Genetic Investigation of MHC-Independent Missing-Self Recognition by Mouse NK Cells Using an In Vivo Bone Marrow Transplantation Model

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B one marrow (BM) transplantation models have vital importance in studying immunological tolerance and clinical therapies for hematopoietic disorders and cancer.

MHC-I–specific receptors play a vital role in NK cell–mediated “missing-self” recognition, which contributes to NK cell activation. In contrast, MHC-independent NK recognition mechanisms are less well characterized. In this study, we investigated the role of NKR-P1B:Clr-b (KlrB1:Clec2d) interactions in determining the outcome of murine hematopoietic cell transplantation in vivo. Using a competitive transplant assay, we show that Clr-b−/− bone marrow (BM) cells were selectively rejected by wild-type B6 recipients, to a similar extent as H-2Db−/− MHC-I–deficient BM cells. Selective rejection of Clr-b−/− BM cells was mitigated by NK depletion of recipient mice. Competitive rejection of Clr-b−/− BM cells also occurred in allogeneic transplant recipients, where it was reversed by selective depletion of NKR-P1B+ NK cells, leaving the remaining NKR-P1B− NK subset and MHC-I–dependent missing-self recognition intact. Moreover, competitive rejection of Clr-b−/− hematopoietic cells was abrogated in Nkp1b−/− deficient recipients, which lack the receptor for Clr-b. Of interest, similar to MHC-I–deficient NK cells, Clr-b−/− NK cells were hyporesponsive to both NK1.1 (NKR-P1C)–stimulated and IL-12/18 cytokine–primed IFN-γ production. These findings support a unique and nonredundant role for NKR-P1B:Clr-b interactions in missing-self recognition of normal hematopoietic cells and suggest that optimal BM transplant success relies on MHC-independent tolerance mechanisms. These findings provide a model for human NK-P1A:LLT1 (KLRB1:CLEC2D) interactions in human hematopoietic cell transplants. The Journal of Immunology, 2015, 194: 000–000.
described: NK subsets expressing an inhibitory Ly49 specific for either H-2\(d\) or H-2\(b\) alleles, a subset expressing Ly49 specific for both H-2\(d\) and H-2\(b\) alleles, and a subset expressing neither class of Ly49 receptor. Thus, parental (H-2\(d^b\) or H-2\(b^d\)) BM cells transplanted into an F\(_1\) (H-2\(b^d\)) host are perceived to be “missing” at least one self MHC-I allele by NK cell subsets rendered tolerant to non-overlapping allelic specificities. At the molecular level, F1 inhibitory Ly49 receptors that recognize only H-2\(d\) or H-2\(b\) alleles are not engaged; hence, missing-self recognition ensues, leading to selective rejection of parental BM grafts. Hybrid resistance is one of the earliest phenomena delineating a unique role for NK cells in determining BM transplant reaction (8, 12, 13). Further evidence came from observations in which allogeneic BM cells engraft in NK-depleted hosts (14–16) yet are rejected by C.B-17/SCID recipients (17, 18) (which lack B and T lymphocytes but possess intact NK cell activity) (19).

Nonetheless, MHC-independent NK recognition mechanisms have recently emerged, including the NKR-P1B:Clr-b receptor–ligand pair. In rats, viral evasion of this system determines the outcome of viral titers during rat CMV-English infection (RCMV-E; Mhv-8) (20, 21). Briefly, RCMV-infected cells lose surface expression of host Clec2d11, yet RCMV-E encodes an NKR-P1B–specific decoy, RCTL, that functionally replaces Clec2d11 and inhibits NK cells. In addition, mouse tumor lines and stressed cells also lose expression of Clec2d (22, 23), thereby augmenting their susceptibility to NK cells. These findings broaden the significance of MHC-independent NK cell immunosurveillance in the discrimination of pathological versus healthy target cells.

In this study, we investigated whether this system plays a non-redundant role in normal self-nonself discrimination during autologous and allogeneic hematopoietic transplants. Specifically, we assessed the selective rejection of Clec2d11-/- BM cells in comparison with wild-type (WT) and various MHC-I–deficient BM cells, using a competitive in vivo transplantation assay. We show that Clec2d11-/- BM cells are selectively and acutely rejected by B6 and allogeneic recipients in an NK cell–dependent manner, mediated specifically by the NKR-P1Bbw NK cell subset. Furthermore, Clec2d11-/- NK cells are functionally hyporesponsive, similar to MHC-I–deficient NK cells. These results support the importance of MHC-independent mechanisms in regulating hematopoietic transplant rejection and NK cell function in vivo.

**Materials and Methods**

**Cells**

BWZ.36 and P815 cells were obtained from Drs. Nilabh Shastri and David Raulet (University of California, Berkeley, Berkeley, CA); BWZ.CD3\(^{+}\)/NKR-P1B, BWZ.Clec2d11-/- cells were generated previously (22, 24). Cells were cultured in complete DMEM–high glucose, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, 50 \(\mu\)g/ml gentamicin, 110 \(\mu\)g/ml sodium pyruvate, 50 \(\mu\)M 2-ME, 10 mM HEPES, and 10–20% FCS.

