Characterization of a Novel Killer Cell Lectin-Like Receptor (KLRH1) Expressed by Alloreactive Rat NK Cells


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Characterization of a Novel Killer Cell Lectin-Like Receptor (KLRH1) Expressed by Alloreactive Rat NK Cells

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NK cells have the ability to recognize and kill MHC-mismatched hematopoietic cells. In the present study, strain-specific differences in the rat NK allorecognition repertoire were exploited to generate Abs against receptors that may be involved in allogeneic responses. A mAb termed STOK9 was selected, and it reacted with subsets of NK cells and NKR-P1+ T cells from certain rat strains possessing highly alloreactive NK cells. The STOK9+ NK subset was broadly alloreactive and lysed Con A lymphoblast targets from a range of MHC-mismatched strains. The mAb STOK9 precipitated a 75-kDa dimeric glycoprotein from NK lysates. Expression cloning revealed that each monomer consisted of 231 aa with limited homology to other previously characterized killer cell lectin-like receptors (KLRs). This glycoprotein therefore constitutes a novel KLR branch, and it has been termed KLRH1. A gene in the central region of the natural killer gene complex on rat chromosome 4 encodes KLRH1. A mouse homolog appears to be present as deduced from analyses of genomic trace sequences. The function of KLRH1 is unknown, but it contains an immunoreceptor tyrosine-based inhibitory motif, suggesting an inhibitory function. The MHC haplotype of the host appears to influence KLRH1 expression, suggesting that it may function as a MHC-binding receptor on subsets of NK cells and T lymphocytes. The Journal of Immunology, 2002, 168: 5147–5154.

N

atural killer cells play an important role as a first line of defense against infections (1). NK cells are also able to reject MHC-mismatched bone marrow transplants (2–4). They express activating and inhibitory receptors, many of which recognize MHC class I (MHC-I) molecules, and the net outcome of positive and negative signals from these receptors determines the fate of the target cells. Human NK cells use two main superfamilies of structurally divergent receptors, the killer cell Ig-like receptors and the killer cell lectin-like receptors (KLRs) (5, 6). In rats and mice the KLRs, which are encoded by the NK cell gene complex (NKC), appear to dominate. There are three important KLRs that recognize MHC-I-related ligands, the Ly49 homodimers, CD94/NKG2 heterodimers, and NKG2D homodimers. The functions of these receptors may partially explain the observed complexities in target cell recognition and killing by NK lymphocytes. With the expanding number of novel class I-related molecules detected, the possibility remains that other MHC-binding receptors exist.

We have exploited differences in the NK allorecognition repertoires of different rat strains (7, 8) to generate Abs against receptors potentially involved in the control of allogeneic responses. A variety of different Abs have been generated, among them STOK1 and STOK2, which we have previously shown to bind an inhibitory rat Ly49 receptor specific for the classical MHC-I molecule RT1-A1+ (9, 10). We have recently simplified the Ly49 nomenclature in the rat and have termed the STOK2 receptor for Ly49 inhibitory receptor 2 (Ly49I2). In the present study, we have characterized another mAb, termed STOK9, which identifies a member of a novel branch of the KLRs. This membrane glycoprotein has been termed KLRH1 and is expressed by small subsets of NK cells as well as NKR-P1+ T cells from certain rat strains possessing highly alloreactive NK cells. KLRH1 is slightly more related to the Ly49 family of MHC receptors than to other KLR families. This, together with the fact that the rat MHC haplotype influences its in vivo expression, suggests that it may also be a novel receptor for an MHC-encoded molecule.

