microbiota and then tested for *Clr-f* expression. The expression of *Clr-f* could also be verified in the small intestine of mice infected with gut pathogens. The receptor for Clr-f is NKR-P1G and *Nkrp1g* mRNA is detected in ALAKs. The expression of *Clf-f* becomes relevant if an intestinal cell is found to express NKR-P1G. Testing for the presence of *Nkrp1g* mRNA is various intestinal cell types should be attempted.

Ly49 are known to bind to their MHC-I ligands either in *trans* (Ly49 on the NK cell binds to MHC-I on a target cell) or in *cis* (Ly49 on the NK cell binds to MHC-I on the same NK cell). Similar to Ly49, NKR-P1 receptors are C-type lectin molecules and possibly share structural similarities. It is therefore possible that NKR-P1 receptors are able to bind to their Clr ligands in *cis*. This kind of interaction is possible since both NKR-P1 and Clr are expressed by ALAKs (298). The ability of NKR-P1 receptors to bind to their ligands in *cis* should be tested as well as the functional implications of such interactions.

4. Figures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100
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500
600
700
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Clr-h
Clr-f
Nkp1g
Nkp1c
Nkp1b
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Clr-b
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570e7 (147 kb)
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475p24 (117kb)
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519p18 (110kb)
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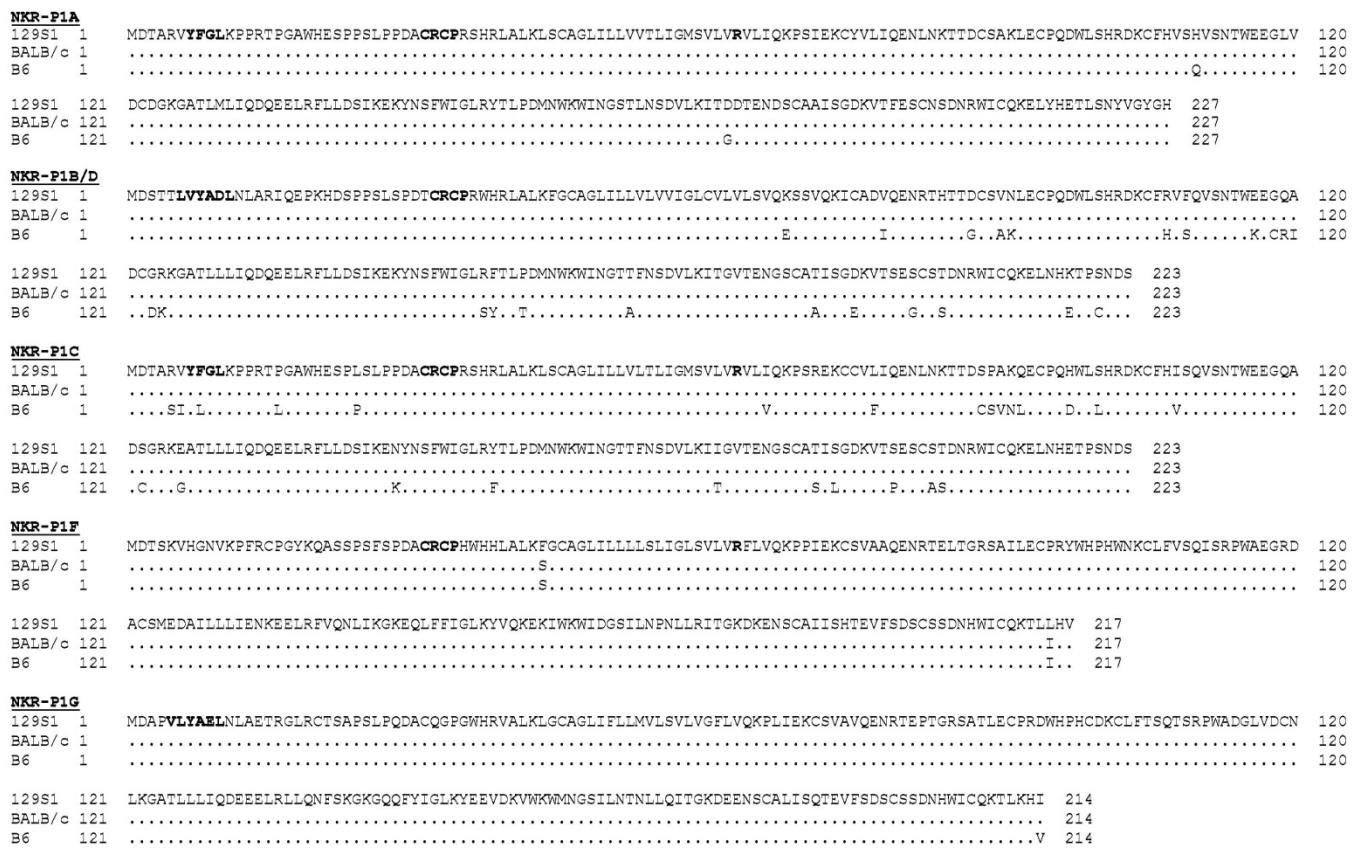
410a1 (172 kb)
330j9 (113 kb)
456c20 (140 kb)
475e14 (108kb)
439c16 (123 kb)
510d17 (155 kb)

