Genetic Investigation of MHC-Independent Missing-Self Recognition

by NK Cells

By

Peter Chen

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Immunology

University of Toronto

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Peter Chen

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ABSTRACT

NK cells are innate lymphocytes involved in the elimination of transformed, infected, stressed, antibody-coated and transplanted cells. The goal of this thesis, as well as the interest of our laboratory is to explore the MHC-independent “missing-self” branch of NK recognition, and more specifically, the NKR-P1B:Clr-b receptor-ligand system and its role in bone marrow transplantation.

In the first results chapter, we generated hybridoma clones producing monoclonal antibodies against another inhibitory receptor in the Nkrp1 receptor family, NKR-P1G via the CELLISA assay. Our results demonstrate that CELLISA is a cost-effective, efficient, and high-throughput screening protocol, and the generation of the α-NKR-P1G 1H8 hybridoma clone enables characterization of the mouse Nkrp1-Clr receptor-ligand system via in vitro reporter cell and transfection approaches.

Next, we show that the Nkrp1-Clr receptor-ligand system exhibits substantial allelic polymorphisms in NKR-P1B, NKR-P1C, and Clr-c loci between three common
laboratory inbred mouse strains (C57BL/6, BALB/c and 129); however, no differential
gene content has been observed in these loci in contrast to the Ly49 and MHC-I loci in
mice. Furthermore, through the usage of BWZ reporter cell assays, we reveal several new
cognate receptor-ligand pairs in addition to the previously reported NKR-P1B:Clr-b and
NKR-P1F: Clr-g interactions; they are: NKR-P1F: Clr-c; NKR-P1F: Clr-d; NKF-P1G:
Clr-d; NKR-P1G: Clr-f; NKR-P1G: Clr-g.

In the final results chapter, we show that Clr-b<sup>−/−</sup> bone marrow cells are rejected
by the recipients’ NKR-P1B+ NK cells. Importantly, through the usage of a 7-colour
membrane labeling technique on the donor cells, we can simultaneously compare the
rejection of Clr-b<sup>−/−</sup> grafts to WT cells and different variants of MHC-I-deficient bone
marrow cells in a single animal. Up to 30-40% of Clr-b<sup>−/−</sup> bone marrow cells were
rejected, which is similar to the rejection phenotype for H-2D<sup>b</sup><sup>−/−</sup> bone marrow cells.
Importantly, this rejection is reverted fully or partially (in B6 vs. Swiss recipients) in NK-
derepleted (total or NKR-P1B+ NK subset depleted) recipients.

Collectively, these results extend our understanding of the Nkrp1-Clr receptor-
ligand family by revealing additional Nkrp1-Clr receptor-ligand pairs; and that the
missing-self recognition of Clr-b in vivo is instrumental in determining the prognosis of
bone marrow transplantation.
DEDICATION

To mom and dad, their limitless love and support
ACKNOWLEDGMENTS

During my Ph.D training, I have met many phenomenal individuals. We laughed and cried together, we created memories, and you guys became my extended family. As we go on our separate ways, I would like to take this opportunity to speak my mind, which I never did in the last half decade that we were together.

Jim, words are not enough to express my appreciation. Thank you for persuading me not to surrender my dreams for science and medicine; thank you for giving me a second chance in science, and the opportunities to journey across Canada to attend all the CSI conferences; and thank you for instilling confidence in me, making me an independent and critical thinker, now ready to tackle on the next challenge in my life. And lastly, thank you for the Bernhard Cinader prize nomination. Receiving this award was the icing on the cake, as there could not have been a better way to conclude my Ph.D career. Thank you, Jim, for everything that you have done for me.

Thank you Dr. Danska, Dr. Zúñiga-Pflücker, and Dr. Poussier, for all the training and words of wisdoms. You taught me not to fear challenges, but to confront them with courage and a positive attitude. These life lessons shall remain with me for eternity.

To my lab family: Dr. David Allan, thank you for finally getting accustomed to my awful jokes. However, since there is Aruz preceding me, I guess it was a subtle change for you. Nonetheless, I would like to thank you for being the “Oracle” of the lab, and providing me with the words of wisdom and the “matrix”, or should I say, matrices, again and again. I am considering changing my name into Neo if I ever undertake a pen name for scientific writing. To the first generation Jimlets: Dr. Aruz Mesci, my undergraduate friend, my Kororean BBQ, all you can eat sushi, and hotpot partner in crime. I will never forget that “ONE” time when I asked you to drive me to Markham, and it ended up being the worst drive of the century in snowmageddon. I guess that evens out all the mileage points that you owed me. But you still left some marks in my car after immunology tonight, so I am still one up. All in all, the laughter and memories are priceless. Dr. Jason Fine, my lab big brother, the founder of using McDonald ketchup holders as jellowshooter containers, and my CSI roommate. You taught me how to loosen up, and showed me the way on how to warm up to people. The evening of my Ph.D defense you mentioned that the concept of family is very important to me. Indeed, this is true; but it is you who incepted this idea in my mind. I only took a good thing that came from you and shared with as many people as possible, which is another thing that you taught me.

To the second generation Jimlets: Miss Tina Kirkham, the lab mommy, the queen of sweets and the elder of the two sisters that I never had. Thank you for keeping the lab in one piece. Also, I meant every word when I said that seeing you smile when you eat sweets makes me smile during my Ph.D defense. I don’t say “nice” things too often, but alas, you were able to bring it out of me. And I would like to reiterate, I DO notice when you trim your bangs, highlight your hair, or paint your nails. I am just too lazy to compliment my own my sister. But now, I do regret not complimenting as often as I should. Mr. Oscar Aguilar, my El Salvadorian brother, and Yoda. Thank you for teaching me the concept of time. People say that time goes by faster when you are enjoying it. Since it seems that everything about you appears to be moving slower than the rest of the world, you must always be radiating happiness to the world around you...? In all seriousness, thank you for always providing happiness to everyone, and thank you for
cheering me up during the darkest hours in my lab life. You are indeed a savior, and I really cherish the times that we have spent in the lab, and the times that we hung out at home, as friends, and not as landlord and tenant. Miss Miho Tanaka, the younger of the two sisters that I never had, and also my teacher that teaches me practical Japanese. Your creativity for the most ridiculous, but heartwarming remarks never ceases to astonish me. I am glad to see you being able to really love Canada, and really enjoy life here in Toronto. Thank you for your reassuring words during our long chats. You are a sweetheart. Jae, my Korean younger brother, your witty one-word comebacks and lyric writing abilities are unparalleled. Your sense of humour, fashion, and friendship are all world-class, and I am blessed to have met you in the last two years of my Ph.D career. For the record, I do agree with many things that you say, both science and non-science stuff. They are all equally illuminating and inspiring. Lastly, I would like to acknowledge Muhammed Ali Akbar, Wayne Chou and Annabelle Chow for the many hours that we spent together in the JRC laboratory, and the continuous source of good coffee. It has been fun.

I am also grateful to my departmental family, and the Immunology Graduate Student Association for all the experiences and memories we share. It was absolutely tremendous to be able to celebrate my birthday and conclusion of my studies with all of you at the retreat and the holiday party. You guys have my eternal love, and I hope that we can always be connected, wherever we are.

I would also like to thank University of Toronto and Sunnybrook Research Institute staff, CIHR and OGS for funding. This work was made possible thanks to your efforts.

Finally, I would like to thank mom, dad, and the rest of my extended family, who never doubted my determination to pursue my passion. You are the foundation of my resolve to chase my dreams, and I am forever grateful for all your love, belief, and support.
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<td>β2m</td>
<td>beta-2-microglobulin</td>
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<td>β-gal</td>
<td>beta-galactosidase</td>
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<td>B6</td>
<td>C57BL/6</td>
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<td>BM</td>
<td>bone marrow</td>
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<td>bone marrow cells</td>
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<td>BWZ</td>
<td>BWZ.36 reporter cell line</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>c-type lectin</td>
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<td>CLP</td>
<td>common lymphoid progenitor</td>
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<tr>
<td>Clr</td>
<td>c-type lectin related</td>
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<td>CMV</td>
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<td>CPRG</td>
<td>chlorophenol-red-β-D-galactopyranoside</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>ERAAP</td>
<td>endoplasmic reticulum aminopeptidase associated with antigen processing</td>
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<td>FcR</td>
<td>Fc receptor</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>green fluorescent protein</td>
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<td>HSC</td>
<td>hematopoietic stem cell</td>
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<td>HTS</td>
<td>high throughput screening</td>
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<td>immunoglobulin</td>
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<td>interleukin</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>internal ribosomal entry site</td>
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<td>immunoreceptor tyrosine-based activation motif</td>
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<td>immunoreceptor tyrosine-based inhibitory motif</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<td>lymphotoxin</td>
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<td>microbe-associated molecular patterns</td>
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<td>natural killer gene complex</td>
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<td>polymerase chain reaction</td>
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<tr>
<td>SH2</td>
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<td>TAP</td>
<td>transporter associated with antigen processing</td>
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CHAPTER I

INTRODUCTION
1.1 NK Cells and the Immune System

1.1.1 Innate and adaptive immunity

The immune system is an intricate and complicated system that is able to distinguish harmless vs. dangerous entities in biological organisms. In general, dangerous entities include infectious microbial agents, altered self such as mutation of host cells, or allo/xenotransplants. More specifically, the immune system facilitates the clearance of these entities, thus ensuring the health and longevity of the host. In mammals, the immune system encompasses both innate and adaptive immunity; in contrast, the immune system in pre/jawless vertebrates and pre-cartilaginous fish is composed by innate immunity. Innate immunity is the first line of defense against pathogen infections. Components of this system include epithelial barriers, enzymes, complement, peptides, cytokines, and immune effector cells, such as granulocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells. Importantly, innate immune effector cells recognize pathogens through germ-line encoded receptors that are specific for MAMPs. These effector cells then initiate inflammatory mechanisms to eliminate infecting pathogens. Furthermore, innate immune cells can also trigger the priming of adaptive immune cells to facilitate sterilizing immunity. The initiation of the adaptive immune response is somewhat delayed in comparison to the innate response; however, it offers a more specific and thorough clearance of these non-self entities. Integral players in the branch of adaptive immunity traditionally include the B and T lymphocytes. B cells produce antibodies, and T cells exercise a variety of activities including cell-mediated immunity, T cell help for B cells and macrophages, polarizing responses (T\textsubscript{H1}, T\textsubscript{H2}, T\textsubscript{H17}, M1, M2), and regulatory and inhibitory roles (Tregs). The hallmark of the
adaptive immune system is the use of rearranging (non-germline) receptor segments for specificity and diversity, which are generated through random and combinatorial mechanisms, and memory.

1.1.2 NK cells

NK cells are innate lymphocytes that specialize in the detection and elimination of infected, transformed, stressed, antibody-coated, and allogeneic transplanted cells. NK cell cytotoxic function is mediated by directed exocytosis of granules containing perforin and granzymes, which in turn polymerize to create pores in the target cellular membrane and activate serine protease-dependent apoptosis, respectively. NK cells are also potent producers of pro-inflammatory cytokines, such as IFN-γ and TNF-α. In turn, cytokine and chemokine production by NK cells promotes largely Th1-type adaptive immunity.

NK cell effector function is determined by a balance of stimulatory and inhibitory signals delivered via cell surface receptors. Healthy cells normally express high levels of inhibitory ligands (e.g., MHC-I) and low levels of stimulatory ligands (e.g., ICAMs, NKG2DL) on the cell surface, which are recognized by inhibitory and stimulatory NK cell receptors, respectively. High-level expression of “self” inhibitory ligands results in strong inhibitory signals, which in turn prevent NK cell activation (Fig. 1.1). However, cells undergoing pathological processes, such as transformation or infection, often down-regulate cell surface expression of inhibitory ligands. A prime example is the down-regulation of MHC-I molecules to evade CD8+ T cell mediated immunity. However, the absence of MHC-I proteins results in the loss of inhibitory signals transduced in NK cells, which can be sufficient to facilitate NK cell activation and cytotoxicity towards
target cells, by a mechanism termed “missing-self” recognition (Fig. 1.1) \(^{76}\). In contrast, “induced-self” recognition occurs when pathological target cells trigger cell surface expression of stimulatory NK ligands, thereby overpowering constitutive inhibitory signals, again resulting in NK-mediated cytotoxicity (Fig. 1.1) \(^{25,40}\). In reality, it is the integration of total inhibitory and stimulatory signals that determine whether a NK cell responds or ignores a given target cell.
FIGURE 1.1

Figure 1.1 Schematic of two modes of NK activation: missing-self versus induced-self recognition
Healthy cells expressing both inhibitory ligands (eg. MHC-I) and low levels of stimulatory ligands (eg. NKG2DL, DNAM) for NK cells, which transduces a balanced net intracellular signal that prevents NK activation. Stressed cells (depicted by the lighting-strike symbol) often downregulate cell surface inhibitory ligand, leading to a predominance in stimulatory signal and NK induced cytotoxicity. Killing by NK cells due to the absence of cell surface inhibitory ligand is termed “Missing-self” recognition. Alternatively, stressed cells can also increase the expression of stimulatory ligands. Here, the upregulation of stimulatory ligands results in an overpowering stimulatory signal, which also results in the activation of NK cells and ultimately target cell death. This mode of NK-mediated cytotoxicity is termed “Induced-self” recognition. Inhibitory and stimulatory NK receptors are indicated as red and green on the NK cell, respectively. Inhibitory and stimulatory ligands are indicated as red and green on the target cell respectively. Dark blue circles with the light blue rim denote NK granules, and the light and dark blue circles denote degranulated perforin and granzymes.
1.1.3 NK receptor overview

NK cell receptors can be classified into two main structural categories: C-type lectin-like and immunoglobulin-like (Ig-like) superfamily members\textsuperscript{163}. C-type lectin-like family members are encoded within the NKC, a syntenic region on human chromosome 12 and mouse chromosome 6\textsuperscript{60}. Members of this category include the Ly49, Nkrp1, and CD94/NKG2 families, wherein each family contains both inhibitory and stimulatory receptor isoforms (Table 1.1). Notably, most C-type lectin-like family receptors are functionally conserved between rodents and hominids, with the exception of the Ly49 family (i.e., only a single \textit{LY49} pseudogene is found in humans)\textsuperscript{20}. Interestingly, mouse Ly49 receptors display great polymorphism, likely due to their recognition of classical MHC-I ligands, which are also extremely polymorphic\textsuperscript{104,114}. In addition to the polymorphism exhibited by the receptors, the overlapping specificities of Ly49 receptors for different MHC-I genes and haplotypes further complicate the biology of this receptor family. Currently, mixed reports have documented ligands recognized by different Ly49s (Table 1.1). By comparison, other C-type lectin-like receptors demonstrate less-pronounced allelic and haplotype polymorphisms\textsuperscript{20,163}. MHC-dependent NK cell recognition in humans is mediated by Ig-like receptors, encoded within the leukocyte receptor complex (LRC), a syntenic region on human chromosome 19 and mouse chromosome 7\textsuperscript{80}. Some members of this receptor superfamily include the killer-cell Ig-like receptors (KIRs), leukocyte Ig-like receptors (LIRs), and natural cytotoxicity receptors (NCRs) (Table 1.2). In parallel with the Ly49 system in rodents, KIRs directly recognize human MHC-I and display significant polymorphism; this constitutes a classical example of convergent evolution. Thus, it is apparent that Ly49s and KIRs are
specialized NK receptors evolved to recognize MHC-I in rodents and humans, respectively. Notably, functional human NK receptors mostly belong to the Ig-like receptor superfamily, whereas rodent NK receptors encompass both the C-type lectin-like and Ig-like receptors. For example, both mice and humans express NK receptors encoded by the LIR, NCR and SAP/SLAM receptor superfamily. Interestingly, ligands for many of the receptor superfamilies listed above are MHC-independent molecules (Table 1.2). This observation thus demonstrates that MHC-independent recognition is an important branch in NK cell biology.

Evidently, identifying the nature of each NK receptor (stimulatory versus inhibitory) sets the foundation to understand and appreciate NK cell biology. In general, stimulatory receptors have short intracellular domains, thus lacking intrinsic signaling motifs for cellular activation. However, stimulatory NK receptors typically contain a positively charged transmembrane residue, thus allowing association with adaptor proteins that contain a negatively charged transmembrane residue. FcRγ, CD3ζ, DAP10 and DAP12 are common adaptor molecules that associate with stimulatory receptors. Importantly, these adaptor molecules all possess ITAMs characterized by the consensus sequence $D/E_{\text{x}xYxxL/I_{6-8}YxxL/I_{125}}$, where x denotes any amino acid. When stimulatory NK receptors bind their ligands, it results in receptor crosslinking which subsequently leads to the phosphorylation of the ITAMs on the adaptor molecules by the associated Src family kinases. Phosphorylated ITAMs then recruit signaling intermediates such as Syk and ZAP70 through their SH2 domains. These intermediates further propagate downstream signaling events, which in turn trigger $Ca^{2+}$ influx, and ultimately activation of NK cell effector function.
Alternatively, inhibitory NK receptors typically possess longer intracellular domains containing ITIMs with the signature sequence I/V/LxYxxL/V, where x represents any amino acid\textsuperscript{112}. Receptor crosslinking results in the phosphorylation of ITIMs by Src family tyrosine kinases, and in turn recruiting phosphatases via their SH2 domains. Examples of recruited phosphatases include SH-2-containing protein tyrosine-phosphatases 1 and 2 (SHP-1 and SHP-2) and SH-2 containing inositol polyphosphate-5 phosphatase (SHIP). The recruited phosphatases dephosphorylate the signaling intermediates in the stimulatory signaling pathway (eg. FcRγ, ZAP70, Syk and PLCγ), in turn dampening and counteracting the stimulatory signaling cascade, resulting in localized inhibition of NK effector function.

Interestingly, the length of the intracellular domain of NK receptors provides clues in predicting the function of many NK receptor isoforms, mostly clearly in the KIR Ig-superfamily. For example, KIRs are categorized into either “short” or “long” isoforms, denoted by either the “S” or “L” in the KIR nomenclature. Typically, short KIR isoforms are stimulatory receptors, and most of the long KIR isoforms are inhibitory receptors (with the exception of KIR2DL4) (Table 1.2). This pattern is extended in the 2B4 nomenclature, where long 2B4 isoforms are inhibitory, and short 2B4 isoforms demonstrate stimulatory potential.
<table>
<thead>
<tr>
<th>Receptors</th>
<th>Species</th>
<th>Strains</th>
<th>+/-</th>
<th>Known Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ly49A 37,59,74,75,111,112,150</td>
<td>M</td>
<td>B6, BALB/c, NOD</td>
<td>–</td>
<td>B6: H-2D(^d), D(^k), D(^p), H-2(^f), q, r, s, v, BALB/c: H-2D(^d)</td>
</tr>
<tr>
<td>*Ly49B 49,112</td>
<td>M</td>
<td>B6, 129, BALB/c, NOD</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ly49C 15,59,97,112,116,122</td>
<td>M</td>
<td>B6, BALB/c, NOD</td>
<td>–</td>
<td>B6: H-2K(^b), K(^d), D(^d), D(^k), H-2(^f), q, r, s, v, D(^b)?, BALB/c: H-2K(^b), K(^d), D(^d), D(^k), H-2(^f), q, r, s, v</td>
</tr>
<tr>
<td>Ly49D 30,51,105</td>
<td>M</td>
<td>B6, NOD</td>
<td>+</td>
<td>B6: H-2D(^d), Hm1-C4</td>
</tr>
<tr>
<td>Ly49E 112,154</td>
<td>M</td>
<td>B6, 129, BALB/c, NOD</td>
<td>–</td>
<td>B6: urokinase plasminogen activator protein?</td>
</tr>
<tr>
<td>Ly49F 59,112</td>
<td>M</td>
<td>B6</td>
<td>–</td>
<td>B6: H-2K(^d)</td>
</tr>
<tr>
<td>Ly49G2 59,71,100,102,141</td>
<td>M</td>
<td>B6, 129, BALB/c, NOD</td>
<td>–</td>
<td>B6: H-2D(^d), 129: H-2D(^d), D(^k), K(^d), D(^b), BALB/c: H-2D(^d), D(^k)</td>
</tr>
<tr>
<td>Ly49H 1,3,142</td>
<td>M</td>
<td>B6, NOD</td>
<td>+</td>
<td>B6: MCMV m157</td>
</tr>
<tr>
<td>Ly49I 3,59,100,112,116,142</td>
<td>M</td>
<td>B6, 129, BALB/c, NOD</td>
<td>–</td>
<td>B6: H-K(^b), D(^d), K(^d), K(^k), H-2(^f), q, r, s, v, D(^b)?, 129: H-2D(^k), K(^b), K(^d), MCMV m157</td>
</tr>
<tr>
<td>Ly49I2 100,112</td>
<td>M</td>
<td>129</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ly49I 103,112</td>
<td>M</td>
<td>B6</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ly49K 112</td>
<td>M</td>
<td>BALB/c</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ly49M 112</td>
<td>M</td>
<td>NOD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ly49N 100,112</td>
<td>M</td>
<td>129</td>
<td>–</td>
<td>129: H-2D(^p), D(^d), D(^k), L(^d)</td>
</tr>
<tr>
<td>Ly49O 62,112</td>
<td>M</td>
<td>129</td>
<td>+</td>
<td>129: H-2D(^b) in conjunction with MCMV m04, D(^d)</td>
</tr>
<tr>
<td>Ly49P 112</td>
<td>M</td>
<td>NOD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ly49P3 112</td>
<td>M</td>
<td>NOD</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>**Ly49Q 112,136,148,149,153</td>
<td>M</td>
<td>B6, 129, BALB/c, NOD</td>
<td>–</td>
<td>B6: H-2K(^b)</td>
</tr>
<tr>
<td>Ly49R 112</td>
<td>M</td>
<td>129</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ly49S 100,112</td>
<td>M</td>
<td>129</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ly49T 100,112</td>
<td>M</td>
<td>129</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Ly49U 112</td>
<td>M</td>
<td>129</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ly49V 100,112</td>
<td>M</td>
<td>129</td>
<td>–</td>
<td>129: H-2D(^b), D(^d), D(^k), K(^b),</td>
</tr>
<tr>
<td>CD94/NKG2 (^{14,155})</td>
<td>M/H</td>
<td>+/−</td>
<td>K(^d), K(^k), L(^d)</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>------</td>
<td>-----</td>
<td>---------------------</td>
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<tr>
<td>NKG2A</td>
<td>M/H</td>
<td>–</td>
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<tr>
<td>NKG2C</td>
<td>M/H</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKG2E</td>
<td>M/H</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKG2D (^{7,17,24,32,39,40,145})</td>
<td>M/H</td>
<td>+</td>
<td>M: Rae-1, MULT-1, H60</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H: MIC-A/B, ULBP-1/2/3/4</td>
<td></td>
</tr>
<tr>
<td>NKR-P1A (^{126})</td>
<td>M/H</td>
<td>–</td>
<td>H: LLT-1</td>
<td></td>
</tr>
<tr>
<td>NKR-P1B (^{18,67})</td>
<td>M</td>
<td>–</td>
<td>Clr-b</td>
<td></td>
</tr>
<tr>
<td>NKR-P1C</td>
<td>M</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NKR-P1F (^{26,89})</td>
<td>M</td>
<td>+</td>
<td>Clr-c, d, g</td>
<td></td>
</tr>
<tr>
<td>NKR-P1G (^{26,89})</td>
<td>M</td>
<td>−</td>
<td>Clr-d, f, g</td>
<td></td>
</tr>
<tr>
<td>NKp65 (^{144})</td>
<td>H</td>
<td>+</td>
<td>KACL (CLEC2A)</td>
<td></td>
</tr>
<tr>
<td>NKp80 (^{160})</td>
<td>H</td>
<td>+</td>
<td>AICL (CLEC2B)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.1 C-type lectin-like NK receptors**

Functional C-type lectin-like NK receptors expressed on mammalian NK cells include the Ly49, CD94/NKG2 and NKR1P1 receptor superfamily. Various Ly49, NKG2 and NKR1P1 receptors and its ligand are listed above. In the species column, M denotes mice, H denotes humans; strains column indicates the mouse strain in which expression of the NK receptor has been reported, which is most relevant for the Ly49 receptor family as polymorphic Ly49 haplotypes and frameworks have been reported in mice; +/− column indicates the type of signal transduced by the NK receptor: inhibitory (−) or stimulatory (+). *Ly49B is not located in the Ly49 cluster, but still resides in the NKC; **Ly49Q is expressed on plasmacytoid DCs, not on NKs despite it being a member of the Ly49 receptor superfamily.
Table 1.2 Ig-like NK receptors
A list of functional Ig-like NK receptors expressed on mammalian NK cells and their corresponding ligands are listed above. In the species column, M denotes mice, H denotes humans; +/- column indicates the type of signal transduced by the NK receptor: inhibitory (−) or stimulatory (+).

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Species</th>
<th>+/-</th>
<th>Known Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KIRs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIR2DL1</td>
<td>H</td>
<td>−</td>
<td>HLA-C2</td>
</tr>
<tr>
<td>KIR2DL2/3</td>
<td>H</td>
<td>−</td>
<td>HLA-C1</td>
</tr>
<tr>
<td>KIR2DL4</td>
<td>H</td>
<td>+</td>
<td>HLA-G</td>
</tr>
<tr>
<td>KIR2DL5</td>
<td>H</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>KIR3DL1</td>
<td>H</td>
<td>−</td>
<td>HLA-Bw4</td>
</tr>
<tr>
<td>KIR3DL2</td>
<td>H</td>
<td>−</td>
<td>HLA-A3, A11</td>
</tr>
<tr>
<td>KIR2DS1</td>
<td>H</td>
<td>+</td>
<td>HLA-C2</td>
</tr>
<tr>
<td>KIR2DS2</td>
<td>H</td>
<td>+</td>
<td>HLA-C1</td>
</tr>
<tr>
<td>KIR2DS3</td>
<td>H</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KIR2DS4</td>
<td>H</td>
<td>+</td>
<td>HLA-C?</td>
</tr>
<tr>
<td>KIR2DS5</td>
<td>H</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>KIR3DS1</td>
<td>H</td>
<td>+</td>
<td>HLA-Bw4</td>
</tr>
<tr>
<td><strong>Other Ig-like NK Receptors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LILR 30,31,96</td>
<td>H/M</td>
<td>−</td>
<td>MHC I, UL18</td>
</tr>
<tr>
<td>PILR 140</td>
<td>M</td>
<td>+</td>
<td>CD99</td>
</tr>
<tr>
<td>KLRG1 59</td>
<td>H/M</td>
<td>−</td>
<td>E−, N− and R− cadherins</td>
</tr>
<tr>
<td>2B4 (short) 95</td>
<td>H/M</td>
<td>+</td>
<td>CD48</td>
</tr>
<tr>
<td>2B4 (long) 95</td>
<td>M</td>
<td>−</td>
<td>CD48</td>
</tr>
<tr>
<td>NKp30 13,120</td>
<td>H</td>
<td>+</td>
<td>BAT-3, HSPG, B7-H6</td>
</tr>
<tr>
<td>NKp44 116</td>
<td>H</td>
<td>+</td>
<td>Viral HA</td>
</tr>
<tr>
<td>NKp46 116</td>
<td>H/M</td>
<td>+</td>
<td>Viral HA</td>
</tr>
<tr>
<td>DNAM-1 12</td>
<td>H</td>
<td>+</td>
<td>PVR (CD155), Nectin-2 (CD112)</td>
</tr>
</tbody>
</table>
1.1.4 MHC I biology and biosynthesis

T cell recognition of antigenic peptides presented in the context of MHC molecules is central to self-nonself discrimination mediated by adaptive immunity. However, MHC molecules (particularly MHC-I) also evolved to function in innate immunity as “self” ligands for NK cell receptors, such as the Ly49 family in rodents, and KIR family in humans. In this setting, a number of factors that affect MHC-I surface expression have been shown, in turn, to regulate NK cytotoxicity.