Materials and Methods

Animals

Breeding pairs of PVG.1U (RT1u* or haplotype u, RT1-A1u-B1u-D1-D2u-C7M0 or u-u-u), PVG.1AV1 (av1 or a-a-av1), PVG.R23 (a-a-av1), PVG (c), AO (a), and DA (av1) were obtained from Harlan U.K. (Bicester, U.K.), whereas PVG.IV1 (h1) and PVG.II (n) were from Dr. G. W. Butcher (Babraham Institute, Cambridge, U.K.). These strains were reared under conventional conditions in Oslo and screened for common rat pathogens. F344 (h1), LEW (f), and BN (u) rats were purchased directly from Mollegaard (Ejby Skensved, Denmark), WAG (u) and BUF (b) were from Harlan (Horst, The Netherlands), and PVG.R8 (a-a-u) were from Harlan U.K. The experimental protocol was approved by the institute veterinary surgeon and registered by the Experimental Animal Board under the Ministry of Agriculture of Norway.

Abs and flow cytometry

For single-color analysis, 50 μl of cells (0.2–2 × 106 cells/ml) were incubated with 50 μl of Ab for 30 min on ice. After three washes, labeled cells were incubated with F(ab)2 of FITC-conjugated goat anti-rat Ig (Jackson ImmunoResearch Laboratories, West Grove, PA). For three-color

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flow cytometry, we used mononuclear spleen cells from 2- to 3-month-old male rats obtained by centrifugation on Lymphoprep (Nycomed, Oslo, Norway), depleted of IgG cells with sheep anti-rat IgG-coated magnetic Dynabeads (Dynal Biotech, Oslo, Norway). Primary labeling of FITC-conjugated anti-NKR-P1A (mAb 3.2.3; from J.C. Hiscott, Portland, MA), FITC-conjugated anti-CD3 (G4.18; from BD Pharmingen, San Diego, CA), and biotinylated STOK9 (purified by Protein A and biotinylated according to standard procedures), followed by PE-indodicarbocyanine (PE-Cy5)-conjugated streptavidin (DAKO, Glostrup, Denmark). The cells were analyzed by standard procedures, followed by PE-indodicarbocyanine (PE-Cy5)-conjugated anti-CD3 (G4.18; from BD PharMingen, San Diego, CA), and directed against mouse heat stable Ag, was obtained from the American Type Culture Collection (Manassas, VA).

Generation of the anti-KLRH1 mAb STOK9

The methods for immunization, cell fusion, and screening were generally as described for mAb STOK2 reacting with the Ly49 receptor (9). Briefly, DA rats (RT1 av1 matched control hybridoma for STOK9, M1/75 (M1/75.16.4.HLK; IgG2c; conjugated streptavidin (DAKO, Glostrup, Denmark). The cells were analyzed by standard procedures), followed by PE-indodicarbocyanine (PE-Cy5)-conjugated anti-CD3 (G4.18; from BD PharMingen, San Diego, CA), and directed against mouse heat stable Ag, was obtained from the American Type Culture Collection (Manassas, VA).

Generation of Generation of KLRH1 NK cultures

An adaptation of a method for generation of cultures of Ly492 NK cells was used (9). KLRH1 NK cells were positively selected from mononuclear spleen cells with streptavidin-coated M280 magnetic Dynabeads (Dynal Biotech) preincubated with mAb STOK9-biotin. In some experiments, nonadherent splenocytes were depleted of NS0 myeloma cells, and hybridoma supernatants were screened for mAbs that bind to subsets of IL-2-activated PVG.1AV1 NK cells by indirect immunofluorescence and flow cytometry. The anti-KLRH1 mAb STOK9 was selected based on its unique staining pattern. Monoclonal Ab STOK9 was of the IgG2c isotype as determined by ELISA (Zymed Laboratories, South San Francisco, CA).