Mouse tissues were processed into single-cell suspensions using 40-\(\mu\)m filters (BD Biosciences), ACK Lysing Buffer (Life Technologies), and/or Lympholyte (Cedarlane). Solid organs were processed using gentleMACS (Miltenyi Biotec). Animals

B6, B6.CD45.1, and B6.NKp46-/- mice were purchased from The Jackson Laboratory; B6.H-2\(k^d\)/Clec2d11-/- and B6.H-2\(k^b\)/Clec2d11-/- mice from Taconic; and National Institutes of Health (NIH) Swiss mice from National Cancer Institute Frederick (25). B6.Clec2d11-/- (Oci-/-; Clec2d11-/-) mice were described previously (26). B6.NKp46-/- mice were generated as described (27). The B6 Clec2d11-/- genetic background was verified from genomic tail DNA via the Illumina Mouse Medium Density Linkage Panel Whole-Genome Genotyping Service (Hospital for Sick Children, Toronto, ON, Canada). B6.Clec2d11-/- and B6.CD45.1.CD45.2 double-congenic mice were generated in-house. All animals were maintained according to approved protocols at Sunnybrook Research Institute or the Donnelly Centre, University of Toronto, Toronto, ON, Canada.

**RT-PCR and quantitative RT-PCR**

Tissue RNA was isolated using an RNA Purification Kit (Invitrogen) and reverse transcribed using oligo-dT primer and a SuperScript III cDNA Synthesis Kit (Invitrogen) (22, 23). Semi-quantitative RT-PCR was performed using 50–125 ng of template cDNA, Expand High Fidelity Enzyme (Roche), and primers in Table I. PCR products were cloned into pGEM3zf and sequenced. Real-time quantitative RT-PCR (qRT-PCR) was performed on a CFX-96 Real-Time PCR Detection System (Bio-Rad) using 50–100 ng of template cDNA, SsoFast EvaGreen Supermix (Bio-Rad), and primers in Table I. Data were analyzed using CFX Manager software.

**Abs**

Abs were purchased from eBioscience, BD Pharmingen, Sigma-Aldrich, or BioLegend, except Clec2d11 mAb, 4A6 (22), and NKR-P1Bbw, 2D12 (28). The mAb clones are as follows: NKG2D (B14); Ly49C/I/F/H (14B11); Ly49C/I (S66); Ly49H (3D10); Ly49Y (YLI-90); Ly49D (4E5); Ly46-Gr1 (Rb6-8C5); FLAG (M2); CD19 (ID3); H-2K\(^d\) (AF6-88.55); H-2D\(^d\) (KH95); CD25 (3C7); NK2G2A (16a11); NK2G2A/C (CE205); CD244.2/B4 (eBio244F4); CD11c (N418); NKp46 (29A1.4); CD44 (18d3); CD44 (IM7); CD44 (GK1.5); CD11b (MI70); CD8a (53.6-7); CD3e (145-15C1); CD45.1 (A20); NK1.1/NKR-P1C/2D (PK136); CD45.2 (104); IFN-\(\gamma\) (XM16.2); and FcγRIII (2.4G2). 2D12 purified mAb and hybridoma were generous gifts from Dr. Koho Iizuka (University of Minnesota, MN). Subcloned 2D12 hybridoma supernatant was purified using protein A resin (Pierce) and biotinylated in-house by the hybridoma facility at Sunnybrook Research Institute.

**Flow cytometry**

Cells were FcR blocked on ice for 10 min using 2.4G2 supernatant, stained with primary mAb for 25 min, and washed and stained for 20 min with secondary streptavidin conjugates. Intracellular staining, fixation, and permeabilization used Cytofix/Cytoperm (BD Biosciences), as well as IFN-\(\gamma\) Ab (30 min in 1× Perm/Wash). Cells were analyzed using an LSRII (BD Biosciences) and FlowJo software (TreeStar). DAPI exclusion was used for live cell viability.

**Competitive BM rejection assay**

Donor cells from various animals were singly or combinatorially labeled using PKH26, PKH67 (Sigma-Aldrich), and/or CellVue Maroon (eBioscience) (Table II), washed, counted, and stoichiometrically pooled prior to transplant. Recipient mice were sublethally irradiated (6.5 Gy; Mark I Cs\(^{137}\) irradiator; J.L. Shepherd & Associates) on day −1. Untreated mice or NK-depleted cohorts (injected i.p. with 150–200 \(\mu\)g PKH136 on day −2) were injected i.v. with 10–21 \(\times\) 10\(^5\) labeled cells on day 0.Recipient spleens were harvested 18–48 h post transplant and analyzed by flow cytometry. Some mice were treated with polyinosinic-polycytidylic acid (poly I:C) (24 h prior) or CpG-B oligodinucleotides (ODN) (6 h prior), where noted, before transplant. For NKR-P1Bbw-deficient recipients, WT and Clec2d11-/- donor cells were labeled with 5 \(\mu\)M or 0.5 \(\mu\)M CFSE in lieu of PKH dyes.

Percent engraftment of experimental donor cells relative to control WT or syngeneic donor cells was determined as follows: \([\%\text{ donor}/(\%\text{ control})\times 100\%]\). Percent Engraftment = 100% – % Engraftment.

**Reporter cell assays**

BWZ cells were retrovirally transduced with CD3\(^{+}\)/NKR-P1 constructs and sorted for GFP+ cells, as described (22, 24). Reporter cells (10\(^5\) per well) were incubated with serial 3-fold titrations of stimulator cells overnight. Control reporter cells were stimulated with 10 ng/ml PMA and 0.5 \(\mu\)M ionomycin. Cells were washed, resuspended in 100 \(\mu\)l of 1× CXPBR buffer (90 mg/ml chlorophenol-red-\(\beta\)-galactopyranoside [Roche], 9 mM MgCl\(_2\), 0.1% NP-40, in PBS), incubated, then analyzed using a microplate reader (Victor 3 MultiSkan) using OD595–655.