Figure 1: BAC contig overlap and construction of a physical map for the 129S6 *Nkrp1-Ocil/Clr* gene cluster. The BAC gene content and sizing data from pulsed-field gel electrophoresis were integrated to produce a map of the relative location of all known 129S6 *Nkrp1/Clr* genes. BACs are represented by *horizontal lines*, with the name and size of each given on the *left side*. *Black circles* represent the start of exon-4 of the indicated genes. The spacing between genes was based on the average of possible maximum and minimum sizes imposed by BAC size and gene content. The *scale bar* (*top*) is demarcated in kilobases. The marker *Clr-i-d-gap*, represented by *empty circles*, is the end sequence of the *Clr-d*$^{+}$ BAC 410a1. After cloning, this BAC end sequence fragment was used as a probe to show that BAC 410a1 overlaps with *Clr-i*$^{+}$ BACs, despite not sharing any *Nkrpl/Clr* genes.

With kind permission from Springer Science+Business Media: *Immunogenetics*, Analysis of the mouse 129-strain *Nkrp1-Clr* gene cluster reveals conservation of genomic organization and functional receptor–ligand interactions despite significant allelic polymorphism, 2011, 63:627-640, Chen P., Bélanger S., Aguilar O.A., Zhang Q., St-Laurent A., Rahim M.M., Makrigiannis A.P., Carlyle J.R., Figure 1.

A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BALB/c 1 120
B6 1 .....Q..... 120
129S1 121 DCDGKGATLMLIQDQEELRFLLDSIKEKYNSFWIGLRYTLPDMNWKWINGSTLNSDVLKITDDTENDSCAAISGDKVTFESCNSDNRWICQKELYHETLSNYVGYGH 227
BALB/c 121 227
B6 121 .....G..... 227
NKR-P1B/D
129S1 1 MDSTTLVYADLNLARIQEPKHDSPPSLSPDTCRCPRWHRLALKFGCAGLILLVLVVIGLCVLVLSVQKSSVQKICADVQENRTHTTDCSVNLECPQDWLSHRDKCFRVFQVSNTWEEGQA 120
BALB/c 1 120
B6 1 .....E.....I.....G..AK.....H.S.....K.CRI 120
129S1 121 DCGRKGATLLLIQDQEELRFLLDSIKEKYNSFWIGLRFTLPDMNWKWINGTTFNSDVLKITGVTENGSCATISGDKVTSESCSTDNRWICQKELNHKTPSNDS 223
BALB/c 121 223
B6 121 ..DK.....SY..T.....A.....A...E....G..S.....E..C... 223
NKR-P1C
129S1 1 MDTARVYFGLKPPRTPGAWHESPLSLPPDACRCPRSHRLALKLSCAGLILLVLTLIGMSVLVRVLIQKPSREKCCVLIQENLNKTTDSPAKQECPQHWLSHRDKCFHISQVSNTWEEGQA 120
BALB/c 1 120
B6 1 ....SI.L.....L.....P.....V.....F.....CSVNL....D..L.....V..... 120
129S1 121 DSGRKEATLLLIQDQEELRFLLDSIKENYNSFWIGLRYTLPDMNWKWINGTTFNSDVLKIIGVTENGSCATISGDKVTSESCSTDNRWICQKELNHETPSNDS 223
BALB/c 121 223
B6 121 .C...G.....K.....F.....T.....S.L.....P...AS..... 223
NKR-P1F
129S1 1 MDTSKVHGNVKPFRCPGYKQASSPSFSPDACRCPHWHHLALKFGCAGLILLLLSLIGLSVLVRFLVQKPPIEKCSVAAQENRTELTGRSAILECPRYWHPHWNKCLFVSQISRPWAEGRD 120
BALB/c 1 .....S..... 120
B6 1 .....S..... 120
129S1 121 ACSMEDAILLLIENKEELRFVQNLIKGKEQLFFIGLKYVQKEKIWKWIDGSILNPNLLRITGKDKENSCAIISHTEVFSDSCSSDNHWICQKTLLHV 217
BALB/c 121 .....I.. 217
B6 121 .....I.. 217
NKR-P1G
129S1 1 MDAPVLYAELNLAETRGLRCTSAPSLPQDACQGPGWHRVALKLGCAGLIFLLMVLSVLVGFLVQKPLIEKCSVAVQENRTEPTGRSATLECPRDWHPHCDKCLFTSQTSRPWADGLVDCN 120
BALB/c 1 120
B6 1 120
129S1 121 LKGATLLLIQDEEELRLLQNFSKGKGQQFYIGLKYEEVDKVWKWMNGSILNTNLLQITGKDEENSCALISQTEVFSDSCSSDNHWICQKTLKHI 214
BALB/c 121 214
B6 121 .....V 214