Immunoprecipitation and deglycosylation of the KLRH1 protein

For surface labeling, cells were washed three times in cold PBS (pH 8) and incubated at 2.5 × 10^5 cells/ml for 30 min at room temperature with 0.5 mg/ml biotin (Pierce, Rockford, IL) in PBS (pH 8). The cells were washed twice before lysis at 4°C in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, protease inhibitors, 1% bovine hemoglobin, and 1% Triton X-100 (Fluka, Buchs, Switzerland). Lysates, obtained by microcentrifugation, were pre-cleared with quenched Sepharose 4B beads and then immunoprecipitated with Sepharose 4B precomplexed with purified Ab STOK9 or the isotype-matched control M1175 for at least 5 h at 4°C. The coupling of cytochrome c oxidase activated Sepharose 4B was performed at 5 mg Ab per milliliter of gel, according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). The immunoprecipitates were washed four times in complete lysis buffer, resolved by 10% SDS-PAGE, blotted onto nitrocellulose membranes, and autoradiographed using [35S]methionine (Amersham). Membrane-bound protein was quantified densitometrically using the MACS (Miltenyi Biotech) and was adapted for in vitro growth in cRPMI. RNK-16 cells were transfected as previously described (13). In brief, a full-length KLRH1 cDNA was subcloned into the XhoI site of the vector EMVC-SRAs with an internal ribosomal re-entry site and twice purified over Qiagen Tip-500 columns. After linearization with ScaI, 20 μg of expression construct was electroporated into RNK-16 cells in 2-mm cuvettes in cRPML at 120 V, 850 μF, and 129 μA using a BTX ElectroCell Manipulator (San Diego, CA). After 24 h, cells were plated in cRPML supplemented with 1 mg/ml G418 in 96-well plates at 10^4 cells/ml. After 14–17 days, G418-resistant cells were subcoloned and analyzed for KLRH1 expression by flow cytometry.

Southern blotting and pulsed-field gel electrophoresis

Genomic DNA was extracted from rat liver, digested with restriction endonuclease (New England Biolabs, Beverly, MA), subjected to horizontal agarose gel electrophoresis, and blotted onto nylon membranes (Biotrans membranes; ICN Biomedicals, Irvine, CA) by conventional methods as previously described (14). Hybridization and washing procedures were performed using an oven and prehybridized with 10 μg/ml denatured salmon sperm DNA (Pharmacia), or a combination of XhoI (5 μg/ml) and 5 μg/ml Clastidium perfringens neuraminidase (Sigma Chemical Co., St. Louis) for 4 h at 37°C. The membranes were exposed to X-ray films (Hyperfilm; Amersham Pharmacia Biotech) for 15 days before development. Briefly, 60% confluent 175-cm² flasks of the human kidney fibroblast line 293T (provided by L. Lanier, DNAX, Palo Alto, CA). After electroporation into supercompetent BRL ElectroMAX DH10B Escherichia coli (Life Technologies), the resultant library was found to have a complexity of 550,000 colonies with >97% of colonies containing cDNA inserts. The library was amplified on 160-mm Luria-Bertani-ampicillin plates (11 in total) at a density of 50,000 PFU/plate, bacterial colonies were harvested by scraping, and plasmids were isolated using Qiagen TIP-500 columns according to the manufacturer’s instructions (Qiagen, Chatsworth, CA).

