Mouse CD94/NKG2A Is a Natural Killer Cell Receptor for the Nonclassical Major Histocompatibility Complex (MHC) Class I Molecule Qa-1 \(^b\)

By Russell E. Vance, Jennifer R. Kraft, John D. Altman, Peter E. Jensen, and David H. Raulet \(^*\)

From the \(^*\)Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California Berkeley, Berkeley, California 94720; and the \(\dagger\)Department of Pathology and Laboratory Medicine and \(\ddagger\)Emory Vaccine Center and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

Summary

Natural killer (NK) cells preferentially lyse targets that express reduced levels of major histocompatibility complex (MHC) class I proteins. To date, the only known mouse NK receptors for MHC class I belong to the Ly49 family of C-type lectin homodimers. Here, we report the cloning of mouse NKG2A, and demonstrate it forms an additional and distinct class I receptor, a CD94/NKG2A heterodimer. Using soluble tetramers of the nonclassical class I molecule Qa-1\(^b\), we provide direct evidence that CD94/NKG2A recognizes Qa-1\(^b\). We further demonstrate that NK recognition of Qa-1\(^b\) results in the inhibition of target cell lysis. Inhibition appears to depend on the presence of Qdm, a Qa-1\(^b\)-binding peptide derived from the signal sequences of some classical class I molecules. Mouse NKG2A maps adjacent to CD94 in the heart of the NK complex on mouse chromosome six, one of a small cluster of NKG2-like genes. Our findings suggest that mouse NK cells, like their human counterparts, use multiple mechanisms to survey class I expression on target cells.

Key words: CD94 • NKG2A • Qa-1 • natural killer cell • major histocompatibility complex class I

Several independent mechanisms have been described by which certain tumors or pathogens downregulate MHC class I expression (1, 2), thereby circumventing immune recognition by cytotoxic T cells. The ability of NK cells to distinguish and lyse targets that express reduced surface levels of MHC class I genes may thus provide protection against such tumors and pathogens (3). In mice, the Ly49 family of class I-specific receptors has provided a substantial molecular basis for the detection of MHC class I by NK cells (4, 5), but some inconsistencies remain. For instance, some murine NK cell lines that lack detectable expression of Ly49 molecules spare class I\(^t\) tumor targets from lysis, but kill their matched class I\(^b\) variants (6, 7).

A further reason for supposing that class I recognition by mouse NK cells may not be solely via Ly49 receptors is that human NK cells express two distinct families of receptors for class I: immunoglobulin-like receptors that directly bind to certain HLA-A, B, and C alleles (reviewed in reference 8) and lectin-like heterodimers of NKG2 family members and CD94 (9–12). The cytoplasmic domain of CD94 is only 10 amino acids in length (13), so the signaling capacity of CD94/NKG2 heterodimers is presumably mediated through the various NKG2 molecules (14). NKG2A contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs)\(^1\) and transmits inhibitory signals (15). NKG2C and E lack ITIMs, and at least NKG2C activates NK cells, probably through its association with a small signaling homodimer, DAP12 (16). CD94/NKG2A and CD94/NKG2C heterodimers were recently shown (17–20) to bind directly to HLA-E, a nonclassical MHC class I molecule. No definitive explanation has been offered as to why the human class I specific receptors should differ so strikingly from those of the mouse, but a popular supposition has been that class I-specific receptors on NK cells are a recent evolutionary phenomenon that has been driven by the rapid evolution of class I molecules themselves (21). Alter-

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\(^1\)Abbreviations used in this paper: APC, allophycocyanin; BAC, bacterial artificial chromosomes; HA, hemagglutinin; ITIM, immunoreceptor tyrosine-based inhibitory motif; LAK, IL-2 cultured NK cell; nt, nucleotide; TAP, transporter associated with antigen presentation.
natively, it remains possible that humans and mice share most or all families of NK class I receptors, and that these receptors will eventually be discovered and characterized.

