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NKR-P1 Biology

From Prototype to Missing Self

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Abstract

Natural killer (NK) cells represent lymphocytes of the innate immune system capable of recognizing and destroying a broad array of target cells, including tumors, virus-infected cells, antibody-coated cells, foreign transplants, and "stressed" cells. NK cells eliminate their targets through two main effector mechanisms, cytokine secretion and cell-mediated cytotoxicity, which in turn depend on detection of target cells through a complex integration of stimulatory and inhibitory receptor–ligand interactions. The NKR-P1 molecules were the first family of NK cell receptors identified, yet they have remained enigmatic in their contribution to self–nonself discrimination until recently. Here, we outline a brief history of the NKR-P1 receptor family, then examine recent data providing insight into their genetic regulation, signaling function, cognate ligands, and gene organization and diversity.

Key Words

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Introduction: The NK1.1 Antigen(s)

Natural killer (NK) cells are large granular lymphocytes capable of recognizing and killing a wide variety of target cells *(1–4)*. They were initially described as a component of spleen cells exhibiting "non-specific", "spontaneous," or "natural" cytotoxicity toward syngeneic or allogeneic tumor cells infected with murine leukemia virus (MuLV) *(5–12)*. Thus, while defined functionally by their "natural killer" activity *(8),* phenotypically NK cells were only known to comprise a small fraction of "null" (non-T/non-B) lymphocytes *(1)*. This necessitated a selective method of purifying NK cells for further analysis of their characteristics and specificity *(1)*.

One of the early methods to purify NK cells arose from a fortuitous discovery that involved immunization of C3H mice with CE thymocytes to produce α-Ly1.2 antiserum: this C3H α-CE antiserum was capable of selectively depleting NK activity from C57BL/6 (B6) and NZB splenocytes against RL1 tumor targets *(1)*. Subsequently, it was discovered that a Ly1.2-independent anti-NK cell specificity could be isolated: (a) serologically, by selectively adsorbing the C3H α-CE antiserum onto BALB/c (Ly-1.2$^+$) thymocytes or splenocytes; (b) genetically, by introducing the BALB/c genotype into the host strain immunized, (i.e, [C3HxBALB]F_1 mice) *(1)*. The resulting antisera, designated "α-NK", did not react with T or B cells, but selectively eliminated NK activity from B6 splenocytes upon complement-mediated lysis *(1)*.

Thereafter, the "NK" alloantigen, now called "NK-1," was broadly used as a criterion to define and purify NK cells *(13,14)*. This utility prompted Koo and Peppard to refine the specificity of the α-NK-1 antisera *(1)* with the development of an α-NK-1.1 monoclonal antibody (mAb), PK136 *(15)*. Using PK136 mAb, the strain-specific expression of the NK1.1 alloantigen was further investigated and found to identify NK cells from CE, B6, NZB, C58, Ma/My, ST, and SJL mice, but not BALB/c, AKR, CBA, C3H, DBA, or 129 mice *(1,15)*. Notably, while the PK136 hybridoma was derived from a (C3HxBALB)F_1 α-CE immunization protocol *(1,15),* α-NK1.1 reactivity has since become popularized as representing a BALB/c α-B6 specificity due to conventional use of the latter strains (see below).

The advent of the PK136 mAb accelerated the study of murine NK cell biology. It was shown that the NK1.1 antigen was expressed on the vast majority of developing and mature NK cells from B6 mice (as well as some T cell lines) *(14–16),* that blocking of NK1.1 did not alter natural killing (in B6 mice) *(15),* and that the NK1.1 antigen corresponded to an approx 39 kDa glycoprotein *(17)* that stimulated NK cell function upon crosslinking *(18)*. Identification of other NK alloantigens (NK2.1, NK3.1, NK4.1, 2B4, etc.), and determination of their genetic relationship to NK1.1, demonstrated that several NK alloantigens were encoded by genes mapped to a single region of distal chromosome 6 *(17,19)*. This finding and subsequent genetic linkage of NK1.1 with another group of NK cell antigens, encoded by the *Ly49* genes, prompted the designation of this region as the "NK gene complex" (NKC) *(20,21),* following the precedent of the major histocompatibility complex (MHC) on mouse chromosome 17.

Expression cloning of an NK1.1 antigen in B6 mice determined that it was encoded by the *Nkrp1c* gene *(22),* a member of a multigenic *Nkrp1* family *(23)*. The NKR-P1 receptors were previously known from studies in rats to represent approx 60 kDa homodimeric disulfide-linked C-type lectin-like molecules involved in stimulation of NK-mediated cytotoxicity *(24,25)*. The signal transduction capacity of the NKR-P1 receptors *(25,26)* and

the existence of multiple *Nkrp1* transcripts in NK cells *(22,23),* suggested they might be involved in target cell recognition *(27,28)*.