Expression cloning of KLRH1 cDNA

cDNAs encoding the KLRH1 molecule were cloned using a variation of the eukaryotic expression method described by Aruffo and Seed (12). Briefly, 60% confluent 175-cm² flasks of the human kidney fibroblast line 293T (provided by L. Lanier, DNAX) were transfected with 20 μg of KLRH1 NK expression library using Lipofectamine/Opti-MEM according to the manufacturer’s instructions (Life Technologies). After 48–72 h, the transfected 293T cells were harvested from the culture dish, washed in panning solution (PBS (pH 7.4) 10 mM EDTA, 2% FCS), and incubated with primary STOK9 mAb. After incubation for 60 min at 4°C, mAb-labeled cells were washed three times with panning solution and incubated at RT for 90 min in Falcon 1001 dishes that had been precoated with rabbit anti-rat Ig followed by BSA (100 μg/ml). Pans were washed three times, and adherent cells were lysed (1 ml/dish) using 2× PBS, 0.1% SDS, 10 mM EDTA. After lysis for 30 min at RT, NaCl was added to a final concentration of 1 M, and the reaction mixture was precipitated overnight at 4°C. After centrifugation, the Hirt supernatant was subjected to phenol/chloroform extraction, chloroform extraction, and precipitation. The recovered sublibrary episomes were transfected in ElectroMAX DH10B E. coli (Life Technologies) and amplified on 160-mm Luria-Bertani-ampicillin plates as described above. The cDNA was purified on Qiagen Tip-500 columns. This primary sublibrary was subjected to two additional rounds of immunoenrichment and Hirt recovery. The tertiary sublibrary induced the expression of KLRH1 on 15–20% of 293T cells after transient transfection. Eight individual bacterial clones from the tertiary sublibrary induced the surface expression of KLRH1 on 293T cells. Both strands of these eight clones were sequenced and analyzed. Approved Klrh1 gene symbol was obtained from the Human Genome Organization Gene Nomenclature Committee. Further details are available at http://www.gene.ucl.ac.uk/nomenclature.

Stable transfection of the RNK-16 NK cell line

RNK-16, a spontaneous NK cell leukemia from F344 rats, was from C. Reynolds (National Cancer Institute, Frederick, MD) and was adapted for in vitro growth in cRPML. RNK-16 cells were transfected as previously described (13). In brief, a full-length KLRH1 cDNA was subcloned into the XhoI site of the vector EMVC-SRAs with an internal ribosomal re-entry site and twice purified over Qiagen Tip-500 columns. After linearization with ScaI, 20 μg of expression construct was electroporated into RNK-16 cells in 2-mm cuvettes in cRPML at 120 V, 850 μF, and 129 μA using a BTX ElectroCell Manipulator (San Diego, CA). After 24 h, cells were plated in cRPML supplemented with 1 mg/ml G418 in 96-well plates at 10^4 cells/ml. After 14–17 days, G418-resistant cells were subcoloned and analyzed for KLRH1 expression by flow cytometry.

Production of a cDNA expression library from KLRH1 NK cells

Messenger RNA was purified from a culture of KLRH1 NK cells (10^7 cells) from PVG rats using the Invitrogen Fast Track mRNA isolation protocol (Invitrogen, San Diego, CA). The cDNA was synthesized from template mRNA in the presence of methyl-ICTP by using a poly dT primer containing a 3′-XhoI site using the Stratagene ZAP-Express cDNA library synthesis kit (Stratagene, La Jolla, CA). After blunt-end ligation of precut EcoRI adapters, the [methyl-dCTP]-cDNA was digested with XhoI. Digested cDNA was separated from unligated adapters and short oligonucleotide cleavage products using Sephadex G25 column chromatography and directionally ligated into the EcoRI (5′) and XhoI (3′) sites of the pME7 Eukaryotic expression vector (provided by L. Lanier, DNAX, Palo Alto, CA). After electroporation into supercompetent BRL ElectroMAX DH10B Escherichia coli (Life Technologies), the resultant library was found to have a complexity of 550,000 colonies with >97% of colonies containing cDNA inserts. The library was amplified on 160-mm Luria-Bertani-ampicillin plates (11 in total) at a density of 50,000 PFU/plate, bacterial colonies were harvested by scraping, and plasmids were isolated using Qiagen TIP-500 columns according to the manufacturer’s instructions (Qiagen, Chatsworth, CA).
Gels were run in a Rotaphor R22 electrophoresis chamber (Biometa, Göttingen, Germany), with microprocessor-controlled cooling and ramping of voltage, switch time, and field angle. DNA was transferred to a nylon membrane by exposing the gel to 302 nm of UV light for 45 s, denaturing in 0.5 M NaOH, 1.5 M NaCl, and neutralizing in 3 M sodium acetate (pH 5.5), followed by capillary transfer in 20× SSC buffer for 24 h before hybridization, which was performed as for ordinary Southern blots.