Previously, we and others identified a gene homologous to human CD94 that is expressed by mouse NK cells (22, 23). We demonstrated that mouse CD94 maps within a region of chromosome six dubbed the NK complex (24), near other NK-expressed genes including CD69, NKR-P1, and Ly49. CD94 and NKG2A/C genes have also been identified in the rat (25, 26), and map to the corresponding rat NK complex. Rodent NKG2D-like sequences also exist (22, 23, 27), but the significance of these sequences is uncertain because human NKG2D is not in the same homology cluster as other NKG2 genes and does not appear to associate with CD94. Unlike human CD94, rodent CD94 molecules contain a positively charged amino acid in their transmembrane domains, suggesting that the structure of any CD94-containing complexes may differ among species. Moreover, no interaction of rodent CD94/NKG2 molecules with MHC class I has been reported to date, leaving open the question of whether rodent and human CD94/NKG2 molecules are true functional homologues.

The class I ligand for human CD94/NKG2A and C heterodimers, HLA-E, is interesting because it appears to associate predominantly with peptides derived from the signal sequences of classical class I proteins (28, 29). In the mouse, the nonclassical class I molecule Qa-1b has been found preferentially associated with a peptide called Q dm (AMAPRTLLLL), also derived from class I signal sequences (30–32). Although Qa-1 and HLA-E bind signal peptides that are cleaved in the ER, proper loading of these peptides into Qa-1 or HLA-E appears to depend on the transporter associated with antigen presentation (TAP), a complex that shuttles peptides from the cytoplasm into the ER (28, 33). As a consequence, NK cells could detect defects in class I expression or TAP function resulting from infection or tumorigenesis simply by monitoring the surface expression of Qa-1/HLA-E and associated peptides.

Although Qa-1b and HLA-E both exhibit a preference for TAP-dependent signal peptides, it should be emphasized that sequence alignments do not make a compelling case that HLA-E and Qa-1b are true homologues (33). Such a case would be strengthened if it could be demonstrated that HLA-E and Qa-1 play similar functional roles. Here, we report the cloning of mouse NKG2A and document the direct and functional interaction of CD94/NKG2A heterodimers with Qa-1b/Q dm complexes on target cells. Our results demonstrate for the first time the existence of a functionally conserved mechanism by which NK cells can monitor class I expression on target cells.

Materials and Methods

Primers and PCR. PCR was performed with either Pfu Polymerase (Stratagene, La Jolla, CA) or Taq Polymerase (Promega Corp., Madison, WI) using 30–35 cycles as follows: 92°C for 60 s, 55°C for 60 s, and 72°C for 30 s. Primers were as follows (nucleotide [nt] 1 is the first translated base; lower case bases are restriction enzyme sites, Kozak sequences, etc.): mNK2C 3’ no. 1 = tacccaagTCAGATGGGAAATTTACACCTTAAAGATTTG (nt 703–735); mNK2A 3’ no. 4 = CTGGAGATCCGG-TATGCTCC (nt 150–170); mNK2A 5’ UTR = CTTGC-CTCAGCACATCCTAGCTG (nt 373–731); CD94 3’ STOP = atgtggacggctgccattCAAAGGCAGTTTCTTCTACATAGT (last 25 nt of mCD94); mNK2A 5’ = atatcgcaatccgtcaccagTCATGCTAAATGCGTACCT-ATGCAAGATTGGTGCAAAAGAATCAAGGG (enodes a Kozak sequence and the first 18 amino acids of mouse NK2A, although some of the nucleotide wobble positions are not correct); mNK2A HA N ots = taatagagggctTCAGGCTAAGTCGGGCACGTGTCAGGGGATGAGATGGGAATTACCT-TACAAAG (encodes a COOH-terminal hemagglutinin epitope tag [YPYDVPDYA; underlined]; mNK2A 5’ ex5 = GCACAGCGTTCGGTTGCAGATGGGGAATTTACACTTACATAGTG (nt 590–616); mNK2A 3’ ex6 = GGTITGGAATTTGAGACTCTTCTTCC (nt 590–614). Based on the C to T missile dam changes (BAC) clones derived from C57BL/6 genomic DNA were screened on six high density nylon filters (Genome Systems, St. Louis, MO) with mCD94 3’ and mNK2D cDNA probes (22). We focused on three positive BACs (Genome Systems clone addresses: 189c16, 9119, and 89b4). BACs were grown in LB supplemented with 12.5 μg/ml of chloramphenicol (C-0378; Sigma Chemical Co., St. Louis, MO) and were purified over midi columns (QIAGEN Inc., Chatsworth, CA). The sizes of the BAC inserts (released by NotI digestion) were estimated by pulsed field gel electrophoresis.