Subsequently, studies in other mouse strains identified a second NK1.1 antigen, the *Nkrp1b* gene product, which inhibits NK cell function *(29–32)*. While *Nkrp1b* was reportedly cloned from B6 mice *(23),* recent studies have shown that B6 mice do not express the *Nkrp1b* transcript *per se (31,32)*. Instead, B6 NK cells express a related *Nkrp1d* transcript that encodes a divergent but functionally analogous inhibitory receptor *(31,32)*. While NKR-P1D does not react with PK136 mAb, it is now clear that NKR-P1B is responsible for NK1.1 reactivity of a number of inbred and outbred mouse strains, including NIH-Swiss (Sw) *(31),* SJL *(32),* FVB *(33),* and CD-1 *(34)*. The relationship of these mouse strains (and the B6 strain) to the original CE strain used for PK136 hybridoma production *(1,15)* is not clear, thus the identity of the "original" NK1.1 antigen remains obscure at present. Furthermore, it is unclear why certain strains of mice, including C3H and BALB/c *(1,15),* lack NK1.1 alloantigen expression; nor is it known whether NK cells from these strains express the *Nkrp1b*/*c* gene products.

It is important to note that the recent identification of cognate NKR-P1 ligands *(4,34,35)* requires a reexamination of these issues to determine the importance of NKR-P1 receptor–ligand interactions in NK cell biology and innate immunity. Moreover, the existence of both stimulatory- and inhibitory-class NKR-P1 receptor and ligand isoforms suggests a novel role for these receptors in class I MHC-independent self–nonself discrimination by NK cells. The remainder of this review will examine the regulation and function of the NKR-P1 receptor–ligand system in NK cell recognition across strain and species boundaries.

Genetic Regulation: The *Nkrp1c* Promoter and Enhancer Region

Recently, the upstream region of the of the mouse *Nkrp1c* gene (approx 10 kb) was cloned and analyzed in detail to identify regulatory elements responsible for controlling lineage-specific expression of the B6 NK1.1 antigen *(36)*. This work revealed the presence of three functional promoters and an enhancer-like region *(36)*. The major promoter, promoter 1, is located immediately upstream of the first coding exon and represents the dominant transcriptional initiation site, one that is active at all stages of NK cell development *(36)*. The promoter 1 region lacks a canonical TATA-box, but contains an Initiator (Inr) element and a downstream promoter element (DPE) *(36)*. Both the Inr and DPE elements are required for sequence-specific binding of TFIID to TATA-less promoters *(37)*. Furthermore, promoter 1 contains an upstream A/T-rich sequence that may contribute to the assembly of the transcription preinitiation complex *(36)*. Notably, this region is also depleted in CpG dinucleotides and contains a number of consensus transcription factor binding sites, including Ikaros, GATA, TCF-1, and multiple Ets-1 sites *(36)*. Thus, it is likely that these and other transcription factors required for normal NK cell differentiation *(38–41)* contribute to the lineage-specific expression of *Nkrp1c*.

Upstream of promoter 1, two additional promoters were identified that appear to be responsible for the developmental regulation of *Nkrp1c* expression *(36)*. Promoter 2 is located immediately upstream of promoter 1 and appears to be active only in mature NK cells, whereas promoter 3 is located approx 9 kb upstream of promoter 1 and appears to be active only in fetal NK cells *(36)*. Furthermore, the promoter 3 region contains a DNaseI hypersensitive site (HS1) that exhibits

enhancer-like activity *(36)*. Neither of the transcripts initiated by promoter 2 or promoter 3 represent coding exons, as both contain multiple stop codons following the proximal ATG codons *(36)*. Thus, these promoters presumably function to initiate developmental stage-specific expression of *Nkrp1c*. In this light, it is interesting to note that the *Nkrp1b* gene is active in early fetal blood T/NK progenitors *(30,31,42),* prior to entry into the fetal thymus *(30,42),* whereas the *Nkrp1c* gene is only active after entry of progenitors into the fetal thymus *(30,42–45)*. This suggests that the inhibitory NKR-P1 receptors are expressed earlier than the stimulatory receptors *(30,42)*. Because the promoter/enhancer regions of the *Nkrp1c* and the *Nkrp1d* genes are conserved *(46),* the fetal-specific promoter of the inhibitory-class *Nkrp1b/d* genes (promoter 3) may be active earlier than the *Nkrp1c* promoter *(30,42)*. In addition, while *Nkrp1c* is expressed by virtually all NK lineage cells in B6 mice *(16,22),* expression of the *Nkrp1b* gene is variegated, such that it is expressed on only approx 60–70% of NK cells in various mouse strains, including Sw *(31),* SJL *(32),* and FVB mice *(33)*. The recent identification of probabilistic switches in the *Ly49* promoters *(47)* suggests that similar elements may be present in the *Nkrp1* promoters *(46)*. Thus, the proportion of NK cells that express a given *Nkrp1* gene may be a product of the relative strength and orientation of upstream bidirectional promoter elements *(46,47)*. These issues require further exploration.