**Chromium release assay**

Cytotoxicity assays were performed as described (22). Splenic lymphokine-activated killer (LAK) effector cells were cultured in 10% complete RPMI
1640 plus 2500 U/ml human IL-2 (Proleukim; Novartis) for a total of 6 d. On day 4, these cells were FACs sorted for NKp46+ and NKR-P1B+ exons-2 through -4 for the coding sequence of Clr-b relative to GAPDH as an internal loading control. The smaller fragment observed for 3′–5′ Ci Na2

Clr-b/F(e2)

TAG TCC CAC AGG CAG CCC GC

FL-Clr-b/F(e1)

GAG AGC AAT GCC AGC CCC GG

GAPDH-F

ATC TCC GCC CCT TCT GCC GA

GAPDH-R

CTG GGA AGC CCA ACT TCT GCA C

qTBP-F

ATG AGT GCT GCA AAG GTT GA

qTBP-R

TAG CGA CTC TCT GTG CAG GCC A

qGAPDH-F

GAG GGA GAT GGT TCC GTG CCT TT

qGAPDH-R

TGA CGA CTC TCT GTG CAG GCC A

qClr-bF

AGC TCC TCA GCT CTG AGA TGT GTG

qClr-bR

AGG GGA GAT GGT TCC GTG CCT TT

Mutant Clr-b transcripts were detected by RT-PCR and qRT-PCR in all Clr-b−/− tissues analyzed (Fig. 1A and data not shown). Most tissues containing hematopoietic cells expressed Clr-b transcripts, with nearly undetectable levels in brain (data not shown). However, as expected, transcripts from Clr-b−/− mice were smaller than WT Clr-b transcripts, and sequencing of Clr-b amplification products confirmed exon-3 deletion in Clr-b−/− mice. Nonetheless, transcriptional regulation of the mutant Clrb (Ocil/Clec2d) gene appears to be intact in Clr-b−/− mice.

To confirm a lack of Clr-b surface protein, we evaluated Clr-b expression on cells from B6 and Clr-b−/− tissues ex vivo, using Clr-b mAb, 4A6 (22). As shown in Fig. 1B, Clr-b−/− cells lacked detectable Clr-b surface protein, in turn validating the lack of cross-reactivity of 4A6 mAb with other Clr family members. To confirm a loss of Clr-b ligand function, ex vivo Clr-b−/− cells were used as stimulator cells in BWZ.CD3γ/NKR-P1B reporter cell assays. Here, the cognate NKR-P1B receptor recognized intact Clr-b ligand on WT cells, but not Clr-b−/− cells (Fig. 1C). In turn, the absence of BWZ.CD3γ/NKR-P1B reporter stimulation by Clr-b−/− cells confirms the specificity of NKR-P1B for Clr-b, and suggests that Clr-b is the only ligand recognized by NKR-P1B on normal B6 cells ex vivo. Taken together, these data demonstrate a lack of Clr-b surface protein and a loss of NKR-P1B ligand function on Clr-b−/− cells.

FIGURE 1. Phenotypic confirmation of the Clr-b−/− mouse genotype. (A) Semiquantitative RT-PCR analysis of Clr-b transcript expression using primers specific for exons-2 through -4 for the coding sequence of Clr-b relative to GAPDH as an internal loading control. The smaller fragment 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 (26). We confirmed the deficiency of these Clr-b−/− animals via three approaches: 1) RT-PCR analysis of Clr-b transcripts, using multiple primers (Table I); 2) flow cytometric analysis of Clr-b surface protein, using 4A6 mAb (22, 29); and 3) reporter analysis of Clr-b ligand function, using BWZ.CD3γ/NKR-P1B cells (22, 29).

### Results

#### Phenotypic and functional characterization of Clr-b−/− mice

B6.Ocil/Clec2d−/− (hereafter, Clr-b−/−) mice exhibit a mild defect in osteoclast development and function (26). These animals were generated directly on a B6 background (verified by Illumina genotyping) by targeted deletion of exon-3 of the Clec2d gene; this leads to a frame-shifted transcript and the absence of a functional ectodomain (26). The computed deficiency of these Clr-b−/− animals via three approaches: 1) RT-PCR analysis of Clr-b transcripts, using multiple primers (Table I); 2) flow cytometric analysis of Clr-b surface protein, using 4A6 mAb (22, 29); and 3) reporter analysis of Clr-b ligand function, using BWZ.CD3γ/NKR-P1B cells (22, 29).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′–3′)</th>
</tr>
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<tbody>
<tr>
<td>Clr-b-F(e2)</td>
<td>TAG TCC CAC AGG CAG CCC GC</td>
</tr>
<tr>
<td>Clr-b-R(e4)</td>
<td>TGA CGA CTC TCT GTC CAG GCA A</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>ATC TCC GCC CCT TCT GCC GA</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GAG AGC AAT GCC AGC CCC GG</td>
</tr>
<tr>
<td>FL-Clr-b-F(e1)</td>
<td>ctc ggc gcc cta TCC GGG ATT TAC AAT TA</td>
</tr>
<tr>
<td>FL-Clr-b-R(e5)</td>
<td>ggc gcc gc clt tcc ggg att tac aat ta</td>
</tr>
<tr>
<td>qClr-bF</td>
<td>AGC TCC TCA GCT CTA GAA GTG GA</td>
</tr>
<tr>
<td>qClr-bR</td>
<td>AGG GGA GAT GGT TCC GTG CCT TT</td>
</tr>
<tr>
<td>qGAPDH-F</td>
<td>TGT GCA GTG CCA GCC TCG TC</td>
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<tr>
<td>qGAPDH-R</td>
<td>TGA AGG GGT CGT TGA TGG CAA CA</td>
</tr>
<tr>
<td>qTBP-F</td>
<td>AGA GCC AGC GAC AAC TGG TTT G</td>
</tr>
<tr>
<td>qTBP-R</td>
<td>CTG GSA AGC CCA ACT TCT GCA C</td>
</tr>
</tbody>
</table>