B

Clr-b

```
129S1   1   MCVTKASLPMLSPTGSPQEVEVGKILQGKRHGTISPESCAKLYCYYGVIMVLTVAVIALSVALSATKTEQIPVNKTYAACPQNWIGVENKCFYFSEYPSNWTFAQAFCMAQEAQLARFDN  120
BALB/c  1   ....................................................................................................................  120
B6      1   ....................................................................................................................  120

129S1   121 QDELNFLMRYKANFDSWIGLHRESSEHPWKWTDNTEYNNTIPIRGEERFAYLNNNGISSTRIYSLRMWICSKLNSYSLHCQTPFFPS  207
BALB/c  121 .......................................................................  207
B6      121 .......................................................................  207
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Clr-c

```
129S1   1   MTASQDEVAHERLLNTEVTITDHLENGETGKKIQEKFRGIVSSNSHCRLFCCYGVIMVLTVAV--IALSVALWTTKTEQISTKTTYDACPKKWIGVGNKCFYFSENSKNWTVAQNCCMAQ  118
BALB    1   ................T.............C.....K....R.R.C..T...I.V.VL.VVV...SAIMIK.M...N..A..LQN.T.....Y.....YTS...F..AF....  120
B6      1   ................T.............C.....K....R.R.C..T...I.V.VL.VVV...SAIMIK.M...N..A..LQN.T.....Y.....YTS...F..AF....  120

129S1   119 EAQLARFHNQDELNFLKRHMNSSHWIGLHRDSSEHPWRWTDNTEYNNTFLIQGDGECGFLSDNGISSSRDYIPRKWICSRSSNYMLQC  206
BALB    121 .......D.EE..............................................................  208
B6      121 .......D.EE..............................................................  208
```

Clr-d

```
129S1   1   MNAQCLKKPEEGEGSPGTGGVQCYKILQRKSLRAISPESSAKLYCCYGVIMVLTVAVVALSVALSVTKTEQILINKTYAACPKNWIGVGNKCFYFSEYTSNWTFAQTFCMAQEAQLARFD  120
BALB    1   ....................................................................................................................  120
B6      1   ............S.......HS......N................C.................PA.....................................................  120

129S1   121 NEKELNFLMRYKANFDSWIGLHRESSEHPWKWTDNTEYNNMIPIQGVETCAYLSGNGISSSRHYIPRIWICSKLNNYSLHCPTPVPV  207
BALB    121 .......................................................................  207
B6      121 .......................................................................  207
```

Clr-f

```
129S1   1   MLQRADLTAADCLQEGEMGKKIQGKCFRIISTVSPVKLYCCYGVIMVLTVAVIALSVALSVRNKIPVMEDREPCYTACPRGWIGFGSKCFYFSEDMGNWTFSQSSCVASNSHLALFHSLE  120
BALB/c  1   ....................................................................................................................  120
B6      1   ..............................................................A.............S.....................................  120

129S1   121 ELNFLKRYKGTSDHWIGLHRASTQHPWIWTDNTEYNNLVLTRGGGECGFLSDNGISSGRSYTHRKWICSKFVSSCKSRVGSVPRHV  206
BALB/c  121 .......................................................................  206
B6      121 ..............................S........................................  206
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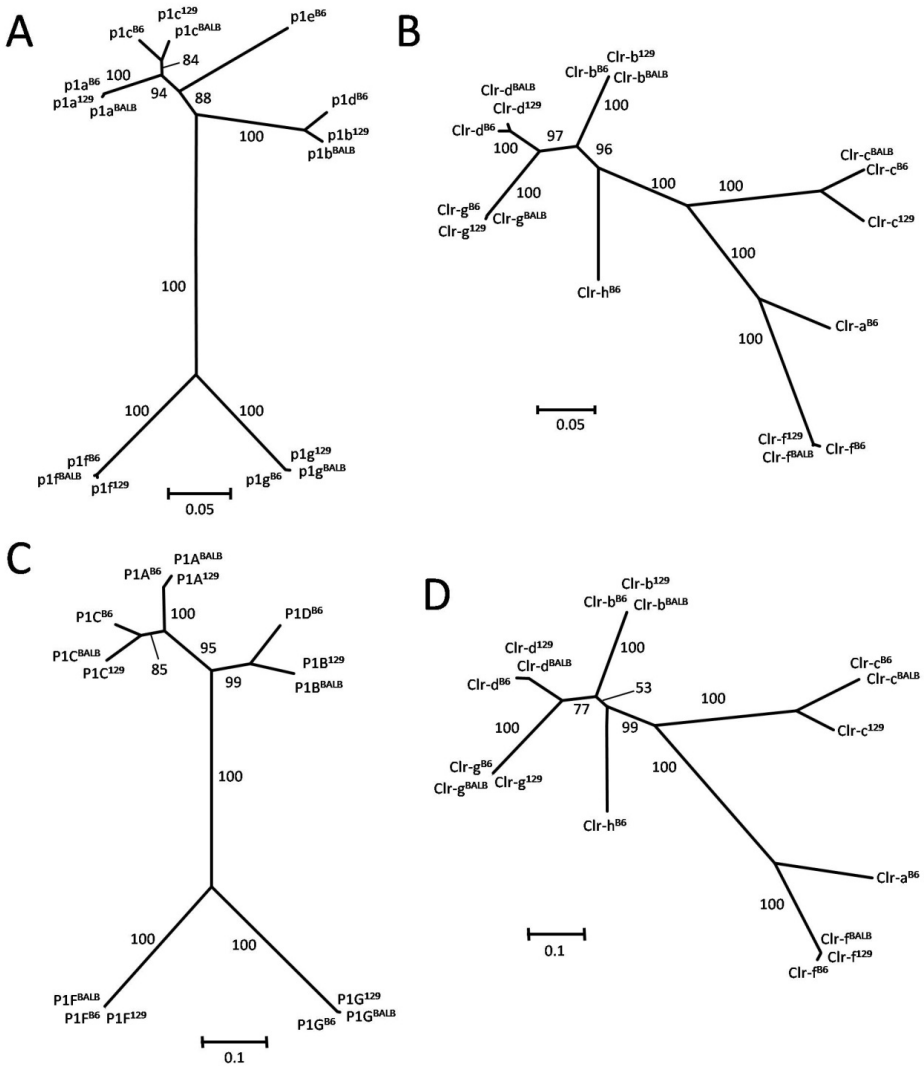
Clr-g

```
129S1   1   MNAQCVQKPEEGNGPLGTGG----KIVQGKCFRIISTVSPVKLYCCYGVIMVLTVAVIALSVALSTKKTEQIIINKTYAACSKNWTGVGNKCFYFSGYPRNWTFAQAFCMAQEAQLARFD  116
BALB/c  1   ..................VQCC..............................................................................  120
B6      1   ..................----..............................................................................  116

129S1   117 NEEELIFLKRFKGDFDCWIGLHRESSEHPWKWTNNTEYNNMNPILGVGRYAYLSSDRISSSRSYINRMWICSKLNNYNLHCQTPPV  202
BALB/c  121 .......................................................................  206
B6      117 .......................................................................  202
```