Collectively, the above findings suggest that the mouse *Nkrp1c* gene shares a number of similar features with genes encoding other NK cell receptors *(46)*. Indeed, the existence of a TATA-less promoter in the *Nkrp1c* gene is not unusual, as most of the NKC-encoded and NK-related genes lack a canonical TATA-box in their promoters, including human *NKG2A (48)* and murine *2b4 (49)*. Moreover, it has been shown that the *Ly49* genes are also regulated by three distinct promoter elements: the major downstream promoter (Pro-3) is preceded by a proximal upstream promoter (Pro-2), active in mature NK cells, and a distal upstream promoter (Pro-1), primarily active in immature NK cells *(46,47,50–54)*. The usage of multiple promoters has also been described for other NKC-encoded genes, including *Cd94 (55)*. Furthermore, the presence of an upstream DNase I HS site has been demonstrated in the *Cd94 (55), NKG2A (56),* and *Ly49* genes *(57)*. Finally, the intron–exon organization is similar for the *Nkrp1c* and *Ly49* genes, including conservation of the non-coding, cytoplasmic, transmembrane, stalk, and lectin-like domain exons *(36,50)*. Taken together, these findings suggest a commonality in the structure and transcriptional regulation of NK-related genes that has been conserved throughout evolution in order to maintain important features of NK cell function.

Integration of NKR-P1–Mediated Signaling

Initial examination of the signaling capacity of the NKR-P1 receptors utilized mAb-dependent crosslinking of the stimulatory rat NKR-P1A receptor [using 3.2.3 mAb *(24–27,29)*] or mouse NKR-P1C receptor [using PK136 mAb *(18,22,58)*]. Ligation of rat NKR-P1A was shown to induce antibody-induced redirected lysis (AIRL) *(24),* phosphatidylinositol (PI) turnover, and Ca^{2+} flux *(26)*. Similarly, ligation of mouse NKR-P1C on B6 NK cells was observed to mediate AIRL and IFN-γ cytokine production *(18,59)*. It was later shown that the mouse NKR-P1C receptor stimulated NK cells via association with the FcRγ adaptor molecule, previously known to transduce signals for the FcγRIII (CD16) and FcεRI receptors for immunoglobulin *(58)*. The association between NKR-P1C and FcRγ is thought to involve the charged

transmembrane R residue in NKR-P1C and the corresponding transmembrane D residue in FcRγ *(31)*. In turn, NKR-P1C crosslinking presumably results in phosphorylation of the FcRγ immunoreceptor tyrosine-based activation motif (ITAM) Y residues, leading to downstream recruitment of the Syk tyrosine kinase, and activation of NK cytotoxicity *(31,58)*.

In contrast to NKR-P1C, the mouse NKR-P1B receptor inhibits NK function *(31,32)*. Notably, co-crosslinking of NKR-P1B and NKR-P1C on (B6xSw)F_1 LAK cells using PK136 mAb revealed a dominance of NKR-P1B–mediated inhibition over NKR-P1C–mediated stimulation, although the NKR-P1B signal was not sufficient to abrogate CD16-mediated ADCC *(31)*. Recently, the rat NKR-P1B receptor was also shown to mediate dominant inhibitory function over 2B4-mediated stimulation *(60)*. Similarly, mouse NKR-P1B overexpression in human YTS NK cells exerted dominant inhibition over 2B4 function *(61)*. Sequence alignment revealed that the mouse NKR-P1B receptor lacks a charged transmembrane R residue due to a single amino acid deletion in its sequence relative to that of NKR-P1C *(31)*. Conversely, NKR-P1B possesses a consensus immunoreceptor tyrosine-based inhibition motif (ITIM; LxYxxL) in its cytoplasmic domain *(29,31,32)*. Like other ITIM-bearing receptors expressed by NK cells, mouse NKR-P1B binds SHP-1 in a phosphorylation-dependent manner, suggesting a molecular mechanism for inhibition of NK cell cytotoxicity through NKR-P1B *(31,32)*. Indeed, mutation of the NKR-P1B cytoplasmic ITIM Y residue (to F) was found to abolish both SHP-1 recruitment and inhibitory signaling, providing direct evidence for the importance of the ITIM in inhibitory NKR-P1 receptor function *(61)*.