MHC-I heterotrimers are composed of a polymorphic 45 kD heavy chain, an invariant 12 kD β2m light chain, and diverse ~8-11 amino acid peptides. MHC-I is pivotal in the presentation of peptides processed from cytosolic proteins. Briefly, nascent and cytosolic proteins undergo constitutive processing by the proteasome into peptide fragments. These antigenic peptides are then transported into the ER by the heterodimeric transporter associated with antigen processing (TAP-1/2) complex. Here, the peptides are subsequently loaded into the empty peptide-binding groove of nascent MHC-I heterodimers, whose folding is facilitated by the peptide-loading complex, a series of molecular chaperones including calnexin, calreticulin, ERp57, and tapasin. Peptide-loaded MHC-I is then transported via the Golgi complex to the cell surface.

To study the role of MHC-I in vivo, a gene-targeting strategy was implemented, whereby the β2m locus was disrupted. Lack of β2m association with MHC-I heavy chains prevents cell surface expression of MHC-I (i.e., both H-2Kb and Db on a B6 background), and all β2m-associated molecules such as CD1 and Qa-1. In turn, β2m−/− animals are grossly deficient in CD8+ T cells, due to a requirement for MHC-I expression during positive selection. In contrast, NK cells from β2m−/− animals are functional,
albeit hyporesponsive\textsuperscript{9,123}. That is, β\textsubscript{2}m\textsuperscript{−/−} NK cells are “educated” differently such that they do not recognize MHC-I molecules as “self”, and cannot mediate “missing-self” recognition of cells lacking MHC-I (i.e., they are rendered tolerant during development to avoid autoimmunity)\textsuperscript{123} (N.B., NK education and development will be further discussed in subsequent sections). Nonetheless, β\textsubscript{2}m\textsuperscript{−/−} cells are readily eliminated by wild-type NK cells, both \textit{in vitro} and \textit{in vivo}\textsuperscript{9}.

Other genetically modified animals have been engineered to further study missing-self recognition of MHC-I by NK cells. For example, the deletion of TAP-1/2\textsuperscript{41,122,124,131,132} and the removal of the individual H-2 genes\textsuperscript{117} are all successful strategies in eliminating cell surface expression of MHC-I. Indeed, TAP-1/2 deficient cells are readily eliminated by NK cells both \textit{in vivo} and \textit{in vitro}; and cells devoid of H-2K, H-2D, or both genes are also killed by NK cells (although to a less degree in comparison to cells completely deficient of cell surface MHC such as β\textsubscript{2}m\textsuperscript{−/−}, more on this later). These early studies thus demonstrate a prominent role of MHC-dependent missing-self recognition by NK cells. Similar to the Ly49 receptor family, MHC genes from different mouse strains can be grouped into different haplotypes, thus providing some sort of order in the vast number of documented MHC alleles. Table 1.3 is a tabulated list of corresponding MHC-I haplotypes of commonly used laboratory mouse models for \textit{in vivo} modeling and experimentation.

On a different note, it has been well established that physiological recognition of MHC molecules by the TCR requires a specific antigenic peptide/MHC complex. However, it is unknown whether the recognition of MHC-I molecules by Ly49 or KIR by NK cells is modulated in a peptide-specific manner. Previous approaches to investigate
this question have involved manipulation of the peptide loading complex, such as the deletion of TAP-1/2 \textsuperscript{41,122,124,132}. However, empty MHC-I molecules are unstable and thus are not expressed significantly at the cell surface. Consequently, although TAP1/2-deficient cells are eliminated by NK cells, we cannot differentiate the cause of elimination (either missing-self recognition of MHC-I, or peptide-dependency) of TAP1/2-deficient cells. Interestingly, recent studies have shown that genetic deletion of ERAAP, an ER-resident enzyme that trims the N-termini of loaded antigenic peptides, results in an vastly altered repertoire of extended-length self peptides presented by MHC-I \textsuperscript{57,58,137,138}. This repertoire shift not only affects T cell recognition and tolerance, but it also affects B cell tolerance, as wild-type B and T cells are reactive to ERAAP\textsuperscript{+/−} cells \textit{in vitro} and \textit{in vivo}, and vice versa \textsuperscript{58}. Importantly, ERAAP\textsuperscript{+/−} animals exhibit approximately normal expression levels of cell surface MHC-I molecules, at least within a 2-fold range \textsuperscript{57}. Yet, while ERAAP\textsuperscript{+/−} MHC-I molecules present a distinct peptide antigen repertoire, it is currently unknown whether NK recognition is altered in these mice. Interestingly, recent work has shown that ERAAP-silenced lymphoma cells are readily rejected by NK cells \textit{in vivo} \textsuperscript{29}, and replacement of the untrimmed peptide with high affinity peptide averaging 8-11 amino acid in length reversed the phenotype. Thus, disruption of ERAAP appears to be an effective strategy to address the question of peptide-dependency in MHC-I-dependent missing-self recognition by NK cells.
<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>H-2^a</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>H-2^b</td>
</tr>
<tr>
<td>129</td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>H-2^d</td>
</tr>
<tr>
<td>NOD/LtJ</td>
<td>H-2^g, H-2K^d, H-2D^b</td>
</tr>
<tr>
<td>AKR</td>
<td></td>
</tr>
<tr>
<td>C3H/He</td>
<td>H-2^k</td>
</tr>
<tr>
<td>CBA/J</td>
<td></td>
</tr>
<tr>
<td>Ma/My</td>
<td></td>
</tr>
<tr>
<td>NIH/Sw</td>
<td>H-2^d</td>
</tr>
<tr>
<td>FVB/N</td>
<td></td>
</tr>
<tr>
<td>SJL/J</td>
<td>H-2^k</td>
</tr>
</tbody>
</table>

Table 1.3 MHC-I haplotypes in commonly used laboratory mouse strains
A compiled list of the corresponding MHC-I haplotypes with their mouse strains.112
1.1.5 The Nkrp1-Clr receptor-ligand family

The NKR-P1 (a.k.a., Ly55, Klrb, CD161) receptors are encoded by the Nkrp1 (Klrb) genes located within the NKC. Structurally, these receptors consist of disulfide-linked homodimers. Previously, it was thought that only a single functional inhibitory isoform existed in mice, designated NKR-P1B. However, another putative inhibitory isoform, NKR-P1G, was recently identified, the orthologue of which was recently reported to be functional in rats. In contrast, there are multiple putative stimulatory isoforms, designated NKR-P1A, NKR-P1C, and NKR-P1F. Notably, the NKR-P1D gene product appears to represent a highly divergent allele of NKR-P1B, while NKR-P1E appears to represent a pseudogene, at least in some mouse strains.

Intracellular signaling through rodent NKR-P1 isoforms is initiated via association with the Src-family tyrosine kinase, p56\textsuperscript{Lck}, which binds to a cytosolic CXCP motif found in some NKR-P1 receptors. The functional outcome following receptor cross-linking is then determined by distinct intracellular motifs such as ITAMs and ITIMs, as described previously. Specifically, stimulatory NKR-P1 isoforms contain a positively-charged transmembrane arginine residue that has been shown to promote association with the FcRγ adaptor protein via a complementary negatively-charged transmembrane aspartic acid residue. Phosphorylation of FcRγ ITAMs by Lck leads to recruitment of the S\textsubscript{H}2-containing Syk tyrosine kinase, facilitating downstream stimulatory signals. In contrast, inhibitory NKR-P1 isoforms lack a charged transmembrane residue, but instead contain a cytosolic ITIM motif, which upon phosphorylation by Lck, leads to recruitment of the S\textsubscript{H}2-containing SHP-1 tyrosine phosphatase, thereby preventing induction of co-localized stimulatory signals.
Recent published works have demonstrated that the NKR-P1 receptors bind to structurally-related ligands known collectively as the Clr family\(^{18,67,118}\). The \textit{Clr} (\textit{Clec2d}) genes are interspersed among the \textit{Nkrp1} (\textit{Klrb}) genes, which ensure co-inheritance of both loci, due to tight genetic linkage\(^{20,60,163}\). To date, many receptor-ligand pairs have been identified: the inhibitory NKR-P1B receptor binds to Clr-b\(^{18}\); in contrast, the putative inhibitory NKR-P1G receptor binds to three different ligands, Clr-d, Clr-f, and Clr-g, while the stimulatory NKR-P1F receptor binds to Clr-c, Clr-d, and Clr-g,\(^{26}\). Interestingly, work in the rat system has recently confirmed these interactions\(^{89,90}\), although nomenclature issues exist due to the expansion of the Clr family in rats\(^20\). Thus, rat NKR-P1B binds the Clr-b orthologue, Clec2d11, or Clr11\(^{60,157}\), while rat NKR-P1F binds multiple homologs of Clr-c, d, and g (including Clr2, 3, 4, 6, 7)\(^{89}\). Recently, rat NKR-P1G was also reported to share a partially overlapping ligand specificity (Clr-d2, 6, 7) with NKR-P1F\(^{89}\). Interestingly, mouse NKR-P1F also recognizes rat Clr2, 3, 4, 6, 7, and mouse NKR-P1G cross-reacts with Clr2, 4, 6, 7\(^{89}\). The cross-reactive nature of mouse receptors to rat ligands, and the overlapping homology between the different Clrs in the two species makes it extremely difficult to decipher the orthologous versus homologous relationships between mouse and rat Clrs. Table 1.4 is a compiled list of mouse Clr ligands with corresponding Clec2 nomenclature, as well as an attempt of organizing the rat Clr ligands into orthologous pairs or homologous molecules in comparison to the mouse Clr members. The situation in humans is much simpler than the rodent systems, as there is only a single receptor, NKR-P1A (CD161)\(^{93}\), which binds to the LLT1 (CLEC2D2) gene product and inhibits NK cell function\(^{2,126}\). This specificity was thought to be functionally orthologous to the rodent NKR-P1B:Clr-b interaction.
However, since human NKR-P1A lacks the murine CXCP motif, analogous to mouse NKR-P1G, and the expression pattern of LLT-1 is different than the rodent Clr-b, it is also possible that human NKR-P1A is an ortholog to rodent NKR-P1G. Moreover, the human KLRF1/2 stimulatory receptors, which bind genetically linked CLEC2B/A ligands have been postulated to be distant homologs of the murine KLRB (NKR-P1) stimulatory receptors.

Expression of rodent NKR-P1B is largely restricted to NK cells and appears to be variegated (i.e., limited to a subset of NK cells, ~60-70%)\textsuperscript{19,88,98,107}. This expression pattern is similar to that of the Ly49 receptors for MHC-I molecules, and promotes the development of distinct functional NK cell subsets with unique specificities (i.e., each NK cell is educated to recognize a different array of ligands, creating a diverse NK repertoire, more on this in the next section)\textsuperscript{123}. Importantly, Clr-b is broadly displayed on most nucleated cells in a pattern quite similar to that of MHC-I molecules\textsuperscript{18}. However, unlike MHC-I expression, Clr-b expression is β\textsubscript{2}m- and peptide-independent\textsuperscript{18,67}. This allows the ligand to function in an MHC-independent mode of missing-self recognition\textsuperscript{86} in the immunosurveillance of tumour cells\textsuperscript{18}, infected cells\textsuperscript{157}, and perhaps transplanted cells\textsuperscript{20,107}.
**Table 1.4 Rodent Clr genes**

A compiled list of mouse Clr genes (left column) with their corresponding Clec2 nomenclature (middle column), as well a comparison to rat Clr genes organized in assumed orthologous pairs or homologs based on phylogenetic analyses.  

<table>
<thead>
<tr>
<th>Mouse Clr Gene</th>
<th>Clec2 Nomenclature</th>
<th>Rat Ortholog/Homologs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clr-a</td>
<td>Clec2-e</td>
<td>Ortholog: Clr10</td>
</tr>
<tr>
<td>Clr-b</td>
<td>Clec2-d</td>
<td>Ortholog: Clr11</td>
</tr>
<tr>
<td>Clr-c</td>
<td>Clec2-f</td>
<td>Homolog to Clr1-7?</td>
</tr>
<tr>
<td>Clr-d</td>
<td>Clec2-g</td>
<td>Homolog to Clr1-7?</td>
</tr>
<tr>
<td>Clr-e</td>
<td>– (pseudogene)</td>
<td>– (Clr8? Also pseudogene)</td>
</tr>
<tr>
<td>Clr-f</td>
<td>Clec2-h</td>
<td>Ortholog: Clr9</td>
</tr>
<tr>
<td>Clr-g</td>
<td>Clec2-i</td>
<td>Homolog to Clr1-7?</td>
</tr>
<tr>
<td>Clr-h</td>
<td>Clec2-j</td>
<td>Homolog to Clr1-7?</td>
</tr>
<tr>
<td>Clr-i</td>
<td>– (pseudogene)</td>
<td>– (Clr8? Also pseudogene)</td>
</tr>
<tr>
<td>(Qiang et al. Submitted Manuscript)</td>
<td></td>
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</tr>
<tr>
<td>Clr-j</td>
<td>– (pseudogene)</td>
<td>– (Clr8? Also pseudogene)</td>
</tr>
<tr>
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</tbody>
</table>
1.1.6 NK cell development and education

Often, NK cell development and education are referred to interchangeably; here, development refers specifically to the stages a hematopoietic stem cell (HSC) undergoes to become a NK cell. These stages are characterized by the expression of cell surface markers and transcription factors. In contrast, education is the process in which immature NK cells acquire the capacity to differentiate self versus non-self entities, once committed to the NK lineage.

The primary site of adult NK cell development is the bone marrow (BM), although a unique population of NK cells originate from the thymus that may be functionally distinct compared to BM derived NKs \(^{22,23,38}\). In general, the murine NK development pathway starts with the HSCs (CD34+) in the BM progressing into common lymphoid progenitors (CLPs), triggered by the expression of the transcription factor, Ikaros \(^{63}\). Subsequently, CLPs develop into NK precursors (NKPs) accompanied by the expression of Ets-1 and PU.1 \(^{63}\). NKPs are characterized by cell surface expression of IL-2Rβ (CD122) and the absence of lineage markers (CD3–, CD19–, CD4–, CD8–, Gr-1–, CD11b–) and most NK cell receptors \(^{65}\). The key transcription factor that drives the transition of NKPs into immature NK (iNK) cells is E4BP4, encoded by the gene, *Nfil3* \(^{73}\). Progression into iNKs is characterized by the acquisition of the inhibitory receptors, NKR-P1B and CD94/NKG2A heterodimers, and by the expression of NKR-P1C (NK1.1), NKG2D, and NKP46 \(^{65}\). The hallmark of the iNK to mature NK transition is the expression of the Id2 transcription factor (Id2 is also expressed in other stages along with other members of the Id TF family, but Id2 expression is further augmented here). During this differentiation stage, Ly49 receptors, CD49b (DX5) and CD11b (Mac-1) appear on
The cytokine requirements for NK cell development explain the relevance of the expression of cell surface receptors and the transcription factors mentioned previously. NK development in vivo heavily depends on IL-15 65. Logically, NK developmental intermediates must express IL-15R. IL-15R is a heterotrimer: a unique IL-15Rα, IL-15Rβ (CD122), which is the IL-2Rβ expressed by the NKPs, and IL-15Rγ (CD132) 65. Interestingly, NKPs express IL-15R and bind IL-15 that is transpresented by cytokine presenting cells (CPCs), likely DCs or activated monocytes that express IL-15 and IL-15Rα 134. Specifically, CPCs secrete IL-15 and binds it with IL-15Rα, then subsequently transpresent the IL-15 to NKPs. One of functions of IL-15 signal transduction is to activate the E4BP4 transcription factor, since it has been shown that IL-15R−/− BMCs fail to differentiate into NK cells, but transgenic expression of E4BP4 can revert this phenotype 47. In addition, NK precursor cells express lymphotoxin β (LTβ), which can interact with lymphotoxin β receptors (LTβR) on stromal cells. This interaction triggers IL-15 secretion by the stromal cells, thus feeding into the NK developmental pathway 65. Lastly, the appearance of E4BP4 triggers the expression of Id2, thus concluding the murine NK developmental cascade 63.

Notably, human NK development can be organized into the similar stages observed in murine NK differentiation. However, transcription factors involved in the stages of human NK development are mostly based on mouse modeling; hence, it is not as well characterized as the mouse system. Briefly, human HSCs (CD34+, Lin−) acquire c-kit and Flt3 and develop into lymphoid stem cells, which subsequently progresses into the bi-potential T/NK progenitor via the expression of IL-15Rα 139. The T/NK progenitors
then express CD62L and differentiate into iNKS, characterized by the appearance of the inhibitory receptor, NKR-P1A \(^{65}\). Subsequently, these NKR-P1A+ iNKS express CD122, CD56 (human NK marker), and CD94/NKG2A heterodimer, followed by the expression of the stimulatory receptors NKp46 and NKG2D \(^{65}\). The appearance of these receptors and the expression of KIRs denote the mature NK stage in human NK cell development. Interestingly, CD56 expression is modulated in this final stage of NK cell development resulting in CD56\(^{\text{bright}}\) and CD56\(^{\text{dim}}\) NK subsets. The CD56\(^{\text{dim}}\) NK subset is believed to be the more mature subset of the two, and possesses a higher cytotoxic effector function and co-expression of CD16 (Fc\(\gamma\)RIII).

The transition from iNKS to mature NKS is where NK cell education occurs \(^{146}\). Conceptually, NK cell education is the process where NK receptors interact with their ligands to set an “activation threshold” for each individual NK cell. At least two rivaling theories have been proposed to explain the process of NK education: the disarming and licensing models \(^{123}\). The disarming model proposes that stimulatory receptors are constantly engaged by endogenous-self ligands, thus transducing a perpetual activating signal into the iNKS. If this signal persists, it will render the iNK to an anergic state. However, if an inhibitory receptor on the iNK can bind its ligand, it can rally an inhibitory signal thus reducing the strength of the activating signal in the iNKS, resulting in a functional, mature NK cell.

Alternatively, the licensing model assumes the initial state of the iNK to be hyporesponsive, unless an inhibitory receptor expressed on the iNK recognizes and binds its ligand during the window allotted for NK education. If this event takes place, the iNK will be activated or “licensed”, thus progressing into a mature NK cell \(^{123}\). However, this
model is a little counter-intuitive, since it does not consider the role of stimulatory receptors, and the notion of an inhibitory signal that activates cells is also unprecedented. Nonetheless, both the disarming and licensing models accentuate on one important concept, which is a mature NK cell must express at least one inhibitory receptor. By extension, the education process can occur via both MHC-dependent and MHC-independent inhibitory receptors. This notion can be exemplified by the following case: it is unlikely for any individual to inherit and express all of the KIRs that will recognize the array of MHC-I-molecules expressed by that person, because MHC-I and KIRs are not genetically linked. Consequently, there will always be orphan KIR inhibitory receptors expressed on the iNKs during the education process. Therefore, to prevent autoreactivity, these cells are either rendered hyporesponsive, or other MHC-independent inhibitory receptors participate in their education. This model agrees with previous works showing that “unlicensed” NK cells (no MHC-I dependent ligand engagement) still possess the ability to eliminate MCMV-infected cells, thus suggesting that MHC-independent NK education may result in the cytotoxic effector function observed in this study. In fact, NK cells in β2m-deficient mice are more responsive to MCMV-infected cells compared to WT NK cells. MCMV infected cells express m157, which is the ligand to the stimulatory NK receptor Ly49H. In B6 mice, MCMV-infected cells will be eradicated by NK cells through the predominant stimulatory signal generated by the recognition of m157 by Ly49H. However, the presence of MHC-I on the surface of MCMV-infected cells will be recognized by inhibitory NK receptors Ly49C/I, thus generating an inhibitory signal into NK cells, which will counteract the stimulatory signal generated by the recognition of m157. In contrast, MCMV-infected β2m-deficient mice will express
m157, but lack cell surface MHC-I. Therefore, NK cells will be activated through Ly49H, but not inhibited through Ly49C/I\textsuperscript{113}, thus providing rationale explaining the heightened activation of “unlicensed” NK cells in response to MCMV-infected targets. Since MCMV is known to promote downregulation of MHC-I molecules from the cell surface, the net effect on WT and β2m-deficient cells recognized by NK cells may be similar\textsuperscript{62}.

Interestingly, NK cell education may also occur in mature NK cells. Previous studies have shown that B6 splenocytes that were adoptively transferred to β2m-deficient recipients become hyporesponsive to MHC-I deficient targets\textsuperscript{43,72}. In contrast, splenocytes from β2m-deficient animals that were transferred into NK-depleted B6 recipients acquired the ability to respond to the MHC-I deficient targets\textsuperscript{43}. Thus, it appears that mature NK cells retain some functional plasticity and can be “re-educated”, since the “self” versus “non-self” entities recognized by mature NK cells can be altered by the environment in which the NK cells are situated.
FIGURE 1.2

HSC

CD34+ Sca-1+ Lin–

Ikaros*

CLP

CD34+
c-Kit+Sca-1+ IL-7Rα+ Flk2+ Lin–

Ets–1*
PU.1
Runx1/Runx3/CBFβ*

NKP

c-Kit+ IL-2Rβ+ Lin–

E4BP4
Id-2

iNK

IL-2Rβ+ NKR-P1B+ CD94/NKG2A+ NKG2D+ NK1.1+ NKp46+

Id-2 Gata-3*
MEF* IRF-2*
T-bet

Mature NK

IL-2Rβ+ NKR-P1B+ CD94/NKG2+ NKG2D+ NK1.1+ NKp46+ Ly49+
**Figure 1.2 Schematic of NK cell development from HSCs**
A generalized schematic of the NK cell developmental pathway starting with HSCs [CD34+ Sca-1+ Lin$^-$ (CD3$^-$, CD19$^-$, CD4$^-$, CD8$^-$, Gr-1$^-$, CD11b$^-$)] progressing into CLPs triggered by the expression of Ikaros. CLPs are characterized by the cell surface expression of CD34+ Sca-1+ c-Kit+ IL-7R$\alpha$ Flk2+ Lin$^-$. Subsequently, CLPs develop into NKPs, and this transition is initiated by the expression of Ets-1, PU.1 and Runx/CBF$\beta$ transcription factors. The hallmark of NKPs is the cell surface expression of IL-2R$\beta$ (CD122). In turn, NKPs express E4BP4 and become iNKs. Conventional NK receptors such as NKR-P1B, CD94/NKG2A heterodimer, NK1.1, NKG2D and NKp46 are expressed by iNKs. Lastly, many transcription factors (Id-2, MEF, T-bet, Gata-3, IRF-2) are expressed in iNKs facilitating its maturation into mNKs and acquire Ly49 expression. Transcription factors and cell surface markers are denoted in red and blue text, respectively. Asterisks indicate that the transcription factor may be involved in other stages of NK development in addition to its designated stage.
1.2 Bone Marrow Transplantation

1.2.1 Hybrid resistance

Transplantation biology can be divided into two main branches: solid organ transplantation and BM transplantation. The effector cells involved in determining acceptance or rejection of solid organ grafts are largely T cells. In contrast, NK cells largely mediate the acute rejection of BM grafts, and T cells are responsible for the chronic rejection. In addition, in solid organ transplantation, parental donor grafts can be accepted by their offspring; however, parental BM grafts will be readily rejected. The observation where parental BM is rejected by their offspring is termed “hybrid resistance”. In a laboratory setting, hybrid resistance refers to the phenomenon whereby F1 “hybrid” animals, the products of crossing two MHC-disparate strains of inbred mice, reject or are “resistant” to bone marrow grafts derived from either parent. As alluded to earlier, this phenomenon is restricted to BM grafts and hematopoietic cell transplants, since solid organ transplants from parental into F1 hybrid mice are accepted according to the rules of T cell tolerance. Hybrid resistance is known to be NK cell-mediated, because BM graft rejection occurs similarly in SCID recipients (which lack mature T and B cells), whereas NK depletion of recipient mice alleviates BM graft rejection. The postulated mechanism for hybrid resistance is as follows: the F1 hybrid animal (ie. C57Bl/6 x BALB/c) will co-dominantly express both parental MHC-I haplotypes. Consequently, mature NK cells from the F1 hybrid animal are “educated” to express a Ly49 receptor repertoire specific for, and tolerant to, both “self” haplotypes. However, since NK receptor expression is variegated, at least four subsets of F1 NK cells can be described: a subset expressing inhibitory Ly49
receptor for H-2^d, a subset expressing the inhibitory Ly49 receptor for H-2^b, another subset that expresses both Ly49 receptors described above, and a final subset that expresses neither Ly49 receptors. Thus, parental (H-2^b or H-2^d) BM cells transplanted into an F1 (H-2^{b/d}) host will be perceived to be “missing” a “self” allele by the NK cell subsets rendered tolerant to non-overlapping allelic/haplotype specificities. At the molecular level, this means that certain F1 inhibitory Ly49 receptors that recognize only H-2^b or H-2^d alleles will not be engaged, hence “missing-self” recognition will ensue, leading to the eradication of the parental BM graft. In addition, stimulatory receptors on the F1 NK cells (eg. NKG2D) can recognize the stimulatory ligands (eg. NKG2D ligands) on the parental cells, which will activate the “induced-self” recognition, ultimately leading to an exacerbated elimination of the parental BM graft. Therefore, it is evident that NK cells have a prominent role in mediating the rejection of allogeneic BM grafts, at least in the context of MHC-dependent NK recognition.

1.2.2 Bone marrow transplantation overview

The first successful bone marrow transplantation procedure was performed in 1968 involving an allogeneic BM transplantation for an infant with X-linked lymphopenic immune deficiency and for another with Wiskott-Aldrich syndrome. In modern healthcare, bone marrow transplantation is a common, yet important regimen used to treat patients diagnosed with leukemia, aplastic anemia, Hodgkin’s Disease, multiple myeloma, immune deficiency disorders and some solid tumors such as breast and ovarian cancer. Many of the diseases listed above involve the malfunctioning of stem cells in the BMCs of the patient, and BM transplantation offers a healthy set of stem cells to replace
the diseased ones.