Figure 2: Coding sequence alignment of novel 129S1 NKR-P1 and Clr alleles. The putative amino acid sequences of the (A) NKR-P1 and (B) Clr cDNAs cloned from 129S1 LAK cells are shown as alignments with the BALB/c and B6 alleles, where available. The alignments were produced using ClustalX. A *period* indicates identity, and a *dash* indicates a gap in sequence alignment. NKR-P1 functional features are highlighted in *boldface*, including: ITIM (L/V/IxYxxL/I/V); YxxL motif; CxCP Lck-recruitment motif; charged transmembrane residue. *Numbers* are indicative of relative sequence position.


A
p1c129
p1cB6
p1cBALB
p1eB6
84
p1a100B6
p1a129
p1aBALB
94
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100
p1b129
p1bBALB
100
100
100
p1fB6
p1fBALB
p1f129
p1g129
p1gB6
p1gBALB
0.05
B
Clr-b129
Clr-bB6
Clr-bBALB
Clr-dBALB
Clr-d129
100
Clr-dB6
97
100
96
Clr-cBALB
Clr-cB6
100
100
Clr-gB6
Clr-g129
100
Clr-gBALB
Clr-c129
Clr-hB6
100
Clr-aB6
100
0.05
Clr-f129
Clr-fBALB
Clr-fB6
C
P1ABALB
P1AB6
P1A129
100
P1C100B6
P1DB6
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P1CBALB
P1C129
85
99
P1B129
P1BBALB
100
100
100
P1FBALB
P1FB6
P1F129
P1G129
P1GB6
P1GBALB
0.1
D
Clr-b129
Clr-bB6
Clr-bBALB
100
Clr-d129
Clr-dBALB
Clr-cB6
Clr-dB6
53
Clr-cBALB
77
100
100
99
Clr-c129
100
Clr-gB6
Clr-gBALB
Clr-g129
Clr-hB6
Clr-aB6
100
Clr-fBALB
Clr-f129
0.1
Clr-fB6

Figure 3: Phylogenetic analysis of novel 129S1 NKR-P1/Clr gene products. The coding region cDNAs (A, B) or putative protein translations (C, D) of all known 129S1, BALB/c, and B6 *Nkrp1* and *Clr* alleles were aligned using ClustalX. The putative coding sequence for *Nkrp1e*^{B6} was artificially spliced together from genomic data. Bootstrap analysis of 1,000 data sets was performed using PHYLIP and a dendrogram representative of the bootstrap analysis was produced with TreeView. The *phylogram branch lengths* indicate the similarity between different cDNAs or protein sequences and the scale bar indicates the percent divergence. *Numbers on branches* indicate the percentage of bootstrap replicates that agree with the branching.

CONCLUSION

Sequencing of the NOD *Ly49* cluster confirmed the low degree of conservation of *Ly49* gene clusters in different mouse strains. This novel cluster has interesting features including its size, the largest known to date, and the large number of gene coding for putative activating receptors. This mirrors the increased incidence of activating KIR in diabetic patients. This knowledge can be applied to understanding the role played by NK cells in the development of diabetes in NOD mice.

The NKC^{KD} mouse provides us with an invaluable tool to study the impact of Ly49 receptors in the control of NK cell functions in various diseases: cancer, viral infections and autoimmune disorders. Characterization of the NK cells from these mice revealed that they possess a defective "missing-self" response and that this defect can be rescued by re-introduction of an Ly49 receptor with self-MHC-I specificity indicating a lack of MHC-I education in NKC^{KD} mice. Further studies will address the resistance of these mice to various cancer models, viral infections and autoimmune disorders.