Cloning of Mouse NKG2A and Expression Constructs. Exons 5–7 of human NKG2A were amplified by RT-PCR from IL-2 cultured PBL RNA, and used as a probe on Southern of three BACs (see above) previously shown to contain mouse CD94 and/or mouse NK2D. Exceedingly weak hybridization to three different HindIII fragments of ~3.5 kb, 9 kb, and ~20 kb was detected. The 3.5- and 9-kb fragments were cloned into pBluescript SKI(+) to generate plasmids B6/H3.5 and B6/H9, respectively, that were partially sequenced using the T3 and T7 primers. Sequence with significant homology to NKG2C (exons 4 or 7) was obtained and used to design a 3’ primer (NGK2C 3’ no. 1) for amplification of full-length cDNA by the same strategy we used to clone mouse CD94 (22). In brief, 100 ng of a CB17.SCID lymphocyte plasmid cDNA library (gift of P.A. Matthew and V. Kumar, University of Texas, Dallas, TX) was used as the template in a PCR reaction with a 5’ primer that recognizes the plasmid vector and a 3’ primer that is specific for the cDNA of interest. PCR products were cloned into the pGEM T-easy vector (Promega Corp.) but none of the resulting clones contained full-length cDNA and were either lacking the extreme 5’ end, or internal exons. To obtain the 5’ end of mouse NKG2A, we sequenced BAC 89004 in the 3’ direction using the mNK2A 3’ no. 4 primer, and the resulting sequence was used to design a primer (mNK2A 5’ UTR) which could be used with the mNK2A 3’ UTR primer to amplify the complete ORF of mNK2A from B6 LAK cDNA. Expression constructs were based on the pmE18S expression vector. pmE-CD94-HA has been described previously (22). pmE-CD94 (untagged) was produced using primers CD94 5’ Xho-Koz (22) and CD94 3’ STOP. pmE-NKG2A was produced using primers mNK2A 5’ and mNK2A 3’ no. 1. pmE-NKG2A-HA was produced using primer mNK2A 5’ and mNK2A HA N ots3’. All inserts were amplified with Pfu polymerase, cloned into the XhoI and NotI sites of pmE18S, and confirmed by sequencing. Transfections into COS7 cells were as described previously (22).
Southern Blotting and Probes. Southern blotting and the CD94 3’ probe were also described previously (22). The 95R probe has been described (24). The NKG2D probe corresponds to the insert in IMAGE consortium clone 621324 (these data are available from GenBank/EMBL/DDJB under accession number AF030313). The NKG2A exon 5/6 probe corresponds to nucleotides 364-614 of mNKG2A (predicted exons 5 and 6) and was generated by PCR with primers mNKG2A 5’ex5 and mNKG2A 3’ex6. The NKG2A 5’ probe was generated from the 5’ end of the NKG2A cDNA (nucleotides 1-398, numbering starting at ATG).

Synthesis of O-a1Tetramers. Expression vectors were constructed using PCR to amplify soluble Qa-1 with primers mNKG2A 5’ ex5/6, and was generated by PCR with primers mNKG2A 5’ex5 and mNKG2A 3’ex6. The NKG2A 5’ probe was generated from the 5’ end of the NKG2A cDNA (nucleotides 1-398, numbering starting at ATG).

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The oligonucleotide primers gagatactagt-gagccaccactgctgctggt and gcagggatcc (T2-BB; reference 43) were obtained from Dr. Ned Braunstein (Columbia University College of Physicians and Surgeons, New York, NY). The Qdm (AMAPRTLLL) and Ova (SINFEKL) peptides were synthesized and purified by the University of California Berkeley Microchemical Facility, and were verified by mass spectrometry. Peptides were added to target cells overnight at 26°C at a concentration of 30 μM, and were also included during incubations at 37°C required to label targets with 32P (1 h) or to perform the cytolysis assay (4 h).

Results and Discussion

A human NKG2 probe was employed to detect weakly cross-hybridizing fragments on several CD94-containing BACs isolated from a B6 genomic library. These cross-hybridizing fragments were subcloned, partially sequenced, and were found to contain sequence homologous to human NKG2A exons. A PCR-based strategy was then used to obtain cDNA encoding full length homologues of NKG2A (Fig. 1). Mouse NKG2A has sequence similarity to the group of C-type lectin-like receptors on NK cells, which includes CD69, NKR-P1, and Ly49, but most resembles human and rat NKG2A (60.5 and 85.7% similarity, respectively, at the protein level; references 14, 25). An inhibitory signaling function for mouse NKG2A is suggested by the presence of one and possibly two conserved motifs in the cytoplasmic tail (V(T)YAEI and I1YSDF) that resemble the consensus ITIM (W/IxYxxV/L). This motif is present in all known inhibitory NK receptors, and has been shown to interact with tyrosine phosphatases such as SHP-1 (44).