There are multiple forms of BM transplantation: syngeneic BM transplant is when the donor is the monozygotic twin of the recipient; allogeneic BM transplant is the most common, which is when the donor is not genetically identical to the recipient; and finally autologous BM transplant is harvesting BM of the patient and re-infusing into the patient. Due to the vastly polymorphic HLA genes in humans, the odds of finding an unrelated, but genetically matching BM donor is 1 in 20,000 to 1 in 100,000. The minimum requirement that defines a “suitable” donor is to search for a donor that exhibits no polymorphism in the expressed HLA-A, -B, -C (MHC-I) and –DRB1 (MHC-II) alleles compared to the recipient, and needs to be CMV serologically negative. However, donor BMCs that are also genetically matched at other HLA loci (eg. HLA-DQ, -DP) demonstrate better prognosis and engraftment post-transplantation. Clearly, this search is extremely difficult, and is one of the key-limiting steps in successful BM transplantation therapy. When a matching BM donor is available, the patient will undergo the “conditioning” step, which is the administration of both chemo and radiation therapy to eradicate the defective BM, followed by the BM transplantation. The recipient is then monitored closely for signs of graft-versus-host disease (detail described below) and given antibiotics in the next 2-4 weeks post-transplantation to prevent opportunistic infections (ablation of BM during the conditioning stage leaves the patient in a temporary immune-compromised state).

1.2.3 The flipside of the coin: graft-versus-host disease (GVHD) and graft-versus-tumour (GVT) effects
Searching for suitable allogeneic BM donors is now the standard approach for BM transplantation therapies. However, prevention of allogeneic graft rejection by the recipient remains a challenge. In addition, there are cases where the allogeneic donor BM graft can attack the recipient, leading to a life-threatening state in the host. This phenomenon is known as the graft-versus-host disease (GVHD), and is thought to be caused primarily by the T cells that reside in the donor BM graft \(^44\). In fact, for GVHD to occur, several conditions must be met. Firstly, the donor graft must contain T cells. Secondly, the recipient must express cell surface molecules that are considered “foreign” by the donor. Lastly, the T cell of the recipient must not be functional \(^44\). Evidently, a majority of these criteria are observed in an allogeneic “conditioned” recipient who received chemo and radiation therapy, where they are immune compromised and likely express some allogeneic HLA alleles. Solutions to circumvent GVHD include T cell depletion of the donor graft and finding HLA-matched donors and recipients. As previously mentioned, finding 100% matching donors is extremely difficult, and T cell depletion from the donor graft increases the potential for engraftment failure and the chance of disease relapse \(^55\). Engraftment failure of the transplanted graft is likely a result of the elimination of Tregs from the donor graft during T cell depletion. In addition, Tregs appear to have an instrumental role in preventing GVHD. Interestingly, the principles of GVHD have been applied for therapeutic purposes in which the graft-versus-tumour (GVT) approaches are used to eliminate the residual tumour for disease relapse scenarios. The final piece of the puzzle remains: is it possible to control GVHD, yet allow the GVT effect to prevail and thus improve the health status of the patient? Previous work in mouse models have demonstrated that infusion of autologous NK cells
in conjunction with an allogeneic BM graft into mouse recipients carrying a metastatic colon adenocarcinoma can facilitate the GVT effect, and GVHD was not reported\textsuperscript{5}. Further investigations showed that the effector cytokine produced by the autologous NK cells that promoted GVT and prevented GVHD is TGFβ\textsuperscript{5}, as neutralization of TGFβ ablated GVT effects, and the recipient mice died from GVHD.

Ultimately, NK cells appear to have an instrumental role in improving the quality of clinical therapeutics, specifically in BM transplantation. They can determine the outcome of BM engraftment; they may also be the solution to the ramification and complications that arise from allogeneic BM transplantation such as GVHD; and finally they can exert a GVT effector function while preventing GVHD. Despite the great therapeutic potential and clinical applications that NK cells possess, more research is required to fully comprehend the biology of NK cells. A thorough understanding of NK biology should increase the success in transplantation regimens, as well as options for current cancer therapies.
1.3 Thesis Goals

The roles of MHC-dependent missing-self recognition by NK cells in tumour surveillance, infectious diseases and bone marrow transplantation have been well established. In contrast, the functional importance of MHC-independent missing-self recognition by NK cells in the immunological sense has only recently emerged, shown by data delineating that the missing-self recognition of Clr-b by NK cells leads to eradication of transformed, or virally-infected cells \(^{45,157}\). However, the role of this MHC-independent missing-self recognition axis in determining the fate of transplanted cells remains to be elucidated. In addition, there are still many Nkrl receptors and Clr ligands in the mouse genome that require characterization, with the possibility of additional Nkrl-Clr receptor-ligand pairs, such as NKR-P1B:Clr-b, and NKR-P1F-Clr-g \(^{67}\).

The goals of my thesis were to characterize NKR-P1G, the other inhibitory receptor in the Nkrl receptor family, to determine additional novel Nkrl-Clr receptor-ligand pairs, and to evaluate the role of missing-self recognition of Clr-b by NK cells in transplanted graft. This will further our understanding of the MHC-independent recognition arm of NK cell biology. Importantly, we hope this study will contribute positively to the prediction of bone marrow transplantation engraftment outcomes in clinical settings.
CHAPTER II

METHODS
2.1 Cells

BWZ.36 cells (Sanderson and Shastri, 1994) were a gift from Dr. Nilabh Shastri (University of California, Berkeley, USA). YB2/0 rat myeloma cells, 293T human embryonic kidney cells and P3XAg8.653.1 (P3) mouse myeloma cells were obtained from Dr. David Raulet (University of California, Berkely, USA).

Ex vivo femurs, lymph nodes, spleen and thymus were harvested from either Lewis rats, 129S1, B6, B6.Clr-b\(^{-/-}\), B6.\(\beta\)2m\(^{-/-}\), B6.H-2K\(^{b/-}\), B6.H-2K\(^{b/-}\), B6.2D\(^{b/-}\), B6.Clr-b\(^{-/-}\)/\(\beta\)2m\(^{-/-}\), and ERAAP\(^{-/-}\) mouse strains and processed into a single cell suspension. Cells were then treated with ACK lysis buffer (Life Technologies, Burlington, ON) for RBC removal and passed through a 40\(\mu\)m filter (BD Biosciences, CA, USA).

Brain, kidney, liver, lung and heart were also harvested from the mouse strains mentioned above, and then processed into a single-cell suspension using GentleMACS tissue dissociator (Miltenyi Biotech, MA, USA) via protocols provided by the manufacturer. The single-cell suspension was treated with the RBC removal protocol described above to obtain viable cells.

All immortalized cell lines were cultured in Dulbecco’s Modified Eagle’s Medium-High Glucose (DMEM-HG), with 2 mM glutamine, 10 U/mL penicillin, 10 mg/mL streptomycin, 100 mg/mL gentamicin, 110 mg/mL sodium pyruvate, 50 mM 2-mercaptoethanol, 10 mM HEPES, supplemented with either 10% or 20% FCS.

Plastic-adherent LAKs were made by culturing B6 or Clr-b\(^{-/-}\) splenocytes in 10% RPMI medium with 2500 U/mL of rhIL-2 (Proleukin; Novartis) for 6 days. Fresh medium was supplemented every 48 hours.
2.2 Animals

B6, B6.CD45.1, 129S1, B6.β2m–/– mice and Lewis rats were purchased from the Jackson Laboratory (Bar Harbor, ME, USA); B6.H-2KbDb–/–, B6.H-2Kb–/–, B6.H-2Db–/– mice were purchased from Taconic (Hudson, NY, USA); NIH.Swiss mice were purchased from NCI Frederick (Frederick, MD, USA); B6.ERAAP–/– mice were provided by Dr. Nilabh Shastri (University of California, Berkeley, USA); B6.Clr-b–/– mice were provided by Dr. Matthew Gillespie (University of Melbourne, Australia); B6.Clr-b–/–/β2m–/– double knockout mice were generated by crossing B6.Clr-b–/– and B6.β2m–/– mice resulting in F1 progeny heterozygous for both genes, and subsequently intercrossing the F1 mice to achieve homozygosity for both loci; CD45.1/CD45.2 double congenic mice were generated by crossing B6 (CD45.2) and B6.CD45.1 mice resulting in F1 progeny expressing both CD45.1 and CD45.2 antigens. All animals were housed and maintained in the in-house facilities at Sunnybrook Research Institute and Donnelly Centre for Cellular and Biomolecular Research (CCBR), University of Toronto.

2.3 RT-PCR

RNA was isolated from bone marrow, lymph node, spleen, thymus, brain, kidney, liver, colon, lung, heart and d6 LAKs from B6, B6.Clr-b–/– and 129S1 animals using an RNA purification kit (Invitrogen, Burlington, ON). RT reactions were performed using a Superscript III cDNA synthesis kit (Invitrogen, Burlington, ON) according to manufacturer’s instructions.

2.4 PCR and cloning
PCR amplification procedures for cloning and transcript analyses were executed using ExpandPLUS High Fidelity enzyme (Roche Applied Science, Laval, QC). For cloning purposes, PCR products were directly cloned into pcDNA3.1/V5/His/TOPO (Invitrogen, Burlington, ON), and sequenced to confirm identities (Macrogen Inc., Seoul, South Korea). Ectodomains of Nkrp1a/b/c/f/g from either B6 or 129S1 strain were cloned by using the confirmed Nkrp1 full-length clones in the pcDNA3.1/V5/His/TOPO vector as template and the corresponding primers indicated in table 2.1. Semi-quantitative PCR experiments were controlled by using threefold serial dilution of equal amounts of cDNA (125 ng) as a template for the amplification process. All primers used are listed in Table 2.1.

**TABLE 2.1**

<table>
<thead>
<tr>
<th>Primer Specificity</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
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<td>GCG GCC GCT CAG TGT CCA TAA CCC ACA TAG</td>
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<tr>
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<td>GCG GCC GCT CAG GAG TCA TTA CTC GGG GT</td>
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| NKR-P1F<sup>B6</sup> | CTC GAG GCC ACC ATG  
GAC ACA TCA AAG GTC  
CAT G | GCG GCC GCT CAG ACA TGT  
ATC AGG GTC TTT TG |
|-------------------|-------------------------------------------|
| NKR-P1G<sup>B6</sup> | CTC GAG GCC ACC ATG  
GAT GCA CCA GTG CTC  
TAT G | AGC GGC CGC TCA GAC GTG  
TTT CAG TGT CTT TTG |
| NKR-P1A<sup>129</sup> | Same as NKR-P1A<sup>B6</sup> | Same as NKR-P1A<sup>B6</sup> |
| NKR-P1B<sup>129</sup> | Same as NKR-P1B<sup>B6</sup> | AGC GGC CGC TCA GGA GTC  
ATT ACT CGG GGT TTT ATG |
| NKR-P1C<sup>129</sup> | CTC GAG ATG GAC ACA  
GCA AGG GTC TAC TTT G | Same as NKR-P1C<sup>B6</sup> |
| NKR-P1F<sup>129</sup> | Same as NKR-P1F<sup>B6</sup> | GCG GCC GCT CAA ACA TGT  
AGC AGG GTC TTT TG |
| NKR-P1G<sup>129</sup> | Same as NKR-P1G<sup>B6</sup> | AGC GGC CGC TCA GAT GTG  
TTT CAG TGT CTT TTG |
| NKR-P1A<sup>B6</sup>-EC | CTC GAG CGA GTC CTA  
ATA CAA AAA CCA TC | Same as NKR-P1A<sup>B6</sup> |
| NKR-P1B<sup>B6</sup>-EC | CTC GAG TCA GTA CAA  
AAA TCA TCA GTA CAA  
AAA ATC | Same as NKR-P1B<sup>B6</sup> |
| NKR-P1C<sup>B6</sup>-EC | CTC GAG CGA GTC TTA  
GTA CAA AAA CCA TCA AG | Same as NKR-P1C<sup>B6</sup> |
<p>| NKR-P1F&lt;sup&gt;B6&lt;/sup&gt;-EC | CTC GAG AGA TTC CTA | Same as NKR-P1F&lt;sup&gt;B6&lt;/sup&gt; |</p>
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<tr>
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<td>Same as NKR-P1F&lt;sup&gt;H6&lt;/sup&gt;-EC</td>
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<td>ATC AC</td>
<td>TA</td>
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</table>
Table 2.1 Primers used throughout this work

Primers used for cDNA cloning, retroviral vector sub-cloning and semi-quantitative RT-PCR. EC indicates the extracellular domain of the corresponding gene; Δ indicates stop-codon (TCA)-deleted EC primer sequence. Underlined sequences indicate restriction enzyme sites used for retroviral vector subcloning. (XhoI site: CTC GAG; NotI site: GCG GCC GC).

2.5 Retroviral constructs

The pMSCV2.2-CMV-IRES-GFP, the type II transmembrane CD3ζ-fusion and the type II transmembrane CD3ζ-fusion-flag retroviral expression vectors were generated and provided by Dr. Aruz Mesci (University of Toronto). Full-length inserts of Nkrp1a/b/c/f/g and Clr-b/c/d/f/g cDNA constructs from either B6 or 129S1 strains (amplified from d6 LAK cDNA and cloned into pcDNA3.1/V6/His/TOPO) were sub-cloned into the pMSCV2.2-CMV-IRES-GFP retroviral expression vector; EC sequences of Nkrp1a/b/c/f/g were sub-cloned into the type II transmembrane CD3ζ-fusion retroviral expression vector; the EC sequence of Nkrp1g with the stop-codon deletion was sub-cloned into the type II transmembrane CD3ζ-fusion-flag retroviral expression. All of the sub-cloning used XhoI and NotI restriction sites.

2.6 Retroviral transduction

Retroviral supernatants were generated by triple transfection of the proviral vector (containing the gene of interest, eg. NKR-P1B<sup>B6</sup>) with gag/pol + VSV-G/env constructs into 293T cells using Effectene reagent (Qiagen, Mississauga, ON). The culture medium was replaced after 24 hours, and the supernatants were collected at 48 hours post transfection. The retroviral supernatants were used to spin-fect approximately 2x10<sup>5</sup>
BWZ or YB2/0 cells for 90 minutes at 1000xg in 20% FCS plus 8µg/mL polybrene. Stable transductants were sorted 72 hours post spinfection for high GFP expression via FACS.

2.7 Flow cytometry

10 µl of 2.4G2 supernatant (approximately 0.5mg/mL) was added to ex vivo cells or cell lines that expressed FcR for 10 minutes on ice to prevent non-specific staining from the interaction of FcR with Fc portions of mAb used for staining. Post Fc block, cells were stained with primary mAbs on ice for 25 minutes, followed by washing and incubation in secondary reagents (25 minutes for anti-Ig mAb, 20 minutes for streptavidin conjugates). Stained or membrane-labeled cells (refer to subsequent sections of the methods chapter) were analyzed using FACSCalibur flow cytometer, BD LSR II, or BD LSR II Special Order System (BD Biosciences, San Jose, CA, USA). For data acquired on the FACSCalibur flow cytometer, cells were gated on FSC and SSC, followed by propidium idodide dye exclusion for cell viability; for data acquired on LSR, cells were gated on FSC-A and SSC-A, followed by doublet exclusion (FSC-W x FSC-H, SSC-W x SSC-H) and DAPI exclusion for cell viability.

Various mAbs were used to verify cell-surface protein expression, or to analyze immune composition of the Clr-b–/– animals and the long-term mixed bone marrow chimeras in comparison to the B6 wild type animals. The complete list of the mAbs used for this work is listed in table 2.2.
TABLE 2.2

<table>
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<tr>
<th>Clone</th>
<th>Recognized Ag</th>
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<th>Conjugated Fluorophore</th>
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<td>AlexaFluro700</td>
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Table 2.2 mAb used for flow cytometric analysis

4A6, 2D12, 2.4G2, and 1H8 mAb were purified in-house; the production of 1H8 will be described in detail in chapter 3. PKH67, PKH26 and CellVue® Maroon are membrane dyes that were used to track different bone marrow populations for the bone marrow rejection assay. Usage of the dyes is described below.

2.8 Cell labeling and competitive BM rejection assay

Various membrane dyes were used to label donor bone marrow cells according to manufacture’s instructions. Briefly, BMCs derived from B6, Clr-b−/−, Clr-b−/−/β2m−/−, ERAAP−/− and various MHC-I-deficient animals (β2m−/−, H-2K−/−, H-2D−/−, H-2K−/−, H-
2D<sup>b−/−</sup>) were either singly or combinatorily labeled with PKH26 (Sygma-Aldrich, Oakville, ON), PKH67 (Sigma-Aldrich, Oakville, ON) or CellVue® Maroon (eBioscience, CA, USA). All of the labeled BMCs are then counted and pooled together to generate a BM mixture that has approximately equal proportions of each individual WT and genetically modified BMCs.

To assess acute BM rejection, mixed BM chimeras were generated as follows: the NK cell-depleted cohort (either B6 or NIH.Swiss) was intraperitoneally injected with 150- 200 µg of PK136 mAb (Sunnybrook Hybridoma Core Facility, ON) on day –2, and both the NK-depleted and wild type cohorts were sublethally irradiated (6.5 Gy) with a cesium- sourced Mark I irradiator (J.L. Shepherd & Associate, CA, USA) on day –1. Subsequently, both cohorts were intravenously injected with the membrane-labeled bone marrow mix on day 0. Each recipient received approximately 21x10<sup>6</sup> BMCs. The recipient spleens were harvested 24-48 hours post BM transplant and analyzed by flow cytometry for the presence of membrane-labeled cells.

The % rejection of the experimental candidate donor relative to the autologous donor (or which ever donor origin desired) was determined by the following equation:

\[
\left\{1 - \frac{\left(\frac{\text{proportion of donor BM of interest}}{\text{proportion of autologous BM}}\right)_{\text{input}}}{\left(\frac{\text{proportion of donor BM of interest}}{\text{proportion of autologous BM}}\right)_{\text{output}}}\right\} \times 100\%
\]

Long-term mixed bone marrow chimeras were made by administering 9.5 Gy of radiation to a double-congenic cohort (CD45.1+ CD45.2+) on day –1 followed by transplanting 10<sup>7</sup> cells of the donor bone marrow mix (comprised of 5 x 10<sup>6</sup> B6.CD45.1 BMCs and 5 x 10<sup>6</sup> Clr-b−/− (CD45.2+) BMCs] into the lethally irradiated recipients. Three to five weeks after transplantation, lymphoid tissues (BM, spleen, thymus and lymph
nodes) as well as liver were harvested and analyzed via flow cytometry.

2.9 BWZ Reporter Cell Assays

Reporter cells were made by retrovirally transducing BWZ cells with the various 
Nkrp1-EC proviral constructs and sorted for GFP+ cells as described above; stimulator 
cells were generated by transfecting 293T cells with the various Clr constructs using 
Effectene reagent. Transfection efficiency was verified by examining IRES-GFP 
expression via flow cytometry 24 – 48 hours post-transfection. Stimulator cells were 
added at $10^5$ cells per well followed by a threefold serial titration across a 96-well round- 
bottom plate, then cocultured with $3–5 \times 10^4$ reporter cells overnight. The next day, cells 
were spun down in the plates, and the supernatant was decanted. Cells were then 
resuspended in 100 µL of 1× CPRG buffer [90 mg/L chlorophenol-red-β-D-
galactopyranoside (Roche Diagnostics, Indianapolis, IN), 9 mM MgCl2, 0.1% NP-40, 
in PBS]. Plates were incubated at 37°C or at room temperature, and then analyzed using a 
microplatereader (Varioskan) with a signal background subtraction set at OD (595–655 
nm). ΔOD values represent the specific BWZ.CD3ζ/NKR-P1 responses to 293T.Clr 
stimulator cells following subtraction of the baseline responses of parental BWZ cells to 
identical stimulator cells.

For ex vivo-based cell stimulation assays, $3–5 \times 10^4$ reporter cells were cocultured 
overnight with ex vivo BMCs or splenocytes at $10^6$ cells per well followed by serial 
threefold titrations. For PMA/ ionomycin stimulation, the cells were stimulated overnight 
with 10 ng/mL PMA and 0.5 µM ionomycin. Plates were developed as above.

For plate-bound stimulation assays, capturing antibodies (eg. Anti-ratIgG/IgM)
were immobilized on 96-well flat-bottom plate at 10µg/mL (in 50-75 µL) overnight. The antibody-coated plates were washed 2-5 times with 200 µL of PBS, then 100 µL hybridoma supernatants or capture mAb (10 µg/mL) of interest were added. Plates that contained hybridoma supernatant were incubated overnight, and plates that captured the mAb of interest were incubated for one hour. Plates were then washed 2-5 times with 200 µL of PBS followed by the addition of 3–5 × 10⁴ reporter cells to each well and incubated overnight. Plates were developed as previously described.

2.10 Hybridoma Generation, Screening and Hybridoma Supernatant isotyping.

Lewis rats were injected intraperitoneally with cells expressing mouse NKR-P1G on the cell surface (Z.G Full Length, Z.G EC, Z.G EC-Flag, YB.G Full Length, YB.G EC, YB.G EC-Flag; 10⁶ of each cell type was injected). 3-4 week intervals after the initial injection, the second and third immunizations were carried out using protocols described above. The first boost was performed with the same cell mix as the initial immunization; however, the final boost consisted only rat cell lines expressing the mouse antigen (YB.G Full Length, YB.G EC, 3x10⁶ each). Three days after the final boost, the rat spleen was harvested and processed into splenocytes, filtered through a 40 µm strainer and separated by density gradient centrifugation following underlay of the cell suspension with Lympholyte-Mammal cell separation medium (Cedarlane Laboratories, Hornby, ON, Canada) according to the manufacturer’s instructions. The rat splenocytes were fused with mouse P3XAg8.653.1 (P3) cells at a 1:1 ratio via standard techniques ⁷⁹. The fused cells were resuspended in 20% media and distributed in 96-well plates (100 µL per well) for overnight recovery in the incubator. The next day, 100 µL of 20% media supplemented
with 2x HAT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and selection was carried out for one week (total 200 µL supernatant per well). On day 8 (referencing from day of fusion), 100 µL of 20% HT media was added to each well (total 300 µL supernatant per well) and hybridomas were allowed to grow further for approximately five-seven more days with feeding and media changes. 12-14 days after fusion, hybridoma supernatants were collected and screened via plate-bound stimulation of BWZ.NKR-P1G cells via a CELLISA protocol described above. Fresh 20% media replaced the collected supernatant, and the supernatant was collected and screened again 24-48 hours after the first screen. The hybridoma cells were frozen down in freezing medium (90% FCS, 10% DMSO) in 96-well format.

Rat hybridoma supernatants were tested with an ELISA-based rat immunoglobulin isotyping kit (BD Biosciences), according to manufacturer’s instructions.

2.11 Chromium Release Assay

Effect LAK cells were generated as above. Effector cells were added at 1.5-2.5x10^5 cells per well (in 100 µL) followed by a threefold serial titration across a 96-well round-bottom plate.

1-3 x10^6 target cells were incubated with 100mCi of sodium chromate (Perkin Elmer) in 100 µL of FCS at 37°C for one hour followed by at least three washes with 1mL 10% media. The target cells were then resuspended in 10% media and plated into round-bottom plates that contained the effector cells at a concentration of 3-5 x 10^4 cells (in 100µL) per well. For maximum chromium release, 100 µL of 2% NP40 was added to
the target cells in place of the effector cells; for spontaneous chromium release, 100 µL of 10% media was added to the target cells in place of the effector cells. The plates were incubated at 37°C for four hours. Post incubation, 100 µL of the supernatant from the experimental plates was transferred on to scintillation plates (Perkin Elmer) to air-dry overnight and counts were read using a Top Count NXT Microplate Scintillation Counter (Packard Instrument Company) the next day.

Specific lysis of the target cells was calculated as follows: % specific lysis =

\[
\frac{\text{experimental chromium released} - \text{spontaneous chromium released}}{\text{maximum chromium released} - \text{spontaneous chromium released}} \times 100\%
\]
CHAPTER III

CELLISA: Reporter Cell-Based Immunization and Screening of Hybridomas
Specific for Cell Surface Antigens

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All assays were performed by P. Chen; hybridoma fusion was performed with the assistance of Dr. J.R. Carlyle; manuscript preparation was assisted by Dr. A. Mesci.

This work has been published in Immune Receptors: Methods and Protocols, Methods in Molecular Biology 748 (2011) 209-225.
3.1 ABSTRACT

mAbs specific for cell surface antigens are an invaluable tool to study immune receptor expression and function. Here, we outline a generalized reporter cell-based approach to the generation and high-throughput screening of mAbs specific for cell surface antigens. Termed CELLISA, this technology hinges upon the capture of hybridoma supernatants in mAb arrays that facilitate ligation of an antigen of interest displayed on BWZ reporter cells in the form of a CD3ζ-fusion chimeric antigen receptor (zCAR); in turn, specific mAb-mediated cross-linking of zCAR on BWZ cells results in the production of β-galactosidase enzyme (β-gal), which can be assayed colorimetrically. Importantly, the BWZ reporter cells bearing the zCAR of interest may be used for immunization as well as screening. In addition, serial immunizations employing additional zCAR or native antigen-bearing cell lines can be used to increase the frequency of the desired antigen-specific hybridomas. Finally, the use of a cohort of epitope-tagged zCAR (e.g., zCAR\textsuperscript{FLAG}) variants allows visualization of the cell surface antigen prior to immunization, and coimmunization using these variants can be used to enhance the immunogenicity of the target antigen. Employing the CELLISA strategy, we herein describe the generation of mAb directed against an uncharacterized natural killer cell receptor protein.
3.2 INTRODUCTION

Monoclonal antibodies (mAbs) can be instrumental in studying protein expression and function, receptor signaling pathways, ligand specificities, protein–protein interactions, and a myriad of other cellular and molecular properties. Conventional immunization approaches often require a large amount of purified, nonnative (e.g., refolded) protein or the synthesis of several small peptide fragments of the antigen of interest. Such approaches frequently yield mAbs specific for improperly folded forms of the antigen of interest or mAbs with only weakly reactive or highly cross-reactive properties. In addition, subsequent hybridoma screening can be laborious, frequently involving sequential analysis of duplicate samples. For example, flow cytometric analysis of cells ectopically expressing an antigen usually involves parallel analysis of mAb reactivity toward a parental cell line for each individual hybridoma supernatant. This screening can involve costly secondary reagents, and the identification of mAbs with a desired specificity can be obscured by oligoclonal hybridoma outgrowths within single wells.