Results

Generation of the anti-KLRH1 hybridoma STOK9

Except for the use of Ly49i2 (STOK2)-depleted NK cells for immunization, the immunization strategy for generation of the anti-KLRH1 mAb STOK9 was the same as for mAb STOK2 (9). In short, NK alloreactivity-deficient DA rats, which are low in Ly49 transcripts (14), were immunized with alloreactive Ly49i2-negative NK cells from MHC-matched PVG.1AV1 rats. Spleen cells from immunized rats were fused with NS0 myeloma cells, and hybridomas were screened by indirect immunofluorescence and flow cytometry for patterns of NK staining different from mAb STOK2. One hybridoma, STOK9 (IgG2c, Igκ isotype), labeled a smaller proportion of NK cells than the anti-Ly49i2 mAb STOK2 and was shown to react with the KLRH1 molecule (see below). In the experiment shown in Fig. 1A, 24% and 33% of IL-2-activated NK cells from PVG.1AV1 were KLRH1⁺ (STOK9⁺) and Ly49i2⁺, respectively. Like Ly49i2 (9), relative numbers of KLRH1⁺ cells generally increased as a result of IL-2 culture (between 3- and 10-fold), as can be deduced from a comparison with freshly isolated NK cells (see below). Two-color analyses showed that mAbs STOK9 and STOK2 defined two distinct, but overlapping NK subpopulations. Roughly one-half of the KLRH1⁺ IL-2-activated NK cells in PVG.1AV1 rats were Ly49i2⁺, whereas about one-third of the Ly49i2⁺ population were KLRH1⁺ (Fig. 1B). Less than 1% of cervical LN cells stained with mAb STOK9 (Fig. 1C), indicating that expression of KLRH1 is limited to NK cells and not to the majority of conventional T and B lymphocytes.

The anti-KLRH1 mAb STOK9 labels small subsets of NKR-P1⁺ NK cells and T lymphocytes from certain high NK-alloresponder rat strains

Freshly isolated splenocytes from PVG.1AV1 rats were depleted of Ig⁺ cells and stained with mAb STOK9, in combination with mAb 3.2.3 (anti-NKR-P1) and G4.18 (anti-CD3). As shown in Fig. 2, KLRH1 expression was confined to small subpopulations of NKR-P1⁺ NK cells and T lymphocytes, but not to NKR-P1⁻ T cells. Thus, although 3.2 and 1.9% of the respective CD3⁺ NKR-P1⁺ and CD3⁺NKR-P1⁻ populations costained with the anti-KLRH1 mAb STOK9, CD3⁺NKR-P1⁺ cells were negative. Similarly, in the panel of inbred rat strains tested, only NK cells from PVG, AO, and WAG stained positively for KLRH1, whereas DA, F344, LEW, BN, and BUF were all negative (Table I). Interestingly, this distribution was similar to that previously observed for Ly49i2 (9), showing that, like Ly49i2, KLRH1 was only expressed on NK cells from rat strains with a broad NK allorecognition repertoire (7, 8).

Biochemical characterization of the KLRH1 molecule as a homodimeric membrane glycoprotein

KLRH1⁺ NK cells were surface-biotinylated and lysed with Triton X-100. Cell lysates were subjected to immunoprecipitation with mAb STOK9 or the M1/75 isotype-matched control followed by SDS-PAGE, transfer to polyvinylidene difluoride membrane, and development of the Western blot with streptavidin-HRP and ECL reagents. The mAb STOK9 precipitated bands migrating at ~35 and 75 kDa under reducing and nonreducing conditions, respectively (Fig. 3A), whereas there were no bands in the control (data not shown). The 35-kDa band was reduced to ~30 kDa after PNGase F treatment, suggesting that KLRH1 contains N-linked glycosylation sites. A small decrease in size was also observed by

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Reactivity of the anti-KLRH1 mAb STOK9 with minor subsets of NK cells (CD3⁺ NKR-P1⁺) and NKR-P1⁻ T cells (CD3⁺ NKR-P1⁻), but not other T cells (CD3⁺ NKR-P1⁻). Freshly isolated splenocytes, depleted of Ig⁺ cells, from PVG.1AV1 rats were analyzed by three-color flow cytometry. One representative experiment is shown.