![Figure 1. Comparison of mouse (m), rat (r), and human (h) NKG2A protein sequences, as deduced from the cDNA sequences, using the Clustal W algorithm (v.1.6; reference 52). Identical residues are joined by a vertical line and similar residues are joined by a colon. The exon structure of the human NKG2A gene is shown, as determined previously (53). Potential or actual ITIM are shaded, as are the predicted transmembrane domains and potential N-linked glycosylation sites (*). The mouse NKG2A nucleotide sequence is available from GenBank under accession number AF095447.](image-url)
Northern blot analysis showed strong NKG2 expression in IL-2 cultured NK cells (LAKs), but not in total lymph node, spleen, kidney, thymus, heart, or testis, suggesting that NKG2 expression is relatively NK-specific (data not shown).

To address whether there are multiple NKG2 genes in the mouse, a Southern blot of total genomic DNA from several inbred strains of mice was probed with predicted exons 5 and 6 of mouse NKG2A. In the human, exons 5, 6, and 7 are highly (>90%) conserved among NKG2A, C, and E, so we expected the probe to cross-hybridize with additional mouse NKG2 genes, should they exist. Indeed, hybridization of the probe to multiple genomic fragments was observed, consistent with the possibility that mice, like humans, might contain a small family of NKG2-related molecules (Fig. 2 A). Several strains of mice, including BALB/c, DBA/2J, and CBA/J, exhibited a Southern blot pattern different than that of B6, demonstrating that the NKG2 locus is polymorphic. Direct evidence that the multiple bands represent multiple genes was obtained by partially sequencing two cloned nonoverlapping B6 genomic fragments (B6/H3.5 and B6/H9; see Materials and Methods). Each fragment contained an additional NKG2-related exon most similar to human NKG2C (exon 4). Partial cDNAs corresponding to the sequences in these exons have been isolated (data not shown), suggesting that these additional genes are also transcribed by mouse NK cells.

The exon 5/6 probe, as well as probes specific for additional NK complex markers, were then hybridized to a series of BACs that overlap in the region of the NK complex on mouse chromosome six (Fig. 2 B). The exon 5/6 probe detected multiple fragments of sizes similar to those detected on Southern blots of total mouse genomic DNA. In contrast, the NKG2A 5' probe appeared to be relatively specific for a single locus, suggesting that, as in the human, the 5' (cytoplasmic) domains of mouse NKG2 genes are relatively divergent. Because each BAC contained a different number of NKG2 exon 5/6 hybridizing fragments, and only BAC 89b04 produced strong hybridization to an NKG2A 5' probe, a proposed structure for the mouse CD94/NKG2 locus could be determined (Fig. 2 C). Without NKG2C or NKG2E-specific probes, the number of NKG2-related sequences remains uncertain, but there appears to be at least two. Interestingly, the mouse NKG2 genes are tightly clustered within an ~100-kb region between CD69 (centromerically) and the Ly49 family (telomERICALLY), with NKG2A being the most telomeric NKG2 gene.

To investigate whether mouse CD94/NKG2A recognizes Qa-1β, soluble complexes of Qa-1β/β2m were tetramerized with streptavidin aliphophycocyanin (APC), permitting their use as high avidity staining reagents (37). COS7 cells were then transiently transfected with hemagglutinin (HA) epitope-tagged versions of NKG2A and/or CD94. A appreciable surface expression of individually transfected NKG2A-HA or CD94-HA was detected with anti-HA mAb on a subset of transfectants (Fig. 3), but the Qa-1β tetramers did not bind to these cells, nor to COS7 cells expressing either untagged CD94 or NKG2A. Strikingly, however, cotransfection of CD94 and NKG2A resulted in strong binding to Qa-1β tetramers, providing direct support for the notion that CD94 and NKG2A together form a receptor for Qa-1β. The tetramer did not bind to COS7 cells transfected with any of the known Ly49 cDNAs (Hanke, T., and D.H. Raulet, unpublished results).