On the other hand, whole cell-based immunization approaches avoid large-scale production and purification of the antigen of interest, and can be used to generate an enriched library of hybridomas and mAbs specific for native antigenic epitopes. Furthermore, when the immunogen is coupled to a reporter cell that generates endogenous enzyme, hybridoma selection can be simplified into an efficient high-throughput screening (HTS) technology. Here, we report an enzymatic reporter cell-based adaptation of hybridoma screening technology, termed CELLISA. This method involves central use of the BWZ reporter cell line bearing a CD3ζ-fusion
chimeric antigen receptor (zCAR)\textsuperscript{18,21,106,133,148,157}. The cell surface zCAR serves as a target for the desired antibodies, both in vivo during immunization, as well as in vitro during hybridoma screening, since the reporter cell produces β-gal upon ligation of the antigenic epitopes presented by the mAb-immobilized zCAR\textsuperscript{106}.

CELLISA offers several advantages: (1) large-scale production of the protein of interest is unnecessary; (2) antigens can be presented in their native form at the cell surface; (3) a single cell line can be used for immunizations as well as screening, since BWZ cells produce their own β-gal enzyme in response to zCAR ligation (thus, obviating the need for expensive secondary reagents); (4) HTS of hybridoma supernatant arrays can be achieved in a single day using an inexpensive colorimetric substrate; (5) duplication of effort is not required during primary HTS, although secondary validation of hybridoma specificities can be performed using the parental BWZ cell line; and (6) mAb of desired specificity can be uniquely identified among oligoclonal hybridoma populations, since only positive ligation events are visualized even in the presence of mixed mAb specificities.

In addition, we recently modified our CELLISA immunization strategy in order to optimize the generation of mAbs of desired specificity and quality. In order to amplify the response to the antigen of interest, we took advantage of several previously observed immune phenomena. First, to encourage linked recognition (the promotion of T:B collaboration during an immune response by inclusion of both B and helper T cell epitopes into a single immunogen), we constructed an epitope-tagged variant of our original zCAR vector (zCAR\textsuperscript{FLAG})\textsuperscript{106}. Here, the inclusion of a strongly immunogenic and highly charged FLAG epitope sequence (DYKDDDDK) serves to enhance the
presentation of helper T cell epitopes in the linked antigen following the uptake by primary FLAG-specific B cells. Of course, the FLAG epitope offers several additional advantages, including the ability to detect surface expression of the tagged antigen, thus \textit{by proxy} inferring similar surface expression of the native (nontagged) antigen of interest. Furthermore, the FLAG tag serves to encourage intramolecular epitope spreading (the directed expansion of antibody responses from a dominant primary epitope to secondary or cryptic epitopes on a linked protein sequence). This can enhance the overall immunogenicity or breadth of the antibody response to an otherwise weak target antigen, amongst a multitude of cellular antigens. Finally, to encourage immune focusing toward the target antigen itself \cite{18}, and thereby discourage the expansion of antibody responses toward nontarget cellular antigens, the same zCAR vectors are used to transduce distinct cell lines (for example, from different species). The use of different zCAR-bearing cell lines during the priming and boosting immunizations focuses the antibody response toward the shared antigen of interest, while at the same time promoting antibody class switching to IgG isotypes. Importantly, following hybridoma generation, supernatants are screened using the native (nontagged) zCAR-bearing BWZ reporter cells in order to visualize only desired specificities. Taken together, these flexible adaptations were used to derive novel mAbs specific for an uncharacterized and conserved (i.e., weakly immunogenic) NK cell receptor, mouse NKR-P1G \cite{20,107}. 
3.3 RESULTS

3.3.1 Generation of cell lines expressing native NKR-P1G\textsuperscript{B6} and its fusion receptor (zCAR) variants

NKR-P1B and NKR-P1G are the only inhibitory receptors in the NKR-P1 gene family for both mice and rats. Reports on the receptor:ligand pair of NKR-P1B:Clr-b and its importance in infectious disease models have recently emerged \textsuperscript{157}, whereas studies of NKR-P1G and its cognate ligands are less well characterized, thus making it a good candidate to generate mAb to shed light on the biology of this inhibitory NK receptor.

Construction of BWZ reporter cells expressing a NKR-P1B/CD3\(\zeta\) fusion receptor was vital in the generation of hybridoma clone, 4A6 \textsuperscript{18}, which secreted mAb against mouse Clr-b. Thus, we followed a similar approach to generate cell lines expressing NKR-P1G. A preexisting pCR2.1 plasmid clone encoding the full-length NKR-P1G molecule of C57BL/6 origin was used as the PCR template to clone out NKR-P1G\textsuperscript{B6} full-length, NKR-P1G\textsuperscript{B6}-ectodomain and NKR-P1G\textsuperscript{B6}-EC\(\zeta\) (TGA-deleted) using primer pairs indicated in table 2.1 (The two latter inserts were used to generate zCAR+ cell lines). The PCR products were visualized (Fig. 3.1B), excised, purified, and directly cloned into the pcDNA3.1/V6/His/TOPO vector, then sub-cloned into the pMSCV2.2-CMV-IRES-GFP vector or its variants according to methods outlined in section 2.5 (Fig. 3.1A). Retroviral supernatants for these constructs were subsequently generated and used to transduce BWZ \textsuperscript{135} and YB2/0 cells, which were then sorted for GFP+ to enrich for NKR-P1G+ or zCAR+ cells.

In order to visualize cell surface expression of NKR-P1G on the newly generated cell lines, epitope-tagged zCAR+ cell lines (BWZ.CD3\(\zeta/P1G\textsuperscript{B6.FLAG} and
YB.CD3ζ/P1G^{B6.FLAG} were stained with biotinylated α-FLAG mAb and streptavidin-conjugated secondary reagent, then analyzed via flow cytometry. As shown in Fig. 3.2, all sorted transductants express high levels of GFP, and FLAG epitope expression is confirmed on BWZ.CD3ζ/P1G^{FLAG} cells, indicating that the fusion receptor is expressed on the cell surface.
**Figure 3.1** PCR amplification and cloning of the NKR-P1G coding sequence variants into the native and CD3ζ-fusion chimeric antigen receptor (zCAR)-based retroviral vectors

(A) Schematic diagram of the three NKR-P1G variants. Rectangles indicate protein domains; bold text indicates NKR-P1G insert sequences; italics indicate sequences present in the pMCIG, zCAR, and zCARFLAG fusion vectors; Flag, sequence encoding the DYKDDDK epitope tag; X, Xho I restriction site; N, Not I restriction site; and TGA, stop codon. (B) PCR products amplified for subcloning of the mouse NKR-P1G inserts. Full-length (P1GFL), extracellular domain (P1GEC), and stop-codon-deleted ectodomain (P1GECΔ) variants are shown with their corresponding sizes (in bp) below the bands. Standard bands from the 1 kb Plus DNA Ladder and their sizes are given on the left.
Figure 3.2 Flow cytometric analysis of BWZ transductants of the NKR-P1G variants

$10^6$ cells were stained with α–FLAG-biotin mAbs followed by secondary streptavidin-APC. Cells were gated on according to the size and lack of propidium iodide to exclude dead cells and cellular debris.
3.3.2 Use of NKR-P1G+ and zCAR+ cells for immunization and hybridoma generation

Lewis rats were immunized with a total of $6 \times 10^6$ cells according to procedures described in section 2.10. Here, we used $10^6$ of each cell line generated (BWZ.NKR-P1G$^{B6,FL}$, BWZ.CD3ζ/P1G$^{B6,EC}$, BWZ.CD3ζ/P1G$^{B6,FLAG}$, YB.NKR-P1G$^{B6,FL}$, YB.CD3ζ/P1G$^{B6,EC}$, and YB.CD3ζ/P1G$^{B6,FLAG}$) for the initial immunization. Boosts were administered in 3-4 week intervals after the initial immunization, and the first boost used the identical cell mix as the primary immunization. The final boost, however, used only $3 \times 10^6$ of YB.NKR-P1G$^{B6,FL}$ and YB.CD3ζ/P1G$^{B6,EC}$ cells to minimize antibody responses mounted against endogenous BWZ cell antigens or to the FLAG epitope tag. Furthermore, since YB2/0 was derived from rats, the immunization strategy was designed so the antibody response would be focused on the ectopic mouse antigen on the rat cells, in this case, NKR-P1G$^{B6}$. Three days after the final boost, the rat spleen was harvested to generate hybridomas via procedures outlined previously. The immunization timeline is outlined in Fig. 3.3.
**Figure 3.3 Schematic diagram of the immunization protocol used**

Time elapsed following the first immunization is shown above the line. Black arrows indicate dates of immunizations performed and dotted arrow indicates date of harvest. Transductants used for immunization are given below each injection. All injections were performed intraperitoneally.

**Cells:**
- BWZ.NKR-P1G^{FL}
- BWZ.CD3ζ/P1G
- BWZ.CD3ζ/P1G^{FLAG}
- YB.NKR-P1G^{FL}
- YB.CD3ζ/P1G
- YB.CD3ζ/P1G^{FLAG}

**#Cells:**
- 1x10^6 each
- 1x10^6 each
- 3x10^6 each

Figure 3.3 Schematic diagram of the immunization protocol used
Time elapsed following the first immunization is shown above the line. Black arrows indicate dates of immunizations performed and dotted arrow indicates date of harvest. Transductants used for immunization are given below each injection. All injections were performed intraperitoneally.
3.3.3 CELLISA Screening

In order to test for the presence of NKR-P1G\textsuperscript{B6} specific antibodies in the hybridoma supernatants, the “CELLISA” screening strategy was employed where antibodies within hybridoma supernatants were captured by immobilized goat anti-rat IgG/IgM capture antibody on 96-well high-binding chemistry plates, followed by the addition of BWZ.CD3ζ/P1G\textsuperscript{B6,EC} reporter cells (refer to section 2.9 and 2.10 for detailed procedure). Only wells that contain α–NKR-P1G\textsuperscript{B6} antibody will be able to stimulate the BWZ.CD3ζ/P1G\textsuperscript{B6,EC} reporter cell. The crosslinking of the zCAR (BWZ.CD3ζ/P1G\textsuperscript{B6,EC}) by α–NKR-P1G\textsuperscript{B6} antibody on will activate the zCAR bearing BWZ cells through the conventional T cell activation pathway. This ultimately leads to the activation of the AP1, NFκB, and NFAT pathways. Since the BWZ parental cell line have been transgenically modified to encode the LacZ gene downstream of a triple tandem NFAT promoter element, the expression of NFAT will activate LacZ expression thus producing β-galactosidase, which can then be assayed colourimetrically\textsuperscript{106}.

As shown in Fig. 3.4, NKR-P1G\textsuperscript{B6} specificities were detected in multiple wells. Specifically, in plate G1, one hybridoma, corresponding to plate 1, row H, column 8, was selected for further sub-cloning resulting in a monoclonal population denoted 1H8. Interestingly, the majority of the wells on column 8 of the plate G1 showed positivity for NKR-P1G\textsuperscript{B6}, although signal gets progressively weaker as it distanced away from row H. This “comet” effect can be attributed to the fact fresh tips were not used for each well per plate when using a 12-well multichannel pipette to harvest the original supernatants from the 96-well plates to transfer them into capturing plates. Therefore, depending on the direction of supernatant transfer (in this case it is row H-A), small amounts of
contaminating supernatants that originated from the well H8 were carried over to every 8th well of each row. Consistently, if a strong mAb was carried over during handling, a linear series of activated wells in a single column (a “comet” effect) can be observed during CELLISA development, where the degree of activation followed a titration series starting at the well containing the actual specificity. This phenomenon had been observed in this instance (Fig. 3.4) and with other antibodies made by our group18,106, and usually indicates strong mAb specificity in the initial supernatant well.

To confirm the specificity of this mAb, the BWZ and YB transductants used in the immunizations were stained with 1H8 and analyzed via flow cytometry. In addition, transient 293T transfectants of NKR-P1G B6 were also analyzed in parallel. Here, we stained the cells with primary 1H8 supernatant followed by a secondary goat anti-rat-IgG-PE or goat anti-rat-IgM-PE reagent. As shown in Fig. 3.5, all three NKR-P1G B6 transductants were recognized by 1H8 mAb+anti-rat-IgG-PE; however, the untransduced parental cells, or cells expressing an alternate fusion receptor (BWZ.CD3ζ/P1F B6.EC; a related molecule belonging to the NKR-P1 gene family20,107), did not react with 1H8 mAb. In addition, the NKR-P1G transductants were not recognized by 1H8+anti-rat-IgM-PE secondary reagent. Subsequently, we determined that 1H8 mAb is a rat IgG2ακ isotype antibody. These results collectively show that 1H8 selectively recognizes the mouse NKR-P1G B6 molecule expressed at the surface of the transductants, and the staining of transient 293T transfectants with 1H8 mAb + anti-rat-IgG-PE confirms this specificity (Fig. 3.5).

To further demonstrate functional specificity of the mAb, the reporter cell assay was repeated with captured supernatant as described above. In parallel, 100 µL of α-
FLAG mAb was directly immobilized on a plate at a concentration of 30 µg/mL. Reporter cell stimulation using 0.5 µg/mL PMA and 10 µM ionomycin is included as a positive control. As expected, in our example, 1H8 mAb-mediated stimulation was observed for BWZ.CD3ζ/P1G<sup>B6,EC</sup> and BWZ.CD3ζ/P1G<sup>B6,FLAG</sup> cell lines (but neither parental BWZ cells, nor BWZ.NKR- P1G<sup>B6,FL</sup> transductants lacking the CD3ζ-fusion moiety) while α-FLAG mAb selectively activated only the epitope-tagged BWZ.CD3ζ/P1G<sup>B6,FLAG</sup> transductants (Fig. 3.6). These results indicate that 1H8 mAb recognizes the native mouse NKR- P1G<sup>B6</sup> molecule, independently of the FLAG epitope, on the surface of the reporter cells.
Figure 3.4 Primary CELLISA screen using BWZ.CD3ζ/P1G reporter cells
Polyclonal goat anti-rat-IgG/M antibodies were plate-bound overnight, washed, used to capture hybridoma supernatants overnight, then 75 x 10^3 reporter cells were added per well and stimulated overnight. Plate numbers are indicated in the top right corners, rows are indicated by letters (A–H), and individual bars are shown for each column within a row (1–12; numbers not shown). OD_{595/655}, differences of optical density values at 595–655 nm. Clone 1H8 was selected for subcloning and further analysis. Note the reverse “comet” effect for plate 1, rows H–A, column 8, as the 1H8 supernatant was inadvertently carried over in the direction of pipetting (in this case, from row H to row A, as one set of tips was used per plate, direction of pipetting noted); this effect usually indicates a strong mAb specificity and the sensitivity of the CELLISA method.
FIGURE 3.5

A. Flow cytometric analysis of NKR-P1G expression on various cell lines using 1H8 mAb. Cells were stained with 1H8 supernatant at a 1/10 dilution, followed by washing and secondary goat anti-rat-IgG-PE at a 1/200 dilution. Shaded area, 1H8 staining; thin line, goat anti-rat-IgG-PE secondary reagent alone. RCN, relative cell number.

B. Flow cytometric analysis of NKR-P1G expression on different cell lines, stained and analyzed as in (A).

C. Flow cytometric analysis of NKR-P1G expression on 293T transfectants, stained as in (A); top plot, goat anti-rat-IgG-PE versus IRES-GFP reporter expression; bottom plot, 1H8 mAb plus secondary goat anti-rat-IgG-PE versus IRES-GFP reporter expression.

D. Flow cytometric analysis of NKR-P1G expression on BWZ.CD3ζ/P1G transductants using 1H8 mAb plus two distinct secondary reagents: goat anti-rat-IgG-PE (shaded area) versus goat anti-rat-IgM-PE (thin line). This indicates that 1H8 mAb is a rat IgG isotype mAb.
Figure 3.5 Secondary flow cytometric analysis of 1H8 mAb specificity for NKR-P1G on various transductants and transfectants

(A) Flow cytometric analysis of NKR-P1G expression at the cell surface on BWZ transductants using 1H8 mAb. Cells were stained with 1H8 supernatant, followed by washing and secondary goat anti-rat-IgG-PE. Shaded area, 1H8 staining; thin line, goat anti-rat-IgG-PE secondary reagent alone. RCN, relative cell number. (B) Flow cytometric analysis of NKR-P1G expression on YB2/0 transductants, stained and analyzed as in (A). (C) Flow cytometric analysis of NKR-P1G expression on 293T transfectants, stained as in (A); top plot, goat anti-rat-IgG-PE secondary reagent versus IRES-GFP reporter expression; bottom plot, 1H8 mAb plus secondary goat-anti-rat-IgG-PE secondary reagent versus IRES-GFP reporter expression. (D) Flow cytometric analysis of NKR-P1G expression on BWZ.CD3ζ/P1G86 transductants using 1H8 mAb plus two distinct secondary reagents: goat anti-rat-IgG-PE [F(ab')2 fragment, Fcγ-specific] (shaded area) versus goat anti-rat-IgM-PE [F(ab')2 fragment, Fcµ-specific] (thin line). This indicates that 1H8 mAb is a rat IgG isotype mAb.
Figure 3.6 Secondary reporter cell analysis of 1H8 mAb specificity for NKR-P1G
Immobilized antibody-mediated stimulation of BWZ transductants using 1H8 mAb or α-FLAG mAb. In the left panel, goat anti-rat-IgG/M antibody was immobilized on wells, and 10µL of 1H8 supernatant was captured overnight. Following washing, 75 × 10³ reporter cells were added to each well. OD$_{595/655}$ indicates the difference of the optical density values at 595–655nm; samples were analyzed in triplicate, and error bars indicate standard deviation. In the middle panel, purified mouse α-FLAG mAb (30µg/mL) was immobilized on wells overnight without capture, and reporter cells were stimulated overnight as above. In the right panel, reporter cells were stimulated nonspecifically using PMA/ionomycin overnight as a positive control.
3.4 DISCUSSION

“CELLISA” offers many advantages over the conventional immunization and screening procedures. Firstly, by using zCAR+ cell lines for immunization, we avoid the task of antigen purification, and importantly, the antigen can be presented in its native form on the cell surface. Secondly, by using the YB transductants as the immunization mix for the final boost, we hypothesized that this approach will facilitate an “immune-focusing” effect, where the rat host will mount a strong response to the mouse antigen (NKR-P1G\textsuperscript{B6}) as the cell line expressing the mouse antigen was rat-derived. Thirdly, zCAR+ BWZ cells (BWZ.CD3ζ/P1G\textsuperscript{B6}) provide an invaluable tool as an efficient method to screen antibodies secreted by the hybridomas that are specific for the zCAR (in this case, NKR-P1G\textsuperscript{B6}) in a high-throughput manner. Lastly, the BWZ.CD3ζ/P1G\textsuperscript{B6,EC} reporter cells can subsequently be used to unravel novel receptor – ligand interactions \textsuperscript{26}. Importantly, the BWZ parental cell line does not appear to express any Nkrp1 receptor nor C1r ligands based on semiquantitative PCR. A more thorough analysis via qPCR will further solidify this notion. Ultimately, the “CELLISA” assay is a powerful tool that can be applied to generate and screen antibodies against a broad scope of cell surface antigens.
CHAPTER IV

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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cDNA cloning of all Nkrp1 genes and Clr-b/d/f/g, sequence alignment, semi-quantitative RT-PCR and reporter cell assays were performed by P. Chen; cDNA cloning of Clr-c was performed by O. A. Aguilar; evaluation of BAC gene content (section 4.3.1), phylogenetic analyses (section 4.3.3), and sequence alignment were performed by S.Bélanger; independent experiments of BAC evaluation and phylogenetic analyses (biological replicates) were contributed by Q. Zhang, A. St-Laurent and M. M. A. Rahim; project was co-funded by Dr. A. P. Makrigiannis and Dr. J. R. Carlyle

This work has been published in Immunogenetics. 2011 Oct;63(10):627-40.
4.1 ABSTRACT

The Nkrp1 (Klrb) family of NK cell receptors and their genetically linked Clr (Clec2) ligands are conserved between rodents and humans. Nonetheless, certain mouse and rat Nkrp1 genes exhibit significant allelic polymorphism between inbred strains. We previously demonstrated that the Nkrp1–Clr recognition system is genetically and functionally conserved between the B6 and BALB/c strains, with focused sequence divergence evident in certain genes (e.g., Nkrp1b,c). Here, we extend this finding by mapping the 129-strain Nkrp1–Clr gene cluster, which is structurally conserved yet displays significant sequence divergence relative to the B6 haplotype. In addition, we show that 129-strain NK cells possess comparable Nkrp1 and Clr transcript expression, and characterize several NKR-P1:Clr interactions that are functionally conserved between the B6 and 129 strains, including documented and novel receptor–ligand pairs. Thus, despite significant allelic polymorphism observed in the Nkrp1–Clr region, the overall genetic organization and functional repertoire appear to be conserved among mouse strains, in contrast to the striking variation observed in the corresponding Ly49 region. These data extend our knowledge of the complex genetically linked Nkrp1–Clr NK recognition system in mice.
4.2 INTRODUCTION

NK cells are innate lymphocytes capable of recognizing and killing a wide variety of pathological target cells. In mice, NK cells were first phenotypically characterized in selected strains using the alloantigen-specific anti-NK1.1 mAb, PK136. Strain-dependent NK1.1 reactivity occurs as a result of the allogeneic immunization protocol used to generate the PK136 mAb, specifically CE splenocyte immunization into (C3HxBALB)F1 host mice. Thus, BALB/c and C3H NK cells are characteristically NK1.1−, while CE and related strains are NK1.1+. Initial cDNA expression cloning experiments revealed that an NK1.1 antigen is encoded by the Nkrp1c gene in B6 NK cells. However, subsequent investigations have demonstrated that the Nkrp1b gene also encodes an NK1.1 antigen in the SJL, FVB, and related strains. Whereas nearly 100% of NK cells from B6 mice are NK1.1+ due to expression of the NKR-P1C gene product, only ~60–70% of NK cells from SJL and related mice are NK1.1+ due to the expression of the NKR-P1B gene product. Conceptually, this reveals that the stimulatory NKR-P1C receptor exhibits broad expression, similar to that of NKG2D and NKp46, whereas the inhibitory NKR-P1B receptor displays variegated expression, similar to that of the Ly49 and CD94/NKG2A receptors.

Interestingly, early genomic RFLP experiments and more recent aCGH analysis of the Nkrp1 region of numerous inbred mouse strains revealed that expression of the NK1.1 alloantigenic epitope can be predicted at the genetic level, with most strains segregating into one of four groups. First, as expected, NK cells from the BALB/c and C3H strains lack NK1.1 expression. Second, CE and B6 cells are broadly NK1.1+ due to NKR-P1C alloantigen expression. Third, the SJL and FVB strains display
variegated NK1.1+ and NK1.1− subsets, due to differential NKR-P1B alloantigen expression \(^{19,87,98}\). Finally, the SWR and NOD strains appear to be NK1.1− yet segregate as a distinct group from the BALB-related strains, perhaps signifying an unknown combination of NKR-P1B/C polymorphisms \(^{64,164}\).

Recently, the underlying molecular basis for the lack of NK1.1 reactivity of BALB/c-strain NK cells was shown to be due to the existence of allelic polymorphisms present in the NKR-P1B\(^{BALB}\) and NKR-P1C\(^{BALB}\) gene products, and not due to deletion of Nkrp1 genes, or defective or low- level Nkrp1 gene expression \(^{21}\). In part, a residue conserved in the NK1.1 reactive NKR-P1C\(^{B6}\) and NKR-P1B\(^{Sw/SJL}\) receptors exists as an NK1.1-nonreactive residue in the NKR-P1B/C\(^{BALB}\) receptors. Indeed, a directed T191S substitution in the ectodomain of the NKR-P1B\(^{BALB}\) receptor was sufficient to confer NK1.1 reactivity, whereas all other NKR-P1 gene products lacking NK1.1 reactivity possess a non-S residue at this position. Interestingly, this study also demonstrated that the NKR-P1D\(^{B6}\) gene product represents a highly divergent allele of the Nkrp1b gene (aka, NKR-P1B\(^{B6}\)). This in turn suggests that distinct combinations of Nkrp1 alleles found in different groups of mouse strains provide an underlying genetic basis for the four divergent RFLP/aCGH groups outlined above \(^{20,64,164}\).

Historically, the genetic linkage of the first identified mouse Nkrp1 gene (Nkrp1a; Klrb1) with the first identified Ly49 family member (Ly49a; Klra1) on mouse chromosome 6 resulted in the designation of this region as the NK gene complex (NKC) \(^{164,165}\). Since then, a number of other killer cell lectin-like receptor genes have been assigned to the centre of this region, including the Cd94 (Klrd1), Nkg2a (Klrc1), and Nkg2d (Klrk1) loci. While the Ly49 multigene family is highly polymorphic with distinct
haplotypes being composed of vastly different types and numbers of genes, the Nkrp1 gene region appears to be quite stable in mice \(^2^0\). Private and public sequencing of the B6 mouse genome have collectively indicated that the Nkrp1 gene family is composed of several genes and pseudogenes (Nkrp1a-g) within the centromeric end of the NKC \(^2^1,1^1^8,1^1^9\). The only other mapped Nkrp1 haplotype that exists is from BALB/c mice and has a very similar genetic organization and gene order to that of B6 mice, with the caveat that nonconservative nucleotide polymorphisms are present in the coding regions of several genes, most prominently the Nkrp1b/d and Nkrp1c alleles, as noted above \(^2^1\).

Cell-based reporter assays have identified ligands for several NKR-P1 receptors as products of the Clr gene family, which was found to be physically intermingled with the Nkrp1 genes in the NKC \(^1^8,6^0,6^7,1^1^8,1^6^3,1^6^6\). The lectin-like morphology of the Clr ligands for the NKR-P1 receptors was surprising, considering that all ligands previously identified for NKC–based receptors were MHC-Ia, MHC-Ib, or MHC-related proteins \(^1^6^3\). The reason for the tight genetic linkage of the Nkrp1 and Clr genes (in contrast to the genetically segregating Ly49 and Mhc genes) is unknown, but it likely functions to conserve self–self interactions through coinheritance of receptor and ligand alleles. Mapping studies in the B6 and BALB/c strains have established the following gene order in both strains: Nkrp1a, Clr-h, Clr-f, Nkrp1g, Nkrp1c, Nkrp1b/d, Clr-g, Clr-d, Clr-e, Clr-c, Nkrp1f, Clr-a, Nkrp1e, and Clr-b \(^2^0,2^1\). Among these, the allelic NKR-P1B/D gene products recognize Clr-b, while NKR-P1F recognizes Clr-g \(^1^8,6^7\). In addition, recent surface receptor downmodulation assays using transfected cell lines have also tentatively identified Clr-x (identical to Clr-d) as a ligand for NKR-P1F \(^6\). This study also showed that the stimulatory NKR-P1 receptors (NKR-P1A, C, F) are expressed on the vast
majority of bona fide NK cells, although NKR-P1A and NKR-P1F appear to be expressed at low levels. In addition, the NKR-P1D (NKR-P1B\textsuperscript{B6}) allele is expressed in a variegated fashion such that 55% of B6-strain NK cells are positive, and this subset appears to be more responsive in terms of cytotoxicity and cytokine production towards Clr-b ligand-negative target cells \textsuperscript{6}. To date, the expression pattern of the NKR-P1G inhibitory receptor remains unknown, while NKR-P1E appears to represent a pseudogene, at least among the strains analyzed thus far \textsuperscript{20,21}.