Lastly, study of the *Nkrp1-Clr* gene cluster of 129 mice demonstrated that this gene cluster is well conserved in three different mouse strains unlike the *Ly49*. Sequencing the *Nkrp1* and *Clr* genes permitted the analysis of newly discovered NKR-P1/Clr interactions. Collectively, these studies revealed the critical role of NK cell receptors such as Ly49 in the direct control of NK cell cytotoxicity and the uniqueness of the NKC which possess a highly diverse gene family (*Ly49*) and a second, well conserved, gene family (*Nkrp1-Clr*).

APPENDICES

1. Materials and methods

Isolation of splenocytes

Spleens were crushed on a 70μM cell strainer and the cells were transferred into a tube and spun down. Red blood cells were lysed by resuspending the cell pellet in 5mL of ACK lysis solution (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM Na_2EDTA; pH 7.2-7.4) for 5 minutes at room temperature before the addition of 10mL of PBS followed by centrifugation. Cell pellets were resuspended in PBS or cRPMI (RPMI supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin and 100μg/mL streptomycin).

Collagenase digestion of spleen

Spleens were digested with collagenase for flow cytometric analysis of pDC. Spleens were slowly injected with 5mL of HBSS containing 0.5mg/mL collagenase D (Roche) until they lost their dark red colour. Spleens were then cut in small pieces in a Petri dish and incubated at 37ºC for 20 minutes. 10μL of 0.5M EDTA was added (final 1mM EDTA) followed by a 5 minute incubation at 37ºC. Cells were harvested, filtered through at 70μm cell strainer and spun down. Red blood cells were lysed as previously described. Cell pellets were resuspended in PBS or cRPMI and then used for flow cytometry analysis.

Isolation of lymphocytes from bone marrow, liver and lung

Both femurs were isolated and muscle was carefully removed. Femurs were cut at both ends and marrow was flushed from the bone over a 70μm cell strainer with 5mL of PBS by using a syringe equipped with at 25G needle. Cells

were spun down and red blood cells were lysed as described above. Cell pellets were resuspended in cRPMI, counted and used for flow cytometry analysis.

Livers were perfused *in situ* with 5mL of PBS using a syringe equipped with a 25G needle. The needle was inserted in the hepatic portal vein located between the small intestine and the liver. The heart was punctured before beginning the infusion. PBS was slowly injected in the liver until it changed colour and became paler. After perfusion, liver was removed and crushed on a 70μm cell strainer placed in a Petri dish. The cell strainer was thoroughly washed with PBS (three times with 10mL of PBS) and the cell suspension was transferred to a 50mL tube. Cells were spun down, resuspended in PBS and spun down again. The pellet was then resuspended in 1mL room temperature 37.5% isotonic Percoll (37.5% Percoll in 1x PBS, GE Healthcare: 33.75mL Percoll, 10mL 10x PBS, 56.25mL ddH_2O) and transferred to a 15mL conical tube. Tubes were filled with 37.5% isotonic Percoll and spun down at 700g for 12 minutes (room temperature). The supernatant containing hepatocytes was discarded; the cell pellet was resuspended in PBS and spun down followed by lysis of red blood cells as described earlier. Cell pellets were resuspended in cRPMI, counted and used for flow cytometry analysis.

Lungs were removed and minced in 5mL cRPMI containing 0.5mg/mL collagenase D (Roche) followed by incubation for 25 minutes at 37 ºC with agitation. The minced pieces were crushed on a 70μm cell strainer, transferred to a tube and spun down. The cell pellet was resuspended in 1mL room temperature 67.5% isotonic Percoll, transferred to a 15mL tube followed by addition of 3mL

of 67.5% isotonic Percoll. 2mL of room temperature 45% isotonic Percoll was carefully layered on top of the 67.5% isotonic Percoll solution containing the lung cells. 45% and 67.5% isotonic Percoll solutions were prepared by diluting 90% isotonic Percoll (9:1 Percoll in 10x PBS) with PBS 1:1 and 3:1, respectively. Tubes were spun down for 15 minutes at 800g with minimal acceleration and deceleration (room temperature). The lymphocytes can be seen at the interphase located between the layers of 67.5% and 45% isotonic Percoll. Lymphocytes were harvested and transferred to a new tube filled with PBS followed by a centrifugation. If required, red blood cell lysis was performed as described earlier. Cell pellets were resuspended in cRPMI, counted and used for flow cytometry analysis.

Flow cytometry

$2.5x10^6$ splenocytes were transferred to FACS tubes containing 2mL FACS buffer (PBS, 0.5% BSA and 0.02% NaN_3) and then spun down. Rat serum (2μL) was added to the cells, and the cell pellet was resuspended by gentle vortexing following by incubation at 4ºC for 15 minutes. Antibodies directly conjugated to fluorochromes were added without washing off the rat serum and cells were incubated in the dark at 4ºC for 20 minutes followed by a wash with FACS buffer. If required, a secondary antibody stain was performed as above. After all staining and washes, cells were resuspended in 450μL FACS buffer and 1μL of propidium iodide (BD Biosciences) was added to the cells right before acquisition with a FACScan or a FACsCalibur using the CellQuestPro software (BD Biosciences) or the CyAN-ADP using Summit software (Beckman Coulter).

