Molecular and Genetic Basis for Strain-Dependent NK1.1 Alloreactivity of Mouse NK Cells[1]

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NK1.1 alloantigen expression can be used to define NK cells in certain mouse strains, such as B6 (NKR-P1C) and SJL (NKR-P1B). However, BALB/c NK cells do not react with the anti-NK1.1 mAb, PK136. To investigate the NK1.1$^-$ phenotype of BALB/c NK cells, we have undertaken NK1.1 epitope mapping and genomic analysis of the BALB/c *Nkrp1* region. Bacterial artificial chromosome library analysis reveals that, unlike the *Ly49* region, the *Nkrp1-Ocil/Clr* region displays limited genetic divergence between B6 and BALB/c mice. In fact, significant divergence is confined to the *Nkrp1b* and *Nkrp1c* genes. Strikingly, the B6 *Nkrp1d* gene appears to represent a divergent allele of the *Nkrp1b* gene in BALB/c mice and other strains. Importantly, BALB/c NK cells express abundant and functional *Nkrp1* transcripts, and the BALB/c NKR-P1B receptor functionally binds Ocil/Clr-b ligand. However, the BALB/c NKR-P1B/C sequences differ from those of the known NK1.1 alloantigens, and epitope mapping demonstrates that directed mutation of a single amino acid in the NKR-P1B^{BALB} protein confers NK1.1 reactivity. Thus, PK136 mAb recognizes, in part, a distal C-terminal epitope present in NKR-P1B$^{Sw/SJL}$ and NKR-P1C^{B6}, but absent in NKR-P1A/D/F^{B6} and NKR-P1B/C^{BALB}. Allelic divergence of the *Nkrp1b/c* gene products and limited divergence of the BALB/c *Nkrp1-Ocil/Clr* region explain a longstanding confusion regarding the strain-specific NK1.1 alloantigen reactivity of mouse NK cells. ***The Journal of Immunology,*** **2006, 176: 7511–7524.**

NK cells are large granular lymphocytes capable of recognizing and killing transformed, infected, Ab-coated, transplanted, and stressed cells (1, 2). Historically, NK cells from selected mouse strains have been phenotypically defined using NK-1 alloantigen-specific antisera (3) or the anti-NK1.1 mAb, PK136 (4). The NK1.1 alloantigen is now known to identify NK cells from CE, B6, NZB, C58, Ma/My, ST, SJL, FVB, and Swiss outbred mice, but not BALB/c, AKR, CBA, C3H, DBA, or 129 mice (3–7). In fact, the NK1.1 alloantigen was originally identified by immunizing BALB/c-background [(C3H $\times$ BALB)F_1] host mice with donor CE splenocytes (3), and the same immunization protocol was used to generate the PK136 mAb (4). Immunologically, this precludes the possibility that BALB/c (self) NK cells would react with anti-NK1.1 alloantibodies or PK136 mAb; thus, by definition, BALB/c NK cells are NK1.1$^-$ (3, 4, 8). Due to conventional use of the B6 mouse strain and initial molecular cloning of an NK1.1 Ag from B6 NK cells (9), anti-NK1.1 reactivity has since become popularized as representing the product of the *Nkrp1c* gene; however, the *Nkrp1b* gene product also reacts with PK136 mAb (5, 6).

Nonetheless, the underlying molecular basis for the lack of NK1.1 reactivity of NK cells from BALB/c and other mouse strains remains an enigma. Previous studies have suggested that BALB/c NK cells either lack or possess low-level expression of the *Nkrp1* genes, as detected by Northern blotting (10); however, no genetic basis for a BALB/c defect in *Nkrp1* expression has been established. Thus, lack of NK1.1 reactivity could be due to deletion of *Nkrp1* genes, defective gene expression, or allelic polymorphism in BALB/c mice. Extreme variation in gene content between the BALB/c and B6 haplotypes has been observed previously for the related *Ly49* gene family (11). This suggests that other NK gene complex (NKC)[4] regions, including the *Nkrp1-Ocil/Clr* region, also may be subject to rapid evolutionary divergence and/or polymorphism.

Importantly, since cognate NKR-P1 ligands have recently been identified (12–14), a BALB/c defect in NKR-P1 expression could be functionally significant for NK cell function and innate immunity. Moreover, while ligands for the stimulatory NKR-P1A/C receptors remain elusive, ligands for the inhibitory NKR-P1B/D receptors (12) and stimulatory NKR-P1F (13) receptors have been identified as products of the *Ocil/Clr* family of genes (15–17), which are intermingled with the *Nkrp1* genes themselves in the NKC (18). Thus, determination of the gene content of the BALB/c *Nkrp1-Ocil/Clr* region and the basis of the BALB/c defect in NK1.1 expression could have implications for the importance of the NKR-P1–Ocil/Clr system in self-nonself discrimination in mice and other species. Therefore, we have undertaken NK1.1

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[4] Abbreviations used in this paper: NKC, NK gene complex; BAC, bacterial artificial chromosome; EC/UTR, extracellular/3′-untranslated region.

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epitope mapping and genomic analysis of the BALB/c *Nkrp1* region to determine the functional significance of the NKR-P1 recognition system in NK1.1$^-$ mouse strains.

In this study, we demonstrate that the BALB/c genome possesses a full complement of *Nkrp1* and *Ocil/Clr* genes, including novel family members present in both B6 and BALB/c mice. Furthermore, BALB/c NK cells possess normal *Nkrp1* expression relative to B6 NK cells, and the BALB/c NKR-P1B receptor functionally binds cognate Ocil/Clr-b ligand. Absent NK1.1 reactivity of BALB/c NK cells can be explained by *Nkrp1* allelic divergence, specifically a single amino acid substitution (S191T) present in the BALB/c NKR-P1B/C receptors. In fact, divergence between the B6 and BALB/c *Nkrp1-Ocil/Clr* regions appears to be localized to the *Nkrp1b/c* genes, and the B6 *Nkrp1d* gene appears to be an allele of the *Nkrp1b* gene found in BALB/c and other mouse strains. These results confirm the importance of the NKR-P1–Ocil/Clr recognition system across strain boundaries, and suggest that this mode of self-nonself discrimination is conserved in other NK1.1$^-$ mouse strains, as well as other species.

Materials and Methods

Mice

Mice were purchased from the Charles River Laboratories National Cancer Institute–Frederick Animal Production Area (Frederick, MD), and/or maintained in our own animal facilities.

Cells

BWZ.36 cells (19) were obtained from Dr. N. Shastri (University of California, Berkeley, CA). The 293T cells were obtained from Dr. D. Raulet (University of California, Berkeley, CA). Cells were grown in complete DMEM-HG (high glucose, 10% FCS). All cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Expression constructs and NKR-P1 mutants

Mouse *Nkrp1b* (National Institutes of Health–Swiss–Sw) and *Nkrp1a/c/d* (C57BL/6–B6) constructs were generated previously (5). Mouse *Nkrp1e/f* cDNAs were amplified from NK libraries using the following primers: P1E-F, ATGGACACAGAAAGGATCTACCTC; P1E-R, TCAGGAGT CATGAAATATGGTTTC; and P1F-F, ATGGACACATCAAAGGTC CATG; P1F-R, TCAGACATGTATCAGGGTCTTTTG. Rat *Nkrp1a/b* cDNA clones (20) were generously provided by Dr. R. G. Miller (Ontario Cancer Institute, Toronto, ON). Site-directed mutagenesis was performed by the method of gene splicing by overlap extension of DNA ends, as described previously (12). Briefly, known *Nkrp1* sequences were amplified by PCR using specific 5′ or 3′ outside primers and complementary internal primers with specified point mutations; the resulting products were then mixed and amplified using the outside primers alone to obtain the full-length mutant cDNA. All PCR products were directly cloned into pcDNA3.1/V5/HIS/TOPO (Invitrogen Life Technologies), and constructs were sequenced to confirm their identities. The resulting constructs were cotransfected into 293T cells using Effectene reagent (Qiagen) with a pCMV-GFP-nuclear localization sequence reporter, or subcloned into the pMSCV2.2-CMV-IRES-GFP (pMCIG) retroviral vector (12) before transfection into 293T cells. Transfection efficiencies were monitored by GFP expression, and transfection results are shown gated on GFP$^+$ cells for all contstructs tested.

Flow cytometry and cell sorting

Cells were stained as described previously (21). Stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) or were sorted on a FACSDiVa (BD Biosciences). Sorted cells were >99% pure, as determined by postsort analysis.

Receptor fusions, retroviral infections, and BWZ assay

A pMSCV2.2-CMV-IRES-GFP (pMCIG) retroviral vector (12) was modified to include a CD3ζ/NKRP1B cassette (intracellular region of CD3ζ, membrane proximal and transmembrane regions of NKR-P1B). The CD3ζ/NKRP1B fusion cassette was generated by gene SOEing using the following primers: SALCD3Z-F, GTCGACATGAGAGCAAAATTCAGCAG GAGTG; CD3ZP1B-R, GCGAGGGCACCGACAGCGAGGGGCCAGGG TCTGC; CD3ZP1B-F, ACCCTGGCCCCTCGCTGTCGGTGCCCTCGCT GGCATC; XHOP1BTM-R, CTCGAGTGATGATTTTTGTACTGATAG. The resulting product was TOPO TA-cloned (Invitrogen Life Technologies) and cut with *Sal*I and *Xho*I (New England Biolabs), then ligated into pMCIG that had been linearized with *Xho*I and treated with CIP. cDNA inserts were subcloned into the modified cassette vector, pMC3BIG, using *Xho*I and *Not*I sites. BWZ.36 cells were infected with retroviral supernatants (24–48 h) from transient triple-transfected 293T cells, as described previously (12), then sorted at day 3–4 following infection. Stable BWZ transductants were analyzed using plate-bound mAb or cell mixtures, as described previously (12).

cDNA libraries

BALB/c DX5$^+$ NK cell (22) and BALB/c-congenic C.B-17/SCID LAK (23) cDNA libraries were reported previously. The NKC of C.B-17/SCID (BALB/c.C57BL/Ka-*Igh-1*^{b} *scid/scid*) mice has been confirmed to be BALB/c in gene content (Ref. 24 and data not shown). A (B6 × BALB/c)F_1 LAK library was generously provided by Dr. R. G. Miller (Ontario Cancer Institute, Toronto, ON). A B6 DX5$^+$ NK cell cDNA library was provided by Dr. H. Arase (Osaka University, Japan).

Evaluation of BAC gene content

BAC clones from the CHORI-28 BALB/c library (BACPAC Resources) containing *Nkrp1* or *Ocil/Clr* genes were identified and size-estimated as described previously (25). The gene content of BACs was determined by PCR and sequencing of products. Briefly, ~10 ng of BAC DNA was subjected to PCR (94°C 30 s, 57°C 30 s, 72°C 1 min, 30 cycles) with primers capable of amplifying known and predicted BALB/c fragments of *Nkrp1* and *Ocil/Clr* gene sequences (primers are shown in Table I). PCR products were separated on 1% agarose gels and visualized with ethidium bromide. PCR products of each size were cloned by TOPO TA-cloning into pCR2.1 (Invitrogen Life Technologies) and sequenced in-house using T7 and M13 reverse primers. All BAC PCR amplifications were performed using *Taq* polymerase on enriched, high-copy BAC DNA. Importantly, no mutations were detected between distinct PCR (i.e., at least two clones of each product from separate PCR were sequenced). Partial exon sequence allowed the identification of the *Nkrp1* or *Ocil/Clr* gene that was amplified from each BAC clone. The partial gene sequences from the BALB/c BACs were deposited in GenBank under the following accession numbers: *Nkrp1a* (exons 3–5, DQ143102); *Nkrp1b* (exons 3–5, DQ143103); *Nkrp1c* (exon 1, DQ143106); *Nkrp1c* (exons 3–5, DQ336140); *Nkrp1e* (exon 2, DQ143104); *Nkrp1f* (exons 3–5, DQ143105); *Nkrp1g* (exons 3–5, DQ336141); *Clr-a* (exons 3–4, DQ143108); *Clr-b* (exons 3–4, DQ143111); *Clr-c* (3′ end, DQ143114); *Clr-d* (exons 4–5, DQ143107); *Clr-e* (exons 4–5, DQ143109); *Clr-f* (exons 3–4, DQ143110); *Clr-g* (exons 3–4, DQ143112); and *Clr-h* (exons 3–4, DQ143113). Sequence-confirmed gene fragments were used in Southern blot analyses of BAC DNA to confirm *Nkrp1-Ocil/Clr* gene content as described previously (25).