<table>
<thead>
<tr>
<th>Strain</th>
<th>CD3⁺ NKR-P1⁺</th>
<th>CD3⁺ NKR-P1⁻</th>
<th>CD3⁺ NKR-P1⁻</th>
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<tbody>
<tr>
<td>PVG</td>
<td>1.9</td>
<td>1.9</td>
<td>0</td>
</tr>
<tr>
<td>AO</td>
<td>1.4</td>
<td>2.1</td>
<td>0</td>
</tr>
<tr>
<td>WAG</td>
<td>0.7</td>
<td>2.9</td>
<td>0</td>
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<tr>
<td>DA</td>
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<td>F344</td>
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*Mononuclear spleen cells, depleted of Ig⁺ cells, were analyzed by three-color flow cytometry for the percentage of KLRH1⁺ cells. Values represent medians of two to four individual observations.*
treatment with neuraminidase, indicating the presence of terminal sialic acids, whereas gel migration was not affected by treatment with O-glycosidase (Fig. 3B). Taken together, these data show that the KLRH1 exists as a dimeric membrane glycoprotein in IL-2-activated NK cells.

A gene constituting a novel KLR branch encodes the KLRH1 receptor

A cDNA library from KLRH1+/PVG NK cells was screened for the induction of transient surface expression of the KLRH1 molecule after transfection in 293T cells. Eight clones were selected for further analysis. Sequencing indicated that they were all from the same gene, with minor differences in their 3' polyadenylation site. One cDNA clone (sequence data available from GenBank/EMBL/DDBJ under accession no. AF416564) was 863 bp long and contained an open reading frame encoding a polypeptide of 231 aa (Fig. 4A) with a predicted m.w. of 26.6. Transient expression in 293T cells confirmed that mAb STOK9 bound the protein product of the KLRH1 cDNA as determined by flow cytometry (Fig. 4B). An alternative start codon is present 5 codons downstream of the first ATG. The amino-terminal methionine of the short variant has been marked with an asterisk in Fig. 4A. The flanking sequences of both ATGs are in accordance with the requirements for eukaryotic initiation codons (15). Notably, one of the eight selected cDNA clones lacked the first ATG, likely due to early termination of the first strand reaction during reverse transcription, but still led to the expression of the KLRH1 after transfection in 293T cells, suggesting at a minimum that the second ATG is functional.

The predicted KLRH1 molecule is a type II integral transmembrane protein consisting of a cytoplasmic domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) (LTYAEL; conserved amino acid underlined), a transmembrane region lacking a charged amino acid, a stalk region, and a C-terminal domain with homology to C-type lectins. Sequence comparison demonstrated that it is closely related to the KLR family of receptors, constituting a novel branch with 35% amino acid identity to other KLR families. The new branch has been termed KLRH and the STOK9 Ag has been designated KLRH1 (approved Klrh1 gene symbol was obtained from the Human Genome Organization Gene Nomenclature Committee). A sequence alignment analysis showed that KLRH1 is somewhat more related to the Ly49/
KLRA multigene family than to the others (Fig. 4C). In common with other KLRs, the lectin-like domain of KLRH1 lacks the motifs required for Ca\(^{2+}\) binding and shows little sequence similarity to the ligand-binding loops of classical C-type lectins.