- **Table I.** RT-PCR and qRT-PCR primers

### Material

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Steady-state Clr-b<sup>−/−</sup> hematopoietic characterization

We next characterized Clr-b<sup>−/−</sup> mice in comparison with WT and MHC-I-deficient animals, such as B6.<sub>β<sub>2m</sub></sub><sup>−/−</sup> mice (30); although these latter mice are deficient in CD8<sup>+</sup> T cells (31), their NK cells are functional (32), albeit hyporesponsive (33, 34). Because Clr-b expression resembles that of MHC-I, we investigated the influence of Clr-b absence during hematopoietic development. Various Clr-b<sup>−/−</sup> tissues were compared with age- and sex-matched WT tissues for both lymphoid and myeloid cell composition. A nearly identical leukocyte distribution (neutrophils, macrophages, dendritic cells, B cells, T cells, NK cells, and NK cells) was observed (data not shown). This result might be expected, as Clr-b is the only known ligand for NKR-P1B (also known as NKR-P1D in B6 mice) (22, 28).

We further characterized the Clr-b<sup>−/−</sup> NK cell subset composition and receptor repertoire. Of interest, NKR-P1B<sup>B6</sup> expression levels (i.e., NKR-P1D, assessed using 2D12 mAb) (28) were significantly higher on Clr-b<sup>−/−</sup> NK cells versus WT NK cells (Fig. 2A, 2B; Supplemental Figs. 1–3). This was expected because elevated Ly49 levels are observed on NK cells from MHC-I-deficient mice (35). Somewhat unexpectedly, NKp46 levels were significantly lower on Clr-b<sup>−/−</sup> NK cells versus WT NK cells; similar trends were also observed for other stimulatory receptors (Fig. 2A 2B; Supplemental Figs. 1A, 1B, 1E, 1F, 3). Next, the subset-restricted expression of NKR-P1B was examined in greater detail with respect to that of the self–MHC-I-specific B6 strain NK cell receptors Ly49C/I and NKG2A, on splenic and BM NK cells from WT and Clr-b<sup>−/−</sup> mice (Fig. 2C, 2D; Supplemental Fig. 1C, 1D). Of note, NKR-P1B was enriched on self–MHC-I-specific (“educated”) NK cells expressing either Ly49C/I or NKG2A. Moreover, gating on NKR-P1B<sup>+</sup> NK cells revealed an enrichment of the classical “hyporesponsive” subset lacking expression of Ly49C/I and NKG2A; notably, however, a significant fraction of this subset did express NKR-P1B (~30–50%), suggesting these NK cells are indeed educated and not entirely hyporesponsive, at least to MHC-independent signals. Only subtle differences were noted in the NK repertoires between WT and Clr-b<sup>−/−</sup> mice; specifically, fewer NKR-P1B+ cells among the hyporesponsive Ly49C/T/NKG2A<sup>−</sup> subset, and a corresponding increase among the Ly49C/I<sup>+</sup> subsets, were observed among Clr-b<sup>−/−</sup> NK cells (Fig. 2C, 2D; Supplemental Fig. 1C, 1D). Similar observations were made for NK subsets grouped according to CD94 (i.e., NKG2A/C/E) in lieu of NKG2A (Supplemental Fig. 2A–D). Interestingly, NKR-P1B<sup>+</sup> NK cells were almost entirely 2B4<sup>+</sup>, suggesting a cosegregation of NK cells responsive to MHC-independent ligands (Supplemental Fig. 2E–H).

Notably, trends toward higher expression of inhibitory NK receptors (e.g., NKR-P1B, Ly49) and lower expression of stimulatory NK receptors (e.g., Ly49C/I) were observed in Clr-b<sup>−/−</sup> mice compared to WT mice. These findings suggest that the absence of Clr-b may lead to an increase in the expression of inhibitory receptors and a decrease in the expression of stimulatory receptors on NK cells, potentially altering their functional responsiveness.