RT-PCR and cDNA library PCR

Full-length cDNA clones were obtained using fresh cDNA isolated from d6 BALB/c LAK cells (plastic-adherent IL-2 lymphokine-activated killer cells). RNA was isolated using TRIzol (Invitrogen Life Technologies) and reverse-transcribed into cDNA with SuperScript first-strand cDNA synthesis kit (Invitrogen Life Technologies). PCR conditions, visualization, cloning, and sequencing were performed as outlined above. Library cDNA PCR was performed using primers specific for either the full-length *Nkrp1a/b/c* coding regions (5) or the corresponding extracellular/3′-untranslated regions (21), under limiting cycle conditions (25 cycles PCR with 50 ng cDNA library, representing ~10–20 ng cDNA equivalents). For mammalian expression studies, PCR products were cloned directly into pcDNA3.1/V5/HIS/TOPO (Invitrogen Life Technologies) and sequenced to confirm identity and orientation. All cDNA PCR amplifications were performed using Expand High Fidelity enzyme (Roche Diagnostics), and sequences were determined from multiple clones from at least two separate PCR. Sequences were deposited in GenBank under the following accession numbers: *Nkrp1a*^{BALB}, DQ237927; *Nkrp1b*^{BALB}, DQ237928; *Nkrp1c*^{BALB}, DQ237929; *Nkrp1e*^{BALB}, DQ237930; and *Nkrp1f*^{BALB}, DQ237931). Phylogenetic analysis was performed as described previously (26).

BAC end-sequence characterization

BAC DNA (1 μg) was sequenced in-house with T7 and SP6 primers. Based on end-sequence results, primers were designed to PCR-amplify the respective BAC ends. Products were cloned using a TOPO-pCR2.1 kit (Invitrogen) and sequences were confirmed. Cloned end-sequence fragments were radioactively labeled and used as probes in Southern blots of *Eco*RI-digested BAC DNA.

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Table I. *Primers used to amplify Nkrp1-Ocil/Clr-related genes from BAC clones and cDNA*

Primer Specificity	Sense Primer	Antisense Primer	*BALB/c* Gene(s) Detected
Nkrp1a^{B6}	CGTTAATCTCTCTCTGTGTTTTCTCATTTAT (intron 2)	TGTCAGTGTTAGAGCCCTAC (intron 5)	*Nkrp1a*
Nkrp1b^{SJL}	GTCAAGTCCCTCCATCTACA (exon 2)	AATGGGGATGGGATTCACAG (exon 7)	*Nkrp1b* (cDNA)
Nkrp1c^{B6}	TTAGCGTTAATCCTCTCTG (intron 2)	CAATTAGCCCCAAGGGGAAT (intron 5)	[a]NP
Nkrp1c^{B6}	AATCAGTGGGTGTGGGAACA (exon 1)	TGCTCAAGTTTCACACTGAC (exon 1)	*Nkrp1e, Nkrp1c*
Nkrp1c^{B6}	CAGCCTGCTTTATCAAAGCAAACAG (intron 1)	TCCAGGCCCAAGGGTCCCA (intron 2)	*Nkrp1e*
Nkrp1c^{BALB}	TGCGAGTCCTAATACAAAAACCATCAAG (exon 3)	CCTTAGTCCAATCCAAAATGAATTGTAA (exon 5)	*Nkrp1c* (cDNA)
Nkrp1d^{B6}	CTTCTGCCTGTGTTTTCTTATC (intron 2)	ACTATTAGCCCCAAGGGGAC (intron 5)	*Nkrp1b*
Nkrp1f^{B6}	CCTGAAGTGAGAACTAGAATATCC (intron 2)	TTCAGCTGAACCTTTCAAGA (intron 5)	*Nkrp1f*
Nkrp1g$^{B6/BALB}$	CACTCCACCATTCATTTCAG (exon 3)	CCCTCTCAAGAATTCATAAGG (exon 5)	[a]NP (cDNA)
Clr-a^{B6}	CTGAAGTTTGGATGGCTAGA (intron 2)	GCATCTCTTACAGATACAATGC (intron 4)	*Clr-a*
Clr-b^{B6}	AAGACCTGCTTGCCATGATC (intron 2)	TGACACTGCCTTTCATCTCC (intron 4)	*Clr-b, Clr-h, Clr-d*
Clr-b^{B6}	ACTCAGCTCCTCAGCTCTGA (exon 1)	GGCTAAAAAGCGTCTCTTGG (exon 5)	*Clr-b* (cDNA)
Clr-c^{B6}	TGTAATGGGATGTGTTAACCTATTAGG (3′ UTR)	GTACTTCATCAACCTTCATTTGTTATGC (3′ UTR)	*Clr-c*
Clr-d^{B6}	TCCTAATGAGATACAAGGCA (exon 4)	CAGGAACAGGAGTTGGGCAG (exon 5)	*Clr-d*
Clr-e^{B6}	GGCCTGCACAGAGTCGTCAA (exon 4)	GACAGGACACTAGCATTAGT (exon 5)	*Clr-e*
Clr-f^{B6}	GTGAGATTTGGATGGCTAGG (intron 2)	GGCTTCACATGATATAGTCA (intron 4)	*Clr-f*
Clr-g^{B6}	TCAGGAGGGTGTGCCAAAAA (intron 2)	CTCATAGCCACGACACACAT (intron 4)	*Clr-g*
Clr-h^{B6}	CTCCATCCTAGTGGGTACAA (exon 1)	TGCTACAAATCCACTTCCTT (exon 5)	[a]NP (cDNA)
Cd69^{B6}	ACAAATGTCCTTTCTGTCCAC (intron 2)	AAAGTGTCAGCCTAGAGAGG (intron 4)	*Cd69*
BAC 358o7 SP6-end	CACCTCTCTTGTTTAGAAGAGCCCT	CTGAGGAGAACTTACATGATGAA	[a]NA
BAC 387p19 SP6-end	TACAGCCTCTGCTTTCCTTC	CTGAAGCAGCGATCAGAAAG	[a]NA

[a] No product detected for BALB/c BAC DNA cited in this study. A positive control (B6 genomic DNA) was included in every BAC PCR and always resulted in the amplification of the correct-sized product. In the case of *Nkrp1c* and *Nkrp1d,* the B6-derived product was confirmed by sequencing to verify the specificity of the primers. NP, No product; NA, not applicable.

Results

Divergence of the BALB/c and B6 Nkrp1-Ocil/Clr *regions*

One simple explanation for the NK1.1$^-$ phenotype of BALB/c NK cells is that the two genes known to confer NK1.1 reactivity, *Nkrp1b* and *Nkrp1c*, like *Ly49d* and *Ly49h*, may not be present in the BALB/c genome (11). BALB/c NK cells have been reported to be deficient in *Nkrp1* transcripts, as detected by Northern blotting (10). Thus, lack of NK1.1 reactivity of BALB/c NK cells could be due to deletion of *Nkrp1* genes or defective gene expression.

To investigate this possibility, we constructed a physical map of the BALB/c *Nkrp1-Ocil/Clr* region. To this end, a BALB/c genomic library, CHORI-28, was probed with a mixture of known *Nkrp1* and *Ocil/Clr* cDNAs. Positive BAC clones were isolated and further screened by PCR using gene-specific primers for the known *Nkrp1* and *Ocil/Clr* genes (Table I). PCR products were sequenced to confirm gene identity, and Southern blots of *Eco*RI-digested BACs were probed to confirm the presence of specific genes in each BAC clone (Table II). In some instances, the primer pairs cross-amplified closely related sequences (see Table I), resulting in the discovery of new gene fragments. One such novel sequence, cross-amplified using *Clr-b* primers, corresponds to an exon-3/intron-3/exon-4 (e3-i3-e4) genomic fragment resembling a near-identical (>99%) match to a sequence available from the latest B6 genome assembly (designated *Clr-h*; GenBank accession no. XM487965). The matching region in the B6 genome does not correspond to the location of any other known *Clr* gene. This suggests that the *Clr-h* sequence represents a new *Clr* family member conserved in the B6 and BALB/c genomes. In addition, a new gene was detected in the region between *Clr-f* and *Nkrp1c* based on Southern hybridization using an *Nkrp1f* probe. This sequence also resembles a near-identical (>99%) match in the B6 genome (designated *Nkrp1g*; GenBank accession no. XM355818) that does not correspond to the location of any other known *Nkrp1* gene. Thus, two new genes, *Clr-h* and *Nkrp1g*, are conserved in the B6 and BALB/c genomes.

With the exception of the *Nkrp1c* and *Nkrp1d* primers, all primer pairs amplified the BALB/c allele of the intended gene. As a positive control, all B6-derived primers amplified the intended target sequence when B6 genomic DNA was used as a template. Sequencing of the BALB/c *Nkrp1a/e/f* and *Clr-a/b/c/d/e/f/g* gene fragments showed that all were highly related to their B6 counterparts (>98% identity). Three different primer pairs specific for *Nkrp1c*^{B6} gave variable results when BALB/c BACs were used as templates (see Table I): one set failed to produce any product; a second set cross-amplified *Nkrp1e*; and a third set cross-amplified both *Nkrp1e* and an exon-1 sequence identical with a known BALB/c *Nkrp1* gene fragment, designated as gene-40 (GenBank accession no. X64720) (10). Although this latter fragment resembles *Nkrp1a* exon 1 in coding sequence, it was distinct from the *Nkrp1a*^{BALB} sequence that we identified; therefore, because the

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Table II. *Gene content of BALB/c BAC clones*[a]

Gene/Marker	*Nkrp1a*	*Clr-h*	*Clr-f*	*Nkrp1g*	358o7-end	387p19-end	*Nkrp1c*	*Nkrp1c*	*Nkrp1b*	*Clr-g*	*Clr-d*	*Clr-e*	*Clr-c*	*Nkrp1f*	*Clr-a*	*Nkrp1e*	*Clr-b*	*Cd69*
Probe Region	Exons 3–5	Exons 3–4	Exons 3–4	Exons 3–5	SP6	SP6	Exons 3–5	Exon 1	Exons 3–5	Exons 3–4	Exons 4–5	Exons 4–5	3′ End	Exons 3–5	Exons 3–4	cDNA	Exons 3–4	Exon 2
Fragments (kb)	10.5	8	4.5	5.8	2.6	2.0	5.3/ 4.6	9.0	3.3	5.8	5.75	3.0	1.8	3.9/ 3.25	7.1	4/0.7/ 11.5	10.5	PCR
368i24	X																	
299i20	X	X	X															
517h18	X	X	X															
331e2	X	X	X	X	X													
457k23	X	X	X	X	X													
387p19	X	X	X	X	X	X												
296i7		X	X	X	X													
358o7					X	X	X	X	X	X								
493d24							X	X	X	X								
521c8								X	X	X	X							
501d7								X	X	X	X	X						
451l24										X	X	X	X	X	X			
444p13													X	X	X	X		
299f24													X	X	X	X		
349l18													X	X	X	X		
371k12													X	X	X	X	X	
510j5															X	X	X	
306n15															X	X	X	
334k17															X	X	X	
370a24															X	X	X	
445a21																	X	X
445k14																	X	X

[a] BAC clones containing *Nkrp1* and *Ocil/Clr*-related genes were initially identified from high-density spotted BAC library filters. Positive BACs were screened by PCR with gene-specific primers. One of each kind of product was sequenced to confirm gene identity. These PCR gene fragments then served as probes in Southern blots of *Eco*RI-digested BAC DNA. In some cases, cDNAs were used to identify positive BACs.

gene-40 designation is synonymous with the *Nkrp1c* transcript in B6 mice, this sequence was designated *Nkrp1c*^{BALB}. Primers specific for *Nkrp1d* amplified a product with a coding sequence very similar to that of the known *Nkrp1b* gene from Sw and SJL mice. This was surprising, considering that the *Nkrp1b* gene product is responsible for NK1.1 reactivity in Sw, SJL, and other mouse strains (5–7, 12).