Further sequence analysis of the mouse and rat NKR-P1 proteins reveals that all isoforms possess a cytoplasmic CxCP(R/H) motif *(29,62)* similar to that found in the cytoplasmic domains of the CD4 and CD8 coreceptors *(63)*. This motif is known to mediate association with the Src-related protein tyrosine kinase, $p56^{lck}$ *(63)*. Using the yeast 2–hybrid approach, Campbell and Giorda *(62)* demonstrated a direct physical association between $p56^{lck}$ and the rat NKR-P1A cytoplasmic domain sequence. This association was dependent on the CxCP motif, as mutation of both C residues (to A) abrogated the interaction. Recent studies have in also analyzed the functional requirements for the CxCP motif in mouse NKR-P1 signaling *(61)*. This analysis revealed that both the stimulatory NKR-P1C and inhibitory NKR-P1B receptors functionally associate with $p56^{lck}$. In addition, mutation of a single C residue (to S) in the CxCP motifs of mouse NKR-P1B/C either abolished or greatly reduced the capacity of the receptors to mediate intact signal transduction *(61)*. Moreover, NK cells from B6 *lck*$^{-/-}$ mice displayed greatly reduced NK1.1-mediated AIRL of FcR^+ P815 mastocytoma targets, demonstrating a direct functional requirement for Lck in NKR-P1C-mediated stimulation *(61)*. Notably, Lck-deficient bone marrow–derived LAK cells were more severely impaired in NK1.1-mediated AIRL than splenic LAK cells, suggesting that additional signaling pathways can compensate for the Lck deficiency during NK cell maturation in vivo (unpublished observations). These data implicate Lck in the establishment of NKR-P1 receptor tyrosine phosphorylation, as well as the recruitment and activation of the Syk tyrosine kinase or SHP-1 tyrosine phosphatase effector molecules. Thus, both the stimulatory and inhibitory NKR-P1 receptors utilize the same tyrosine kinase to initiate their signaling pathways. Interestingly, the human NKR-P1A

receptor lacks the CxCP Lck-recruitment motif *(29,64),* suggesting an alternate signal initiation pathway for this receptor.

Identification of Non-MHC Ligands for the NKR-P1 Receptors

The search for physiological ligands for the NKR-P1 receptors has spanned three decades. Early investigations into the binding of bacterially produced extracellular domains of the rat NKR-P1A receptor demonstrated high-affinity binding of the protein to oligosaccharide carbohydrate ligands in a Ca^{2+}-dependent manner *(65,66)*. While this initially suggested that the NKR-P1 receptors possess intact lectin function, further investigations into carbohydrate recognition by the rat NKR-P1A extracellular domain failed to reproduce previous results *(67,68)*. Subsequently, it was shown that the stimulatory rat NKR-P1A receptor could functionally recognize a xenogeneic ligand expressed on several mouse tumor lines, including IC-21 macrophages, C1498 T lymphoma cells, and B16 melanoma cells *(27)*. Furthermore, a role for the mouse NKR-P1C receptor in allorecognition was suggested by studies of the NK-mediated F1 anti-parent "hybrid resistance" phenomenon *(28)*. However, the significance and underlying basis of these putative stimulatory interactions remains unclear to date.

The identification of inhibitory-class isoforms of the NKR-P1 proteins *(29,31,32)* revitalized the search for physiological ligands, as this finding predicted the existence of "self" protein ligands encoded by the mouse genome *(31)*. The initial cloning of the mouse *Nkrp1* cDNAs resulted in the isolation three transcripts *(23),* only one of which was speculated to possess an intact ITIM motif *(Nkrp1b) (29)*. However, the lack of available antibodies to the inhibitory receptors precluded an early investigation into their expression and function *(29)*. Nonetheless, studies in mouse strains other than B6 (Sw and SJL) demonstrated binding of the α-NK1.1 PK136 mAb to a cell surface receptor that was functionally distinct from the *Nkrp1c* gene product *(31,32)*. It was shown that the mouse *Nkrp1b* gene product was responsible for this NK1.1 reactivity, while a novel B6-derived inhibitory *Nkrp1d* gene product did not react with PK136 *(31,32)*. Importantly, NKR-P1B was shown to possess inhibitory function in PK136-mediated AIRL assays *(31,32)*. This discovery lead to the hypothesis that a novel class of "missing self" ligands existed for the NKR-P1 orphan receptors *(31)*.