Notably, the 129, B6, BALB/c, and NOD inbred strains of mice all possess unique Ly49 haplotypes with distinct numbers of genes and gene content \textsuperscript{20}. To further test the hypothesis that the Nkrp1–Clr region in mice is genetically stable, as is thought for the corresponding human region, a third murine Nkrp1 haplotype was deduced from 129 mice. Here, we show that the gene order and gene content of the 129-strain Nkrp1–Clr region are remarkably conserved in comparison to the previously characterized B6 and BALB/c regions, with the exception of focused allelic polymorphisms within selected Nkrp1 and Clr loci. We also identify a novel gene or gene fragment within this region, adding to the known complexity of the mouse Nkrp1–Clr gene content. Finally, we show that NKR-P1:Clr interactions are conserved and functional in the 129-strain, including novel interacting receptor–ligand pairs. Thus, in sharp contrast to the extreme variability observed for the mouse Ly49 region, the Nkrp1–Clr region appears to be structurally and functionally conserved with regions of localized polymorphisms.
4.3 RESULTS

4.3.1 Production of a gene map of the 129S6 Nkrp1–Clr region using bacterial artificial chromosomes

The BALB/c and B6 Nkrp1-Clr gene clusters are similar in terms of gene content, size, and organization, based on previous mapping studies of the relative positioning of the Nkrp1–Clr genes in the BALB/c inbred mouse strain genome. Individual cDNA cloning and sequencing efforts showed that some gene family members are highly conserved (Clr-b), while others have diverged significantly, leading to highly polymorphic alleles at specific loci (Nkrp1b/d and Nkrp1c). To further investigate the genetic organization and overall polymorphism of this region, we analyzed the Nkrp1–Clr gene cluster of the 129S6 inbred mouse strain, which is known to possess an Ly49 gene cluster distinct from either of the B6 and BALB/c strains.

To this end, cloned BALB/c and B6 Nkrp1 and Clr cDNAs were radioactively labeled and used as probe mixtures on BAC library filters produced from the 129S6 genome. Positively hybridizing 129S6 BAC clones were ordered from BACPAC Resources and grown to produce BAC plasmid DNA preparations, which were subsequently used as templates in PCR amplifications using primers specific for a segment (intron-2 to intron-4) of each of the known members of the Nkrp1–Clr gene families. All PCR products were cloned and sequenced to identify the genes present within each BAC. BAC sizes were determined by pulsed field gel electrophoresis.

As almost all the BACs overlapped, this allowed a map to be assembled of the relative gene order of the Nkrp1–Clr region in the 129S6 genome (Fig. 4.1). All the genes present in B6 and BALB/c mice were also identified in 129S6 mice (i.e., Nkrp1a,
Nkrp1b, Nkrp1c, Nkrp1e, Nkrp1f, Nkrp1g, and Clr-a, Clr-b, Clr-c, Clr-d, Clr-e, Clr-f, Clr-g, Clr-h). In addition, a novel Clr-related gene segment, designated Clr-i, was amplified from several BACs and shown to be located between the Clr-g and Clr-d genes. At present, only limited sequence data are available for this gene fragment (exon-3 and exon-4), so the functional status and relatedness of this gene to the other Clr are awaiting elucidation via BAC sequencing. The partial Clr-i\textsuperscript{129} exon sequence was used to confirm that similar exons were also present in the B6 genome using the genome browser (data not shown). Due to the random nature of genomic DNA restriction enzyme digestion and cloning during production of the 129S6 BAC library, the order of the Clr-h and Clr-f, Nkrp1f and Clr-a, and Clr-b and Cd69 gene pairs was inferred from the closely related BALB Nkrp1/Clr gene order\textsuperscript{21}. Therefore, inversions of these gene pairs in the 129-strain cannot be formally ruled out at this time, but are unlikely.

Since we were unable to identify any BAC containing both the Clr-i and Clr-d sequences, the continuity of the BAC map was verified by end-sequencing of a Clr-d-containing BAC (410a1). This sequence was subsequently used as a probe in Southern blots to show that all Clr-i-containing BACs also contained this sequence (data not shown). Thus, the BAC map of the 129S6 gene region is complete and without gaps in coverage. The predicted length of the Nkrp1–Clr region in 129S6 mice is approximately 600 kb, similar to that predicted for the BALB/c Nkrp1–Clr region and the known Nkrp1–Clr region of the B6 mouse genome reference sequence\textsuperscript{21}. In summary, the gene content and overall organization of Nkrp1–Clr cluster in the 129S6 mouse strain are highly similar to that found for the BALB/c and B6 strains.
**Figure 4.1**

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, situated on chromosome 6. 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. (Contributed by Bélanger, S. et al.).
4.3.2 Cloning and characterization of 129-strain Nkrp1–Clr cDNAs

To assess the functionality of the Nkrp1–Clr genes in 129 strain mice, an effort was made to amplify, clone, and sequence intact cDNAs from this inbred mouse strain. As the BAC gene sequences were only partial (2 exons for each gene), we isolated mRNA from 129S1 LAK and performed RT-PCR using primers capable of amplifying the coding regions of each of the known Nkrp1 and Clr genes (Table 2.1). Although gene mapping was performed using a 129S6 BAC library and cDNA cloning was performed in 129S1 mice, all comparable sequences were identical in the two substrains. In total, we successfully amplified the cDNA of the 129-strain alleles of Nkrp1a, Nkrp1b, Nkrp1c, Nkrp1f, and Nkrp1g, as well as Clr-b, Clr-c, Clr-d, Clr-f, and Clr-g (Fig. 4.2). Full-length cDNA clones were sequenced to determine their identities. The amino acid sequences of these 129-strain alleles are aligned to the known B6 and BALB/c alleles in Fig. 4.3 for comparison.

With regard to the NKR-P1 gene products, we have previously reported the high degree of divergence observed for the NKR-P1B/D and NKR-P1C alleles between the BALB/c and B6 strains 21. However, the putative 129S1 NKR-P1B and NKR-P1C coding sequences were found to be identical to those of the BALB/c alleles (Fig. 4.3A). Similarly, the NKR-P1A sequences are identical in the BALB/c and 129S1 strains and differ from the B6 allele by only 2 amino acid substitutions. Interestingly, unlike most of the NKR-P1 alleles, which are similar if not identical between 129S1 and BALB/c mice, the NKR-P1F alleles in BALB/c and B6 mice are identical but differ from the 129S1 allele by 3 nucleotides, resulting in 2 amino acid substitutions (Fig. 4.3A). Finally, the 129S1 allele of NKR-P1G is identical to the BALB/c version, but these differ from the
B6 allele by 1 nucleotide difference resulting in 1 amino acid substitution (Fig. 4.3A). Interestingly, of all the mouse NKR-P1 receptors, the NKR-P1G isoform appears to lack a consensus cytosolic CxCP motif, previously suggested to be involved in recruitment of the Lck kinase \(^{16,99}\). In addition, the mouse NKR-P1F isoform lacks an N-terminal YxxL motif found in the other NKR-P1 receptors. Finally, NKR-P1C displays evidence of alternative splicing within the stalk region \(^{52,87,130}\), and NKR-P1B/C possess differential cysteine content \(^{128,143}\). The functional significances of these divergences remain to be determined.

Among the Clr gene products, the Clr-b amino acid sequence is identical in all three mouse strains (Fig. 4.3B). In contrast, the Clr-c alleles from 129S1 and B6 are highly divergent, with 38 amino acid differences over the total coding region. Interestingly, the BALB/c Clr-c allele has been reported to be identical to the B6 Clr-c allele \(^{89}\). On the other hand, the 129S1 and BALB/c Clr-f alleles are identical, yet differ from the B6 Clr-f allele by five nucleotide differences resulting in 3 amino acid substitutions. Similarly, the three alleles of Clr-g were nearly identical, with the exception of an extra four residues present in the cytoplasmic region of the BALB/c Clr-g allele, perhaps due to an alternative splicing (Fig. 4.3B) \(^{151}\). The functional ramifications of such alternative splice variants remain unknown at present.

Limited cDNA cloning and sequencing performed in 129X1 and 129Sv mice resulted in NKR-P1B and NKR-P1C alleles identical to those of 129S1. In summary, most 129-strain NKR-P1 and Clr alleles are highly similar to the BALB/c alleles, whereas the B6-strain alleles are more divergent. The exceptions are NKR-P1F and Clr-c, which appear to be conserved in the BALB and B6 strains and divergent in the 129-
strain. Thus, overall, the BALB/c and 129-strain Nkrp1–Clr regions are more similar to one another than they are to the B6 haplotype, suggesting this part of the genome may share a more recent common ancestry in BALB/c and 129. Moreover, there appears to be highly focused polymorphism within the Nkrp1b/d, Nkrp1c, and Clr-c genes, as compared to the remaining Nkrp1 and Clr loci.
FIGURE 4.2

Figure 4.2 RT-PCR analysis of NKR-P1 and Clr transcript expression in B6 and 129-strain d6 LAK cells
Amplification of the indicated RT-PCR products was optimized across a range of 30–35 cycles (except G3PDH, 23 cycles) using threefold serial dilutions of equal amounts of cDNA (125 ng) and the gene-specific primers indicated in Table 2. Each of the RT-PCR products was TOPO-cloned and sequenced to confirm their respective identities.
Fig. 3 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.

Figure 4.3 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. (Work contributed by Chen, P. et al. and Bélanger, S. et al.)
4.3.3 Phylogenetic analysis of the 129S1 Nkrp1 and Clr nucleotide and protein sequences

Alignment of the cDNA sequences of all known mouse Nkrp1 and Clr genes followed by bootstrap analysis results in dendrograms highlighting their evolutionary relationships (Fig. 4.4). The Nkrp1 dendrogram is split into two lineages, in which the Nkrp1a/b/c/e and Nkrp1f/g gene products are clustered and segregate from each other (Fig. 4.4A). In the former Nkrp1 cluster, Nkrp1a/c/e are most closely related, with the Nkrp1b/d alleles being more distant. These results suggest that the Nkrp1a and Nkrp1c genes, which give rise to stimulatory receptor isoforms, arose by gene duplication and in turn share a common ancestry, perhaps via gene recombination with the Nkrp1b/d locus, which encodes an inhibitory receptor isoform. Likewise, the Nkrp1f and Nkrp1g genes share a recent ancestry and likely encode stimulatory and inhibitory receptor isoforms, respectively.

In contrast, the Clr gene cluster is more complicated and the evolutionary history is less obvious in comparison to the Nkrp1 gene cluster. Clr-f and Clr-a are clearly related (Fig. 4.4B). Additionally, Clr-b, d, and g form a strongly related cluster as assessed by bootstrap replicate values. Similarly, the Clr-c and Clr-h genes appear to be more divergent and intermediate in relatedness between the above two clusters, although the Clr-h cDNA and putative protein sequence tends to group with the Clr-b/d/g group. Dendrograms of the putative coding sequences of these cDNAs support the relationships discussed above (Fig. 4.4C, D).
Figure 4.4

Duplication and in turn sharing of loci, which encode an inhibitory receptor isoform. Likewise, the Nkrp1 and Nkrp1f genes share a recent ancestry and likely encode stimulatory and inhibitory receptor isoforms, respectively.

The Clr gene cluster is more complicated and the clustering or evolutionary history is less obvious.

Clusters or evolutionary history is less obvious.

Conserved and novel NKR-P1:Clr interactions in the 129 strain

To investigate functional interactions between the various NKR-P1 and Clr gene products, chimeric NKR-P1/CD3ζ-fusion receptors were ectopically expressed on AB cells.

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 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.
Figure 4.4 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<sup>B6</sup> was artificially spliced together from genomic data. Bootstrap analysis of 1,000 data sets was performed using PHYLIPI 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. (Contributed by Bélanger, S. et al).
4.3.4 Conserved and novel NKR-P1:Clr interactions in the 129 strain

To investigate functional interactions between the various NKR-P1 and Clr gene products, chimeric NKR-P1/CD3ζ-fusion receptors were ectopically expressed on the BWZ reporter cell line, as previously described \(^{18,21,106}\). These reporter cells were then stimulated with 293T cells transiently expressing various full-length Clr ligands. Sorting for equivalent IRES-GFP levels on the transduced BWZ reporter cells was employed to normalize expression levels of the NKR-P1 fusion receptors. Furthermore, the expression of the various Clr molecules was confirmed by IRES-GFP expression in the transfected 293T stimulator cells. In addition, equivalent NKR-P1G expression levels were further verified by flow cytometry using a novel monoclonal antibody, 1H8, which was generated previously and specifically recognizes NKR-P1G transfectants \(^{27}\).

Using the reporter cell assay, we first confirmed the specificity of the 129-strain allele of NKR-P1B. As shown in Fig. 4.5A, the NKR-P1B\(^{129}\) receptor specifically recognizes the known cognate ligand, Clr-b. This is not surprising, since the NKR-P1B\(^{129}\) allele is identical in sequence to the previously reported NKR-P1B\(^{BALB}\) allele, and the Clr-b coding sequences between these two strains and the B6 strain are identical. Interestingly, however, when compared to BWZ.NKR-P1B\(^{B6}\) reporter cells, the BWZ.NKR-P1B\(^{129}\) reporter cells responded more robustly to titrated doses of 293T.Clr-b stimulator cells, despite equivalent expression levels of the two NKR-P1B gene products (as supported by similar IRES-GFP levels). A similar trend has been observed before using BWZ.NKR-P1B\(^{B6}\) (NKR-P1D) reporter cells in comparison to BWZ.NKR-P1B\(^{Sw/SJL}\) reporter cells, suggesting that the highly divergent NKR-P1B\(^{B6}\) allele may possess a differential affinity for Clr-b ligand compared to the other alleles tested to date.
As expected, neither reporter cell line was stimulated by 293T.Clr-b cells in the presence of blocking 4A6 anti-Clr-b mAb, demonstrating the specificity of the interaction (Fig. 4.5B).

Next, we verified the previous observation that the NKR-P1F receptor functionally interacts with Clr-g ligand. Interestingly, the single amino acid substitution in the 129-strain NKR-P1F ectodomain relative to the B6 allele does not confer a differential response of BWZ.NKR-P1F^{129} versus BWZ.NKR-P1F^{B6} reporter cells when titrated with 293T.Clr-g stimulator cells (Fig. 4.5A). Notably, the Clr-g sequence, like that of Clr-b, is identical between the B6 and 129 strains. Thus, the NKR-P1F:Clr-g interaction, similar to the NKR-P1B:Clr-b interaction, is functionally conserved between the B6 and 129 strains.

To further investigate interactions between the NKR-P1 and Clr family members, we extended reporter cell analysis to test interactions between other known receptor and ligand family members. Interestingly, BWZ.NKR-P1F reporter cells were also found to respond to 293T.Clr-c and 293T.Clr-d stimulator cells, but not to 293T cells expressing Clr-b or Clr-f (Fig. 4.5A). This demonstrates that the NKR-P1F stimulatory receptor recognizes Clr-c, Clr-d, and Clr-g. Furthermore, BWZ.NKR-P1G reporter cells responded well to 293T.Clr-d, 293T.Clr-f, and 293T.Clr-g stimulator cells, but not to 293T transfectants expressing the Clr-b or Clr-c family members (Fig. 4.5A). Thus, the NKR-P1G inhibitory receptor recognizes Clr-d, Clr-f, and Clr-g. Importantly, the responses of BWZ.NKR-P1G^{B6} and BWZ.NKR-P1G^{129} reporter cells to 293T.Clr-f stimulators were either fully or partially blocked by 1H8 anti-NKR-P1G mAb (Fig. 4.5B), demonstrating the specificity of the interactions. Interestingly, unlike the B6 and
129 NKR-P1B alleles, the polymorphisms found between the B6 and 129-strain NKR-P1F and NKR-P1G alleles appeared to have little effect on the reporter cell responses to any of the ligand-bearing stimulator cells. Notably, BWZ.NKR-P1A and BWZ.NKR-P1C reporter cells failed to respond to any of the stimulator cells tested.

To survey the expression of functional endogenous Clr ligands, ex vivo cell populations harvested from B6 and 129 mice were used as stimulators for the above panel of BWZ reporter cells. Interestingly, ex vivo hematopoietic cells from B6 and 129 mice were equally able to stimulate only BWZ.P1B\textsuperscript{129} reporter cells (Fig. 4.5C). It should be noted that two independent BWZ.P1B\textsuperscript{B6} reporter cell lines were tested, and neither exhibited significant reactivity to ex vivo cells, even though both responded to 293T.Clr-b transfectants; the reason for the discrepancy between these results is unknown, although NKR-P1B\textsuperscript{B6} may possess an altered affinity for Clr-b compared to NKR-P1B\textsuperscript{129}, either in cis or in trans\textsuperscript{68}. Also, the expression level of Clr-b on 293T transfectants may be higher than ex vivo cells, thus providing rationale why 293T Clr-b transfectants are able to activate BWZ.P1B\textsuperscript{B6} reporter cells, whereas ex vivo cells cannot. This hypothesis can be tested through Western blot analysis. As expected, the response of BWZ.P1B\textsuperscript{129} reporter cells to ex vivo stimulator cells was blocked in the presence of 4A6 mAb, confirming the expression of Clr-b on normal hematopoietic cells.
The overall organization, content, and number of genes in this region seems to be quite conserved between these strains, with genetic diversity limited to localized regions of highly focused allelic polymorphisms in selected genes (e.g., *Nkrp1b*, *Nkrp1c*). Here, we investigate a third *Nkrp1*–*Clr* haplotype, of the 129 strain, and show that the trend of overall genetic conservation with focused allelic polymorphism holds true. Moreover, we expand the known gene content of the region to include *Clr-i*, a putative new ligand gene, and show that all NKR-P1–Clr interactions are conserved.

**FIGURE 4.5**

A

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<th>NKR-P1B&lt;sup&gt;B6&lt;/sup&gt;</th>
<th>NKR-P1B&lt;sup&gt;129&lt;/sup&gt;</th>
<th>NKR-P1F&lt;sup&gt;B6&lt;/sup&gt;</th>
<th>NKR-P1F&lt;sup&gt;129&lt;/sup&gt;</th>
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**Clr-b**<sup>129/B6</sup>  
**Clr-c**<sup>129</sup>  
**Clr-d**<sup>129/B6</sup>  
**Clr-f**<sup>129</sup>  
**Clr-g**<sup>129/B6</sup>

Number of Stimulators (X1000)

**β**

Antibody-mediated blockade of BWZ.NKR-P1B reporter cell responses to 293T.Clr-b stimulator cells using 4A6 anti-Clr-b mAb (upper panel), and blockade of BWZ.NKR-P1G reporter cell responses to 293T.Clr-f stimulator cells using 1H8 anti-NKR-P1G mAb (lower panel). Control (Con) samples indicate mock blockade using equivalent concentrations of the above two mAb with opposite specificity (i.e., 4A6 for NKR-P1G, 1H8 for NKR-P1B).

**b**

Responses of BWZ.NKR-P1B<sup>129</sup> reporter cells to fresh ex vivo bone marrow (BM) or splenocyte (SPL) stimulator cells from B6 or 129-strain mice, either with no treatment (left) or with 4A6 mAb blockade (right).
Figure 4.5 Reporter cell analysis of functional receptor–ligand interactions between the B6 and 129-strain NKR-P1 and Clr alleles

(A) Stably transduced BWZ reporter cell lines bearing strain-specific CD3ζ/NKR-P1 fusion receptors were mixed with titrated doses of 293T stimulator cells expressing various Clr ligand alleles, and responses were analyzed overnight. Reporter cells were normalized for receptor expression by prior sorting for equivalent IRES-GFP levels (NKR-P1B, NKR-P1F), and/or by staining using 1H8 mAb (NKR-P1G). Stimulator cells were also validated for ligand expression by parallel analysis of IRES-GFP levels. (B) Antibody-mediated blockade of BWZ.NKR-P1B reporter cell responses to 293T.Clrb stimulator cells using 4A6 anti-Clr-b mAb (upper panel), and blockade of BWZ.NKR-P1G reporter cell responses to 293T.Clrd, f, and g stimulator cells using 1H8 anti-NKR-P1G mAb (lower panel). Control (Con) samples indicate mock blockade using equivalent concentrations of the above two mAb with opposite specificity (i.e., 4A6 for NKR-P1G, 1H8 for NKR-P1B). (C) Responses of BWZ.NKR-P1B129 reporter cells to fresh ex vivo bone marrow (BM) or splenocyte (SPL) stimulator cells from B6 or 129-strain mice, either with no treatment (left) or with 4A6 mAb blockade (right).
4.4 DISCUSSION

The NKC is one of the most highly polymorphic genetic regions in the mouse genome, rivaling the diversity of the Mhc region. To date, four distinct Ly49 haplotypes have been investigated (B6, BALB, 129, and NOD), and each appears to be unique in terms of gene content, gene numbers, gene organization, and allelic polymorphism, with only a limited set of framework genes conserved between all strains. In contrast, only two Nkrp1–Clr haplotypes are currently known (B6, BALB), and the overall organization, content, and number of genes in this region seems to be quite conserved between these strains, with genetic diversity limited to localized regions of highly focused allelic polymorphisms in selected genes (e.g., Nkrp1b, Nkrp1c). Here, we investigate a third Nkrp1–Clr haplotype, that of the 129 strain, and show that the trend of overall genetic conservation with focused allelic polymorphism holds true. Moreover, we expand the known gene content of the region to include Clr-i, a putative new ligand gene, and show that all NKR-P1:Clr receptor–ligand interactions identified thus far are functionally conserved between the B6 and 129 strains, including novel interacting receptor and ligand pairs. Thus, despite significant polymorphism observed in the Nkrp1–Clr region, the overall structure appears to be remarkably conserved, in contrast to the striking diversity observed in the corresponding Ly49 region.

In particular, the Nkrp1–Clr regions of the strains evaluated thus far (B6, BALB, 129) are predicted to be approximately 600 kb in length with the following gene content and order: Nkrp1a, Clr-h, Clr-f, Nkrp1g, Nkrp1c, Nkrp1b/d, Clr-g, Clr-i, Clr-d, Clr-e, Clr-c, Nkrp1f, Clr-a, Nkrp1e, and Clr-b. In line with previous Southern blot analyses, the 129-strain Nkrp1 genes remain highly conserved in comparison to the corresponding
BALB-strain genes (including Nkrp1b and Nkrp1c), yet these two haplotypes have diverged quite significantly from the more unique B6 haplotype. In addition, the degree of allelic polymorphism, in terms of coding sequence substitutions, varies dramatically across the Nkrp1–Clr region between the B6 and 129-strain alleles, as follows: NKR-P1A (2 substitutions), NKR-P1B/D (23 substitutions), NKR-P1C (25 substitutions), NKR-P1F (2 substitutions), NKR-P1G (1 substitution), Clr-b (conserved), Clr-c (38 substitutions), Clr-d (7 substitutions), Clr-f (3 substitutions), and Clr-g (conserved). As mentioned above, the vast majority of these polymorphisms appear to be unique to the B6 strain, with the 129 and BALB sequences (where known) being largely identical. Exceptions to this rule are NKR-P1F and Clr-c, where the B6 and BALB/c alleles appear to be identical, but differ from the corresponding 129 allele.

Notably, the most highly polymorphic genes are Nkrp1b/d, Nkrp1c, and Clr-c. Previous analysis of the BALB/c haplotype has shown that Nkrp1b/c polymorphisms underly the differential NK1.1 alloreactivity of the B6 and BALB strains; this observation can now be extended to the 129-strain, which shares identical (NK1.1 non-reactive) NKR-P1B and NKC-P1C alleles with the BALB strain.

Unexpectedly, however, our analysis of the 129-strain Clr haplotype reveals an additional, highly focused set of polymorphisms localized to the Clr-c gene. The functional consequences and driving force behind the accumulation of these polymorphisms remain unknown. Nonetheless, it has been speculated that pathogen-driven selection pressure arising during infection, combined with viral immune evasion strategies, may provide a mechanism behind the evolution of selected genes within a conserved framework. The existence of a rat CMV decoy immunoevasin targeting the
NKR-P1 recognition system (RCTL) provides support for this notion\textsuperscript{157}, although no similar viral gene products have been characterized in the mouse or human CMV genomes to date.

The discovery of a novel putative ligand gene conserved in the B6 and 129 mouse strains, Clr-i, adds to the repertoire of an already complex recognition system consisting of at least six receptor loci (Nkrp1a,b,d,c,e,f,g) and nine ligand loci (Clr-a,b,c,d,e,f,g,h,i). While not all of these loci encode functional gene products, at least in the strains studied to date, the potential for diversity in recognition facilitated by combinations and permutations of receptor–ligand interactions in this system is daunting. The tight genetic linkage of the Nkrp1 receptor and Clr ligand genes within the NKC has supported the notion that many unidentified interactions remain to be characterized\textsuperscript{18,67,163}. Indeed, we and others\textsuperscript{6,18,67,89} have expanded the number of known interactions to seven, as follows: NKR-P1B/D:Clr-b, NKR-P1F:Clr-c,d,g, and NKR-P1G:Clr-d,f,g. Notably, these functional interactions are conserved between the B6 and 129-strain [and presumably BALB;\textsuperscript{21,89}] alleles of each receptor and ligand combination, despite the considerable polymorphism observed in certain gene products, as noted above. Interestingly, whereas the divergence of the NKR-P1B (NKR-P1D) receptor alleles has been documented previously for the B6 and BALB strains\textsuperscript{21}, the polymorphism observed for the Clr-c ligand alleles between the B6 and 129 strains is novel and quite striking. In turn, this suggests that the Clr-c locus may be under significant selection pressure and that both the Nkrp1 receptor and Clr ligand genes are capable of rapid evolution despite their close genetic linkage. Because Clr-c is a ligand for the stimulatory NKR-P1F receptor, the sequence diversity of the Clr-c alleles may be functionally constrained by their cognate
NKR-P1F specificity and tight genetic linkage. On the other hand, since Clr-c is not a ligand for the inhibitory NKR-P1G receptor, yet NKR-P1F and NKR-P1G share sequence similarity and otherwise overlapping ligand specificities (i.e., Clr-d,g), the divergence of the B6 and 129 Clr-c alleles may have evolved under selection pressure specifically targeting NKR-P1F function. In turn, it is possible that Clr-c expression may be modulated during virus infection, and that an unidentified viral immunoevasin may alter Clr-c expression or function in order to evade NKR-P1F-mediated recognition. These possibilities require further investigation.