In contrast with genomic PCR, PCR of cDNA libraries derived from BALB/c NK cells revealed the existence of an *Nkrp1c*-like transcript; however, only a partial extracellular domain fragment could be amplified, due to the location of the primers (data not shown). Southern analysis using this cDNA product, new fragments derived from the cDNA, and the previous genomic *Nkrp1c*^{BALB} exon-1 fragment as probes (Fig. 1 and data not shown) revealed that the *Nkrp1c*-like gene resided centromeric to *Nkrp1b*^{BALB}, approximating the location of *Nkrp1c* relative to *Nkrp1d* in the B6 genome (27). Hybridization with *Nkrp1b*^{BALB} and *Nkrp1c*^{BALB} probes showed that several BACs contained these genes (Fig. 1). Similarly, hybridization of the same BALB/c BACs with *Nkrp1d*^{B6} and *Nkrp1c*^{B6} probes revealed the same bands, albeit with weaker intensity (Fig. 1).

A physical map for the BALB/c Nkrp1-Ocil/Clr *gene cluster*

A relative gene order of *Nkrp1a*, *Clr-h*, *Clr-f*, *Nkrp1g*, *Nkrp1c*, *Nkrp1b*, *Clr-g*, *Clr-d*, *Clr-e*, *Clr-c*, *Nkrp1f*, *Clr-a*, *Nkrp1e*, *Clr-b*, and *Cd69* was revealed after assembling the BACs in order of gene

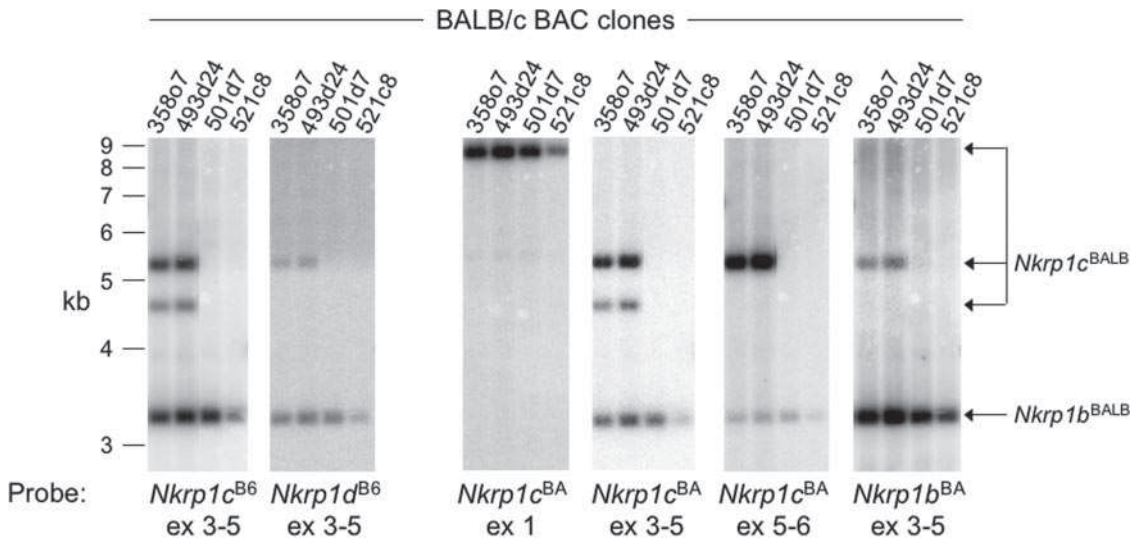

FIGURE 1. Identification of BALB/c genomic clones containing *Nkrp1b/c*. BALB/c BAC clones previously found to contain *Nkrp1* sequences (by PCR) were confirmed in their gene content by Southern blotting. BAC DNA was digested with *Eco*RI, gel-separated, and transferred. The blot was consecutively hybridized with the indicated probes. For simplicity, only BACs positive for *Nkrp1b* or *Nkrp1c* are shown. Probes *Nkrp1c*^{B6} exons 3–5, *Nkrp1d*^{B6} exons 3–5, *Nkrp1c*^{BA} exon 1, and *Nkrp1b*^{BA} exons 3–5 are derived from BAC or genomic DNA. Probes *Nkrp1c*^{BA} exons 3–5 and *Nkrp1c*^{BA} exon 5–6 are derived from cDNA. BA, BALB/c; ex, exon.

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content (Table II). There are some ambiguities in this assembly. Notably, the order of *Clr-h* and *Clr-f* in the BALB/c genome is unknown because of their identification on shared BACs; however, in the B6 genomic database, *Clr-h* lies centromeric to *Clr-f* and is transcribed in the opposite orientation. Similarly, *Clr-c* and *Nkrp1f* also were found on the same BACs, but their order is known in the B6 haplotype, with *Clr-c* lying centromeric to *Nkrp1f*.

To generate a physical map of the approximate relative location of each gene on the chromosome, the size of each BAC clone was ascertained by pulsed-field gel electrophoresis (Fig. 2 and data not shown). Using the gene content and size of each BAC, the approximate location of each gene on chromosome 6 of BALB/c mice was predicted (Fig. 2). The BALB/c *Nkrp1-Ocil/Clr* region is predicted to be ~640 kb in length (*Nkrp1a* exon 3 to *Clr-b* exon 4), with a possible range from 575 to 700 kb (Fig. 2). In previous gene-mapping studies, the entire B6 *Nkrp1-Ocil/Clr* cluster was predicted to be ~700 kb in length (16, 27); however, the exact length is unknown, as the B6 genome assembly for this region contains several gaps.

Determination of Nkrp1-Ocil/Clr *haplotypes in different inbred mouse strains*

Direct comparison of the gene content between BALB/c and B6 mice shows that at least 13 genes are conserved, including *Nkrp1c* and the novel, but shared, *Clr-h* and *Nkrp1g* genes (Fig. 3). The only BALB/c-specific gene was *Nkrp1b*, but this gene corresponded to the approximate location of *Nkrp1d* in the B6 genome (Fig. 2), and both the $Nkrp1b^{BALB}$ and $Nkrp1d^{B6}$ probes hybridized to the same genes (Fig. 1). Thus, it is likely that the two designations actually represent different alleles of the same gene, especially since their transmembrane and cytoplasmic coding sequences are identical. Therefore, like the B6 and BALB/c *Ly49* gene clusters, the *Nkrp1-Ocil/Clr* regions represented in these mice possess shared genes and have presumably diverged from an ancestral haplotype. However, unlike the *Ly49* gene repertoire, that of the *Nkrp1-Ocil/Clr* genes appears to be highly conserved, perhaps with the exception of a seemingly directed divergence of *Nkrp1b* and *Nkrp1c*. These similarities and differences are apparent in Southern analyses of genomic DNA (Fig. 4A). The RFLP patterns generated by the *Nkrp1f* and *Clr-f* probes are similar in the two mouse strains, but the banding patterns resulting from the $Nkrp1c^{B6}$ probe are distinct (Fig. 4*A*). Hybridization of the $Nkrp1c^{B6}$ and *Clr-f* probes was conducted at lower stringency than that of *Nkrp1f* and confirms that the number of *Nkrp1* and *Ocil/Clr* genes, as evidenced by cross-hybridizing genes, is similar in these two strains.

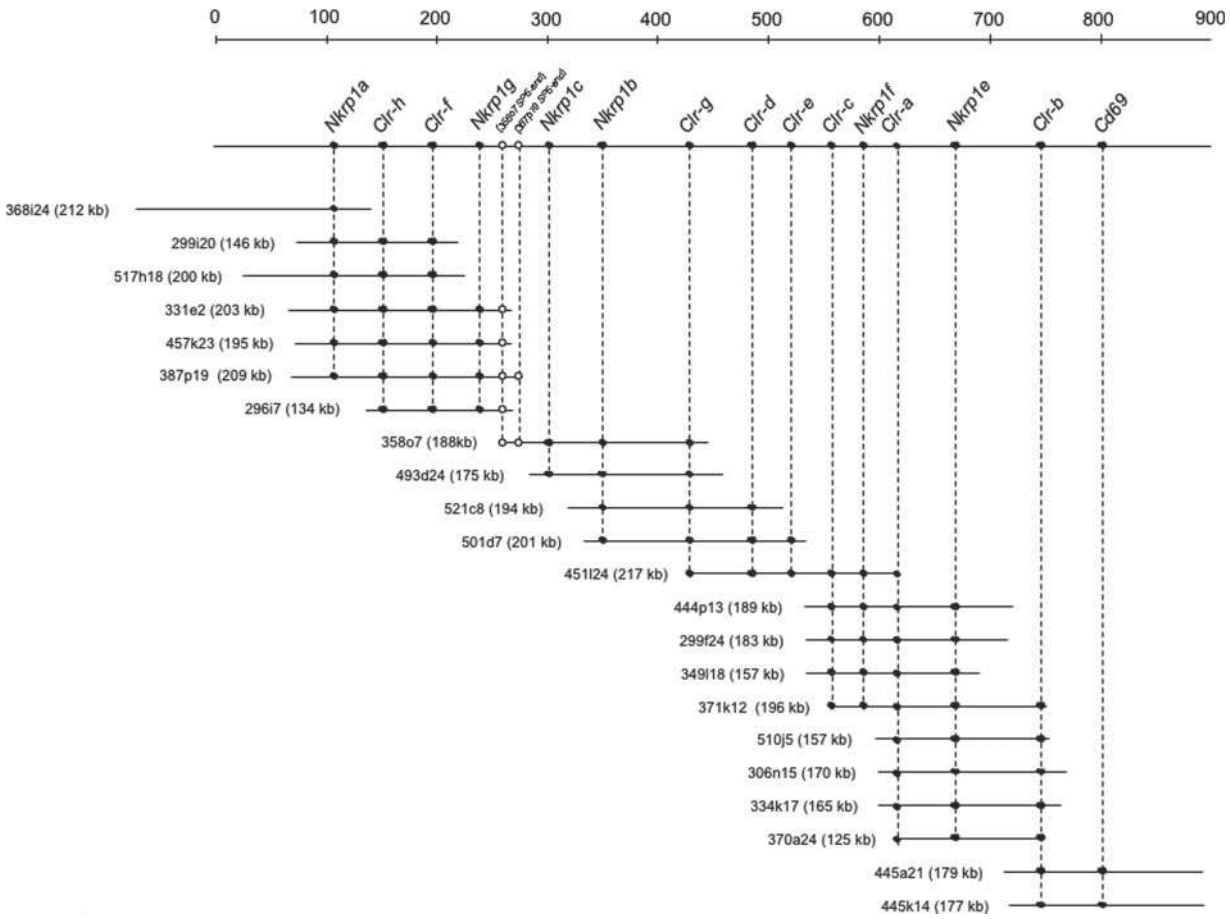

FIGURE 2. BAC contig overlap and construction of a physical map for the BALB/c *Nkrp1-Ocil/Clr* gene cluster. The BAC gene content from Table II and BAC sizing data (pulsed-field gel electrophoresis; data not shown) were integrated to produce a map of the relative location of all known BALB/c *Nkrp1-Ocil/Clr* genes. BACs are represented by horizontal lines, with the name and size of each given on the left side. ●, 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 the BAC size and gene content. The scale bar (*top*) is demarcated in kilobases. The markers 387p19-SP6′ and 358o7-SP6′, represented by empty circles, are the end sequences of BACs 387p19 and 358o7, respectively. After cloning, these BAC end sequence fragments were used as probes to show that BACs 387p19 and 358o7 overlap, despite not sharing any *Nkrp1-Ocil/Clr* genes.