Recently, physiological protein ligands for the mouse NKR-P1 receptors were identified using a multifaceted array of techniques *(34,35)*. Remarkably, the NKR-P1 ligands are also C-type lectin-like in structure, and the genes encoding the ligands are interspersed among the *Nkrp1* receptor genes themselves within the NKC *(34,35,69)*. The cDNAs encoding the ligands were previously known from osteoclastogenesis and NKC mapping studies to be members of a gene family referred to as osteoclast inhibitory lectin *(Ocil) (70,71),* or C-type lectin-related *(Clr) (72),* respectively. Only two ligands have been identified: Ocil/Clr-b is recognized by the inhibitory NKR-P1B/D receptors *(34),* while Ocilrp2/Clr-g/Dcl-1/LCL-1 is recognized by the stimulatory NKR-P1F receptor *(35)*. Currently, little is known about the stimulatory NKR-P1 receptor–ligand interactions *(73,74);* however, the function of the inhibitory ligand has been elucidated in greater detail (see Fig. 1).

The inhibitory NKR-P1B/D receptors have been shown to bind Ocil/Clr-b by a number of approaches; however, there are discrepancies in the results *(34,35)*. Tetramers of bacterially produced and refolded NKR-P1D extracellular domains bind to the surface of Ocil/Clr-b transfectants, but not untransfected cells or normal cells ex vivo *(35)*. However, CD3ζ/

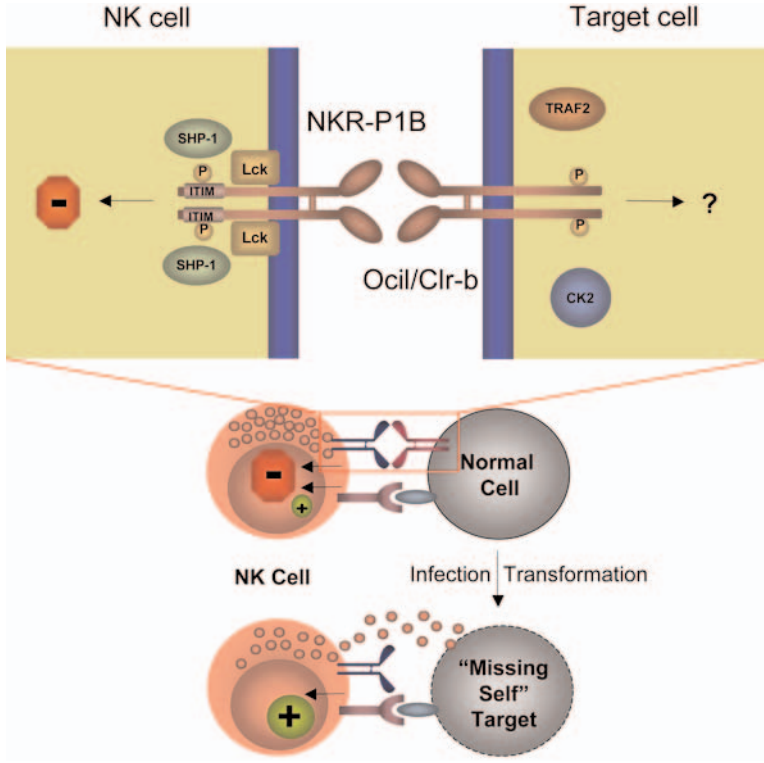

Fig. 1. NKR-P1B-mediated "missing self" recognition of abnormal target cells by NK cells. Close up view (top) of the NKR-P1B:Ocil/Clr-b interaction between an NK cell and a target cell under surveillance (middle). The NKR-P1B receptor, upon binding of Ocil/Clr-b ligand, mediates inhibitory signals to the NK cell. Upon receptor ligation, Lck tyrosine kinase (associated with NKR-P1B via a cytoplasmic CxCP motif) phosphorylates the ITIM (LxYxxL) tyrosine residue, which facilitates recruitment of SHP-1 tyrosine phosphatase. Reciprocally, signals may be transmitted to the target cell via the Ocil/Clr-b cytoplasmic tail, which contains potential motifs for CK2 phosphorylation (SxxE), TRAF2 binding (SPQE), ubiquitinylation and endocytosis. Upon transformation or infection (bottom), target cells lose Ocil/Clr-b surface expression, resulting in loss of NK cell inhibition, granule exocytosis, and induction of target cell lysis/apoptosis. A basal stimulatory interaction, required for NK:target adhesion and directed granule exocytosis, is also depicted. Other receptor-ligand interactions are not depicted for simplicity.

NKR-P1D fusion receptors expressed on BWZ.36 reporter cells detect functional ligand expressed on ex vivo bone marrow and spleen cells, but not thymocytes *(35)*. This was taken as evidence of restricted Ocil/Clr-b expression, as further analysis of hematopoietic cells revealed that only macrophages and dendritic cells specifically stimulated BWZ.CD3ζ/NKR-P1D reporter cells *(35)*. Moreover, the tetramer results suggest that the NKR-P1D:Ocil/Clr-b interaction is of low affinity, insufficient to visualize ligand expressed at normal, endogenous levels *(35)*.