Data analysis was performed using CellQuestPro, FlowJo (Tree Star) and Kaluza (Beckman Coulter) softwares.

In vitro *Cytotoxicity assays*

ALAKs (Adherent Lymphokine Activated Killer cells) were generated by cultivating splenocytes in 1000U/mL human IL-2 (Peprotech) for 6 days. More specifically, splenocytes from half a spleen were plated in 75cm^2 tissue culture flasks in 15mL of NK medium (RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100U/ml penicillin, 100µg/mL streptomycin, 1mM sodium pyruvate, 100µM non-essential amino acids, 10mM HEPES and 50µM 2-mercaptoethanol) with 1000U/mL human IL-2. After 3 days, medium containing cells growing in suspension was removed and replaced by 15mL of fresh NK medium with fresh IL-2. Adherent cells were grown for 3 more days before being used in a cytotoxicity assay.

On day 6, medium was removed and ALAKs were harvested with PBS-1mM EDTA. Cells were counted and resuspended in NK medium at a concentration of 2.5x10^6 cells/mL. Serial 2-fold dilutions of the ALAKs were performed in triplicates. 100µL of ALAKs were added to the first 3 wells (#1-3, 50:1 ALAK:target ratio) of a row of a 96-well plate (V-bottom). 100µL of NK medium was added to the following 9 wells (#4-12) of the row. 100µL of ALAKs were added to the next 3 wells (#4-6, 25:1 ALAK:target ratio). The contents of the wells #4-6 were mixed and 100µl was moved to wells #7-9 (12:1 ALAK:target ratio). The contents of the wells #7-9 were mixed and 100µl was

moved to wells #10-12 (6:1 ALAK:target ratio). The contents of wells #10-12 were mixed and 100μL was discarded.

Target cells were harvested and labelled with 100μCi of ^{51}Cr (Perkin-Elmer) in the form of Na_2CrO_4 resuspended in PBS for 1 hour in a 37ºC incubator. The labelling was started before harvesting the ALAKs. Every 15 minutes, the cells were gently resuspended. After the incubation, target cells were washed 3 times with cRPMI, counted and finally resuspended in NK medium at a cell concentration of $5.0x10^4$ cells/mL. 100μL of the cells ($5.0x10^3$) were added to the ALAKs in the 96-well plate. 100μL of target cells were also added to 6 more wells (3 containing 100μL of 10% SDS for Maximal release; 3 containing 100μL of NK medium for Minimal release). Plates were incubated at 37ºC for 4 hours.

After the incubation, plates were spun down for 5 minutes at 1500rpm and 100μL of the supernatant was transferred to titer tubes. The radioactivity present (or the ^{51}Cr released by lysed target cells) in the supernatant was determined with a gamma counter.

The % cytotoxicity was calculated for each experimental well as follows:

$$\%\ cytotoxicity = \left[\frac{(Experimental\ release - Average\ of\ Minimal\ release)}{(Average\ of\ Maximal\ release - Average\ of\ Minimal\ release)}\right] x\ 100$$

For antibody blocking experiments, purified anti-NKG2D (clone CX5) was added to the ALAKs plated in the 96-well plate to obtain a concentration of 10μg/mL in the wells and incubated for 30 minutes at 4°C before the addition of target cells.

For antibody-dependent cellular cytotoxicity (ADCC) assays, ^{51}Cr-labelled target cells were washed and counted as above. $2.0x10^5$ ^{51}Cr-labelled target cells were resuspended in 250μL NK medium. Purified anti-Thy1.2 (clone 30-H12) was added to the target cells to obtain a concentration of 10μg/mL. Cells were incubated with the antibody for 30 minutes at 4°C followed by a washing step. Washed cells were resuspended at a concentration of $5x10^4$ cells/mL before plating 100μL of the target cells in the 96-well plate containing the ALAKs.

Concanavalin A (ConA, Sigma) blasts were sometimes used as target cells. Splenocytes from the indicated mice strains were isolated, resuspended in NK medium and counted. Cells were plated in 15mL of NK medium at a concentration of $2.5x10^6$ cells/mL and then ConA was added to obtain a final concentration of 5μg/mL. Cells were grown for 2 days before being used as target cells in cytotoxicity assays.