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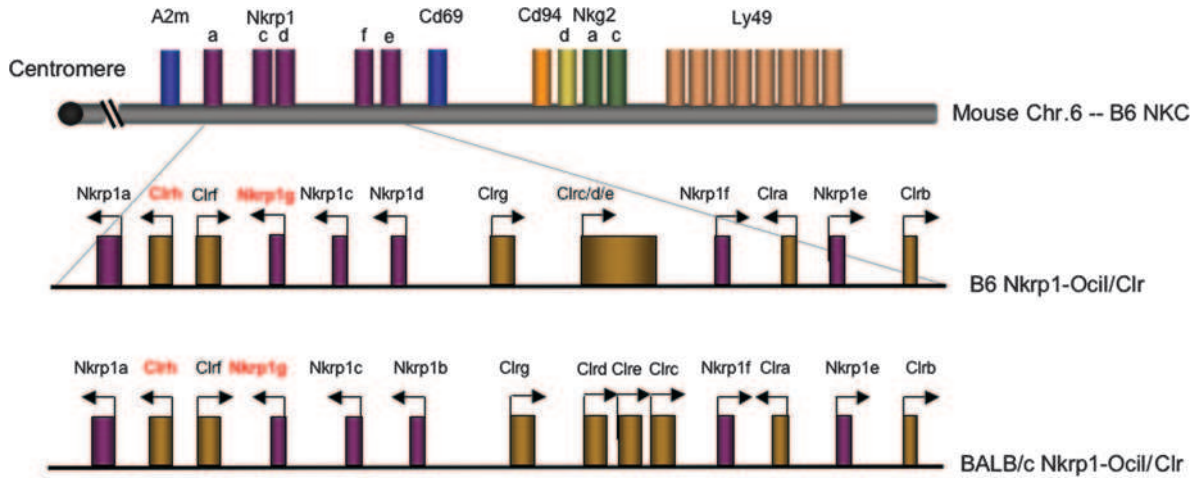

FIGURE 3. Comparison of gene content in the B6 and BALB *Nkrp1-Ocil/Clr* regions. The mouse NKC is depicted at the top, with major groups of NK cell receptor genes shown. Note that additional genes are present but are not shown for simplicity. Immediately below is an expanded view of the known genes in the B6 *Nkrp1-Ocil/Clr* region, and on the bottom, a diagram of the BALB/c region. Rectangles represent genes, and the arrows indicate transcriptional orientation.

The only other well-characterized *Ly49* strain haplotype, represented by the 129 group of inbred mice, is a third distinct haplotype more complex than that of either B6 or BALB/c mice (26). To determine whether the 129 strain *Nkrp1-Ocil/Clr* cluster is similar or divergent, genomic Southern blot analyses were performed (Fig. 4). Hybridization with $Nkrp1b^{BALB}$ and $Nkrp1c^{B6}$ probes revealed that the 129-derived genomic DNA gave an identical RFLP pattern to that of BALB/c, suggesting the 129 and BALB/c strains share a similar *Nkrp1-Ocil/Clr* haplotype, in contrast with their *Ly49* regions (Fig. 4*B*). In a third experiment, hybridization with the $Nkrp1b^{BALB}$ probe suggests that BALB/c, 129/Sv, 129S1, and FVB inbred mice have a similar *Nkrp1* haplotype, one that is distinct from that of B6 mice (Fig. 4*C*). Hybridization with a $Clr\text{-}h^{BALB}$ probe shows that this gene is present in all five mouse strains (Fig. 4*C*). Interestingly, the same probe cross-hybridized to one band at 5.8 kb in the BALB/c and 129-group mice (most likely *Clr-g* and *Clr-d*, see Table II), but in B6 and FVB mice, a doublet (5.9 and 6.0 kb) was detected (Fig. 4*C*). Collectively, these data suggest that the commonly used inbred mouse strains have one of at least three distinct *Nkrp1-Ocil/Clr* haplotypes, represented by B6 (group 1), BALB/c and the 129-group (group 2), and FVB (group 3). These findings extend earlier work showing that the BALB/c and 129-strain *Nkrp1* haplotypes belong to one of four RFLP groups, based on previous analysis of the *Nkrp1a* locus (28). In any case, BALB/c mice are not deficient in *Nkrp1* gene content, so the lack of NK1.1 reactivity of BALB/c NK cells is not due to deletion of either the *Nkrp1b* or *Nkrp1c* genes.

BALB/c NK cells express abundant Nkrp1b *and* Nkrp1c *transcripts*

To obtain cDNA clones of the *Nkrp1* genes from BALB/c mice, RT-PCR was performed on fresh BALB/c A-LAK cDNA and BALB/c NK cell cDNA libraries using multiple primer sets. This approach yielded full-length or near full-length cDNA clones for $Nkrp1a^{BALB}$, $Nkrp1b^{BALB}$, $Nkrp1c^{BALB}$, $Nkrp1e^{BALB}$, and $Nkrp1f^{BALB}$ (Fig. 5 and data not shown). This was unexpected, given that BALB/c NK cells were reported previously to lack detectable *Nkrp1* expression in Northern blotting experiments (10). To determine whether *Nkrp1b/c* expression was specifically deficient in BALB/c mice, RT-PCR was applied to both BALB/c and B6 NK cell cDNA libraries using limited amounts of cDNA and amplification cycles. The *Nkrp1* primer sets used have been previously reported, one set corresponding to the extracellular/3′-untranslated region (EC/UTR) (21), a second set corresponding to the full-length coding sequence (FL/CDS) regions (5). As shown in Fig. 5, *Nkrp1* transcripts were easily detected in both strains. However, while the EC/UTR primers yielded a significant signal for $Nkrp1c^{BALB}$, a FL/CDS $Nkrp1c^{BALB}$ signal could not be detected. This has been observed before for Sw strain NK cells (5, 21), and suggests that the primer sets used may be divergent compared with the $Nkrp1c^{BALB}$ sequence. Interestingly, note that the *Nkrp1b* FL/CDS primer set amplifies $Nkrp1b^{Sw}$ (5), $Nkrp1b^{SJL}$ (6), and $Nkrp1b^{BALB}$ (Fig. 5; see below), whereas it also cross-amplifies $Nkrp1d^{B6}$ (5, 6). Therefore, to confirm the cDNA identities, and determine the discrepancy of the $Nkrp1c^{BALB}$ results, all PCR products were sequenced.

Sequencing results revealed that the EC/UTR $Nkrp1c^{BALB}$ PCR product was most highly related to the known $Nkrp1c^{B6}$ sequence in overall BLAST score, followed by $Nkrp1d^{B6}$, and $Nkrp1b^{Sw/SJL}$. Moreover, it shared 196 bp of nucleotide identity to the gene-40 partial sequence derived from BALB/c LAK cells (GenBank accession no. X64720) (10). As mentioned previously, the gene-40 sequence is also identical over 89 bp to the genomic $Nkrp1c^{BALB}$ exon-1 probe, confirming its $Nkrp1c^{BALB}$ designation. However, this sequence closely resembles *Nkrp1a* in its 5′ region, which could confound attempts to amplify the full-length cDNA by PCR using the *Nkrp1c* 5′ primer. Therefore, to take advantage of the sequence similarity, we used PCR amplification of cDNA libraries using a P1A-5′/P1C-3′ combination of FL/CDS primers.

Interestingly, full-length transcripts for $Nkrp1c^{BALB}$ were detected equally in BALB/c, B6, and F_1 LAK libraries by this method (Fig. 5*B*). Moreover, primers spanning exons 3–5 were capable of specifically detecting $Nkrp1c^{BALB}$ transcripts in fresh BALB/c but not B6 NK cells (Fig. 5*C*). Sequencing confirmed that the $Nkrp1c^{BALB}$ EC/UTR product, the P1A-5′/P1C-3′ product, the $Nkrp1c^{BALB}$ exon 3–5 product, and the gene-40 sequence (GenBank accession no. X64720) (10) were all identical with one another. These results confirm the existence of a full-length coding *Nkrp1c* cDNA in BALB/c NK cells. However, comparison of the $Nkrp1c^{BALB}$ cDNA with the published $Nkrp1c^{B6}$ cDNA reveals that they differ by 20 nonsynonymous substitutions in the extracellular coding region alone (Fig. 6*A*). Such divergent sequences would be expected to confound attempts using Northern blotting to compare expression of *Nkrp1c* transcripts between the two mouse

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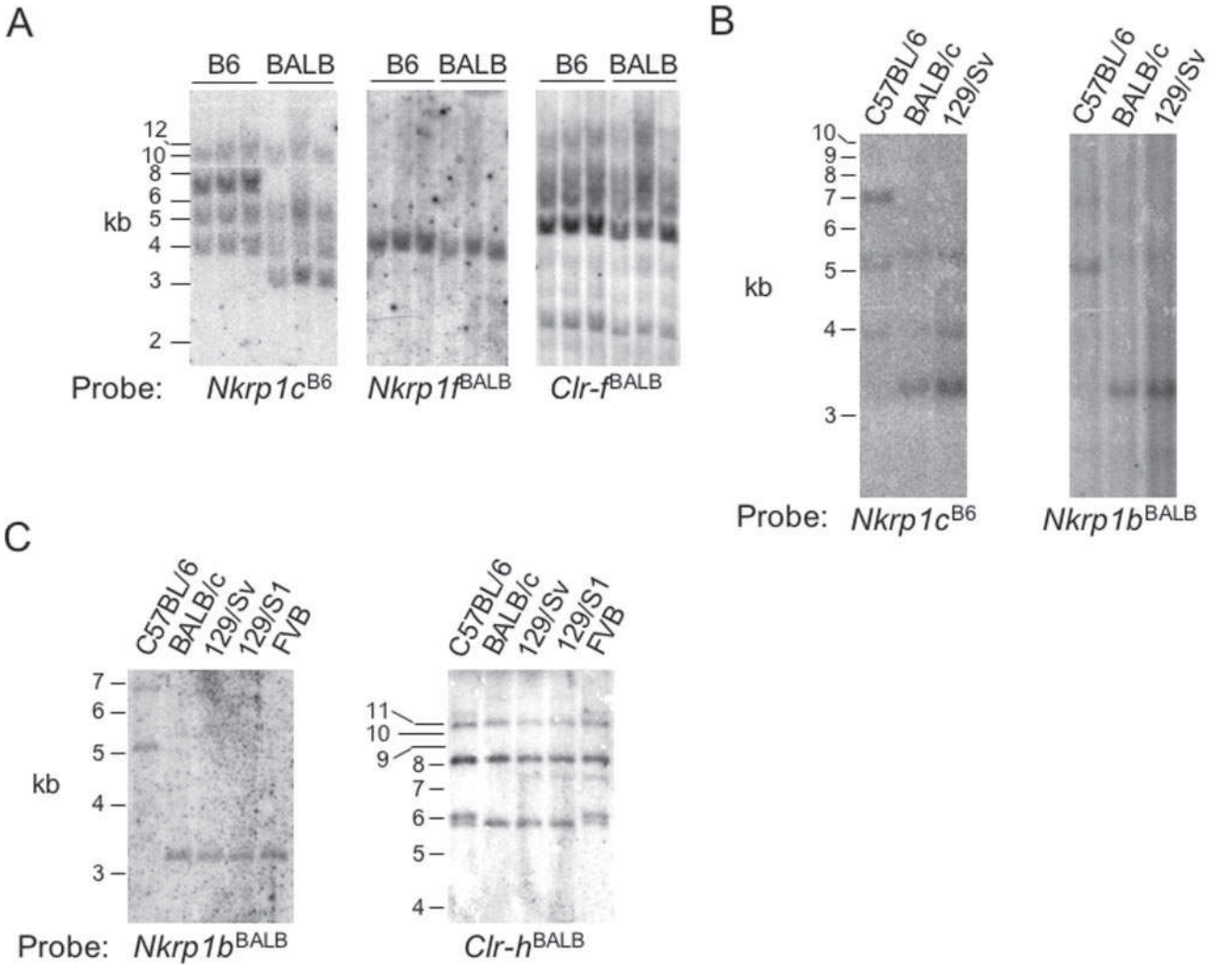

FIGURE 4. Inference of *Nkrp1-Ocil/Clr* haplotypes in different inbred mouse strains by RFLP analysis. (*A–C*) Genomic DNA samples from the indicated mouse strains were digested with *Eco*RI, separated, and transferred to nylon membranes. The membranes were probed with the indicated probes containing exons 3–5. In *A*, DNA samples from three different animals of each strain are shown. Hybridization with the *Nkrp1c*^{B6} and *Clr-f*^{BALB} probes was performed at less stringent conditions so that all *Nkrp1*- and *Ocil/Clr*-related fragments could be detected.

strains (10). Notably, the similarity of the *Nkrp1c*^{BALB} 5′ region to *Nkrp1a*^{B6} is reminiscent of *Ly49o*$^{129/C57L}$, which resembles *Ly49a*^{B6} upstream and *Ly49d*^{B6} downstream (29, 30).