In contrast, NKR-P1B tetramers were shown to bind endogenous Ocil/Clr-b on cell lines, including MNK-1 pre-NK cells and NIH-3T3 fibroblasts, but not several tumor lines *(34)*. Moreover, BWZ.CD3ζ/NKR-P1B reporter cells were capable of detecting ligand on all hematopoietic cell populations ex vivo, including thymocytes *(34)*. This suggests that

the affinity of the NKR-P1B:Ocil/Clr-b interaction may be greater than that of the NKR-P1D:Ocil/Clr-b interaction; indeed, differential binding of NKR-P1B vs NKR-P1D to a single allele of Ocil/Clr-b has been demonstrated in the absence of ligand overexpression *(34)*. Nonetheless, the greater sensitivity of the CD3ζ/NKR-P1B reporter cells may have been due to the inclusion in the fusion receptor sequence of the native NKR-P1B cytoplasmic CxCP motif *(34),* which is responsible for Lck recruitment in the initiation of signaling *(61)*. In any case, the above results demonstrate a broader expression pattern of Ocil/Clr-b, or at least inhibitory NKR-P1 ligands *(34),* than previously reported *(35)*.

Further support for a broad NKR-P1B/D ligand expression pattern came from a direct analysis of surface protein expression *(34)*. Prior to cloning of Ocil/Clr-b as an NKR-P1B/D ligand, a mAb specific for Ocil/Clr-b (4A6, rat IgMκ) was generated via an "immune focusing" protocol *(34)*. Here, mouse cells known to express NKR-P1B/D ligand (i.e., MNK-1, NIH-3T3, BM macrophages, as determined by tetramer/BWZ analysis) were used to serially immunize Lewis rats to focus the antibody response on shared antigens (one of which included the inhibitory NKR-P1 ligand) *(34)*. Hybridomas were screened by supernatant blockade of the interaction between BWZ.CD3ζ/NKR-P1B reporter cells and MNK-1 or NIH-3T3 stimulator cells *(34)*. Importantly, 4A6 α-Ocil/Clr-b mAb results recapitulated staining using NKR-P1B tetramers; indeed, tetramer staining could be blocked by 4A6 mAb *(34)*. Moreover, surface staining of normal hematopoietic cells ex vivo revealed that Ocil/Clr-b was expressed in a broad manner remarkably similar to that of class I MHC *(34)*. This includes high-level expression on most hematopoietic cells, low-level expression on double-positive thymocytes, and little to no expression on terminally differentiated erythrocytes *(34)*. However, Ocil/Clr-b expression and function were independent of β_2m and MHC I expression *(34,35),* thus implicating the NKR-P1B/D:Ocil/Clr-b interaction as an MHC-independent "missing self" recognition system.

Strikingly, this notion was confirmed by the observation that Ocil/Clr-b expression is frequently downregulated or lost altogether on a number of tumor lines relative to normal cells ex vivo, and this "missing self" ligand renders the tumor cells sensitive to NK killing *(34)*. Indeed, a tumor cell line sorted for endogenous Ocil/Clr-b expression (BWZ.4A6$^+$) exhibited dramatically reduced NK lysis relative to the parental line (BWZ), and this inhibition could be reversed using blocking mAb specific for Ocil/Clr-b ligand (4A6 mAb) *(34)*. Furthermore, overexpression of Ocil/Clr-b on tumor cells inhibited killing by B6 NK cells, and this inhibition could be reversed using blocking mAb specific for the NKR-P1D receptor (2D12 mAb) *(35)*. Notably, though, endogenous Ocil/Clr-b expression on BWZ.4A6$^+$ cells was insufficient to inhibit lysis by B6 NK cells (which express NKR-P1D), while lysis was significantly inhibited using NK cells derived from Sw or CD-1 mice (which express NKR-P1B) *(34)*. These results functionally correlate with the relative strength of signal observed for NKR-P1B vs NKR-P1D using the same target cell as a stimulator in the BWZ reporter cell assay *(34)*. This observation may also explain why endogenous Ocil/Clr-b expression on other tumor lines either does not inhibit (e.g., C1498 cells) or only partially inhibits (e.g., RMA/S cells) the function of B6 NK cells *(35),* whereas both C1498 and RMA/S stain using 4A6 mAb and weakly stimulate BWZ.CD3ζ/NKR-P1B reporter cells *(34)*. On this point, even parental BWZ cells exhibit low 4A6 staining, and BWZ.CD3ζ/NKR-P1B reporter cells weakly stimulate themselves in isolation *(34)*.