Significantly, the Qa-1β tetramers were produced in E. coli and are consequently not glycosylated; thus, although CD94 and NKG2A exhibit homology to lectin-like receptors, they appear capable of recognizing carbohydrate-independent epitopes on their ligands, a situation akin to that of human CD94/NKG2 (20) and also mouse Ly49A (45). Carbohydrate recognition may nevertheless play a role in increasing the affinity of the CD94/NKG2A-Qa-1 interaction. As a further note, it has been reported that without CD94, human NKG2 molecules are not efficiently expressed on the cell surface (9, 10). In contrast, we observe substantial surface expression of NKG2A in the absence of CD94. It remains possible that surface expression of mNKG2A without CD94 is the result of the abnormally high levels of expression that occurs in our COS7 transfec-
tants. As reported previously (22), we also see expression of mouse CD94 alone on the surface of COS transfectants, despite the presence of a positive charge in the transmembrane domain of CD94. Again, it is not known whether mouse CD94 is expressed alone on the surface of NK cells, nor is it known whether the positive charge plays a role in mediating associations with other membrane proteins.

The Qα-1b tetramer was also used to stain freshly isolated B6 splenocytes (Fig. 4 A). Specific staining of ~30% of NK cells (NK1.1+CD3−) was observed. This percentage likely represents an underestimate since ~45% of NK1.1+ cells were tetramer positive in preliminary experiments using a fresher tetramer preparation that separated a fully distinct positive population (data not shown). Expression of the Qα-1 receptor on NK cells partially overlaps with Ly49 expression (Fig. 4 B), demonstrating additional complexity in the NK class I receptor repertoire. Weak but reproducible tetramer binding to a fraction of NK1.1+ T cells was also observed (Fig. 4 A), suggesting a possible role for CD94/NKG2 receptors in the regulation of certain T cell subsets (46).

To demonstrate that the interaction of CD94/NKG2A and Qα-1b is of functional significance, the Qα-1b tetramer was used as a reagent to sort Qα-1b tetramer positive and negative NK cell (NK1.1+CD3−) populations from IL-2 cultured B6 splenocytes. Human TAP-deficient T2 cells are normally highly susceptible targets for mouse NK cells. Sorted tetramer positive NK cells were fully lytic, and were not inhibited by the expression of Qα-1b in T2 target cells.

Figure 3. CD94/NKG2A is a receptor for Qα-1. Expression constructs encoding CD94, NKG2A, or HA epitope-tagged versions of these proteins, were transiently transfected individually or together into COS7 cells as indicated. After 48 h, cells were lifted with PBS and 0.02% EDTA, stained either with anti-HA or Qα-1b tetramer, and analyzed by flow cytometry using forward and side scatter to exclude dead cells. The percentage of cells within the indicated gate is shown for each sample.

Figure 4. The Qα-1b tetramer predominantly recognizes a subset of NK cells. (A) B6 splenocytes were enriched for T and NK cells by passage over nylon wool and stained with the indicated monoclonal antibodies and with streptavidin-allophycocyanin (SAv-Apc) complexed Qα-1b tetramer (top). As a control for the specificity of the tetramer, cells were also stained with streptavidin-APC alone (bottom). Left hand panels are gated on CD3 negative cells, whereas right hand panels are gated on CD3 positive cells. The percentages of gated cells in each quadrant are indicated, and are the average of 3 mice ± SD. The percentage of APC+ cells among NK1.1+ cells is given in parentheses. (B) The receptor for Qα-1b on NK cells only partially overlaps in its expression with other class I–specific receptors on NK cells. NK1.1+/tetramer positive or negative cells were electronically gated and analyzed for their binding of FITC-conjugated monoclonal antibodies to Ly49 family members. Again, the percentage of cells within the indicated gate is given ± SD (n = 3 mice).