In contrast with *Nkrp1c*^{BALB}, the *Nkrp1a*^{BALB} sequence differs from its B6 counterpart by only two nonsynonymous substitutions in its extracellular coding region, and the *Nkrp1b*^{BALB} cDNA sequence is ~99% identical with *Nkrp1b*$^{Sw/SJL}$, differing by only three nonsynonymous substitutions in its extracellular coding region (Fig. 6*A*). The ITIM motif in the *Nkrp1b*^{BALB} coding sequence is intact, and it lacks a charged residue in its transmembrane region, so this cDNA is predicted to code for a functional inhibitory transmembrane receptor. A near full-length (exon 5 deleted) in-frame cDNA clone of *Nkrp1e*^{BALB} was also isolated by this method (Fig. 6*A*); in contrast, *Nkrp1e* is only known from single genomic exons in B6 mice (27). The *Nkrp1f*^{BALB} cDNA sequence is identical with the published *Nkrp1f*^{B6} sequence (Fig. 6*A*). Three independent primer sets failed to amplify an *Nkrp1g*$^{B6/BALB}$ cDNA (Fig. 5*C* and data not shown), so this gene may represent a pseudogene, at least in the strains tested.

When these *Nkrp1* sequences (and the B6 *Ocil/Clr* sequences) are compared over their coding regions, the *Nkrp1b*^{BALB} and *Nkrp1b*$^{Sw/SJL}$ sequences are closely associated with each other and with *Nkrp1d*^{B6} (Fig. 6*B*). The *Nkrp1c*^{BALB} sequence is most closely related to the allelic sequence, *Nkrp1c*^{B6}. All other BALB/c *Nkrp1* genes are closely related to their B6 alleles. The novel gene, *Clr-h*, groups with the *Clr-b*/*c*/*d*/*g* clade (Fig. 6*C*). All *Clr* genes are shown without allele designation, as the BALB/c full-length coding sequences were not available for direct comparison.

Thus, while our results using RT-PCR differ from previous Northern blot analysis of *Nkrp1* transcript expression in BALB/c mice, our sequencing analysis confirms and extends previous findings, where our *Nkrp1a/b/c*^{BALB} sequences correspond to the BALB/c partial sequences of gene 2 (GenBank accession no. X64723), gene 34 (GenBank accession no. X64719), and gene 40 (GenBank accession no. X64720), respectively (10). Thus, BALB/c mice are not deficient in *Nkrp1* expression, they simply possess divergent *Nkrp1* sequences compared with those of B6 mice.

BALB/c NKR-P1B/C proteins lack NK1.1 reactivity due to allelic divergence

Our results demonstrate that BALB/c mice possess a full *Nkrp1* gene content and normal *Nkrp1b/c* transcript expression in NK/

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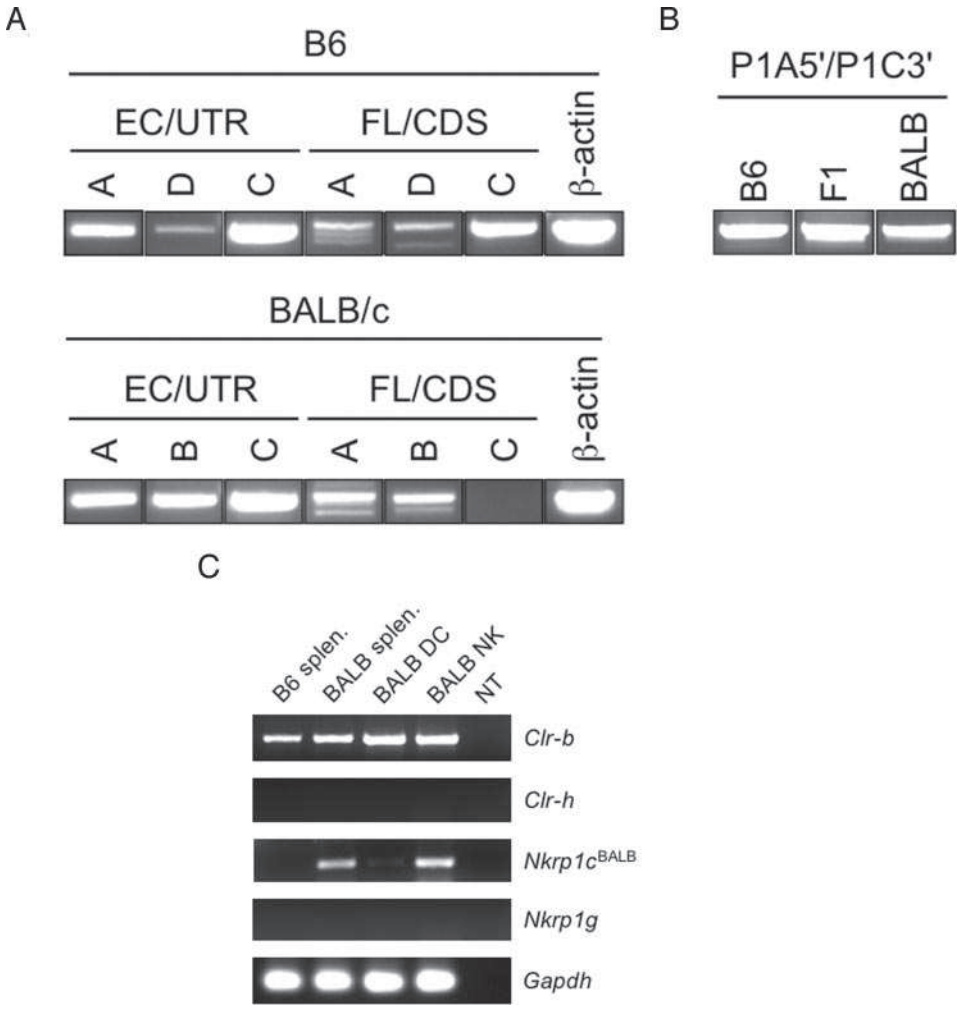

FIGURE 5. PCR analysis of Nkrp1 transcript expression in BALB/c vs B6 NK cDNA libraries and fresh tissues. *A*, Limited cycle PCR analysis with high-fidelity polymerase was applied to clone BALB/c strain *Nkr-p1* cDNA sequences. Shown for comparison are *Nkr-p1* amplifications performed using a B6 NK cell cDNA library in parallel. Two sets of primers were used: EC/UTR primers correspond to the extracellular and 3′-untranslated region; FL/CDS primers correspond to the full-length coding sequence regions. The signals shown for *Nkr-p1b/d* are combined, as the primers amplify both sequences. *B*, Amplification of full-length *Nkrp1c* sequences from B6, BALB/c, and (B6xBALB)F_1 LAK libraries using the upstream 5′-*Nkrp1a* and 3′-*Nkrp1c* primer combination. All PCR products were sequenced to confirm identity. *C*, RT-PCR analysis of *Nkrp1-Ocil/Clr* transcripts in fresh tissues. cDNA from B6 and BALB/c splenocytes (splen.), BALB/c GM-CSF-cultured bone marrow dendritic cells (DC), and BALB/c NK cells enriched from IL-2 cultured splenocytes (ALAK) are shown. NT, no template. All PCR products were cloned and sequenced to confirm their identity. Primers (see Table I) were designed based on available sequence to amplify only the BALB/c allele (*Nkrp1c*^{BALB}, exons 3–5), or both B6 and BALB alleles (*Nkrp1g*, exons 3–5; *Clr-b*, full-length coding region; *Clr-h*, exons 1–5).

LAK cells when compared with NK1.1$^+$ mouse strains, suggesting that the basis of NK1.1 alloreactivity between these mouse strains may be due to divergence in NKR-P1 protein sequences. As shown in Fig. 7, NK1.1 reactivity is specific to NKR-P1B$^{Sw/SJL}$ and NKR-P1C^{B6} among known NKR-P1 proteins from both mice and rats. However, sequence alignment of these proteins does not directly reveal an amino acid sequence unique to NK1.1$^+$ vs NK1.1$^-$ isoforms (Fig. 7*A* and data not shown). Therefore, we limited our analysis to the mouse NKR-P1 isoforms, and further focused on differences between the mouse NKR-P1B$^{Sw/SJL}$ and NKR-P1B^{BALB} sequences, because the two differ in NK1.1 reactivity, but only differ in sequence by three amino acids (see Fig. 7*A*). Of these three substitutions in the NKR-P1B$^{Sw/SJL}$ sequence, two were expected to be nonconservative, resulting in charge alterations in the NKR-P1B^{BALB} isoform (D183V, E217K). However, the D183V substitution also is present in the NK1.1$^+$ NKR-P1C^{B6} isoform, while the E217 amino acid is intact in the NK1.1$^-$ NKR-P1C^{BALB} isoform. This left a seemingly conservative S191T substitution as a candidate determinant for NK1.1 epitope reactivity (Fig. 7*A*). Interestingly, both NK1.1-reactive isoforms (NKR-P1B$^{Sw/SJL}$, NKR-P1C^{B6}) possess an S residue at this position, whereas all other mouse NKR-P1 sequences have substitutions, including NKR-P1B^{BALB} (T), NKR-P1C^{BALB} (T), NKR-P1D^{B6} (A), NKR-P1D^{129} (T), NKR-P1A$^{B6/BALB}$ (A), NKR-P1E$^{B6/BALB}$ (T), NKR-P1F$^{B6/BALB}$ (I), and the predicted NKR-P1G^{B6} (L). Therefore, to test the significance of this residue in conferring NK1.1 reactivity, the NKR-P1B^{BALB} sequence was altered by site-directed mutagenesis.

As shown in Fig. 7*C*, the T191S mutation conferred NK1.1 reactivity to the NKR-P1B^{BALB} protein, whereas a K217E control mutation, found natively in the NKR-P1C^{BALB} sequence, did not alter NK1.1 reactivity. This demonstrates that a single amino acid substitution present in both the BALB/c NKR-P1B and NKR-P1C sequences is sufficient to explain the lack of NK1.1 reactivity of the BALB/c mouse strain. Furthermore, these results suggest that the basis of NK1.1 alloreactivity across mouse strains, including the original (C3H × BALB)F_1 α-CE immunizations used to generate both the polyclonal α-NK-1 (3) and monoclonal α-NK1.1 (PK136; Ref. 4) specificities, is due to a single amino acid substitution in either NKR-P1B, NKR-P1C, or both receptors. Due to a lack of information about NKR-P1 expression in the CE mouse strain, we cannot distinguish these possibilities at present. Nonetheless, these results demonstrate that BALB/c mice are not deficient in NKR-P1 expression, rather they possess divergent NKR-P1 sequences sufficient to generate alloantibodies when immunized with NK cells from other mouse strains.