Nonetheless, a potential caveat to the 4A6 mAb observations is the possibility that the mAb may crossreact with an unknown Ocil/Clr family member or other antigen. However, 4A6 mAb has been shown not to crossreact with Clr-f or Ocilrp2/Clr-g (at least certain alleles) *(34)*. Moreover, only Ocil/Clr-b exhibits a broad expression pattern at the transcript level—both RT-PCR *(72)* and Northern blotting *(70,71)* results recapitulate the Ocil/Clr-b expression pattern observed at the protein level by 4A6 staining *(34)*. Taken together, these results support the notion that Ocil/Clr-b is expressed broadly on normal cells ex vivo, and that it is frequently downregulated on tumor cells *(34)* (see Fig. 1). Thus, Ocil/Clr-b exhibits the expected expression pattern of a "missing self" ligand *(34)*. In any case, it is also possible that "induced self" upregulation of other Ocil/Clr family members, combined with their heterodimerization with the Ocil/Clr-b protein, could lead to a more rapid loss of inhibitory ligand function prior to loss of 4A6 staining during transformation. This issue warrants further investigation.

Significantly, NKR-P1–mediated recognition appears to be conserved in other species. Only a single receptor gene exists in humans [*NKRP1A (3,64,69)*], and only a single ligand gene shares significant homology and positional location with the rodent Ocil/Clr family [*(LLT1 (3,69,75)*]. Recently, two reports have demonstrated that human NKR-P1A functionally binds LLT1 *(76,77)*. Moreover, like the mouse NKR-P1B/D:Ocil/Clr-b interaction, the human NKR-P1A:LLT1 interaction is inhibitory in function *(76,77),* and LLT1 is expressed in a broad manner *(75,78)*. Interestingly, LLT1 was observed to stimulate IFN-γ production on human T cells *(76)* and NK cells *(78),* suggesting that the Ocil/Clr family members may also mediate reverse signaling in rodent cells *(34,71,73,74)* (see Fig. 1). Collectively, these results bolster and extend the significance of NKR-P1–mediated self–nonself discrimination and "missing self" recognition in the immune system. The existence of putative NKR-P1:Ocil/Clr-like genes within the MHC region of the chicken and quail genomes *(79,80)* suggests that the MHC and NKC regions may share a common ancestral origin *(81)*. Moreover, the existence of an Ocil/Clr-like gene in the rat cytomegalovirus genome *(82)* suggests some viruses may have evolved strategies to subvert NKR-P1–mediated recognition during infection.

The NK1.1 Antigen(s) Revisited: Genetic Organization, Allelic Diversity, and Origin of the NK Alloantigen

Mouse strains can be subdivided into two groups based on reactivity of their NK cells with the α-NK1.1 mAb, PK136: NK cells from commonly used strains such as B6 and SJL are NK1.1$^+$, while BALB/c and 129 NK cells are NK1.1$^-$ *(1,15)*. The basis for NK1.1 reactivity of B6 [*Nkrp1c (22)*] and Sw/SJL [*Nkrp1b (31,32)*] NK cells has been established; however, the underlying molecular and genetic basis for the lack of NK1.1 reactivity of BALB/c and 129 NK cells has remained a mystery for many years *(83)*. Previous studies suggested that BALB/c NK cells do not efficiently express *Nkrp1* transcripts *(83);* however, no genetic basis for such a defect has been established. Given the recent elucidation of the NKR-P1:Ocil/Clr receptor–ligand interaction, such a genetic defect could have profound implications. For example, recent sequencing of the BALB/c *Ly49* gene cluster has revealed a complete absence of BALB/c equivalents of the *Ly49h* and *Ly49d* genes *(84),* the consequences of which include uncontrolled MCMV replication and early host death *(85,86),* as well as a greatly decreased efficiency of BALB/c NK cells to destroy specific tumor cells *(87)*. A