Our findings regarding the overall conservation of the mouse Nkrp1–Clr gene content and organization among the B6, 129, and BALB strains support a relatedness of distinct gene family members with one another. Despite considerable and focused allelic polymorphism among the Nkrp1b/d (Nkrp1d = Nkrp1bB6) and Nkrp1c genes, their gene products are quite closely related to one another and even share NK1.1 epitope crossreactivity in certain strains. Nonetheless, Nkrp1c more closely resembles Nkrp1a overall, while Nkrp1e appears to be a pseudogene. Notably, the rat Nkrp1b gene also displays considerable allelic polymorphism between strains and its gene product also shares mAb crossreactivity with its closest stimulatory paralog (NKR-P1A). On the other hand, the mouse Nkrp1f and Nkrp1g sequences are more similar to one another and form a distinct phylogenetic cluster. This has lead to the suggestion that the mouse Nkrp1 genes comprise two distinct groups of related genes, specifically Nkrp1a,b/d,c(e) and Nkrp1f,g. Indeed, we show here that the mouse NKR-P1F and NKR-P1G receptors possess overlapping ligand specificities that are conserved between mouse strains. Notably, the phylogenetic and functional dichotomy is even clearer in the rat system,
where the simpler Nkrp1a,b and Nkrp1f,g clusters are even physically segregated in the genome, separated by a large group of Clr genes \(^{20,89,90}\). Here, the two divergent rat Nkrp1 gene clusters share close sequence similarity within each group, and with their mouse counterparts, and each group codes for a paired (inhibitory/stimulatory) receptor system with overlapping Clr ligand-binding characteristics \(^{89,90}\). Thus, the mouse and rat Nkrp1–Clr systems appear to share a similar (although non-identical) gene content and a functional conservation of receptor–ligand specificities, including documented allogeneic \([\text{this study;}^{89}]\) and xenogeneic \(^{89,129}\) interactions.

Importantly, only a few polymorphisms distinguish the NKR-P1F and NKR-P1G alleles between the B6, BALB, and 129 strains \(^{21,89}\). Hence, NKR-P1F and NKR-P1G may have not undergone the vigorous evolutionary pressure that seems to have occurred for NKR-P1B and NKR-P1C. Notably, NKR-P1F recognizes Clr-c,d,g, whereas NKR-P1G recognizes Clr-d,f,g. This overlap is particularly interesting, since it suggests that NKR-P1F and NKR-P1G can compete for common ligands. For many years, the dogma of NK cell biology has proposed that NK cell effector function is dictated by a balance or integration of stimulatory and inhibitory signals, in turn modulated by surface expression of stimulatory and inhibitory ligands. The unique and overlapping ligand binding characteristics of NKR-P1F and NKR-P1G may serve to complement variegation in receptor expression in order to modulate the effector function of NK cells. In this regard, the differential modulation of common or exclusive ligands under distinct pathological circumstances could impact NK recognition in a pathogen or tissue-specific fashion.

In contrast to the diversity observed in the Ly49–Mhc system, the polymorphisms observed in the Nkrp1–Clr system seem to minimally impact cognate specificity across
mouse strains, or allogeneic recognition. That is, all the functional receptor–ligand interactions identified for the Nkrp1–Clr system thus far seem to be maintained in different mouse strains. Interestingly, this observation extends to NKR-P1 receptor recognition of the BALB/c Clr alleles, as well as a number of xenogeneic mouse–rat receptor–ligand interactions. The only notable exception seems to be the differential interaction observed in reporter cell assays between the NKR-P1B^{129/BALB} or NKR-P1B^{Sw/SJL} alleles versus the NKR-P1B^{B6} (NKR-P1D) allele in terms of recognition of the monomorphic Clr-b ligand. Thus, the genetic linkage of the Nkrp1 and Clr genes may have an impact in preserving the specificity of cognate “self” interactions within the species, since they are not subject to the random pairing that the Ly49 and Mhc haplotypes suffer. For example, random breeding in the wild could lead to extremes in terms of self-MHC recognition governed by the number and strength of self-MHC-binding receptors. Differential ligand-binding strength by the NKR-P1B inhibitory receptor may help to maintain a certain threshold of self-recognition regardless of the MHC haplotype. In this regard, the NKG2A inhibitory receptor for the Qa-1b MHC-Ib ligand may complement NKR-P1B-mediated self-recognition. Sequencing of more Nkrp1-Clr haplotypes and assessing the rate of evolutionary change of each gene will provide further functional insight into this complex system.
CHAPTER V

Genetic Investigation of MHC-Independent Missing-Self Recognition
by NK Cells using an in vivo Mouse Bone Marrow Transplantation Model

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All assays were performed by P. Chen; independent experiments (biological replicates) characterizing the Clr-b$^\pm$ mice and chromium release assays were performed with the assistance of J. Fine and J. Ma; project was funded by Dr. J. R. Carlyle

Manuscript for this work is under preparation.
5.1 ABSTRACT

MHC-I-dependent recognition has been shown to have vital importance in contributing to “missing-self” recognition by NK cells, which ultimately determines the activation state of these innate lymphocytes. In contrast, roles for MHC-independent NK recognition mechanisms are not as well characterized. Here, we investigate the role of the NKR-P1B:Clr-b interactions in determining the outcome of BM transplantation \textit{in vivo}. Using a competitive transplant assay, we show that B6.Clr-b\(^{-/-}\) BM cells are selectively rejected by wild-type B6 recipients, to an extent similar to H-2D\(^{b/-}\) single MHC-I-deficient donor cells. Selective rejection of Clr-b\(^{-/-}\) BM cells was abrogated by NK-cell depletion of recipient mice using PK136 mAb. Furthermore, competitive rejection of Clr-b\(^{+-}\) BM cells was also observed in allogeneic NIH.Swiss recipients, where it could be reversed by selective depletion of the NKR-P1B+ subset of NK cells, while leaving the remaining NKR-P1B− NK subset intact. These findings support a unique and non-redundant role for NKR-P1B:Clr-b interactions in MHC-independent “missing-self” recognition of normal hematopoietic cells, and suggest that the success of BM transplants relies on MHC-independent tolerance mechanisms.
5.2 INTRODUCTION

Bone marrow (BM) transplantation models have been shown to have vital importance in studying immunological tolerance and clinical therapeutic treatments for hematopoietic disorders and cancer. Mixed BM chimeras are now the standard procedure to evaluate the development of the immune system, where donor BM cells are often genetically manipulated and engrafted into either syngeneic recipients or immune-compromised hosts, such as NOD/SCID\textsuperscript{121} or RAG/\gamma c\textsuperscript{54} animals, to ensure engraftment. Clinically, the usage of cellular transplantation has expanded from serving as one of many treatments for immunodeficient and chemotherapy patients, to adoptive cell-mediated immune therapy for combatting malignant cells, characterized by the graft-versus-tumour (GVT) effect\textsuperscript{5}, a cancer regimen inspired by the clinical condition, graft-versus-host disease (GVHD)\textsuperscript{44}. Nonetheless, determinants of success in immune reconstitution are the minimization of hematopoietic graft rejection, and the maximization of the available donor BM stem cells. Naturally, it would be beneficial if healthy foreign BM cells could be readily transplanted into leukemic patients; however, numerous factors attributed to the recipient’s immune system are involved in preventing engraftment of allogeneic BM transplants.

One of the major obstacles that prevent reconstitution is the presence of host NK cell activity. The importance of NK cells in determining BM graft engraftment or rejection surfaced when the biological phenomenon of “hybrid resistance” was reported. Hybrid resistance is the observation wherein a parental BM graft is rejected by their offspring\textsuperscript{34-36}. Hybrid resistance can be simulated using \textit{in vivo} mouse modeling, whereby F1 “hybrid” animals generated by crossing two MHC-disparate strains of inbred
mice, reject or are “resistant” to bone marrow grafts derived from either parent.\textsuperscript{34-36,85} The postulated mechanism for hybrid resistance is as follows: the F1 hybrid animal (ie. C57Bl/6 x BALB/c) will co-dominantly express both parental MHC-I haplotypes (H-2\textsuperscript{b/d}). Consequently, mature NK cells from the F1-hybrid animal are “educated” to express a Ly49 receptor repertoire specific for, and tolerant to, both “self” haplotypes.\textsuperscript{122-124} However, since NK receptor expression is variegated, at least four subsets of F1 NK cells can be described: individual subsets expressing an inhibitory Ly49 receptor specific for either H-2\textsuperscript{d} or H-2\textsuperscript{b} alleles, a subset expressing Ly49 receptors specific for both H-2\textsuperscript{d} and H-2\textsuperscript{b} alleles, and a subset expressing neither class of Ly49 receptor. Thus, parental (H-2\textsuperscript{b/b} or H-2\textsuperscript{d/d}) BM cells transplanted into an F1 (H-2\textsuperscript{b/d}) host will be perceived to be “missing” at least one “self” allele by the NK cell subsets rendered tolerant to non-overlapping allelic or haplotype specificities. At the molecular level, this means that certain F1 inhibitory Ly49 receptors that recognize only H-2\textsuperscript{b} or H-2\textsuperscript{d} alleles will not be engaged, hence “missing-self” recognition will ensue, leading to selective rejection of the parental BM graft. Thus, hybrid resistance is one of the earliest bodies of scientific evidence delineating a unique role for NK cells in determining the fate of transplanted BM grafts.\textsuperscript{8,85,159} Further evidence suggesting the importance of NK cells in BM transplantation biology came from observations where allogeneic BM cells were rejected by C.B-17 scid recipients\textsuperscript{11,46} (which are devoid of both B and T lymphocytes but still possess intact NK cells\textsuperscript{42}), yet were capable of engraftment in NK-depleted hosts\textsuperscript{108,109,152}

Notably, while these studies established the importance of NK cells in MHC-I-dependent “missing-self” recognition, studies on the biology of MHC-independent
recognition by NK cells have recently emerged, including those of the NKR-P1B:Clr-b receptor-ligand pair. In rats, subversion of this system has proven to have a pivotal role in determining the outcome of viral titres during rat cytomegalovirus (RCMV) infection \(^{157}\). In short, RCMV-infected cells lose surface expression of Clr-b, yet RCMV encodes an NKR-P1B-specific decoy ligand, RCTL, that functionally replaces the host Clr-b, thus inhibiting NK cell function. Furthermore, many tumour cells and stressed cells also lose expression of Clr-b \(^{18,45}\), making them susceptible to NK cells. These findings highlight the significance of MHC-independent immunosurveillance by NK cells.

This cumulative support for a non-redundant role of this MHC-independent recognition mechanism in the discrimination of pathological versus healthy target cells drove us to believe that this same system must also be instrumental in distinguishing self versus non-self entities, such as autologous versus allogeneic BM grafts in a transplant setting. Specifically, we assessed the degree of rejection of Clr-b\(^{-/-}\) BM cells, in comparison to wild-type and various MHC-I-deficient BM cells, using a competitive in vivo transplantation assay. Here, we show that Clr-b\(^{-/-}\) mice possess a nearly identical immune cell repertoire compared to wild-type B6 mice, and that Clr-b\(^{-/-}\) BM cells do not have any overt deficiencies in hematopoietic reconstitution. However, Clr-b\(^{-/-}\) BM cells are acutely and selectively rejected by wild-type recipient mice in NK cell-dependent manner within 48 hours, and this rejection is mediated specifically by the NKR-P1B\(^{+}\) NK subset.
5.3 RESULTS

5.3.1 Verification of Clr-b knockout status at the transcript and protein levels

Ocil+/− (Clr-b+/−) mice generated previously have been shown to exhibit a mild defect in osteoclast development and function. These animals were generated by targeted deletion of exon-3 of the Ocil/Clrbi/Clec2d gene (5 exons total), leading to the absence of a functional ectodomain sequence. Since previous methods of authenticating the knockout status of these mice were restricted to only transcript analysis, we re-confirmed the Clr-b-deficient status of these Clr-b+/− animals via three methods: (i) RT-PCR analysis of Clr-b transcripts, using full-length Clr-b coding sequence primers (same method used by the lab that generated the knockout animal); (ii) flow cytometric analysis of Clr-b surface protein on hematopoietic and non-hematopoietic cells ex vivo, using the anti-Clr- b mAb, 4A6; and (iii) reporter cell analysis of Clr-b ligand function, using BWZ.36 cells bearing an CD3ζ/NKR-P1B fusion receptor. Clr-b transcripts were detected at normal abundance in bone marrow, spleen, lymph node, thymus, colon, kidney, liver and lung, and absent from the brain, in both B6 and Clr-b−/− animals (Fig. 5.1A). As reflected by the deletion of exon 3, however, transcripts from the Clr-b−/− animals exhibited a smaller size compared to B6. Next, we evaluated cell surface expression of Clr-b by staining cells harvested from bone marrow, lymph node, spleen, thymus, liver, brain, heart, kidney and lungs from B6 and Clr-b−/− animals using α-Clr-b mAb (4A6) and α-CD45. We verified that Clr-b+ tissues lacked cell surface expression of Clr-b protein (Fig. 5.1B, C). Interestingly, cell surface expression of Clr-b appears to be restricted mainly to CD45+ cells, at least by the methods utilized to dissociate the tissues analyzed protein. Finally, ex vivo cells were also
utilized as stimulator cells in the BWZ.CD3ζ/P1B129 reporter cell assay. Here, we show that NKR-P1B recognizes Clr-b on WT cells, but ex vivo cells from Clr-b−/− mice and brain cells from B6 mice [which do not express cell surface Clr-b (Fig. 5.1C)] fail to activate the BWZ.NKR-P1B reporter cells, demonstrating a lack of cell surface expression of functional NKR-P1B ligand on Clr-b−/− cells (Fig. 5.1D).
FIGURE 5.1

A

B

BM    SP    LN    TH    BR    CO    KD    LV    LG

C57BL/6

B6

Clr-b

CD45.2

BM    LN    SP    TH    LV

CD45.2+

RCN

TH    LV

CD45.2+

FMO

B6

Clr-b−/−
Figure 5.1 Confirmation of Clr-b$^{-/-}$ mouse phenotype

(A) RT-PCR analysis of Clr-b transcript expression using primers specific for the full-length coding sequence of Clr-b (ATG$>$TAG), relative to G3PDH as an internal loading control. Shown are bone marrow (BM), spleen (SP), lymph node (LN), thymus (TH), brain (BR), colon (CO), kidney (KD), liver (LV) and lung (LG). The smaller fragment (459 bp) observed for Clr-b$^{-/-}$ mice corresponds to a transcript deleted for exon 3, which creates a frame-shift resulting in the absence of a functional ectodomain; larger band observed in B6 mice corresponds to wild type Clr-b transcript (638 bp). Relevant band sizes of the DNA ladder are depicted by the red arrow and numbers on the left. (B) Flow cytometric analysis of Clr-b surface expression using 4A6 mAb on cells derived from lymphoid and (C) non-lymphoid tissues of wild-type B6 versus Clr-b$^{-/-}$ mice. Viable cells were gated on forward and side scatter, followed by DAPI exclusion. RCN, relative cell number; FMO, fluorescence minus one. (D) BWZ.NKR-P1B reporter cell analysis of Clr-b ligand function on ex vivo bone marrow, spleen, liver, and brain cells of WT B6 or Clr-b$^{-/-}$ mice cells using BWZ.CD3ζ/NKR-P1B129 reporter cells. Clr-b$^{-/-}$ cells lack NKR-P1B-specific ligand function. Each data point represents the average value of 3 technical triplicates.
5.3.2 Steady state hematopoietic characterization of the Clr-b\(^{-/-}\) mouse

Since the normal Clr-b expression profile is similar to MHC-I, at least on hematopoietic cells, we first investigated whether the absence of Clr-b would influence the development of the hematopoietic system. We next analyzed hematopoietic cells from Clr-b\(^{-/-}\) mice, in comparison to wild-type B6 and MHC-I-deficient animals, such as \(\beta 2m^{-/-}\) mice\(^{82}\); these latter mice are deficient in CD8+ T cells\(^{167}\), while their NK cells are functional\(^9\), albeit hyporesponsive, due to differential NK cell education\(^{161}\). Thus, we harvested cells from BM, lymph node, liver, spleen, and thymus from Clr-b\(^{-/-}\) mice and compared these with age- and sex-matched B6 counterparts for both lymphoid and myeloid cell composition and distribution. We observed nearly identical leukocyte distribution (neutrophils, macrophages, dendritic cells, B cells, T cells, NKT cells, and NK cells) in the various tissues (Fig. 5.2A-F).

We further characterized the NK cell subset composition and receptor repertoire of Clr-b\(^{-/-}\) animals. Interestingly, statistically higher expression levels of the NKR-P1B receptor were observed on splenic NK subsets from Clr-b\(^{-/-}\) mice in comparison to wild-type B6 animals (p=0.012; Fig. 5.2I, J). In extended analysis, a trend toward higher NKR-P1B levels was observed on splenic (p=0.012), BM-derived (p=0.068) and hepatic (p=0.076) NK cells (Fig. 5.2G-J). This was expected, since elevated Ly49 levels are seen on developing and mature NK cells from MHC-I-deficient mice\(^{127}\). Furthermore, lower expression levels of the paralogous NKR-P1C receptor (NK1.1) were observed on Clr-b\(^{-/-}\) BM-derived (p=0.006) and hepatic (p=0.034) NK cells, with a trend towards lower levels on splenic (p=0.106) NK cells (Fig. 5.2J), perhaps suggestive of compensatory changes in stimulatory NK receptor expression due to loss of inhibition through NKR-
P1B. Unexpectedly, there appears to be a trend where the NKp46 receptor is also expressed at a lower level on Clr-b<sup>b<sup>−/−</sup></sup> BM-derived (p=0.184), hepatic (p=0.086), and splenic (p=0.296) NK cells (Fig. 5.2J). Interestingly, in terms of subsets, we observed a slightly smaller proportion of BM-derived NKG2D+ NK cells from Clr-b<sup>b<sup>−/−</sup></sup> animals compared to B6 mice (Fig. 5.2J).
FIGURE 5.2

A

Bone Marrow Leukocyte Distribution

% of Total Viable Cells

Cell Surface Marker

WT

KD

B6 BM

Clr-b−/− BM

B6 BM

Clr-b−/− BM

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+

Bone Marrow Leukocyte Distribution

WT

KD

Cell Surface Marker

% of Total Viable Cells

Gr-1+

NK.1.1+

CD3+

CD11b+

CD11c+

CD19+
B6 LN  Clr-b<sup>−/−</sup> LN  B6 LN  Clr-b<sup>−/−</sup> LN

Gr-1  CD11b  NK.11  CD3

CD11b  CD11c  CD11c

CD19  CD3  CD11b

Lymph Node Leukocyte Distribution

WT  KO
Liver Leukocyte Distribution

C

B6 LV  Clr-b<sup>−/−</sup> LV

Gr-1

CD11b

CD11b

CD11c

CD19

CD3

CD11c

NK1.1

NK1.1

NK1.1

NK1.1

B6 LV  Clr-b<sup>−/−</sup> LV

Cell Surface Marker

% of Total Viable Cells

WT  KO

Liver Leukocyte Distribution

**!
D

Spleen Leukocyte Distribution

Cell Surface Marker

% of Total Viable Cells

WT
KO

Gr-1
CD11b
CD11c
CD19
CD3
Gr-1+ CD11b+
NK1.1+ CD3-
NK1.1+ CD3+
NK1.1+ CD11c+

B6 SP
Clr-b<sup>−/−</sup> SP

B6 SP
Clr-b<sup>−/−</sup> SP

0
5
30
55
80

B6 SP
Clr-b<sup>−/−</sup> SP
Thymus Leukocyte Distribution

% of Total Viable Cells

WT
KO

Cell Surface Marker
T Cell Development

Cell Surface Marker

WT KO

% of Total Viable Cells

DN1 (CD44+)

DN2 (CD44+ CD25+)

DN3 (CD25+)

DN4 (CD44- CD25-)
116
Expression of NK-Receptors on NK1.1+ CD3- Cells

Proportion of Different NK-Subsets

Bone Marrow NK Cells

Hepatic NK Cells

Splenic NK Cells

NK Subsets
Figure 5.2 Steady state immune characterization of the Clr-b^-/- mouse

(A-E) Cells harvested from either B6 or Clr-b^-/- bone marrow (BM), lymph node (LN), liver (LV), spleen (SP) and thymus (TH) were stained with mAbs specific for surface markers depicting monocytes/macrophages (CD11b), dendritic cells (CD11c), granulocytes (Gr-1), neutrophils (Gr-1, CD11b), B cells (CD19), T cells (CD3), NK cells (NK1.1) and NKT (NK1.1 CD3) to provide a generalized assessment of the leukocyte repertoire observed in the Clr-b^-/- animals in comparison to B6 mice. Numbers in each quadrant indicates proportion of total viable cells. Histograms represent tabulated results of three pairs of mice (n = 3); empty bars represent B6, solid bars represent Clr-b^-/-.

(F) Thymocytes from both B6 and Clr-b^-/- mice were stained with α-CD3, α-CD4, α-CD8, α-CD44 and α-CD25 to visualize T cell development. The bottom panels (CD44 x CD25) are gated on CD4- and CD8- double negative population. Similar to (A-E), histogram represents tabulated results of three pairs of mice. (G-I) BMCs, splenocytes and hepatic leukocytes from both Clr-b^-/- and B6 animals were stained with an array of mAb specific for NK receptors to assess the NK repertoire of Clr-b^-/- deficient mice. NK1.1+ CD3- cells were gated, and analyzed for the proportion of the NK subset of interest, and the expression level of the NK receptor of interest. The proportion of each NK subset is listed as % of NK1.1+ cells, directly above each histogram overlay; MFI is the median fluorescent intensity of the gated population, indicated by the bar gate on the histograms. Solid histogram depicts Clr-b^-/-, lined histogram depicts B6, dotted line is the appropriate FMO (fluorescence minus one) control; RCN, relative cell number. (J) Quantitation of both the proportion of different NK subsets and expression of different NK receptors in 3 pairs of mice (n = 3). Viable cells were gated on forward and side scatter, followed by DAPI exclusion. All comparisons between B6 and Clr-b^-/- were analyzed with the two-tailed, paired t-test, In all quantitation histograms, empty bars represent B6, and solid bars represents Clr-b^-/-. ** indicates a statistically significant difference using a two-tailed t-test (p<0.05).
5.3.3 Acute rejection of Clr-b<sup>−/−</sup> BM grafts in wild-type recipients mediated by NK cells

To test the role of MHC-independent “missing-self” rejection of Clr-b<sup>−/−</sup> donor BM cells, we established a short-term (48h) competitive BM transplant assay using an equal mixture of differentially labeled Clr-b<sup>−/−</sup> versus wild-type B6 donor BM cells into sublethally-irradiated B6 recipient mice. To control for NK cell activity, recipient mice were either NK depleted using PK136 mAb, or NK primed using polyI:C (Fig. 5.3A). Since PK136 was used for depletion, staining using PK136 will not be representative of the presence of NK cells, thus we relied primarily on the pan-NK marker CD49b (DX5 clone) to detect the presence of NK cells post NK depletion. As shown in Fig. 5.3B/C, Clr-b<sup>−/−</sup> BM cells were selectively and acutely rejected by wild-type recipient mice, in comparison to internal control B6 wild-type donor cells (p=0.001). This demonstrates that the NKR-P1B:Clr-b system plays a non-redundant role in the recognition of normal BM cells by NK cells.

To establish a rejection hierarchy in the mixed bone marrow chimeras, we used donor BM cells from Clr-b<sup>−/−</sup> mice in comparison to control B6 and experimental MHC-I-deficient BM cells, as follows: H-2K<sup>b−/−</sup>, H-2D<sup>b−/−</sup>, H-2K<sup>b−/−D−/−</sup>, and β2m<sup>−/−</sup> mice. Cells were prepared and labeled using different combinations of three fluorescent dyes (see Methods), then transplanted in equal mixtures into either untreated or NK-depleted recipient mice. The dye labeling patterns and their correspondence to the different BM populations are depicted in Table 5.1. Following BM transplantation (48h), the various MHC-I-deficient BM donors were rejected differentially, similar to observations reported in previous studies<sup>115</sup>. Notably, Clr-b<sup>−/−</sup> BMCs were selectively rejected in the wild-type
recipient mice to an extent similar to, albeit lower, than the H-2D^{b/+} donor BMCs (Fig. 5.3D). Moreover, depletion of NK cells significantly diminished the rejection of all MHC-I-deficient BM cells (p<0.001 for all), as well as Clr-b^{-/-} BM cells (p<0.01) (Fig. 5.3D).

In addition, we also evaluated the rejection of ERAAP^{-/-} donor BMCs to assess whether NK recognition of MHC-I is peptide-dependent. ERAAP is an ER-resident enzyme that trims the N-termini of loaded antigenic peptides, and the deficiency of this gene results in a vastly altered repertoire of extended-length self peptides presented by MHC-I in ERAAP^{-/-} animals. Nonetheless, these mice possess a nearly identical MHC-I expression in comparison to B6 animals 57. Interestingly, ERAAP^{-/-} BMCs rejection was observed in both WT and NK-depleted hosts, suggesting that NK cells play a minimal role in the rejection phenotype of ERAAP^{-/-} BMCs observed in these mixed bone marrow chimera (Fig. 5.3D).
Table 5.1 Dye labeling combination–BM correspondence chart

The various bone marrow cells were labeled by the dye patterns indicated above. The labeled cells are identified via flow cytometry; PKH67 (Green fluorescent dye) is excited at 490nm, and maximally emits at 502nm, which is similar to a FITC fluorochrome; PKH26 (Red fluorescent dye) is excited at 551nm, and maximally emits at 567nm, similar to a PE fluorochrome; CellVue® Maroon (far red fluorescent dye) is excited at 647nm, and maximally emits at 667nm, similar to an APC fluorochrome.

<table>
<thead>
<tr>
<th>Genetic Modification</th>
<th>PKH67</th>
<th>PKH26</th>
<th>CellVue® Maroon</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2m⁻/⁻</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Clr-b⁻/⁻</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>H-2Dᵇ⁻/⁻</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>H-2Kᵇ⁻/⁻</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H-2Kᵇ⁻/⁻ H-2Dᵇ⁻/⁻</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>ERAAPᵇ⁻/⁻</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 5.3

A

NK-depleted (PK136 mAb)  
NK1.1-PE

1.53

NK-primed (poly I:C)

NK1.1-PE

8.89

DX5-APC

B

CD45.2(PE)  
CD45.2(Clr-b-/-)

2.47

2.91

CD45.1(APC)  
CD45.1(B6)

1.77

3.12

P=0.001

CD45.2(Clr-b-/-)
C

% Rejection of BMC in B6 Recipients

% Rejection of Cln-b<sup>−/−</sup> donor BMC relative to syngeneic donor graft

**
Figure 5.3 Clr-b<sup>−/−</sup> BM cells are acutely rejected in an NK cell-dependent manner by wild-type B6 host mice.