The BALB/c NKR-P1B receptor is functional and recognizes cognate Ocil/Clr-b ligand

Although BALB/c mice express functional NKR-P1B/C transcripts, it remains formally possible that BALB/c NK cells may be deficient in NKR-P1-mediated recognition, if substitutions in their NKR-P1 coding sequences lead to loss of ligand recognition. Although cognate ligands for the NKR-P1A/C receptors remain unknown, NKR-P1-mediated missing-self recognition of tumor cells has been shown to depend on functional recognition of Ocil/Clr-b

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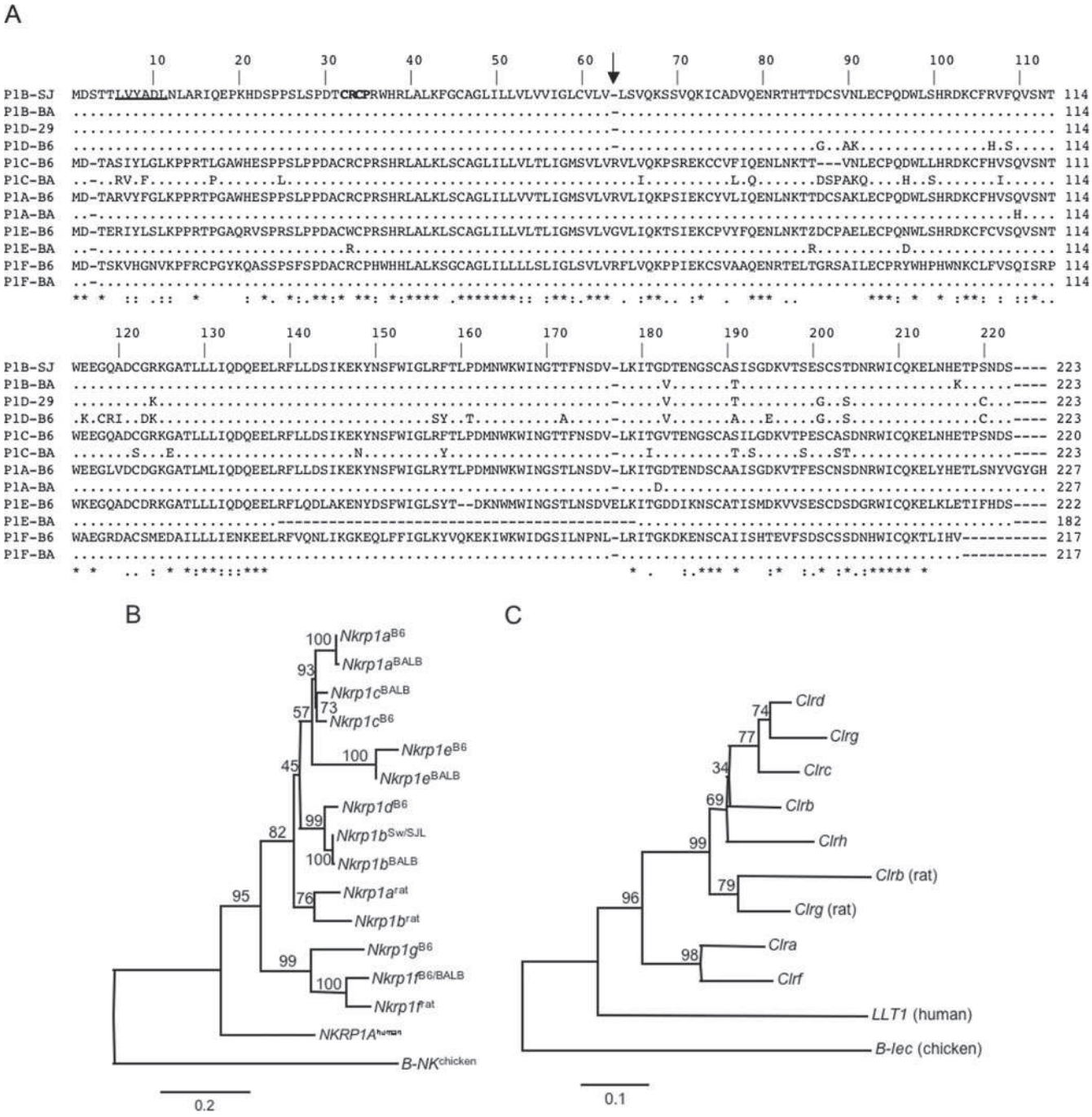

FIGURE 6. Novel BALB/c and B6 *Nkrp1-Ocil/Clr* sequences. *A*, Alignment of the predicted coding sequences for the known *Nkrp1* cDNAs is shown. Included are related sequences from various mouse strains: B6, C57BL/6; BA, BALB/c; SJ, Swiss/SJL; 29, 129. NKR-P1 functional features are highlighted at the top: ITIM, underlined; CxCP Lck-recruitment motif, boldface; charged transmembrane residue position, arrow. *B*, The coding sequence of *Nkrp1*-related cDNAs from mice (B6, BALB, Swiss/SJL), rats, humans, and chickens was aligned using ClustalX software (⟨ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/⟩) and bootstrap analysis of 1000 data sets was performed with PHYLIP. The bootstrap values for each grouping are shown as a percentage. The phylogram branch lengths indicate the similarity between different cDNAs and the scale-bar indicates the percent divergence. The putative coding sequence for *Nkrp1e*^{B6} was artificially spliced together from genomic data. *Nkrp1g*^{B6} is a transcript predicted from the B6 genome (XM355818) and is 100% identical with the available *Nkrp1g* exons (3–5) from the BALB/c mouse. All genes shown are known from newly isolated or previously published cDNAs. The *Nkrp1f* coding sequence in B6 and BALB mice is identical. *C*, All known *Clr*-like cDNA sequences from B6 mice, rats, humans, and chickens were analyzed as described in *B*. *Clr-h* is a transcript predicted from the B6 genome (XM487965) and is 99% identical with the new *Clr*-like exon 3–4 fragment reported in this study.

ligand by the NKR-P1B/D inhibitory receptors in various mouse strains (12). Therefore, we tested whether the NKR-P1B^{BALB} receptor is capable of recognizing Ocil/Clr-b ligand.

To do this, we used our previous modification of the BWZ reporter-cell assay (12, 19). In this study, expression of a chimeric CD3ζ/NKR-P1B fusion receptor on BWZ.36 reporter cells signals functional recognition of ligand expressed on the surface of intact target cells, via NFAT-driven β-galactosidase enzyme expression. As shown previously, BWZ cells expressing the CD3ζ/NKR-P1B$^{Sw/SJL}$ receptor are capable of recognizing target cells (either BWZ or 293T) expressing Ocil/Clr-b ligand, but not target cells alone (BWZ.P1B$^{Sw/SJL}$; Fig. 8*A*). Moreover, this interaction is

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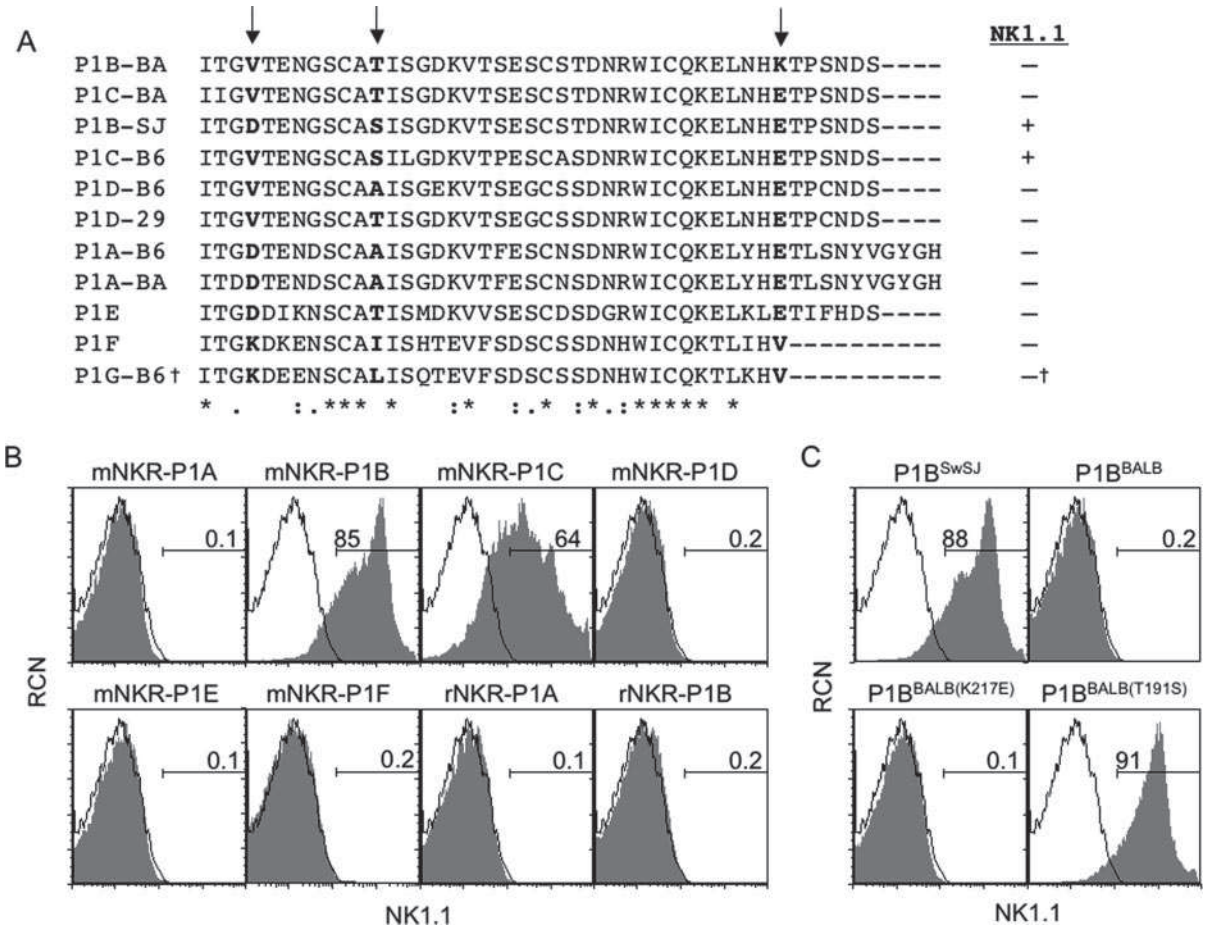

FIGURE 7. NK1.1 epitope mapping of mouse NKR-P1 proteins. *A*, Amino acid alignment of the mouse NKR-P1 distal C-terminal sequences regions is shown. Residues that differ between NKR-P1B^{BALB} and NKR-P1B$^{Sw/SJL}$ are shown in boldface. †Predicted coding sequence from the B6 genomic database (XM355818) (*B*) NK1.1 reactivity of known mouse and rat NKR-P1 proteins. 293T cells were transfected with vectors encoding the indicated cDNA's (plus GFP reporter vector; see *Materials and Methods*), then cells were analyzed by flow cytometry using the PK136 α-NK1.1 mAb. All transfections are shown gated on GFP$^+$ transfected cells. *C*, NK1.1 reactivity of NKR-P1B^{BALB} variants generated by site-directed mutagenesis. Shown are control NKR-P1B$^{Sw/SJL}$ and NKR-P1B^{BALB} transfectants of 293T cells (as in *B*), as well as NKR-P1B^{BALB} (K217E) and NKR-P1B^{BALB} (T191S) mutants (gated on GFP$^+$ transfected cells).

specific for Ocil/Clr-b ligand, as it can be blocked using the α-Ocil/Clr-b mAb, 4A6 (Fig. 8*A*). Importantly, BWZ cells expressing the NKR-P1B^{BALB} receptor are also capable of specifically recognizing Ocil/Clr-b ligand (BWZ.P1B^{BALB}; Fig. 8*A*), while BWZ cells expressing a control CD69 fusion receptor do not recognize Ocil/Clr-b (BWZ.CD69; Fig. 8*A*). Furthermore, BWZ.P1B^{BALB} reporter cells also recognize native Ocil/Clr-b ligand on the surface of various hemopoietic cells ex vivo, including bone marrow (BM), lymph node (LN), spleen (SP), and thymus (TH) (Fig. 8*B*). These results demonstrate that the BALB/c NKR-P1B receptor is functional and recognizes cognate Ocil/Clr-b ligand on the surface of normal cells. Thus, the NKR-P1B–Ocil/Clr-b missing-self recognition system is intact in BALB/c mice despite a single amino acid substitution that is responsible for the lack of NK1.1 reactivity of BALB/c NK cells.