FIGURE 2. Characterization of steady-state NK cell subsets in the Clr-b<sup>−/−</sup> mouse. (A) NK1.1<sup>+</sup>CD3<sup>−</sup> splenocytes harvested from either B6 or Clr-b<sup>−/−</sup> animals were gated and assessed for NK cell subset frequency and receptor expression levels. (B) Quantitation of NK cell receptor expression levels from splenic NK cells. All comparisons between B6 and Clr-b<sup>−/−</sup> mice were analyzed with the two-tailed $t$ test. In all histograms, empty bars represent B6 and solid bars represent Clr-b<sup>−/−</sup> NK cells. * $p < 0.05$, ** $p < 0.01$. (C) Subset-restricted expression of NKG2A and Ly49C/I on splenic NK cells (gated NK1.1<sup>+</sup>CD3<sup>−</sup>) from B6 and Clr-b<sup>−/−</sup> mice, further analyzed for NKR-P1B levels (histogram overlays, below) for each indicated subset. Numbers represent cell percentages (upper histogram gate, B6; lower gate, Clr-b<sup>−/−</sup>); shaded gray histograms, B6; black lines, Clr-b<sup>−/−</sup>. (D) Alternative gating strategy based upon NKR-P1B levels (left plot) for subsetting of splenic NK cells from B6 and Clr-b<sup>−/−</sup> mice, showing NKG2A and Ly49C/I expression on NKR-P1B<sup>−/−</sup> subsets (right plots). All data are representative of at least three independent mice from each genotype. DN, double-negative, hyporesponsive subset; DP, double-positive, educated, total SP + DP subsets; MFI, median fluorescence intensity; SP, single-positive.
latory receptors (e.g., NKp46, NKR-P1C, 2B4, NKG2D, CD94+/NKG2A−) were observed among NK cells from Clr-b−/− mice (Supplemental Fig. 3 and data not shown). These shifts in signal balance suggest that Clr-b−/− NK cells may be hyporesponsive; conversely, WT NK cells may selectively reject Clr-b−/− hematopoietic grafts owing to diminished inhibition via NKR-P1B.

**Acute NK cell–mediated rejection of Clr-b−/− hematopoietic grafts in WT recipients**

To assess MHC-independent missing-self rejection of Clr-b−/− donor cells, we established short-term (18–48 h) competitive transplant assays using equal mixtures of fluorophore-labeled (or congenically marked) Clr-b−/− (CD45.2) versus WT (or CD45.1) donor hematopoietic cells into sublethally irradiated WT (or CD45.1/2) recipient mice. NK cell activity was assessed using NK-depleted (PK136-treated) versus untreated or NK-primed (polyI:C-treated) cohorts. As shown in Fig. 3A, Clr-b−/− BM cells, relative to WT donor cells, were acutely and selectively rejected by WT recipients. This rejection was mitigated in NK-depleted (PK136-treated) versus untreated or NK-primed (polyI:C-treated) cohorts. As shown in Fig. 3A, Clr-b−/− BM cells (B6) were acutely rejected in an NK cell–dependent manner by WT B6 host mice. (Fig. 3A; Supplemental Fig. 4). Moreover, NK depletion reversed the rejection of Clr-b−/− and MHC-I–deficient BM cells (Fig. 3B). In addition, Clr-b−/− and MHC-I–deficient splenocyte rejections mirrored the patterns observed for BM rejections, perhaps with a slightly weaker contribution of Clr-b versus H-2Db (Fig. 3C). Thus, Clr-b−/− hematopoietic cells are selectively rejected in WT mice in an NK-dependent manner, similar in magnitude or slightly weaker than single MHC-I–deficient (H-2Dk−/−) cells. Notably, Clr family members other than Clr-b may play similar roles in transplant tolerance.

**Acute rejection of allogeneic Clr-b−/− BM cells mediated by NKR-P1Bhi NK cells**

To assess whether selective rejection of Clr-b−/− BM cells was mediated by the NKR-P1B+ NK cell subset, we used allogeneic NIH Swiss recipients, in which PK136 mAb treatment selectively depletes the NKR-P1Bhi/+ NK cells intact (37–39). Allogeneic (B6 strain) mixtures of WT, Clr-b−/−, and β2m−/− BM cells were again stoichiometrically transplanted into cohorts that were either untreated or PK136 treated. In this case, depletion of NKR-P1Bhi NK cells is expected to abrogate selective rejection of Clr-b−/− versus WT (B6) BM cells, whereas NKR-P1B+ NK cells should be rejected by the remaining NKR-P1Bhi− NK cells. Indeed, in these allogeneic recipients, both β2m−/− and Clr-b−/− BM cells were again se-

**FIGURE 3.** Clr-b−/− BM cells (BMC) are acutely rejected in an NK cell–dependent manner by WT B6 host mice. (A) Rejection of Clr-b−/− BM cells from B6 mice 48 h after transplantation. Mice shown in the figure were treated with polyI:C or PK136 mAb 24 h before BM transplantation. Symbols represent data from individual mice, and small horizontal bars represent mean values. Statistics were analyzed via an unpaired t test. (B) Rejection of Clr-b−/− and various MHC-I–deficient BM cells 48 h after transplantation. Recipient mice were either untreated or treated with PK136 mAb 24 h prior to BM transplantation. Histograms show the average rejection of three recipients. Data are representative of five independent transplantation experiments. (C) Rejection of Clr-b−/− and various MHC-I–deficient splenocytes 48 h after splenocyte infusion. Recipient mice were either untreated or treated with PK136 mAb 24 h prior to splenocyte transplant. Histograms show the average rejection of three recipients. Data are representative of two independent experiments. Data from (B) and (C) were analyzed via two-way ANOVA; the rejection phenotypes between NK-sufficient and NK-deficient recipients were assessed by the Bonferroni posttest. *p < 0.05; **p < 0.01; ***p < 0.001.
selectively rejected (versus B6 WT cells), whereas elimination of the NKR-P1B/B6+ NK subset (PK136 depletion) reversed the rejection of Clr-b−/− donor cells (Fig. 4A; p < 0.05), but not β3m−/− BM cells (Fig. 4B). This finding suggests that rejection of Clr-b−/− BM cells is mediated specifically by cognate NKR-P1Bhi/+ NK cells, in the absence of which, Clr-b−/− BM grafts are tolerated. In contrast, residual NKR-P1Blo− NK cells in PK136-treated NIH Swiss recipients are sufficient to mediate rejection of β3m−/− BM cells.