(A) Staining of BM graft recipient splenocytes with (left) or without (right) NK depletion using PK136 mAb; NK cells (NK1.1<sup>+</sup>DX5<sup>+</sup>) are absent from NK1.1-depleted recipient mice (PK136-treated 24h prior), and present in NK-primed recipients (polyI:C-treated 24h prior). (B) Competitive transplant of a 1:1 mixture of wild-type (CD45.1<sup>+</sup>) versus Clr-b<sup>−/−</sup> (CD45.2<sup>+</sup>) BM cells into CD45.1<sup>+</sup>CD45.2<sup>+</sup> recipients. Recipients are either NK-primed by polyI:C, or NK-depleted by PK136. Splenocytes were harvested from the recipient mice 48 hours after transplantation and analyzed via flow cytometry. (C) Quantitative analysis of competitive BM rejection shows a statistically significant, selective rejection of Clr-b<sup>−/−</sup> BMC by NK cells (unpaired t-test, p=0.0013). Histograms show the average rejection of 6 recipients. (D) Donor BMCs from B6, Clr-b<sup>−/−</sup>, β2m<sup>−/−</sup>, H-2K<sup>b−/−</sup>, H-2D<sup>b−/−</sup>, H-2K<sup>b−/−</sup>H-2D<sup>b−/−</sup>, and ERAAP<sup>−/−</sup> were injected into sublethally irradiated NK-sufficient or NK-deficient B6 recipients. 48 hours post BM infusion, spleens of the recipient mice were harvested and analyzed via flow cytometry to determine the proportions of the transplanted cell populations. Viable cells were gated on forward and side scatter, followed by DAPI exclusion. % Rejection of the genetically modified BM donor BMC population relative to the autologous BM donor was calculated with the following equation: \(1 - \left(\frac{\text{proportion of donor BM of interest}}{\text{proportion of autologous BM}}\right)\).
BM)\textsubscript{input}/[(\text{proportion of donor BM of interest})/(\text{proportion of autologous BM})]\textsubscript{output} \times 100\%. Histograms show the average rejection of 3 recipients. Data are representative of 3 independent transplantation experiments. Data were analyzed via 2-way ANOVA; the BM rejection phenotypes between NK-sufficient and NK-deficient recipients were assessed by the Bonferroni post-test. * denotes p<0.01; ** denotes p<0.001.
5.3.4 Acute rejection of Clr-b<sup>–/–</sup> BM cells is mediated by NKR-P1B+ NK subset

As NKR-P1B is the only cognate receptor identified for Clr-b, we expected that the rejection of Clr-b<sup>–/–</sup> BM cells would be mediated by NKR-P1B+ NK subset. To address this question, we used wild-type allogeneic NIH.Swiss mice as recipients, since PK136 mAb selectively depletes the NKR-P1B+ NK subset in these mice, leaving the remaining NKR-P1B– NK cells intact<sup>19,21,87</sup>. BM graft mixes consisting of equal proportions of B6, Clr-b<sup>–/–</sup>, and β2m<sup>–/–</sup> cells were used to test rejection. In this scenario, we predicted that depletion of the NKR-P1B+ NK subset should abrogate the rejection of Clr-b<sup>–/–</sup> BM cells, relative to wild-type B6 control BM cells, yet the rejection of β2m<sup>–/–</sup> BM cells should remain unaffected, since residual NKR-P1B– NK cells should eliminate these cells. Indeed, 48 hours later, 90% of transplanted β2m<sup>–/–</sup> BM cells and ~40% of transplanted Clr-b<sup>–/–</sup> BM cells were rejected in the NIH.Swiss recipients relative to B6 donor BM cells, mirroring the phenotype that we observed using the B6 wild-type recipients (Fig. 5.4). Interestingly, elimination of the NKR-P1B+ NK subset in the NIH.Swiss recipients prevented the rejection of Clr-b<sup>–/–</sup> donor cells; on the other hand, the high level of rejection observed for β2m<sup>–/–</sup> BMCs persisted (Fig. 5.4). These results support the notion that rejection of Clr-b<sup>–/–</sup> BMCs is mediated specifically by the NKR-P1B+ NK subset in the recipient mice, and that depletion of this subset results in the acceptance of Clr-b<sup>–/–</sup> BM grafts. In contrast, the NKR-P1B– NK subset remaining in the treated NIH.Swiss recipients effectively mediated the rejection of β2m<sup>–/–</sup> BMCs (Fig. 5.4).
**FIGURE 5.4**

B6, Clr-b<sup>−/−</sup> and β2m<sup>−/−</sup> BMCs were differentially labeled using PKH26, PKH67, and CellVue® Maroon dyes, then mixed equally and transplanted into sublethally irradiated NIH.Swiss recipient mice. Flow cytometric analysis of NIH.Swiss NK-sufficient and NK-depleted recipient spleens were performed 48 hours post-transplant, then analyzed for each donor cell subset. % rejection of the genetically modified BM donor BMC population relative to B6 BM donor was calculated with the following equation: \(\{1 - \left[\frac{\text{(proportion of donor BM of interest)/(proportion of B6 BM)}}{\text{(proportion of donor BM of interest)/(proportion of B6 BM)}}\right]_{\text{input}}/\left[\text{(proportion of donor BM of interest)/(proportion of B6 BM)}}\right]_{\text{output}}\}\) x 100%. Histograms show the average rejection of 3 recipients. Data are representative of 3 independent transplantation experiments. Data were analyzed via 2-way ANOVA; the BM rejection phenotypes between NK-sufficient and NK-deficient recipients were assessed by the Bonferroni post-test. ** denotes p<0.001.

**Figure 5.4 Acute rejection of Clr-b<sup>−/−</sup> BM cells in NIH.Swiss recipient mice.**

* NK-sufficient
* NKR-P1B−

<table>
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<tr>
<th>Genotype of Donor BM</th>
<th>% Rejection of BMC in NIH.Swiss Recipients</th>
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<tr>
<td>Clr-b&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td><strong>50</strong> (NK+)</td>
</tr>
<tr>
<td>β2m&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td><strong>100</strong> (NKR-P1B−)</td>
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5.3.5 Assessing the developmental potential of Clr-b\(^{-/-}\) BM

Successful bone marrow transplantation in clinical settings requires the transplanted graft to be able to repopulate the recipient’s hematopoietic system through various differentiation processes in competition with resident host cells. Thus, we subsequently assessed the developmental capacity of Clr-b\(^{-/-}\) BMCs under competitive pressure with WT BMCs. To this end, we administered 9.5 Gy irradiation to double congenic recipient mice (CD45.1/CD45.2) and transplanted equal proportions of WT (CD45.1) and Clr-b\(^{-/-}\) (CD45.2) derived BMCs. We then allowed reconstitution up to 3-5 weeks, then performed the same immune characterization experiments on these mixed BM chimera outlined in section 5.3.2.

In figure 5.5A, the ratio between WT (CD45.1) and Clr-b\(^{-/-}\) derived cells (CD45.2) after 3-5 weeks deviates from the initial 1:1 ratio (BM: 3:1; LN: 2:1; LV: 2:1; SP: 2:1; TH: 4:1) in NK-sufficient CD45.1/CD45.2 recipients. However, in NK-depleted CD45.1/CD45.2 recipients, the relative reconstitution of Clr-b\(^{-/-}\) donor BMCs to WT BMCs 3-5 weeks post BM infusion remain 1:1 (data not shown), suggesting that Clr-b\(^{-/-}\) cells are eliminated by the recipient NK cells, in agreement with the results from section 5.3. However, NK cells in the recipient will eventually die due to lethal irradiation, and the remaining Clr-b\(^{-/-}\) donor BMCs establish tolerance, and engraft in the double congenic recipients. More importantly, in these long-term mixed bone marrow chimeras, we observed very little difference in the resulting leukocyte distribution in BM, lymph node, spleen, and thymus, and similar patterns in T cell differentiation pathways that stemmed from Clr-b\(^{-/-}\) derived cells (CD45.2) in comparison to WT (CD45.1) (Fig. 5.5.B-G). Notably, a smaller proportion of hepatic and lymph node derived Gr-1+ cells (p
= 0.0035, p = 0.0277, respectively), hepatic CD11b+ (p = 0.0432) and Gr-1+ CD11b+ (p = 0.0373) cells were observed compared to B6 donor BMCs; as well, a greater percentage of hepatic NKT (p = 0.0425) and NK1.1+ CD11c+ (p = 0.0305) cells were seen compared to their WT counterpart (Fig. 5.3D). In terms of NK subset distributions, more BM NKG2A/C/E+ (p = 0.0074) NK cells differentiated from Clr-b−/− BMCs compared to B6 BMCs. In contrast, Clr-b−/− BMCs gave rise to less splenic Ly49CIFH+ (p = 0.0151) and Ly49I+ (p = 0.0473) subsets relative to WT BMCs.

The expression of different NK receptors on various NK subsets is mostly comparable between WT and Clr-b−/− origin (Fig. 5.3H-K), however, higher expression level of NKR-P1B on BM NKR-P1B+ (p = 0.0401) NK cells originated from Clr-b−/− BMCs was still observed (Fig. 5.3H, K), yet this difference is not significant in splenic and hepatic NKR-P1B+ NK cells (p = 0.0514, 0.1697, respectively). In addition to NKR-P1B, Clr-b−/− derived NK cells appear to have higher expression in Ly49H in the BM (p = 0.0465), higher levels of Ly49I (p = 0.0484) in the liver, and finally higher level of Ly49CI (p = 0.0027) and NKR-P1C (p = 0.0081) in the spleen, compared to its B6 counterpart. Importantly, Clr-b−/− BMCs appear to have similar differentiation and developmental capabilities in comparison to the BM of a C57BL/6 donor.
FIGURE 5.5

A

BM  LN  LV
24.9%  20.6%  26.5%
63.7%  58.0%  61.7%

SP  TH
30.1%  15.2%
59.4%  0.234%

CD45.2 (Clr-b−/−)  CD45.1 (WT)
Bone Marrow Leukocyte Distribution

Cell Surface Marker

% Total Viable Cells

WT BM  Clr-b^− BM

Gr-1

CD11b

CD11c

CD19

CD3

Gr-1+

CD11b+

NK1.1+

CD3+

NK1.1+

CD11b+

NK1.1+

CD11c+

WT BM  Clr-b^− BM

NK1.1

CD3

CD11c

CD3

NK1.1

CD11b
Liver Leukocyte Distribution

Cell Surface Marker

WT LV  Clr-b−/− LV  WT LV  Clr-b−/− LV

Gr-1

CD11b

CD11c

CD19

CD3

NK1.1

Liver Leukocyte Distribution

% Total Viable Cells

WT KO

Cell Surface Marker
E

Spleen Leukocyte Distribution

- **WT**
- **KO**

% Total Viable Cells

Cell Surface Marker

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<th>Clr-b⁻/⁻ SP</th>
<th>WT SP</th>
<th>Clr-b⁻/⁻ SP</th>
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Thymus Leukocyte Distribution

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% Total Viable Cells

Cell Surface Marker

WT: White
KO: Black
T Cell Development

Cell Surface Marker

G

T-Cell Development

Cell Surface Marker
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Expression of NK-Receptors on NK1.1+ CD3- Cells

Proportion of Different NK-Subsets

NK Subsets
Figure 5.5 Assessing the developmental capabilities the Clr-b⁻/⁻ BM

(A) Bone marrow (BM), lymph node (LN), liver (LV), spleen (SP) and thymus (TH) were harvested from 3-5 week reconstituted mixed BM chimeras and stained with α–CD45.1 and α–CD45.2 to differentiate donor WT (CD45.1+) and donor Clr-b⁻/⁻ (CD45.2+) derived cells from recipient cells (CD45.1+ CD45.2+). (B-F) mAbs that were used to assess hematopoietic repertoire in section 5.3.2 were used to stain the five organs listed above harvested from the reconstituted mixed BM chimeras. WT derived cells were gated on CD45.1; Clr-b⁻/⁻ derived cells were gated on CD45.2. Histograms represent tabulated results of three independent reconstituted mixed BM chimeras (n = 3); empty bars depict cells developed from WT BM, solid bars depict cells developed from Clr-b⁻/⁻ BM. (G) Thymocytes from the reconstituted mixed BM chimeras were stained with mAb for surface markers that define T cell development (used in section 5.3.2). Similar to (B-F), histogram represents tabulated results of three independent reconstituted mixed BM chimeras (n = 3). (H-J) BMCs, splenocytes and hepatic leukocytes from reconstituted mixed bone marrow chimeras were stained with an array of mAb specific for NK receptors to assess the NK repertoire of Clr-b⁻/⁻ deficient mice. NK1.1+ CD3⁻ cells were gated, and analyzed for the proportion of the NK subset of interest, and the expression level of the NK receptor of interest. The proportion of each NK subset is listed as % of NK1.1+, directly above each histogram overlay; MFI is the median fluorescence intensity of the gated population, indicated by the bar gate on the histograms. Solid histogram represents donor Clr-b⁻/⁻ derived cells (CD45.2), lined histogram represents donor WT derived cells (CD45.1), dotted line is the appropriate FMO (fluorescence minus one) control; RCN, relative cell number. (K) Quantitation of both the proportion of different NK subsets and expression of different NK receptors in three independent reconstituted mixed BM chimeras (n = 3). Comparisons between donor B6 (CD45.1) and donor Clr-b⁻/⁻ (CD45.2) derived cells were analyzed with the two-tailed, paired t-test. In all quantitation histograms, empty bars represent CD45.1 WT derived cells, solid bars represent CD45.2 Clr-b⁻/⁻ derived cells. ** indicates a statistically significant difference using a two-tailed t-test (p<0.05).
5.3.6 The NK receptor NKR-P1B is functional despite the absence of Clr-b during NK education

From section 5.3.2, we observed an elevated cell surface expression of NKR-P1B on Clr-b<sup>−/−</sup> derived NK cells in comparison to its B6 counterparts. In addition to the modulation of NKR-P1B expression level, we wondered if the absence of Clr-b during NK education in Clr-b<sup>−/−</sup> animals would affect the inhibitory function of the NKR-P1B receptor. Here, variants of BWZ.36 cells (Z.Clr-b+ and Z−) were used as target cells for LAKs effector cells generated from B6 and Clr-b<sup>−/−</sup> animals in a chromium release assay. Interestingly, Z.Clr-b inhibited the killing of both B6 and Clr-b<sup>−/−</sup> LAKs, suggesting that cell surface Clr-b inhibits NK effector function (Fig. 5.6A), and this phenotype is reversed upon the addition of the blocking antibody 4A6 (α-Clr-b mAb). Furthermore, we conducted an antibody redirected inhibition assay, where P815 cells were used as target cells for LAKs generated from B6 and Clr-b<sup>−/−</sup> splenocytes sorted for NKp46<sup>+</sup> and NKR-P1B<sup>+</sup>. P815 alone are susceptible to LAK killing from both strains (Fig. 5.6B). However, P815 can bind the Fc portion of the α-NKR-P1B mAb 2D12, thus redirecting the variable domain of 2D12 against the NKR-P1B expressed by the effector cells. Notably, the addition of 2D12 to the target cells decreased the killing of P815 cells via redirected inhibition of NKR-P1B in both B6 and Clr-b<sup>−/−</sup> derived LAKs (Fig. 5.6B).
**Figure 5.6**

**A**

The NKR-P1B receptor is functional on Clr-\(b^{-/-}\) derived NK cells

(A) Clr-b+ (Z.Clr-b) and Clr-b– BWZ (Z–) target cells were used as targets for lymphokine activated killers (LAKs) effector cells cultured from either B6 or Clr-b\(^{-/-}\) derived splenocytes in the chromium release assay. 4A6, an αClr-b mAb was used to block the interaction between the effector and BWZ target cells. (B) P815 with and without 2D12 was used as target cells for antibody redirected inhibition assay, with the same effector cells as part (A). Each point on the plot represents the average value of 3 technical triplicates.
5.4 DISCUSSION

The importance of NK cells in determining BM transplantation success had been delineated with the experimental phenomena observed in hybrid resistance\(^{34-36,85}\) as well as BM allograft acceptance in NK-depleted C.B-17 scid mice\(^{11,42,46,108,109,152}\). Importantly, these conclusions were based on missing-self recognition of class I MHC molecules, which are encoded by a highly polymorphic gene complex in both mice and humans. Class I MHC polymorphisms in turn influenced the evolution of two highly polymorphic receptor families (Ly49 in mice, KIR in humans), with multiple polymorphisms exhibited at both the allelic and haplotype levels\(^{20}\). In contrast, fewer but significant allelic polymorphisms have been reported in the MHC-independent Nkrp1-Clr receptor-ligand family in mice\(^{20,26}\), yet the overall haplotype structure appears to be conserved\(^{20}\). Interestingly, Clr-b is a member of the Clr ligand family that possesses an expression pattern similar to class I MHC, yet demonstrates no allelic polymorphisms across many mice strains studied to date (B6, BALB, 129)\(^{18}\). As prominent roles for missing-self recognition of Clr-b in both tumour immunoserveillance and infectious disease have been documented\(^{45,157}\), we investigated the missing-self recognition of this MHC-independent ligand in BM transplantation via an *in vivo* mouse model, such as the genetically deficient OCIL/Clr-b\(^{-/-}\) mice.

Clr-b\(^{-/-}\) mice were generated by targeted deletion of exon 3 of the *Clr-b* gene (total 5 exons)\(^{77}\), which eliminates the stalk region of the molecule and results in a frame-shift between exon-2 and exon-4/5. Interestingly, unpublished data in our laboratory show that some Clr-b deficient tumor cell lines possess “exon 3-deficient” Clr-b splice isoform transcript, suggesting that a deletion of exon 3 may be a naturally
occurring process during alternative splicing in some cancer cells. In Fig. 5.1A-D, we observed that the presence of shorter Clr-b transcript directly correlates with the absence of Clr-b cell surface staining using 4A6 mAb, and loss of direct activation of NKR-P1B fusion receptor (CD3ζ/NKR-P1B) on BWZ reporter cells. This suggests that the only ligand recognized by NKR-P1B is Clr-b, and that both hematopoietic and non-hematopoietic tissues do not express any other ligand for NKR-P1B.

The steady state immune characterization of the Clr-b−/− mice (section 5.3.2) showed no overt differences in the hematopoietic composition in the BM, lymph node, liver, spleen and thymus compared to B6 animals when analyzed using a two-tailed, paired student t-test. Interestingly, when we analyzed different NK subsets, we observed smaller Ly49G+ and NKG2D NK subsets in the BM. Ly49G+B6 is an inhibitory NK receptor that recognizes the H-2Dd protein, whereas NKG2D is a stimulatory receptor that recognizes many MHC class I-related molecules. The proportion of different NK subsets is unique in each mouse as it is determined through NK education, which was believed to occur through NK receptor and ligand interactions during NK development processes in the BM. Thus, it is possible that Clr-b can indirectly modulate the proportions of these NK subsets by affecting the expression of the ligands for both Ly49G and NKG2D. However, it has been reported that the endogenous MHC-dependent self-ligand for Ly49G is absent in B6 mice (Ly49G+B6 recognizes only H-2Dd). Therefore, Ly49G+B6 may recognize another MHC-independent ligand, and that Clr-b expression may in turn affect the expression of this unknown ligand. Additional work is required to test this speculation. In addition, expression studies of NKG2D ligands in Clr-b−/− animals in comparison to B6 mice may shed light on the subset differences observed
in the NKG2D+ NK subsets in the knockout animals in contrast to its B6 counterpart. Lastly, we saw essentially no differences in the expression levels of various NK receptors by NK cells between the knockout and WT animals, with the exception of NKR-P1B, which is the cognate receptor for Clr-b. Here, we observed a statistically significant higher expression of NKR-P1B on splenic NK cells from Clr-b<sup>−/−</sup> animals relative to WT NK cells. Although not statistically significant, we also observed a trend where BM and hepatic NK cells from Clr-b<sup>−/−</sup> animals exhibit higher expression of NKR-P1B in comparison to WT NK cells. Interestingly, an elevated expression of Ly49 receptors has been observed in β2m<sup>−/−</sup> mice, which are devoid of cell surface class I MHC ligand<sup>127</sup>. Mechanistically, this elevated NKR-P1B expression may be due the lack of receptor recycling, a post-translational process in which receptor-ligand complex is removed from the cell surface following ligation (in cis or in trans) via endocytosis<sup>28</sup>. In Clr-b<sup>−/−</sup> mice, endocytosis of NKR-P1B may not occur since the ligand for NKR-P1B is absent, in contrast to B6 mice where Clr-b on neighboring cells or on the NK cells themselves is available to bind NKR-P1B. Furthermore, the increased expression of NKR-P1B on Clr-b<sup>−/−</sup> NK cells relative to WT was also an expected result from the NK cell education standpoint. Normally, a developing NK cell achieves an integrated or a balanced stimulatory and inhibitory set of signals that are transduced through various receptor-ligand interactions engaged during NK education. In Clr-b<sup>−/−</sup> mice, the NKR-P1B+ NK cell subset cannot transduce an inhibitory signal through the NKR-P1B receptor due to the absence of Clr-b. In attempt to achieve the balanced signaling, the developing NK cell may augment the inhibitory receptor expression and/or decrease the stimulatory signals thus received. In this case, the Clr-b<sup>−/−</sup> NK cells increased the expression of NKR-P1B in
attempt to optimize the balance. Interestingly, BM, and hepatic NK cells from Clr-b<sup>−/−</sup> derived animals exhibit a lowered NKR-P1C cell surface expression, and Clr-b<sup>−/−</sup> derived splenic NK cells also show a similar trend; BM and hepatic NK cells from Clr-b<sup>−/−</sup> animals demonstrate a trend where a lowered NKp46 cell surface expression was observed, in contrast to WT NK cells. Both NKR-P1C and NKp46 are stimulatory NK receptors, and NKp46 has been reported to recognize an unknown self ligand<sup>56</sup>; thus, lowering the expression of this receptor will in turn reduce the net stimulatory signal during NK cell education. Collectively, these data suggest that modulation of NKR-P1B, and perhaps both NKR-P1C and NKp46 are involved in NK education.

The hematopoietic characterization of the developing capabilities of Clr-b<sup>−/−</sup> BMCs in long-term mixed BM chimeras showed no noticeable differences compared to B6 BMCs. However, one important difference when compared to the immune characterization of steady state animals is that in the long-term mixed bone marrow chimera, the developing and differentiating Clr-b<sup>−/−</sup> derived BMCs can now interact with Clr-b <sup>in trans</sup> (ie. Clr-b from either the host or the CD45.1 donor cells). This difference may result in phenotypes that were not detected in the steady state characterization, most profoundly in the NK population as the receptor for Clr-b, NKR-P1B, is predominantly expressed by NK cells. Here, we observed a smaller proportion of Ly49CIFH+ and Ly49I+ splenic NK subsets derived from Clr-b<sup>−/−</sup> compared to their CD45.1 derived counterparts in the long-term mixed BM chimeras. In addition, lower expression levels of NKR-P1C was observed on splenic NK cells that originated from Clr-b<sup>−/−</sup> animals; and higher expression levels of Ly49H, Ly49I, and Ly49C/I were observed on Clr-b<sup>−/−</sup> derived BM, hepatic and splenic NK cells, respectively, in comparison to their CD45.1
derived counterparts. As described previously, NK cell education occurs via receptor modulation to achieve a balanced net inhibitory and stimulatory signal within each individual NK cell subset. Since Clr-b−/− derived NKR-P1B+ NK subset can engage Clr-b in trans during NK cell education in long-term reconstituted bone marrow chimeras, a different NK developmental outcome may be exhibited when compared to Clr-b−/− mice, such as the different percentages and MFI levels of the various NK receptors. Interestingly, significantly higher NKR-P1B expression level was observed only on Clr-b−/− derived BM NK population relative to their CD45.1 derived NK counterparts in the long-term reconstituted mixed bone marrow chimeras. This data may suggest the site in which active NK education takes place: Initially, NKR-P1B level is still getting adjusted through active NK education in the bone marrow, and the lack of cis NK-P1B:Clr-b interaction on Clr-b−/− derived NK cells may result in an elevated level of NKR-P1B. As NK cells migrate to the periphery, their NKR-P1B level will be determined by trans Clr-b interaction, in which there will be no difference experienced by Clr-b−/− or CD45.1 derived NK cells, thus their NKR-P1B expression level are equivalent in the spleen and liver. Additional experimentation is required to validate this hypothesis.