Discussion

It is widely believed that the lack of NK1.1 reactivity of BALB/c NK cells is due to a strain-specific deficiency in the expression of one or more members of the *Nkrp1* family (10). We show here that BALB/c mice are normal with respect to *Nkrp1* gene content and genomic organization (Figs. 1–6), *Nkrp1* transcript expression (Figs. 5–6), and NKR-P1B receptor function (Figs. 7–8). Furthermore, we show that the lack of NK1.1 reactivity of BALB/c NK cells is due to a single amino acid substitution (S191T) found in the extracellular regions of both the BALB/c NKR-P1B and NKR-P1C receptors (the known NK1.1 Ags (5, 6, 9)), which abolishes the epitope recognized by the α-NK1.1 mAb, PK136 (4) (Fig. 7). Despite allelic differences, the BALB/c NKR-P1B receptor is fully functional and recognizes Ocil/Clr-b ligand on the surface of transfected and normal cells (Fig. 8). Our finding that the BALB/c and 129 strains appear to share a related *Nkrp1* haplotype (Fig. 4) suggests that other NK1.1$^-$ mouse strains possess similar allelic differences. These results indicate that the NKR-P1–Ocil/Clr receptor-ligand system plays a broader and more important role in target cell recognition than previously thought.

Insight from the BALB/c Nkrp1 *region:* Nkrp1b *and* Nkrp1d *delineate divergent murine NKC haplotypes*

A significant finding of this study is that the mouse *Nkrp1b* and *Nkrp1d* genes appear to represent alleles of the same genetic locus (Fig. 3). It has long been suspected that this might be the case, for a number of reasons. Although the original *Nkrp1b* cDNA sequence (gene 34; Ref. 31) was reportedly derived from B6 mice, along with *Nkrp1a* (gene 2; Ref. 31); mNKR-P1.7 (9, 28) and *Nkrp1c* (gene 40; Ref. 31); mNKR-P1.9 (9), we and others have been unable to identify an *Nkrp1b* genomic or cDNA sequence, or

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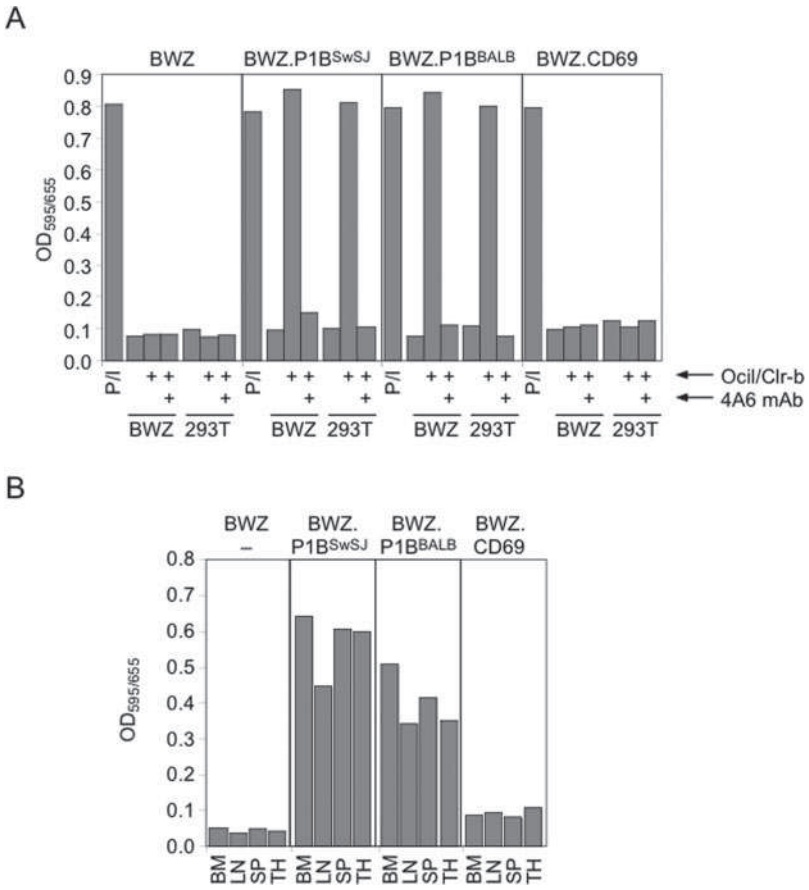

FIGURE 8. Functional analysis of Ocil/Clr-b ligand binding by the BALB/c NKR-P1B receptor. *A*, BWZ reporter cell assay analysis of NKR-P1 transductants vs a panel of target cells. Parental and Ocil/Clr-b-expressing BWZ variants and 293T transfectants with and without 4A6 α-Ocil/Clr-b blocking mAb were tested. *B*, BWZ reporter cell assay analysis of NKR-P1 transductants vs cells from normal mouse tissues ex vivo. Shown are parental BWZ cells, transductants bearing the NKR-P1B$^{Sw/SJL}$ and NKR-P1B^{BALB} receptors, as well as transductants expressing mouse CD69, as a control.

an NKR-P1B protein, from the B6 background (5, 6, 27). However, our group and Kung et al. (6) were able to isolate an identical *Nkrp1b* cDNA sequence derived from the Sw (5) and SJL strains (6), respectively. Thus, it is possible, due to the large numbers of NK cells required for cDNA library preparation and Northern blotting, that a mixed strain may have been included in the original identification of the mouse *Nkrp1* cDNA family. In any case, we and others were successful in cloning an *Nkrp1b*-related cDNA from B6 NK cells by RT-PCR (5, 6). Due to significant coding sequence divergence in the extracellular region (21 nonsynonymous substitutions, a difference similar to that observed between other *Nkrp1* genes), we gave this cDNA a novel designation, *Nkrp1d* (GenBank accession no. AF338321; Ref. 5); however, Kung et al. (6) only detected 15-aa substitutions and designated their cDNA sequence, *Nkrp1b*^{B6} (GenBank accession no. AF354260). Notwithstanding these differences, our collective cDNA findings were later confirmed at the genomic level through random sequencing of the B6 *Nkrp1* region: no evidence for the existence of a B6 *Nkrp1b* sequence could be found, resulting in the designation of the B6 gene, *Nkrp1d* (GenBank accession no. AF324825; Ref. 27). In the absence of information about the *Nkrp1* genomic region from other strains, including Sw or SJL mice, this issue has remained unresolved until now.

Our new data from the BALB/c genome, however, sheds light on the allelic nature of *Nkrp1b* and *Nkrp1d*: (1) only a single inhibitory-class cDNA and/or gene could be identified in BALB/c, similar to B6 and other strains analyzed to date; (2) the genomic location of the BALB/c *Nkrp1b* gene is similar to that of the B6 *Nkrp1d* gene (3); the *Nkrp1b*^{BALB} and *Nkrp1d*^{B6} probes both hybridize to the same bands on genomic Southern blots; and (4) the *Nkrp1b*^{BALB} gene shares a highly conserved e3-i3-e4 organization and sequence with the *Nkrp1d*^{B6} locus (Fig. 9). If *Nkrp1b* and *Nkrp1d* are indeed allelic, they show much greater allelic divergence than other *Nkrp1* genes among B6, BALB/c, and other mouse strains (with exception of the *Nkrp1c* locus). The reason for this specific and directed divergence is unclear, but it is even more intriguing considering that the NKR-P1B/D receptors share a common ligand, the product of the *Ocil/Clr-b* locus, which is identical among the B6, BALB/c, and 129 strains (12, 13). Differential binding of NKR-P1B and NKR-P1D to a single allele of Ocil/Clr-b has been observed before in functional assays (12); however, this could reflect a differential affinity of each gene product, as a consequence of their divergent sequences, for a common ligand. In turn, such differential binding of NKR-P1 receptors to their Ocil/Clr ligands could have implications for the role of this system in missing self recognition and transplantation biology (12). Confirmation of such a role will have to await the elucidation of *Ocil/Clr* alleles and haplotypes, as well as functional analyses of the role of Ocil/Clr proteins in allotransplantation.

Another interesting finding of the genomic analyses is that the BALB/c and 129 strains appear to share a related *Nkrp1* haplotype (Fig. 4 and Ref. 28). In fact, an *Nkrp1b*-like sequence has been identified in 129-strain mice, designated *Nkrp1d*^{129}, that resembles *Nkrp1b*^{BALB} more than *Nkrp1d*^{B6} (13); indeed, the NKR-P1D^{129} protein differs from the NKR-P1B^{BALB} and NKR-P1B$^{Sw/SJL}$ proteins by only 5- or 6-aa, respectively, and possesses the S191T substitution that abrogates NK1.1 reactivity (Fig. 7). As both BALB/c and 129 are well-known NK1.1$^-$ mouse strains, this suggests that other NK1.1$^-$ strains possess similar allelic differences. Interestingly, our classification of *Nkrp1* haplotypes thus far appears to delineate functionally distinct NK1.1 alloantigen expression patterns: B6 strain (group 1) NK cells are known express an NK1.1 alloantigen (*Nkrp1c*) (9); BALB/c and 129 strain (group 2) NK cells do not express NK1.1 (4); while FVB strain (group 3) NK

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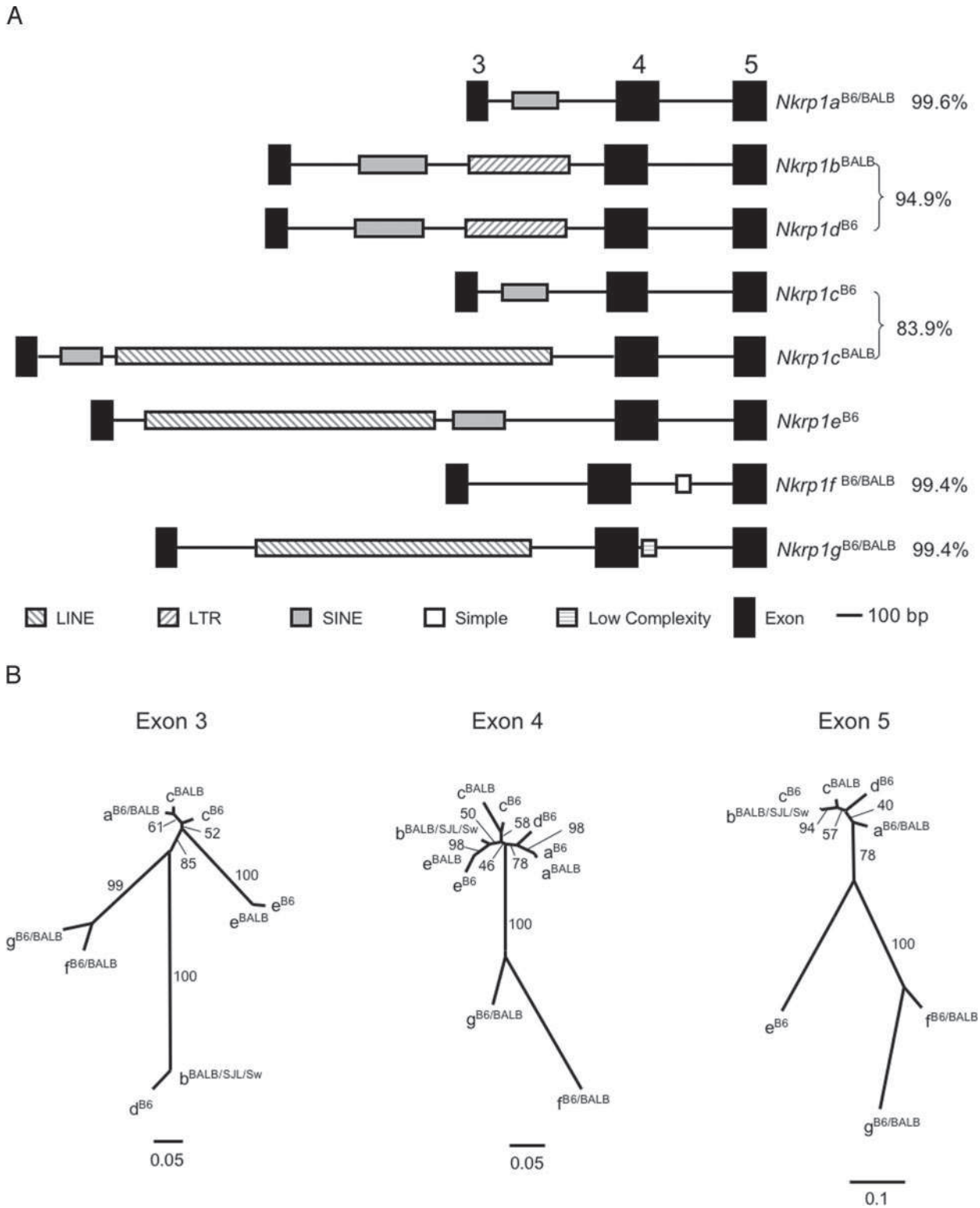