MCMV infection and plaque assays

Mice from the indicated strains were injected intra-peritoneally with 5000 plaque-forming units (PFU) of MCMV Smith strain (a gift from Dr. Silvia Vidal, McGill University). Three days after infections, mice were sacrificed; spleens and livers were removed and homogenized in DMEM 2% FBS (DMEM containing 2% FBS, 2mM L-glutamine, 100U/ml penicillin, 100μg/mL streptomycin and 25mM HEPES). Homogenates were spun down for 15 minutes at 3000rpm, 4 ºC, and homogenates were diluted in DMEM 2% FBS (1:5 for liver, 1:50 and 1:100 for spleens of 129S1 and NOD/ShiLtJ mice, 1:25 and 1:50 for spleens of C57Bl/6 mice). 100μL of the homogenates were layered on top of mouse embryonic

fibroblasts (MEF) (gift from Dr. Silvia Vidal) plated in 48-well plates followed by incubation at 37ºC for 1 hour. After the incubation, the homogenates were removed and cells were covered with 500µL of DMEM containing 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100µg/mL streptomycin, 25mM HEPES and 0.5% low-melt agarose. After 3 days of incubation at 37ºC, cells were fixed with 10% formalin (4% paraformaldehyde) for 10 minutes, stained with 1% methylene blue (dissolved in 70% ethanol) for 10 minutes and washed 3 times with ddH_2O before counting the plaques.

MEF monolayers were cultured in a large tissue culture dish 1 day before the infection of mice (or 4 days before the harvest of the organs of MCMV-infected mice) in 35mL of DMEM containing 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100µg/mL streptomycin and 25mM HEPES. Vials of MEF are obtained from Silvia Vidal (McGill University, Montréal). One vial is thawed for every 6 mice used in the experiment. After 3 days of culture (or 1 day before the harvest of the organs of MCMV-infected mice), cells were harvested by Trypsin treatment (0.25% + 0.2g/L EDTA) and $0.8\text{-}1.0x10^5$ cells were plated in each well of a 48-well plate and grown for 1 day.

Total PFU per organ was calculated as follows:

$$PFU = \#plaques\ x\ dilution\ of\ homogenate\ x\ 10\ x\ total\ volume\ of\ homogenate$$

Generation of the NOD/ShiLtJ Ly49 *gene cluster map*

Repetitive elements were identified in the NOD/ShiLtJ genomice sequence using Repeatmasker version 3.1.8 (available from http://www.cgb.ki.se/groups/sonnhammer/Dotter.html). The sequence (in FASTA format) to be analyzed was copied in the RepeatMasking page. The settings were as follows: Search engine: Cross_match; Speed/Sensitivity: Default; DNA source: Mouse.

A PIP (graphical representation of the genomice sequence) was generated using Advanced Pipmaker (available at http://pipmaker.bx.psu.edu/pipmaker). The sequence was compared to itself with single coverage and one strand search settings. The following optional features were used: First sequence mask: file obtained from the Repeatmasker program; First sequence exons: a file containing the locations of the genes and exons with the gene names and exon numbers; First sequence underlay: the same file as the First sequence exon but with LighYellow and Green written instead of gene names and exon numbers, respectively. The graphical sequence was assembled into a single figure representing the complete *Ly49* cluster of NOD/ShiLtJ with markers for genes, exons and repetitive elements.

Phylogenetic analysis

Sequence alignments were performed using ClustalX version 1.83 to generate a sequence alignment file. The sequence alignment file was modified as shown in Fig.1 and saved as a .txt file. The .txt file is then renamed to “infile” (note: remove .txt extension) and copied in the PHYLIP folder. PHYLIP

(Phylogeny Inference Package) version 3.5c is available for download on the following website: http://evolution.genetics.washington.edu/phylip.html. Sequences are randomized with SEQBOOT program located in the PHYLIP folder. The number of replicates was changed to 1000. SEQBOOT will generate the "outfile" file. Rename this file to "infile" after deleting the original one. DNADIST (for nucleotide sequences) or PROTDIST (for amino acid sequences) were used to calculate the distances between the sequences and the number of datasets were changed to 1000. A new "outfile" is generated; rename it again to "infile" and NEIGHBOUR was used to generate the trees. The number of data sets was increased to 1000. NEIGHBOUR will create an "outtree" file.

The "outtree" file was opened with Tree Explorer version 2.12 (K Tamura, unpublished; available from http://evolgen.biol.metrou/ac.jp/TE/TE_man.html). The file will contain 1000 trees that can be visualized and a consensus tree. A single tree that best fits the consensus tree was selected.

In vivo *cytotoxicity assays*

The ability of NK cells to reject MHC-I^- splenocytes was determined by labelling these cells with CFSE and injecting the cells into recipient mice. The presence of the CFSE-labelled cells in recipient mice was assessed by flow cytometry a few hours after injection. The injected cells were mixed with WT cells labelled with a different concentration of CFSE. WT cells were used as an internal reference since these cells are not rejected by NK cells. This allowed for calculation of the rejection efficacy of injected MHC-I^- cells.