Rejection of Clr-b−/− hematopoietic cells is abrogated in NKR-P1B/B6+ recipients

To further confirm the specificity of rejection in syngeneic mice, we transplanted Clr-b−/− hematopoietic cells into NKR-P1B/B6−/− recipients (27). Notably, Clr-b−/− BM cells and splenocytes were selectively rejected in WT (Nkrp1b−/−) littermates, whereas rejection was mitigated in Nkrp1b+B6+ recipients, which are fully NK sufficient but lack the cognate NKR-P1B receptor (27) (Fig. 4C). Importantly, rejection of Clr-b−/− hematopoietic cells was not observed in Nkrp1B/B6−/− recipient even following in vivo NK cell priming using polyI:C or CpG-B ODN (Fig. 4D), which induce high levels of innate cytokines and augment Clr-b−/− rejection in WT recipients. These results confirm the cognate interaction between NKR-P1Bhi and Clr-b, and further support the allelic nature of the NKR-P1B/D receptors.

Because we observed elevated NKR-P1B levels on Clr-b−/− NK cells versus WT NK cells, we investigated NKR-P1B receptor function in the absence of Clr-b during NK cell education. In this study, tumor variants (BWZ.Clr-b+ and parental BWZ−) cells were used as targets for sorted NKR-P1Bhi WT or Clr-b−/− splenic LAK effectors in Clr-release assays. As shown in Fig. 5A, surface Clr-b on BWZ.Clr-b target cells inhibited cytotoxicity mediated by both WT and Clr-b−/− LAK effectors, relative to control BWZ− targets, an effect blocked using Clr-b mAb (4A6), but not isotype control mAb. We next performed redirected inhibition assays using NKR-P1B/B6+ mAb (2D12) (28) to ligate the receptor on WT or Clr-b−/− NK-LAK effector cells (sorted NKP46+NKR-P1B/B6+) via the FcR on P815 target cells. Notably, P815 targets were susceptible to NK-LAK cells from both genotypes; however, 2D12 mAb (but not isotype control mAb) decreased cytotoxicity of P815 targets via NKR-P1B–mediated redirected inhibition on both WT and Clr-b−/− effectors (Fig. 5B). Notably, redirected inhibition was not observed using sorted NKR-P1B− NK-LAK effectors (data not shown). These data confirm the intact inhibitory function of NKR-P1B, even during Clr-b−/− NK cell education, and suggest that Clr-b−/− NK cells may possess altered responsiveness to some tumor targets.

Table II. Dye label combinations used for competitive hematopoietic transplants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>PKH67</th>
<th>PKH26</th>
<th>CellVue Maroon</th>
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<tbody>
<tr>
<td>β3m−/−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Clr-b−/−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>H-2D−/−</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>
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Hematopoietic cells from the indicated mouse genotypes were singly or co-transplanted into WT and Nkrp1b−/− recipients, 48 h after transplantation. Symbols represent data from individual mice; small horizontal bars represent mean values (± SEM). Data were analyzed via two-way ANOVA; the BM rejection phenotypes between untreated and NK1.1 (NKR-P1B/B6+)-depleted recipients were assessed by the Bonferroni posttest. (C) Rejection of Clr-b−/− hematopoietic cells [(HPC), splenocytes, BM cells] by WT and Nkrp1b−/− B6 recipients, 48 h after transplantation. Mice were treated with 150 μg polyI:C 24 h prior to transplantation. (D) Rejection of Clr-b−/− BM cells by WT and Nkrp1b−/− recipients, 18 h after transplantation. Mice were treated with either 150 μg polyI:C (i.p.) or 5 μg CpG ODN (i.v.) 24 h or 6 h prior to transplantation. *p < 0.05, **p < 0.01, ***p < 0.001.
LAK effector cells in the presence of either isotype control mAb (rIgM, the recipient genotype was being varied, results were plotted as response to IL-12/IL-18 cytokine priming (Fig. 6C). Collectively, linking (Fig. 6A), perhaps in part owing to reduced NK1.1 expression, then measured intracellular IFN-γ production. Clr-b−/− BM cells displayed decreased NKp46 and NKR-P1C levels, and NKR-P1F/G share overlapping Clr ligand specificities (22, 28). To assess this, ex vivo WT or Clr-b−/− splenocytes were used as stimulator cells for BWZ.CD3/NKR-P1(A,C,F,G) reporter cells. Of interest, Clr-b−/−/− cells significantly stimulated BWZ.CD3/NKR-P1F reporter cells, relative to WT stimulators (Fig. 7C). In contrast, no stimulation was observed for BWZ.CD3/NKR-P1(A,C,G) reporter cells (data not shown). This finding suggests that Clr-b−/− cells may display an alternative surface Clr ligand for NKR-P1F. Because Clr-g transcripts are expressed in hematopoietic cells (29, 40–43), antagonistic Clr-b/g heterodimers are possible; alternatively, the absence of Clr-b could unmask another Clr ligand, such as Clr-c (NKR-P1G, but not NKR-P1G ligands), in turn modulating NKR-P1F receptor expression or function during NK cell development and education. The same may be true for NKR-P1C, which is slightly downregulated on Clr-b−/− NK cells; however, NKR-P1C ligands remain unknown to date.