FIGURE 9. Comparison of *Nkrp1* gene structure. *A*, A scale diagram of the organization of exons 3–5 of various mouse *Nkrp1* genes is shown. Differentially shaded boxes indicate exons and various types of repetitive elements in introns as highlighted at the bottom. Numbers indicate the percentage nucleotide identity of the exon 3–5 region between B6 and available BALB/c alleles. The identity score shown for *Nkrp1c*^{B6} and *Nkrp1c*^{BALB} ignores the LINE element in the *Nkrp1c*^{BALB} gene. *B*, Individual exon 3–5 sequences of *Nkrp1* genes from B6 and BALB/c mice were aligned using ClustalX software, and bootstrap analysis of 1000 data sets was performed with Phylip software (⟨http://evolution.genetics.washington.edu/phylip.html⟩). The bootstrap values for each grouping are shown as a percentage. Phylogram branch lengths indicate the exon similarity among different *Nkrp1* genes, and the scale bar indicates the percent divergence. When different strains are grouped together in one gene, this indicates exon identity.

cells are also known to express NK1.1 (*Nkrp1b*) (7). Notably, FVB mice represent an inbred Swiss-related mouse strain (7), perhaps explaining the similar basis for NK1.1 reactivity (*Nkrp1b*) of FVB (7), Sw (5), SJL (6), and CD-1 (12) NK cells; thus, it is likely that these strains all share a related *Nkrp1* haplotype (group 3). Because there are currently no data on the relationship of any of these

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strains to the donor CE strain used for immunizations to generate the original polyclonal (3) and monoclonal (4) anti-NK1.1 alloantibodies, the identity of the original NK1.1 Ag remains unknown.

Nevertheless, before any final conclusions can be made concerning *Nkrp1* (and *Ocil/Clr*) haplotype variation, more haplotypes will have to be characterized. The genomic organization and content of the BALB/c and B6 *Nkrp1-Ocil/Clr* regions are more similar than the *Ly49* regions between these strains. If one assumes that the BALB/c and B6 *Nkrp1-Ocil/Clr* and *Ly49* haplotype association is a natural one and not a consequence of the original derivation of inbred mice, then it would appear that the *Nkrp1-Ocil/Clr* region is evolving less rapidly than the *Ly49* region. Although the reason for this remains speculative at present, a striking difference between the two NK cell receptor systems is that the *Nkrp1* genes are genetically intermingled in the NKC with the *Ocil/Clr* genes that encode their ligands (12, 13); thus, both are coinherited. In contrast, the *Ly49* genes segregate independently of the genes encoding their ligands, because the MHC is located on a different chromosome than the NKC (18). Coinheritance of ligand-encoding genes may negatively affect the rate of accumulated receptor gene mutations (i.e., receptor evolution), and vice-versa, because amino acid changes that decrease receptor-ligand binding affinities might be selected against. In contrast, the observed level of allelic divergence between *Nkrp1b* and *Nkrp1d* (and within the *Nkrp1c* locus) suggests that this may not always be the case. Further characterization of *Ocil/Clr* haplotypes is necessary to clarify the relationship between allelic divergence of the *Nkrp1* and *Ocil/Clr* genes and how that influences their function. Intriguingly, the underlying basis for the evolution of numerous *Nkrp1* genes in rodents and only a single gene (*NKRP1A*; Ref. 32) in humans is unknown. Yet like the *Ly49* receptor system, this dichotomy is probably a functional consequence of differences in receptor usage by rodent vs human NK cells. Only a single *Ocil/Clr-b*-like gene exists in humans, designated *LLT1* (18, 33). Indeed, the recent finding that human NKR-P1A–LLT1 interact and inhibit NK cell function demonstrates that the NKR-P1 missing-self recognition system is intact in humans (34, 35).

Identification of novel Nkrp1 *and* Ocil/Clr *genes*

Southern cross-hybridization and PCR cross-amplification of BAC clones and cDNA libraries identified sequences in the BALB/c genome that appear to represent novel *Nkrp1* and *Ocil/Clr* genes. The first of these, designated *Clr-h*, was identified from BAC analysis using primers intended for *Clr-b* (cross-reactive to *Clr-d*). This new gene is not unique to the BALB/c genome; rather, the sequence is readily identifiable in the latest assembly of the public B6 genome database (99% identical across e3-i3-e4; GenBank accession no. XM487965). Moreover, the corresponding region in the B6 genome does not fit the location of any other known *Clr* gene (18). Such a high degree of sequence conservation suggests that *Clr-h* either represents a ligand for a highly conserved receptor, or that it is not subject to selection pressure and thus may represent a nonfunctional gene/pseudogene (at least in the strains tested; Fig. 5*C*), or encode a ligand for a nonfunctional receptor. In this light, it is interesting to note that the *Nkrp1a* sequence is highly conserved, the *Nkrp1f* sequence is identical between B6 (27) and BALB/c (this study), and the *Nkrp1e* sequence appears to be nonfunctional in both strains (this study, and Ref. 27). As the NKR-P1F receptor binds to Ocilrp2/Clr-g (13, 16, 17, 36, 37), it is likely that at least some of the remaining NKR-P1 receptors and Ocil/Clr ligands bind to one another. Although the *Clr-c/d/e* genes are not novel, this study elucidates their relative order within the NKC (i.e., *Clr-g*, *Clr-d*, *Clr-e*, *Clr-c*, and *Nkrp1f*)

A second new gene, *Nkrp1g*, is predicted to exist in the interval between *Clr-f* and *Nkrp1c*, based upon Southern cross-hybridization with an *Nkrp1f* cDNA probe. Although it is not known whether the sequence encodes a functional receptor, a sequence is currently available for this putative new gene in the B6 genome database (GenBank accession no. XM355818). The coding sequence bears some similarity to the NKR-P1F receptor in the extracellular region (16), but the predicted protein appears to lack known signaling motifs, including a cytoplasmic ITIM or Y residue, a putative CxCP recruitment motif for the Lck tyrosine kinase, and a charged transmembrane R residue (5, 38). Thus, full BAC sequencing may be required to assess the significance of the BALB/c cross-hybridization results, if no related cDNA can be isolated. This approach may also provide a clue to the origin and relationship of this sequence to the other *Nkrp1* genes and/or pseudogenes. Although attempts to isolate an *Nkrp1g* cDNA were unsuccessful (at least in the strains tested; Fig. 5*C*), the *Nkrp1g* sequence appears to be conserved in the rat genome (Ensembl ID ENSRNOT00000035766; ⟨www.ensembl.org⟩), along with *Nkrp1a* (GenBank accession no. M62891), *Nkrp1b* (GenBank accession no. U56936), and *Nkrp1f* (GenBank accession no. X97477).

Conformational nature of the NK1.1 epitope

As mentioned previously, the NK1.1 epitope is specific to NKR-P1B$^{Sw/SJL}$ and NKR-P1C^{B6} among known NKR-P1 proteins from both mice and rats (Fig. 7). However, sequence alignment of these NKR-P1 proteins does not provide a clue as to the context of the NK1.1-reactive sequence. Our identification of a substitution in the NKR-P1B^{BALB} vs NKR-P1B$^{Sw/SJL}$ sequence that confers NK1.1 reactivity by no means indicates that this amino acid alone is sufficient to generate the NK1.1 epitope. In contrast, both the rat NKR-P1A and NKR-P1B sequences contain the NK1.1-reactive S residue at the correct position, yet neither receptor bears the NK1.1 epitope. This is not surprising considering that NKR-P1B/C can be immunoprecipitated but not Western blotted using PK136 mAb (J.R.C., unpublished observations). This suggests that the NK1.1 epitope is conformational in nature, relying on additional context-dependent amino acid residues, and that denaturation of the three-dimensional structure of the epitope destroys its reactivity.

It was surprising that the seemingly conservative S191T substitution found in both NKR-P1B^{BALB} and NKR-P1C^{BALB} could abolish NK1.1 reactivity, whereas reversing this single amino acid substitution could confer NK1.1 reactivity (Fig. 7). The chemical nature of S and T residues is quite similar: both residues contain a hydroxymethyl group, yet the T residue has one additional methyl group. The implications of this are not entirely clear, but since both S and T residues can be posttranslationally modified by O-linked polysaccharides, it is possible that a specific *O*-linked glycosyltransferase may be partially responsible for either conferring or destroying the NK1.1 epitope on these receptors. Carbohydrate modification of the NKR-P1 receptors is not functionally required for ligand binding, because tetramers of NKR-P1 proteins produced in bacteria bind to their cognate ligands (12, 13); however, such modifications could alter the affinity of the receptor–ligand interaction, because bacterially produced NKR-P1 tetramers only bind their ligands weakly (12, 13). This is evident in the findings that NKR-P1D tetramers fail to visualize native Ocil/Clr-b ligand on normal cells ex vivo (13), and NKR-P1B tetramers bind more weakly to Ocil/Clr-b ligand than the α-Ocil/Clr-b mAb, 4A6 (which is a low-affinity IgM) (12). Nonetheless, our present findings using the NKR-P1B^{BALB} receptor in the BWZ reporter system recapitulate our in vitro and ex vivo results using an NKR-P1B$^{Sw/SJL}$ receptor reporter cell (12). These results confirm that

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the cognate Ocil/Clr-b ligand recognized by these inhibitory receptors is expressed in a broad manner reminiscent of MHC class I (12), in contrast with the more restricted expression pattern suggested by other reports (13). The correlation between *Ocil/Clr-b* transcript expression (15, 16) and surface protein staining using α-Ocil/Clr-b 4A6 mAb (12) seems to be in agreement with our current findings.

Thus, the NKR-P1B–Ocil/Clr-b missing self recognition system is intact in BALB/c mice, despite their well-known NK1.1$^-$ strain designation. The generation of mAbs to the BALB/c NKR-P1 proteins should facilitate direct analyses of receptor expression and function. In addition, further analysis of the known and novel NKR-P1–Ocil/Clr interactions in various mouse strains should elucidate the complex nature of this system of at least 6 potential receptor and 8 potential ligand genes linked to one another in the NKC; heterodimerization of these proteins could offer as yet unappreciated additional complexity to NK cell recognition of target cells.

Note added in proof. The genomic sequences and intron–exon structure of the Nkrp1 genes in this study have been modified from Ljutic et. al. (39) to conform to the *Ly49* gene nomenclature (i.e., exon 2, cytoplasmic; exon 3, transmembrane, exon 4, stalk; exons 5–7, C-type lectin-like domain).

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Disclosures

The authors have no financial conflict of interest.

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