WT splenocytes were labelled with 0.5μM CFSE while the MHC-I$^-$ cells were labeled with 5μM CFSE. Splenocytes from WT and various MHC-I$^-$ mice were isolated, depleted of red blood cells with ACK lysis buffer for 7 minutes on ice and PBS was added followed by centrifugation. The cell pellet was resuspended in PBS, counted and spun down. Splenocytes were then resuspended at a concentration of $5.0x10^7$ cells/mL in PBS. The total numbers of cells to be labelled should be at least twice the number of cells that will be required for all injections (i.e. if you inject 10 mice with $5x10^6$ cells each, you need $5.0x10^7$ cells; thus at least $1.0x10^8$ cells should be labelled). 10μM and 1μM solutions of CFSE were prepared in PBS. Equal volumes of splenocytes and of a CFSE solution were rapidly mixed to obtain solutions of $2.5x10^7$ cells/mL with either 0.5μM (WT) or 5μM (MHC-I$^-$) of CFSE. Cells were incubated for 7 minutes in a 37ºC incubator. During the incubation, cells were mixed twice by inverting the tube. After the incubation, the labelling reaction was stopped by quickly adding an equal volume of FBS and incubated for 2 minutes at room temperature. RPMI containing no serum was added and cells were spun down. Cell pellets were resuspended in cRPMI and incubated for 15 minutes in a 37ºC incubator. The cells were spun down, resuspended in PBS, counted and spun down again. After the last centrifugation, cell pellets were resuspended in PBS at a concentration of $5.0x10^7$ cells/mL. WT and MHC$^-$ cells were mixed at a 1:1 ratio. 200μL of the mixed cells (containing $5.0x10^6$ WT cells and $5.0x10^6$ MHC$^-$ cells) were injected in the tail vein of mice of the indicated genotypes. The number of cells of each type to be injected can vary between $2.0x10^6$ and $5.0x10^6$. When this assay was performed

using 129S1-derived mice as recipients, recipient mice were injected intra-peritoneally with 150μg poly I:C in 200μL PBS (Sigma) 24 hours before the injection of the CFSE-labelled splenocytes.

An aliquot of the mixture of CFSE-labelled cells was analyzed by flow cytometry to verify the ratio of WT and MHC-I$^-$ cells (input bracket of the formula). CFSE can be detected using the FITC channel of a flow cytometer. Sixteen hours after the injection of CFSE-labelled splenocytes, the spleens of the recipient mice were harvested and a small aliquot of splenocytes was analyzed for the presence of CFSE-labelled donor cells by flow cytometry (harvest bracket of the formula). The % rejection was calculated using the following formula:

$$\%\, rejection = \left(1 - \frac{\left(\frac{\#\ of\ 0.5\ \mu M\ CFSE\ cells}{\#\ of\ 5\ \mu M\ CFSE\ cells} \right)_{input}}{\left(\frac{\#\ of\ 0.5\ \mu M\ CFSE\ cells}{\#\ of\ 5\ \mu M\ CFSE\ cells} \right)_{harvest}} \right) x\ 100$$

2. Figures

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4    388
i-BALB    ATGAGTGAGCCGGAGGACACTTACTCAACTGTGAGACTTCATAAGTCTTCAGGGTTGCAG
i-B6      ATGAATGAGCCGGAGGTCACTTACTCAACTGTGAGACTTCATAAGTCTTCAGGGTTGCAG
i2-NOD    ATGAGTGAGCCAGAGGTCACTTACTCAACTGTGAGACTTCATAAGTCTTCAGGGTTGCAG
i1-129    ATGAGTGAGCCAGAGGTTACTTACTCAACTGTGAGATTTCATAAGTCTTCAGGGTTGCAG

AAATTAGTAAGGCATGAGGAGACTCAAGGGCCCAGAGAAGCTGGCAACAGAAAGTGTTCA
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GTATCCTGGTAACTCATTGTGAAAGCTCTTGGAATCCTCTGTTTCCTTCTTCTGGTAATA
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TTACTGGATTGGATTGTCTTATGATAAGAAAAAAAAGGAATGGGCATGGATTGACAATGG

CCCATCTAAACTTGACATTAAAATAAGGAAAATGAACTTTAAGCCTAGAGGATGTGTATT
CCAATCTAAACTTGACATGAAAACAAGGAAAATGAACTTTAAGTCTAGAGGATGTGTATT
CCAATCTAAACTTGACATGAAAATAAGGAAAATGATCTTTAAGTCTAGAGGATGTGTATT
CCCATCTAAATTTGACATGAAAATAAGGAAAATGAACTTTAAGTCTAGAGGATGTGTATT

TTTATCTAAATCAAGAATAAAAGATACTGACTGTAATATATCCTACTACTGTATTTGTGG
TTTATCTAAAGCAAGAATAGAAGATACTGACTGTAATATTCCCTACTACTGTATTTGTGG
TTTATCTAAAGCAAGAATAGAAGATACTGACTGTAATATTCCCTACTACTGTATTTGTGG
TTTATCTAAAGCAAGAATAGAAGATACTGACTGTAATATTCCCTACTACTGTATTTGTGG

GAAGAAACTGGATAAATTCCCTAGTTAA
GAAGAAACTGGATAAATTCCCTGATTAA
GAAGAAACTGGATAAATTCCCTGATTAA
GAAGAAACTGGATAAATTCCCTGATTAA
```

Figure 1: Example of a sequence alignment file ready for analysis using PHYLIP software. Each sequence is given a unique name. The sequences begin on the 11th character of the line. The first line has two numbers. The first one, which begins on the 4th character, indicates the number of sequences in the file. The second number beginning on the 9th character indicates the length of the sequences. This figure uses truncated *Ly49i* cDNA sequences from the indicated strains as an example.

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