Figure 5. (A) Inhibition of NK cells by recognition of Qα-1/Qdm. Pure IL-2 cultured NK cells (CD3−NK1.1+) were sorted for the expression (Qα-1b positive) or the lack of expression (Qα-1b negative) of Qα-1b receptor and assayed for cytotoxicity against 51Cr labeled T2 cells stably transfected with Q α-1b (T2-37, top) or HA-1a (T2-BB, bottom). Targets were incubated overnight in media at 26°C with the addition of excess (30 μM) peptide or an equal volume of PBS. Ova (SIINFEKL) is a K b binding peptide; Q dm (AMAPRTLLL) is a Qα-1 binding peptide. The kill was performed in the presence of the peptide/PBS at the indicated E:T ratios. (B) Treatment with Q dm peptide does not detectably enhance surface expression of Qα-1b in T2-α-1b transfecants (T2-37 cells). T2-37 cells were incubated overnight at 26°C in the presence of 30 μM peptide or PBS. To control for the 37°C incubation required to label the targets with 51Cr (see A), the T2-37 cells were then incubated at 37°C for 1 h in the absence of 51Cr but in the presence of appropriate peptide. Cells were then stained with normal rabbit serum (NRS, dotted histograms) or a specific rabbit anti-Qα-1b polyclonal antiserum (filled histograms) at 4°C in the presence of sodium azide (0.02%), followed by staining with FITC-conjugated swine anti-rabbit F(ab)2. Numbers indicate the mean fluorescence intensity of the population.
However, when the T2-Qa-1\(^b\) cells were incubated with excess Qdm peptide (AMAPRTLLL), inhibition of tetramer positive NK cells was observed. As a control, addition of the K\(^b\) binding Ova peptide (SIINFEKL) or PBS did not inhibit lysis. The effect of the Qdm peptide was specific since it had no effect on lysis by the tetramer negative population. Moreover, the inhibition depended on Q a-1\(^b\) expression since no inhibition was observed when Qdm was incubated with T2-IA\(^b\) (T2-BB) transfectants. Based on data from the human system, it might be expected that the sorted tetramer-reactive NK population would include cells expressing the putative activating receptor CD94/NKG2C (20). Our results suggest that Q a-1/Qdm-activated cells must be relatively rare or absent in B6 mice, or else not dominantly activated in our assay conditions.

Two nonexclusive hypotheses can be offered to explain the requirement for peptide addition to observe inhibition. One is that peptide addition results in higher levels of Q a-1\(^b\) on the cell surface, which may be necessary for effective inhibitory signaling by the CD94/NKG2 receptor. The other possibility is that a CD94/NKG2 receptor fails to interact effectively with Q a-1\(^b\) unless the Q a-1\(^b\) molecule has bound peptide. We favor the latter possibility, since neither we nor others (32) could observe a detectable increase in bound peptide. The important related question as to whether the CD94/NKG2A receptor can discriminate between different Q a-1\(^b\)/peptide complexes remains to be addressed. Discrimination of class I peptide complexes has been reported in the case of some human (47) but not mouse (48, 49) class I-specific NK receptors.

Our results are significant for several reasons. First, we provide direct evidence for a physiological receptor for the nonclassical class I molecule, Q a-1\(^b\). Although T cell clones that recognize Q a-1\(^b\) can be obtained by allograft strategies or in other model systems (34, 50, 51), the biological significance of these T cell clones has been unclear, as has the significance of the mysterious observation that Q a-1 binds peptides derived from the signal sequences of classical class I proteins. This latter property of Q a-1 can now be rationalized in terms of NK function. Second, our results demonstrate that mouse NK cells express at least two families of class I-specific receptors, and may consequently help explain numerous complexities in NK recognition, including the prior observations that Ly49-negative NK cell lines can nevertheless detect MHC class I (6, 7). Third, we describe the first instance in which an NK receptor and its class I ligand are shared by mice and humans, and therefore provide the first direct evidence that NK recognition of MHC class I is a primitive function of NK cells, at a minimum predating the divergence of mouse and human ancestors. Finally, our finding that mouse NKG2 genes are clustered within the NKG2 complex on chromosome 6, syntenic with the human MHC complex on chromosome 12, provides additional support for the notion that the NK complex is itself primitive. Finally, our results will allow the study of CD94 and NKG2 function in a well-characterized animal model, and will thus undoubtedly have significance for the study of NK cell responses to pathogens, tumors and tissue transplants.

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Address correspondence to David H. Raulet, Department of Molecular and Cell Biology, 489 Life Science Addition, University of California, Berkeley, CA 94720. Phone: 510-642-9521; Fax: 510-642-1443; E-mail: raulet@uclink4.berkeley.edu.

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More CD94/NKG2A is a Natural Killer Receptor for Qa-1b

1848