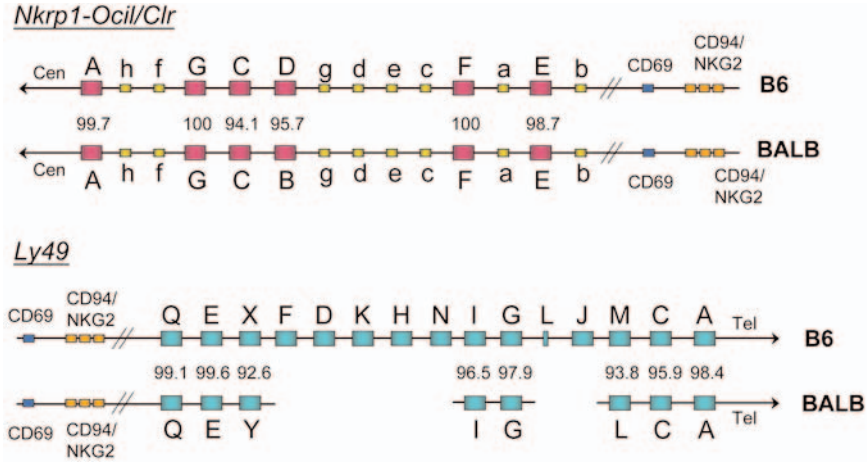

Fig. 2. Comparison of gene content between two divergent murine NKC haplotypes. The gene content in the B6 and BALB/c NKC region on mouse chromosome 6 is compared schematically. The *Nkrp1-Ocil/Clr* gene cluster (top) consists of six *Nkrp1* genes (large boxes; capital letters) intermingled with eight *Ocil/Clr* genes (small boxes; lower case letters). The number of genes in the *Ly49* cluster (bottom) is variable. Probable *Ly49* alleles have been aligned by introducing gaps in the small BALB/c *Ly49* haplotype. The *Cd69* and *Cd94/Nkg2* gene clusters separate the *Nkrp1* and *Ly49* regions in the NKC. The figure is not drawn to scale. The percentage cDNA identity over the known coding region is shown between the B6 and BALB/c alleles. *Nkrp1g* is only known from three exons in BALB/c and the *Nkrp1e*^{BALB} cDNA is missing exon 5. *Ly49l*^{BALB} and *Ly49m*^{B6} are closely related and can be considered alleles, as *Ly49l* in B6 mice is only represented by a single exon 7.

deficiency in NKR-P1 function could have similar implications.

To address this issue, we recently investigated the BALB/c *Nkrp1-Ocil/Clr* gene content *(88)*. Surprisingly, the BALB/c genome possesses a full complement of *Nkrp1* genes, including functional *Nkrp1b* and *Nkrp1c* genes (see Fig. 2). It is important to note that the *Nkrp1d* gene in B6 mice appears to represent a divergent allele of the *Nkrp1b* gene found in BALB/c and other mouse strains (Fig. 2). In addition, this investigation has identified novel genes present in the BALB/c genome that are conserved in the B6 genome (Fig. 2). Counting the *Nkrp1b/d* designation as allelic, this brings the total number of *Nkrp1* genes up to six, arranged in the genetic order *Nkrp1a-g-c-b/d-f-e* (centromere to telomere; see Fig. 2). Likewise, the total number of *Ocil/Clr* genes now stands at eight, ordered *Clr-h-f-g-d-e-c-a-b* (Fig. 2). Thus, unlike the *Ly49* gene content, which is divergent between B6 and BALB/c mice, that of the *Nkrp1-Ocil/Clr* family appears to be quite conserved between these two strains (Fig. 2). Intriguingly, only two receptors and two ligands have been demonstrated to interact, bringing into question the expression and function of the remaining family members. As mentioned previously, heterodimerization of the proteins could add significant complexity to the function of this system.

Given that BALB/c mice possess functional *Nkrp1b* and *Nkrp1c* genes and full-length transcripts, we further investigated the molecular basis for NK1.1 epitope reactivity *(88)*. Alignment of the NKR-P1B protein sequences from Sw/SJL mice (NK1.1$^+$) vs BALB/c mice (NK1.1$^-$) revealed only three amino acid substitutions in the extracellular region. Strikingly, mutation of only a single amino acid was

required to confer NK1.1 reactivity upon the NKR-P1B^{BALB} receptor *(88)*. Moreover, alignment of all available NKR-P1 protein sequences revealed a general trend, such that the amino acid composition of position 191 (in NKR-P1B) explains the presence or absence of the NK1.1 epitope on each receptor. Like the *Nkrp1b* and *Nkrp1d* alleles, the B6 and BALB/c *Nkrp1c* alleles have also diverged significantly. The localized divergence of these two neighboring *Nkrp1* genes is in complete contrast to the surrounding genes, which are quite conserved between the two mouse strains. Yet despite this divergence, the NKR-P1B^{BALB} receptor is functional and binds cognate Ocil/Clr-b ligand *(88)*. Collectively, these results suggest that the *Nkrp1b/c* region may have undergone directed evolution recently, perhaps under selection pressure from viral immune evasion of NKR-P1:Ocil/Clr recognition *(82)*. Such a rapid evolution of receptor function has been observed for the host–pathogen interaction between the Ly49H/Ly49I receptors and the MCMV m157 gene product *(85,86)*. In any case, these results indicate that the NKR-P1:Ocil/Clr recognition system is intact in NK1.1^{-} mouse strains. Together with the recently demonstrated human NKR-P1A:LLT1 interaction, these findings extend the importance of the mouse NKR-P1B:Ocil/Clr-b receptor-ligand system in self–nonself discrimination across strain and species boundaries.

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