**Discussion**

The importance of NK cells in determining the outcome of BM transplantation is highlighted by hybrid resistance experiments (5–8) and BM allograft acceptance in NK-depleted, but not NK-sufficient, SCID mice (14–19). These findings have since been explained by missing-self recognition of MHC-I molecules, which are highly polymorphic. MHC-I polymorphisms in turn have influenced the convergent evolution of two highly diversified receptor families (Ly49 in mice, KIR in humans), which also exhibit multiple polymorphisms at both the allelic and gene content levels (44). In contrast, fewer yet significant allelic polymorphisms exist in the mouse Nkrp1:Clr system (30, 44), in which the overall haplotype structure appears to be conserved (44, 45). Interestingly, among the Clr family, Clr-b possesses an expression pattern similar to that of MHC-I-deficient NK cells, we stimulated splenic NK cells from WT and Clr-b−/− mice, using plate-bound NKR-P1(A,C,F,G) mAb or cytokines, then measured intracellular IFN-γ production. Clr-b−/− NK cells were hyporesponsive to NKR-P1Cstimulation

**Clr-b−/− NK cells are hyporesponsive to cytokine priming and NKR-P1C stimulation**

To assess whether Clr-b−/− NK cells display a generalized hyporesponse phenotype, similar to that of MHC-I-deficient NK cells, we stimulated splenic NK cells from WT and Clr-b−/− mice, using plate-bound NKR-P1(A,C,F,G) mAb or cytokines, then measured intracellular IFN-γ production. Clr-b−/− NK cells were hyporesponsive to NKR-P1Cstimulation

**Clr-b−/− NK cells are self-tolerant despite differential NKR-P1F ligand expression**

To examine self-tolerance, Clr-b−/− mice were used as recipients of WT, Clr-b−/−, β2m−/−, and β2m−/−/− Clr-b−/− BM cells. Because the recipient genotype was being varied, results were plotted as percent engraftment relative to autologous (identical) BM cells. As a control, WT mice transplanted with the same BM mixtures are shown in Fig. 7A (plotted relative to WT grafts). As predicted by intact NKR-P1F function in Clr-b−/− recipients, WT BM cells engrafted slightly better than Clr-b−/− BM cells (autologous graft), whereas β2m−/− BM cells were still rejected (Fig. 7B). Of interest, β2m−/−/− BM cells engrafted slightly better than Clr-b−/−/−/− BM cells in Clr-b−/− recipients (Fig. 7B). Thus, MHC-I-dependent and MHC-independent NK cell tolerance mechanisms are nonredundant and regulated by distinct yet overlapping ligand expression.

Because Clr-b is one of many Clr family members, Clr-b−/− cells may possess differential expression of other Clr ligands, which may affect the balance of recognition by other NKR-P1 or NK cell receptors. Notably, Clr-b−/− mice display decreased NKP46 and NKR-P1C levels, and NKR-P1F/G share overlapping Clr ligand specificities (22, 28). To assess this, ex vivo WT or Clr-b−/− splenocytes were used as stimulator cells for BWZ.CD3/NKR-P1(A,C,F,G) reporter cells. Of interest, Clr-b−/−/− cells significantly stimulated BWZ.CD3/NKR-P1F reporter cells, relative to WT stimulators (Fig. 7C). In contrast, no stimulation was observed for BWZ.CD3/NKR-P1(A,C,G) reporter cells (data not shown). This finding suggests that Clr-b−/− cells may display an alternative surface Clr ligand for NKR-P1F. Because Clr-g transcripts are expressed in hematopoietic cells (26, 40–43), antagonistic Clr-b/g heterodimers are possible; alternatively, the absence of Clr-b could unmask another Clr ligand, such as Clr-c (NKR-P1G, but not NKR-P1G ligands), in turn modulating NKR-P1F receptor expression or function during NK cell development and education. The same may be true for NKR-P1C, which is slightly downregulated on Clr-b−/− NK cells; however, NKR-P1C ligands remain unknown to date.

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To examine self-tolerance, Clr-b−/− mice were used as recipients of WT, Clr-b−/−, β2m−/−, and β2m−/−/− Clr-b−/− BM cells. Because the recipient genotype was being varied, results were plotted as
that Clr-b is the only constitutive ligand recognized by NKR-P1B. However, other developmentally regulated or activation-induced ligands or antagonists may exist.

The steady-state immune and hematopoietic composition of Clr-b−/− mice showed no major differences. In addition, we observed few significant differences in NK cell receptor expression. However, significantly higher expression of the inhibitory NKR-P1B receptor was observed on BM, splenic, and hepatic NK cells from Clr-b−/− animals, along with significantly lower expression of stimulatory Nkp46 receptor. Other trends were observed toward decreased expression of stimulatory NK receptors (NKR-P1C, 2B4, NKG2D, CD94/NKG2C/E) and perhaps slightly increased expression of inhibitory NK receptors (Ly49, CD94/NKG2A).

These changes in balanced signaling are expected to elicit hyporesponsiveness. Elevated Ly49 receptor expression has been observed on NK cells from β2m−/− mice, which lack MHC-I ligands (35). Mechanistically, elevated NKR-P1B expression is likely posttranslational and due to a lack of receptor ligation or internalization following interaction with cognate ligand (on NK cells in cis or on neighboring cells in trans) (47, 48). Furthermore, increased NKR-P1B on Clr-b−/− NK cells may occur during education, as developing NK cells integrate stimulatory and inhibitory signals. To achieve balance, developing Clr-b−/− NK cells may augment inhibitory NKR-P1B expression and/or decrease that of stimulatory receptors. As highlighted above, Clr-b−/− NK cells exhibit reduced Nkp46 receptor levels. Nkp46 is reported to recognize an unknown self-ligand (49); thus, lowering Nkp46 expression may reduce the net threshold of activation to compensate during NK cell education.