As previously mentioned, acute rejection of class I MHC-deficient BM grafts by NK cells has been widely studied in mouse models 9,115. Thus, we developed a novel modification of an assay where we can simultaneously compare the degree of rejection of Clr-b+/− BM grafts and different class I-deficient BM grafts relative to B6 donor graft in a single mouse. This assay employs three different membrane dyes that fluoresce in different channels, thus by using different combinations, we can differentially label up to seven different donor populations. This approach conserves the number of recipient
animals used, but more importantly, it reduces the variability of recipient rejection responses, as we can now visualize the competitive rejection of all donor BMCs by the NK cells from a single mouse, thus, in an internally controlled manner. As expected, all of the MHC-I deficient BM grafts (β2m−/−, KbDb−/−, KbDb−/−, Db−/−) were rejected to different degrees by recipient NK cells 48 hours post transplantation. Notably, β2m−/− and KbDb−/− BMCs were rejected to the greatest extent, since the absence of β2m and the deletion of KbDb−/− genes will prevent the expression of all class I MHC as well as Qa-1, which is a non-classical MHC-Ib molecule that depends on the leader peptides of classical MHC-I molecule for cell surface expression. Therefore, β2m−/− BMCs will disinhibit many Ly49+ NK subsets as well as NKG2A/CD94+ NK subsets which will act in concert to mediate its rejection155. Similarly, high levels of KbDb−/− BMCs were also rejected, since many Ly49+ NK subsets, such as Ly49C and Ly49I112, and NKG2A/CD94+ NK subsets will be disinhibited, mirroring the rejection mechanism of β2m−/− BMCs. Logically, BMCs deficient in only a single MHC-I gene (eg. Kb−/− or Db−/−) would exhibit a lowered rejection compared to KbDb−/− BMCs, and this was indeed reflected in previous reports as well as the results described above. Alternatively, rejection of ERAAP−/− BMCs does not appear to be fully NK-dependent, as rejection of ERAAP+− BMC was only slightly reduced in NK-depleted recipients. However, since it has been shown that ERAAP+− are susceptible to B and T cell mediated killing, and the above transplantation was performed in sublethal irradiated recipients, the effect of NK-dependent cytotoxicity may be masked by the confounding cytotoxic effects exerted by the remaining B and T lymphocytes in the recipient animals. A more robust NK dependent phenotype may be observed if B6.SCID mice are used as recipients instead of B6 WT animals to circumvent the
potential confounding effects posed by B and T lymphocytes. The comparable rejection of Clr-b\textsuperscript{−/−} BMCs to H-2D\textsuperscript{b/−} was quite striking, since only 50-60% of total splenic NK cells express NKR-P1B, the only documented cognate receptor for Clr-b. As previously mentioned, many overlapping NK subsets would be disinhibited by MHC-I deficient BMCs, thus resulting in a robust BM rejection phenotype. In contrast, dis-inhibition of NKR-P1B+ NK subset alone accounts for up to 30-40% rejection. This data thus demonstrate an important role of MHC-independent missing-self recognition of Clr-b in bone marrow transplants. Results using NIH.Swiss as recipients for the mixed BM population further strengthens this claim. In this mouse model, β2m\textsuperscript{-/−} BMCs were still rejected up to 95%, however, rejection of Clr-b\textsuperscript{−/−} BMCs increased up to 50% in comparison to B6 recipients (Fig. 5.4). There are likely 2 factors that contribute to the more robust rejection of Clr-b\textsuperscript{−/−} BMCs in NIH.Swiss recipients. Firstly, both Clr-b\textsuperscript{−/−} and B6 donor BMCs will be rejected since it is an allograft in NIH/Swiss recipients. NIH/Swiss mice harbour the H-2\textsuperscript{a} MHC-I haplotype. Therefore, both Clr-b\textsuperscript{−/−} and B6 donor BMCs (H-2\textsuperscript{b}) are susceptible to the missing-self recognition of H-2\textsuperscript{a} ligands by the NIH/Swiss Ly49+ NK and thus rejected. However, the Clr-b\textsuperscript{−/−} donor cells are rejected by a “second hit”, which is the missing-self recognition of Clr-b. The combination of the MHC-dependent and MHC-independent missing-self recognition may account for additional rejection phenotype in NIH/Swiss recipients in comparison to B6 hosts. Another difference is likely attributed the fact that the NIH.Swiss allele of NKR-P1B has a greater affinity for Clr-b than the B6 allele. Thus, the absence of cell surface Clr-b will result in a greater degree of dis-inhibition of the NKR-P1B+ NK subset in the NIH.Swiss recipient, leading to a more robust rejection phenotype. Importantly, selective depletion
of the NKR-P1B⁺ NK subset in the NIH. Swiss recipient reversed the rejection of Clr-b⁻/⁻ BMCs, whereas the rejection of β2m⁻/⁻ BMCs was not affected. Collectively, these data demonstrate an important and non-redundant role of missing-self recognition of Clr-b in determining the success BM transplantation by NKR-P1B⁺ NK cells.
CHAPTER VI

DISCUSSION
6.1 THESIS GOALS

The field of NK cell biology is rapidly advancing, and our laboratory seeks to understand the role of MHC-independent missing-self recognition by NK cells. There are several MHC-independent inhibitory NK ligands reported including CD48, cadherins, and Clr-b, to list a few. In particular, our laboratory is interested in studying the Nkrp1-Clr receptor-ligand family, as it has been shown previously that Clr-b exhibits an expression pattern similar to MHC-I \(^{18}\). Interestingly, in addition to NKR-P1B, the cognate inhibitory receptor for Clr-b, NK cells may also express another inhibitory receptor of the Nkrp1 receptor family, NKR-P1G. Furthermore, there are seven to ten putatively functional Clr genes reported in mice and rats, respectively (Clr-a-h, Clr-e, i, j are pseudogenes; Clr-1-11, Clr-8 is a pseudogene) \(^{26,89}\). These reports collectively provoked our curiosity about the biology of NKR-P1G and the rest of the Clr ligands. To address these questions, we decided to make a novel mAb against NKR-P1G, followed by the search for NKR-P1G ligands, as well as evaluation of other Nkrp1-Clr receptor-ligand interactions. We believe that the expansion of our understanding of the Nkrp1-Clr receptor ligand system will provide answers and new insights in the understanding of NK recognition. Lastly, with the assistance of our collaborators, we investigated the effects of Clr-b deletion in an \textit{in vivo} mouse model. Here, we postulate that \textit{in vivo} modeling of knocking out a MHC-independent ligand will further our understanding on NK development, education, and NK recognition under physiological conditions. These premises thus define the goals of this thesis, and hopefully will broaden our appreciation of NK cells in the field of immunology.
6.2 APPLICATION OF CELLISA

Chapter III described an application of a methodology of generating and screening for mAb raised against cell surface antigens. Conventional methods of generating mAb can require intensive screening. Here, we proposed an alternative using mammalian cells as an immunizing and screening agent to streamline mAb screening and production.

Using mammalian reporter cell lines expressing the antigen of interest for immunization offers three advantages: Firstly, it circumvents the antigen purification procedures. Purified bacterially expressed antigens often exhibit misfolded conformations compared to native forms expressed on the surface of mammalian cell lines; consequently, usage of purified antigens for immunization may result in undesired specificities of the raised mAb. Secondly, by using an immunogen where the antigen of interest (eg. mouse NKR-P1G) is expressed on cell lines from the same species as the immunized host (eg. rat YB into rat recipients), the immune response to self antigens is minimized and the humoral response is targeted towards the xenogeneic antigen of interest. Thirdly, using reporter cells for both immunization and the screening of hybridoma minimize reagents.

We have shown the use of BWZ cells for hybridoma supernatant screening is highly rapid, cost-effective and efficient. Collectively, this report further showcases another valuable application of the BWZ reporter cell assay in addition to its original use to identify cell surface receptor-ligand interactions.

However, there are caveats; an issue that needs to be addressed is the selection of a suitable host for immunization. Specifically, mAb purified from the 1H8 rat hybridoma was only able to recognize NKR-P1G ectopically expressed on cell lines (eg. Rat YB,
mouse BWZ, human 293T); however, we have not yet been able to detect NKR-P1G on freshly isolated mouse \textit{ex vivo} cells nor LAKs. Interestingly, unpublished results from Kveberg \textit{et al.} reported similar findings described in this work (NK meeting 2012, Asilomar). Notably, when they switched the immunizing host to hamsters, they were able to detect NKR-P1G from rat \textit{ex vivo} NK cells, whereas immunizing mice with rat NKR-P1G was not successful. Perhaps the phylogenetic similarity between mice and rats resulted in a rat humoral response that was raised against an aberrant epitope of mouse NKR-P1G (eg. \textit{ex vivo} NKR-P1G may be differentially glycosylated in comparison to the ectopically expressed NKR-P1G on cell lines); alternatively, epitopes may be masked in \textit{cis} on normal cells (eg. by ligand) \textsuperscript{61}. In contrast, when hamsters were used as hosts, the differences between these two rodent species were great enough to direct an immune response specifically for the mouse NKR-P1G molecule in isolation. Another possibility may be that NKR-P1G is not expressed on the surface of mouse NK cells, where it is on rat NK cells (despite the presence of NKR-P1G mouse LAKs). Ultimately, the idea of “immune-focusing” is still valid; however, like most biological scenarios, the experimental results are empirical, sometimes fortuitous, but trial and error. We postulate that the combinatorial use of rat hosts and an immune-focusing strategy here for a mouse antigen may have raised an antibody that recognized a “finicky” mouse epitope that is either absent or masked on mouse \textit{ex vivo} NK cells. In contrast, hamster hosts may mount an antibody response more specific to a conserved rat epitope, which is evident and easily identified on rat \textit{ex vivo} NK cells.
6.3 ANALYSIS OF THE NKRP1-CLR GENE CLUSTER IN DIFFERENT COMMON INBRED MOUSE STRAINS

Chapter IV examined the genetic polymorphism of the Nkrp1-Clr receptor ligand system in different strains of mice. Importantly, we investigated whether this MHC-independent system exhibited both allelic and structural (haplotype) polymorphisms that were reported in the Ly49 receptor and MHC-I ligand systems \(^2\). Previous work suggested that the BALB/c and 129 strains are more closely related compared to B6 based on their homology observed in the NKR-P1A, B, C, G, Clr-d and f genes. However, the identical alleles of NKR-P1F and Clr-c expressed by BALB/c and B6 (129 expresses different alleles) complicate this notion. The cause of these differences may be pathogen-driven evolution, where an unidentified pathogen may have employed an immunoevasin to subvert this receptor-ligand system of perhaps “induced-self” recognition. However, since both the B6 and 129 Clr-c alleles are able to bind NKR-P1F of either strain with equal affinity, the reason for this allelic divergence requires further investigation that may affect immunoevasin trafficking. Importantly, this work raises interesting questions that may challenge the conventional presumption of categorizing the BALB/c and 129 mice as genetically similar strains in terms of NK biology.

Chapter IV also highlights novel Nkrp1-Clr receptor-ligand pairs in mice (also reported by Kveberg et al), specifically the recognition of Clr-c, d, g by NKR-P1F, and the recognition of Clr-d, f, g by NKR-P1G. Importantly, we showed that the interaction between NKR-P1G and its ligand is blocked either completely (Clr-f, g) or partially (Clr-d) by the \(\alpha\)-NKR-P1G mAb (1H8) that was generated in chapter III. The physiological significance of the overlapping ligand specificity shared by NKR-P1F and NKR-P1G
remains elusive. However, the observation that NKR-P1F and NKR-P1G can compete for common ligands suggests a mechanism where the differential modulation of common or exclusive ligands under distinct pathological circumstances could impact NK recognition in a pathogen or tissue-specific fashion. In addition, this overlapping ligand specificity may have a pivotal role in the integration of signals during NK development. As described previously, NK cell education occurs through receptor-ligand interactions during the transition of iNKs to mature NKs, (eg. characterized by the appearance of Ly49 receptors for MHC-I in mice). The overlapping ligand specificity may be a mechanism to fine-tune or integrate the signals transduced into the NK cell via the Nkrp1 receptors. For example, a developing NKR-P1F+ NKR-P1G+ NK cell upon ligand binding Clr-c could transduce a stimulatory signal. The overlapping ligand specificity exhibited by NKR-P1F and NKR-P1G thus offers several options to “disarm” or educate this NK cell: firstly, engage a NKR-P1G-specific ligand (Clr-f) to rally an inhibitory signal; secondly, engage a common ligand recognized both by NKR-P1F and NKR-P1G, where a net-sum or balanced signal (can either be inhibitory or stimulatory) can be transduced into the developing NK cell. Evidently, having the ability to modulate the signal strength during NK development is advantageous, as it increases the efficiency and success of NK cell education. Of course, these are postulations and require experimental validation.
6.4 MHC-INDEPENDENT MISSING-SELF RECOGNITION OF TRANSPLANTED BM GRAFTS

Chapter V demonstrates the significance of the missing-self recognition of Clr-b in an *in vivo* model, as shown by the rejection of normal Clr-b<sup>+/−</sup> BMCs by WT recipients. Importantly, we show that the rejection of Clr-b<sup>+/−</sup> BMCs is mediated by the NKR-P1B+ NK subset, and that up to 30-40% of the Clr-b<sup>+/−</sup> BMCs are rejected within 48 hours, which is comparable to the H-2D<sup>b</sup><sup>−/−</sup> BMCs. As previously discussed, it is quite striking that the missing-self recognition of Clr-b alone can account up to 40% rejection, since variations of MHC-I deficient BMCs can be rejected by both the Ly49+ and CD94/NKG2+ NK subsets, and Clr-b is only 1 of ~7 Clrs. Since the “missing-self” recognition of Clr-b demonstrates a rather significant rejection phenotype *in vivo*, we want to extend the bone marrow transplantation study into a more physiological setting, where we will assess the effects of genotoxic stress, and opportunistic infections such as MCMV (both have been shown to downmodulate cell surface Clr-b<sup>45,157</sup>) on determining the BM engraftment success of donors and recipients that are both Clr-b sufficient. In addition, conditional knockout mice where the Clr-b gene is deleted in a Cre-dependent fashion (eg. ER-Cre or MX-Cre) could better define the role of Clr-b on cells *in vivo* in the absence of transplantation.

We have begun to investigate the non-redundant roles or MHC-dependent versus MHC-independent recognition in bone marrow transplantation by creating Clr-b<sup>−/−</sup>/β2m<sup>−/−</sup> mice and using their bone marrow cells as donor populations in the bone marrow transplantation system that was implemented in this thesis study. Here, we hypothesized that the combinatorial effect of missing-self recognition of both Clr-b and MHC-I will
exacerbate the rejection phenotype of bone marrow transplants. Interestingly, preliminary results show that up to 90% of both the β2m−/− and Clr-b−/β2m−/− donor BMCs were acutely rejected 24 hours after infusing into a B6 host, and this rejection phenotype was significantly lowered (down to 20%) by NK-depletion in the B6 recipients (Fig. 6.1A). We were surprised that the Clr-b−/β2m−/− donor BMCs were not further rejected in comparison to the β2m−/− donor BMCs 24 hours after the transplantation. Perhaps assessing the rejection phenotype at an earlier time point post transplantation (eg. 12 hours) will reveal a differential degree of rejection between the β2m−/− and Clr-b−/β2m−/− donor cells. In contrast, only 20% of the Clr-b−/− donor cells were rejected by the B6 host, and this phenotype was not reverted by NK-depletion in the recipient. Interestingly, the % rejection of Clr-b−/− BMCs in both NK-sufficient and NK-depleted recipients is similar to the rejection of the β2m−/− and Clr-b−/β2m−/− BMCs in NK-depleted recipients (all at ~20%). Collectively, these data suggest that there is a basal level of rejection of the transplanted bone marrow grafts that is independent of NK cells. This rejection may be mediated by T cells (since it is a sublethal irradiated host), or due to cell stress that occurred through the harvesting of bone marrow cells from its donors. More experimentation is required to confirm these hypotheses.

In addition, we transplanted BMCs harvested from B6, Clr-b−/−, β2m−/−, and Clr-b−/−/β2m−/− into both β2m−/− and Clr-b−/− recipients (Fig. 6.1B-E). In β2m−/− recipients, % rejection of B6, Clr-b−/−, and Clr-b−/β2m−/− BMCs were measured relative to the autologous donor BMCs (β2m−/−) (Fig. 6.1B); and in Clr-b−/− recipients, we assessed % rejection of B6, β2m−/−, and Clr-b−/β2m−/− BMCs relative to donor Clr-b−/− BMCs (Fig. 6.1D). In β2m−/− recipients, ~20% of B6 donor BMCs were rejected (Fig. 6.1B). This
means that the B6 donor BMC was “rejected to a lesser extent relative to the autologous donor graft”. An alternative method to interpret this data is to assess it as % engraftment. For example, a 40% rejection of a donor BM graft can also be defined as a 60% engraftment. Therefore, –20% rejection of the B6 donor BM graft relative to β2m−/− donor graft is can also be perceived as a 120% engraftment of the B6 donor BM graft relative to the autologous β2m−/− donor cells (Fig. 6.1C). In contrast, shown in figure 6.1B, ~30% of Clr-b−/− BMCs were rejected by β2m−/− hosts (or ~70% engraftment, Fig. 6.1C); and Clr-b−/β2m−/− BMCs were not rejected, or engrafted almost identically to β2m−/− autologous grafts (Fig. 6.1B, C). In Clr-b−/− recipients, B6 BMCs engraft better than Clr-b−/− donor BMCs in Clr-b−/− recipients (Fig. 6.1E). As expected, both β2m−/− and Clr-b−/β2m−/− BMCs are rejected relative to Clr-b−/− donor BMCs in Clr-b−/− mice (Fig. 6.1D). Evidently, the absence of both MHC-I and Clr-b from the cell surface results in poor prognosis of the transplanted BM engraftment compared to MHC-I−/− or Clr-b−/− single deficient BMCs. Furthermore, there appears to be an increased engraftment of B6 BMCs in both β2m−/− and Clr-b−/− recipients, relative to the donor BMCs that is syngeneic to the recipient. Since B6 BMCs express both MHC-I and Clr-b, and that NK cells from β2m−/− and Clr-b−/− exhibit elevated inhibitory Ly49 receptors and NKR-P1B, respectively, the presence of the cell surface inhibitory ligands on B6 BMCs will provide an “additional” inhibitory signal to both the β2m−/− and Clr-b−/− recipient NK cells, thus conferring the increased engraftment phenotype. Importantly, results from the chromium release assay (section 5.6) demonstrated that NKR-P1B on NK cells derived from Clr-b−/− animals are indeed functional, and upon crosslinking, it transduces an inhibitory signal into the NK cell. Thus, this supports our rationale for explaining the increased %
engraftment of B6 WT donor cells in both $\beta2m^{−/−}$ and Clr-b$^{+}$ animals. Interestingly, both $\beta2m^{−/−}$ and Clr-b$^{−}$ hosts reject Clr-b$^{−}$ and $\beta2m^{−/−}$ donor cells, respectively, but their response to the Clr-b$^{−}$/\beta2m$^{−/−}$ donor cells are very different. The double knockout donor cells are engrafted almost equally as well as the autologous graft for $\beta2m^{−/−}$ hosts, whereas an exacerbated rejection of the double knockout donor population is seen in the Clr-b$^{−}$ host in comparison to $\beta2m^{−/−}$ donor cells.

Previous data suggest that Clr-b may heterodimerize with other Clr family members $^{67}$, and the absence of Clr-b from the cell surface may then promote the homodimerization of the other Clr members. Unpublished data from our laboratory has demonstrated that ex vivo cells from Clr-b$^{+/−}$ animals are able to weakly stimulate BWZ reporter cells expressing the NKR-P1F/CD3ζ chimeric receptor, whereas B6 ex vivo cells do not, hence supporting the above hypothesis (Fig. 6.2). Thus, the absence of cell surface Clr-b may promote homodimerization of cell surface ligands to NKR-P1F (Clr-c, d, g, $^{26}$). Therefore, the missing-self recognition of MHC-I by Ly49+ NK subsets, and the induced-self recognition of Clr homodimers by NKR-P1F+ NK population may explain the exacerbated rejection phenotype of the double knockout graft in comparison to $\beta2m^{−/−}$ BMCs in the Clr-b$^{+/−}$ recipients. In contrast, the absence of Clr-b may modulate the expression of other MHC-dependent inhibitory ligands (eg. Qa-1) or MHC-independent ligands (eg. CD48), which may explain the engraftment phenotype of the double knockout observed in the $\beta2m^{+/−}$ host. A more thorough characterization of the expression of ligands for NK recognition will provide further insight to elucidate the mechanism of this complex system.

There are a many questions that remain unanswered in the Clr-b$^{+/−}$ mice. For
instance, from the flow cytometric-based mouse characterization experiments, Clr-b−/− mice are essentially identical to B6 mice in terms of the homeostatic immune system. However, the relationship of Clr-b deletion and the expression of the various Nk rp1 receptors and Clr ligands are unknown, since mAbs specific for NKR-P1A, NKR-P1F, Clr-a, c, d, f, g are unavailable. In addition, recent in situ hybridization data from our collaborators has revealed that Clr-f is expressed in intestinal epithelial cells. In this thesis, we have not examined the effect of Clr-b deletion in mucosal immunity. Examination of both inhibitory Clr ligands (Clr-b, Clr-f) in the intestinal mucosa may prove interesting and shed light on the NK biology in the mucosa. Finally, reaffirming the Clr homodimerization versus heterodimerization model (Fig. 6.3) may further our understanding about the molecular interactions that occurs between NK cell receptors and its ligands, as mentioned in section 6.3.
FIGURE 6.1

A

![Graph showing % Rejection of genetically modified donor BMC relative to syngeneic graft.](image)

- **Genotype of Donor BM**
  - Clr-b–/–β2m–/–
  - Clr-b–/–β2m–/–
  - Clr-b–/–β2m–/–

- **% Rejection of BMC in B6 Recipients**
  - NK+
  - NK–

**Notes:**
- Significant differences are indicated by **(**.
B

% Rejection of BMC in β2m−/− Recipients

% Engraftment of genetically modified donor BMC relative to syngeneic donor graft

Genotype of Donor BM

C

% Engraftment of BMC in β2m−/− Recipients

% Engraftment of genetically modified donor BMC relative to syngeneic donor graft

Genotype of Donor BM
D

% Rejection of BMC in Clr-b\(^{-/-}\) Recipients

\[\text{% Engraftment of genetically modified donor BMC}\]

relative to syngeneic donor graft.

Genotype of Donor BM

E

% Engraftment of BMC in Clr-b\(^{-/-}\) Recipients

\[\text{% Engraftment of genetically modified donor BMC}\]

relative to syngeneic donor graft.

Genotype of Donor BM
Figure 6.1 Assessing MHC-dependent and independent missing-self recognition using in vivo mouse models

(A) % Rejection of Clr-b−, β2m−, and Clr-b−/β2m− donor BMCs relative to B6 BM donor in NK-sufficient or NK-deficient B6 hosts 24 hours post transplantation. (B) % Rejection of donor B6, Clr-b−, and Clr-b−/β2m− BMCs relative to the autologous donor graft in β2m− recipients. (C) % Engraftment of donor B6, Clr-b−, and Clr-b−/β2m−/β2m− BMCs relative to the autologous donor graft in β2m− recipients. (D) % Rejection of donor B6, β2m−, and Clr-b−/β2m− BMCs relative to the autologous donor graft in Clr-b− recipients. (E) % Engraftment of donor B6, β2m−, and Clr-b−/β2m− BMCs relative to the autologous donor graft in Clr-b− recipients. % Rejection is calculated with the following equation: \( \{1 - [(\text{proportion of donor BM of interest})/(\text{proportion of autologous donor BM})]_{\text{input}} / [(\text{proportion of donor BM of interest})/(\text{proportion of autologous donor BM})]_{\text{output}} \} \times 100\% \); % Engraftment is calculated with the following equation: \( [(\text{proportion of donor BM of interest})/(\text{proportion of autologous donor BM})]_{\text{input}} / [(\text{proportion of donor BM of interest})/(\text{proportion of autologous donor BM})]_{\text{output}} \times 100\% \). Histograms show the average rejection/engraftment of 3 recipients. Data were analyzed via 2-way ANOVA; the BM rejection phenotypes between NK-sufficient and NK-deficient recipients were assessed by the Bonferroni post-test. ** denotes p<0.001.
Figure 6.2 Identification of ex vivo cell surface ligands of NKR-P1F using reporter cell analysis

BWZ reporter cells bearing CD3ζ/NKR-P1F fusion receptors were mixed with titrated doses of ex vivo stimulator cells harvested from either B6 or Clr-b−/− mice.
Figure 6.3 Proposed model of NK cell activation through molecular interactions of Clr ligands

**Left Panel:** Healthy cells expressing high levels of Clr-b homodimers (red leaflet pairs, on target cells) inhibits NK effector cell function by binding to its cognate receptor, NKR-P1B (depicted in red, on NK cells). In contrast, low levels of stimulatory Clr homodimers (eg. Clr-c, d, g; green leaflet pairs, on target cells) are expressed on healthy cells. While it interacts with its cognate stimulatory NK receptor (eg. NKR-P1F, depicted in green, on NK cells), the stimulatory signal is masked by the high levels of the inhibitory signal as a result of the NKR-P1B:Clr-b receptor-ligand interaction. In addition, high levels of Clr-b may disrupt the formation of stimulatory Clr-homodimers, by sequestering Clr-c, d, g monomers and preferentially form Clr-b:Clr-c/d/g heterodimers (red/green leaflet pairs, on target cells), which is unable to interact with the inhibitory receptor, NKR-P1B, nor the stimulatory receptor, NKR-P1F. 

**Middle Panel:** Stressed cells downregulate Clr-b, resulting in the missing-self recognition of Clr-b by NK cells through the NK cell receptor NKR-P1B. This leads to the killing of the stressed target cell. In addition, absence of cell surface Clr-b will result in the dissociation of Clr heterodimers, thus liberating the Clr-c, d, g monomers, that can potentially form stimulatory Clr homodimers (Single leaflets, on target cells). 

**Right Panel:** formation of stimulatory Clr homodimers (eg. Clr-c, d, g) will substantiate the stimulatory signal in the NK cell via its interaction with the stimulatory NK cell receptor (eg. NKR-P1F), resulting in a further increased NK effector function through the induced-self NK recognition.
6.5 FUTURE DIRECTIONS

The main focus of this thesis is to extend our current understanding of the Nkrp1-Clr receptor-ligand system, as well as further our appreciation for the importance of MHC-independent recognition by NK cells in the context of innate immunity. In this work, we have described in detail an efficient methodology of generating mAbs against cell surface molecules and delineated interesting candidate members in both the Nkrp1 and Clr families that appear to play a role in NK cell biology. Thus, the next logical step in this endeavour will be making hybridoma clones against Clr-c, d, f, g as well as NKR-P1F and NKR-P1G (another clone). By using these mAbs, we can assess the expression profile Clr-c, d, f, g of mice, and test the hypothesis of Clr heterodimerization by performing co-immunoprecipitation experiments on lysates extracted from *ex vivo* cells. These results can be further validated by the co-transfection of different Clr molecules (differentially tagged, flagged, HA etc) in 293T cells followed by a co-immunoprecipitation assay. Furthermore, once we obtain more information on the expression profile of the different Clr molecules, we may attempt additional Clr knockout animal models and study the effects of gene deletion (eg. Clr-f), analogous to the characterization experiments that were performed in chapter V. Currently, our collaborator has generated NKR-P1B−/− mice in the B6 background, and similar experiments performed using Clr-b−/− mice described in this thesis are conducted in parallel using NKR-P1B−/− animals. We foresee a uniformed report on the *in vivo* phenotypes of Clr-b versus NKR-P1B deletion in the near future.
6.6 CONCLUDING REMARKS

NK cells, a population of lymphocytes that was once thought of as “null” cells with background killing activity \(^{81}\) have pivotal roles in both innate and adaptive immunity. Numerous reports have documented the involvement of NK cells in the elimination of tumour cells, protection against infectious diseases, transplantation, reproductive biology \(^{33}\), autoimmunity \(^{56}\), the dichotomy of NK/T cell immunity in response to infections \(^{92}\), and finally NK memory \(^{147}\). The most challenging, yet intriguing aspect of NK cell biology lies within the large repertoire of NK cell receptors, which permits fidelity in self-nonself discrimination. Of the many NK receptor-ligand pairs, the NKR-P1B:Clr-b system in mice represents a functional homolog of the human CD161:CLEC2D receptor-ligand interaction, whose significance in MHC-independent NK recognition remains to be fully defined. We believe a thorough understanding of the NKR-P1B:Clr-b recognition axis will offer insights that can precisely define what is “self”, which is imperative in predicting the acceptance or rejection of BM grafts, thus facilitating the success of BM transplants in humans. In the future, we hope that our understanding of the MHC-independent recognition system can facilitate the success of clinical treatments, such as BM transplantation and graft-versus-tumour therapies, ultimately contributing to everyday health care.
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