The presence of an ITIM in the cytoplasmic domain of KLRH1 suggested that it might perform inhibitory functions (16, 17). A classical method to define NK inhibitory function is the redirected inhibition assay, wherein receptor-specific inhibitory effects can be mimicked by Ab-mediated cross-linking of an inhibitory receptor and the use of FcR\(^{+}\) target cells in cytotoxicity assays. We tested the ability of KLRH1 in redirected inhibition experiments against FcR-bearing P815 and P388D1 targets using KLRH1\(^{+}\) IL-2-activated NK cells or KLRH1 transfectants of the NK line RNK-16 as effectors. As shown in Fig. 5A, RNK-16 cells stably transfected with the KLRH1 cDNA expressed high levels of KLRH1. However, the addition of mAb STOK9 had no inhibitory effect on cytotoxicity against the susceptible target P388D1 compared with the isotype-matched control mAb M1/75. In addition, there was no augmentation of cytotoxicity against the NK-resistant line P815 (Fig. 5B).

Similar results were obtained using KLRH1\(^{+}\) IL-2-activated NK cells, i.e., there was no inhibition or augmentation of lysis after the addition of mAb STOK9 (data not shown). Thus, the results of these redirected inhibition experiments failed to provide any conclusive evidence regarding the signaling of the KLRH1 molecule.

The Klrh1 gene is localized in the central portion of the NKC on rat chromosome 4

We have previously mapped expression of the Ly49i2 receptor to the NKC on rat chromosome 4 by genetic linkage with NKR-P1 (9). We performed a similar backcross expression analysis of KLRH1 using the Ly49i2\(^{+}\) KLRH1\(^{+}\) PVG rat strain and the Ly49i2\(^{-}\) KLRH1\(^{-}\) DA rat strain. (DA \(\times\) PVG)F\(_1\) animals, which possessed Ly49i2\(^{+}\) and KLRH1\(^{+}\) NK cells, were backcrossed to DA rats. Peritoneal NK cells (8) from the (DA \(\times\) PVG)F\(_1\) \(\times\) DA offspring were typed for expression of Ly49i2 and KLRH1. Of the 13 backcrossed rats tested, six animals possessed both Ly49i2\(^{+}\) and KLRH1\(^{+}\) NK cells, whereas seven lacked both subsets (data not shown). Thus, in our backcross analysis, independent assortment of expression of Ly49i2 and KLRH1 did not occur in meiosis (\(p < 0.01\) by \(\chi^2\)), implicating that these two phenotypes inherited as single autosomal dominant traits that were genetically linked.

The Klrh1 locus was further mapped to the centromeric region of the NKC by an RFLP linkage analysis of a panel of 223 (DA \(\times\) PVG)F\(_1\) animals, which we have previously typed for several loci in the NKC and the surrounding chromosomal region (14). Bsal digestion of genomic DNA and hybridization with a full-length KLRH1 cDNA probe yielded two unique bands (4.7 and 3.2 kb) in the PVG strain. The Klrh1 locus showed complete cosegregation with Nkrp1b, Cd94, Nkrp2, Nkg2, as well as some Ly49 RFLP markers, but was separated from the telomeric part of the Ly49 cluster by a single crossover event (results schematized in Fig. 6). Cohybridization analysis on genomic pulsed-field electrophoresis Southern blots indicated that Klrh1 resides in the central part of the NKC, either on the centromeric side of Cd94 (toward the Nkrp1 cluster) or between Nkg2 and the centromeric end of the Ly49 cluster (data not shown).