Acute rejection of MHC-I−deficient hematopoietic grafts by NK cells has been widely documented (32, 36). We developed a competitive assay to assess Clr-b−/− graft rejection relative to control WT and MHC-I−deficient grafts in a single mouse. This assay employs three fluorophores with distinct spectra to enable differential labeling of seven donor populations. This approach is indexed and internally controlled, and inherently conserves recipient animals and reagents, in addition to reducing variability, as competitive rejections of all donors occur simultaneously. As expected, MHC-I−deficient BM grafts (β2m−/−, Kb−/−, D−/−, K−/−, D−/−) were rejected to different degrees by WT NK cells. Notably, β2m−/− and K−/−/− D−/− grafts were rejected vigorously because they lack expression of all MHC-I proteins and Qa-12; in addition, β2m−/− mice lack expression of CD1d and many other

![Graph](image-url)
cell surface molecules (50). Therefore, these transplanted grafts promote disinhibition of both Ly49h and NKGD2/CD94^# NK subsets, which synergize to mediate rejection (51). Functionally relevant B6 NK subsets include those expressing Ly49C/1 and NKGD2/CD94 (52). In contrast, single MHC-I-deficient grafts (K/R^+/−, D^b/−) exhibited reduced rejection compared with double-deficient K/R^+/−D^b/− grafts, as shown previously (36). The hierarchically comparable rejection of Clr-b^−/− BM cells to H-2D^b/−/− BM cells was quite striking because both H-2D^b and Clr-b are single loci, and ~60% of NK cells express NKR-P1B (48). In contrast, many overlapping NK subsets are simultaneously disinhibited by full MHC-I-deficient grafts, resulting in robust rejection relative to disinhibition of the NKR-P1B^hi/^hi NK subset alone (which accounts for ~30–40% of acute BM graft rejection).

Data generated using allogeneic NIH Swiss recipients further strengthen this claim. β-m^−/− grafts were rejected ~95%, whereas Clr-b^−/− grafts were rejected up to ~50%. This difference is likely attributed to enhanced recognition of Clr-b by NKR-P1B^sw versus NK-P1B^hi, compounded with allogeneic Ly49:MHC-I interactions. Importantly, selective depletion of NKR-P1B^hi/^hi NK cells reversed rejection of Clr-b^−/−/− grafts, as did genetic NKR-P1B^−/− deficiency, whereas β-m^−/−/− graft rejection was not affected.

These findings support an important and nonredundant role for MHC-independent missing-self recognition of Clr-b by NKR-P1B^+ NK cells in hematopoietic transplants. The absence of donor MHC-I, Clr-b, or both results in poorer prognosis for BM transplant engraftment in WT recipients. In addition, Clr-b^−/− recipients revealed a trend toward increased engraftment of WT versus autologous Clr-b^−/−/− grafts, as well as β-m^−/− versus Clr-b^−/−/−/−/β-m^−/− double-deficient grafts. Because Clr-b^−/− NK cells exhibit elevated NKR-P1B levels, donor Clr-b may provide a static inhibitory signal to Clr-b^−/− recipient NK cells, conferring increased engraftment. The cytotoxicity assay results demonstrated that NK-P1B is indeed functional and inhibitory on Clr-b^−/− NK cells. In addition, the IFN-γ stimulation assays demonstrated that Clr-b^−/− NK cells (both NKR-P1B^hi/lo subsets) are generally hyporesponsive to NKR-P1C crosslinking and IL-12 cytokine priming. This generalized hyporesponsiveness may be due to differential NK cell education, which may in turn impact stimulatory receptor expression (NKp46 etc.).

Finally, previous unpublished data suggest that Clr-b may heterodimerize with other Clr, such that loss of surface Clr-b may promote expression of alternative Clr dimers. We showed that NKR-P1F, a putative costimulatory NK receptor, displays enhanced recognition of ex vivo Clr-b^−/−/− hematopoietic cells. Thus, missing Clr-b may promote enhanced dimerization of NK-R1P1F ligands (e.g., Clr-c, d, g) (29). This induced-self recognition of Clr dimers by NKR-P1F^+ NK cells may also partially explain the increased rejection of Clr-b^−/−/β-m^−/−/− double-deficient versus β-m^−/−/−/−/− grafts in Clr-b^−/−/− recipients. Notably, NKR-P1F/G share some overlapping (and some distinct) ligand specificities, suggestive of balanced or integrated recognition. Alternatively, it is also possible that Clr family members may engage in both cis/trans interactions with their cognate receptors (or one another), similar to what has been shown for MHC-I ligands and Ly49 receptors (53–56).

These results suggest a model in which Clr-b expression may modulate surface expression of other Clr ligands. Under normal conditions, Clr-b may form both homodimers, which inhibit NK cells via NKR-P1B, and heterodimers with other Clr, which may not be recognized. Loss of Clr-b during stress, transformation, or infection may promote both missing-self recognition of Clr homodimers (via NKR-P1B), as well as induced-self recognition of other Clr (e.g., Clr-c/d/g dimers, via NKR-P1F). The Clr homo/heterodimerization model may further our understanding about the molecular interactions that occur at the NK cell/target synapse during cellular pathological states, via integration of the missing-self and induced-self recognition axes.

It remains to be determined if homologous receptor–ligand interactions in humans, including NKR-P1A:LLT1 (encoded by KLRL1:CLEC2D polymorphisms, play similar roles in NK cell recognition and rejection of normal syngeneic and allogeneic hematopoietic cell grafts.

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References