Selection of the KLRH1\(^{+}\) NK subset is influenced by MHC haplotype: control by the class II-nonclassical class I (RT1-B/D-C/E/M) regions

The localization of the Klrh1 gene to the NK complex near to known, structurally similar NK alloreceivers suggested that KLRH1 might recognize MHC-I-like cellular ligands. Polymorphic MHC-I molecules function as ligands for the Ly49 family of receptors and also markedly influence their repertoire selection in vivo. In mice, extensive studies have shown that the cell surface expression of Ly49 molecules is reduced in the presence of cognate ligands (18, 19). In the rat, this has been shown for the Ly49i2 receptor. Ly49i2 levels on the surface of individual NK cells are 3- to 5-fold lower in the presence of its classical class I ligand RT1-A\(^{1}\) (10). In addition, relative numbers of Ly49i2\(^{+}\) NK cells vary considerably, being 5-fold higher in avl and lv1 than in u MHC haplotype rats, possibly due to the presence of additional Ly49i2 ligands encoded by the nonclassical class I RT1-C/E/M region (10). Comparable results were obtained for KLRH1 using age- and sex-matched rats from a panel of MHC congenic strains. As can be seen from Table II, only 1.1% of CD3\(^{+}\) NKR-P1\(^{+}\) NK cells in u (PVG.1U) rats were KLRH1\(^{+}\), whereas 2.8 and 2.5%, respectively, were KLRH1\(^{+}\) in avl (PVG.1AV1) and lv1 (PVG.1LV1) rats. Intermediate values were observed in two other haplotypes, c (PVG) and n (PVG.1N). Thus, it appears that the relative number of KLRH1\(^{+}\) NK cells is reduced in the presence of a u-encoded ligand or, alternatively, increased in the presence of avl- or lv1-encoded ligands. Unlike Ly49i2 (10), no influence of MHC was observed on the expression levels of KLRH1 on the surface of NK cells (data not shown). Also, there was no clear evidence for an MHC influence on KLRH1 expression by NKR-P1\(^{+}\) T cells (data not shown). In our analysis of avl vs u intra-MHC recombinant rat strains, ~3% of the NK cells were KLRH1\(^{+}\) in PVG.1AV1 (RT1-A\(^{u}\)-B\(^{c}\)-D\(^{u}\)-C/E/M\(^{avl}\) or a-a-avl) and PVG.1AV255 (a-a-avl), whereas only ~1% of NK cells were KLRH1\(^{+}\) in PVG.R8 (a-a-u) and PVG.1U (u-u-u) (Table II). Thus, the gene(s) that influences relative number of KLRH1\(^{+}\) NK cells was mapped to the telomeric...
region of the rat MHC. This corresponded with the class II (RT1-B/D) and nonclassical class I (RT1-C/E/M) regions, but not the classical class I region (RT1-A).

These data indicated that MHC selection of KLRH1 resembled that of the MHC-binding Ly49d2 receptor (10). Therefore, we investigated the coselection of these two receptors on the same NK cells. The data in Table III show an expression of the Ly49d2 receptor on KLRH1+ NK cells that is on the order of 2- to 6-fold over that predicted by random assortment alone. Over-representation was most pronounced in a haplotype rats and in the PVG.R8 intra-MHC recombinant (a-u-u), again mapping the selecting elements to the class II/nonclassical class I region. Based on these data and on previously published observations of the importance of the nonclassical RT1-C/E/M region in NK alloreactivity, it seems reasonable to speculate that an RT1-C/E/M class I molecule(s) is involved in the selection of both the KLRH1 and Ly49i2 receptors during NK development. Alternatively, expression of these two receptors may be under the influence of a common regulatory mechanism.

KLRH1+ NK cells display a broad allorecognition repertoire

Like Ly49d2+ NK cells, we speculated that KLRH1+ NK cells might exhibit broad specificities for allogeneic rat target cells. Alloreactivity of the KLRH1+ subset was tested against Con A-activated lymphoblast targets from a panel of MHC-congenic rat strains. KLRH1+ NK cells from PVG (c haplotype) rats efficiently killed targets from all allogeneic strains tested, including PVG.1AV1 (av1), PVG.1LV1 (lv1), PVG.1U (u), PVG.1N (n), and PVG.1L (l), whereas syngeneic PVG control targets were spared (Fig. 7A and data not shown). The addition of purified anti-KLRH1 mAb STOK9 had no effect on cytotoxicity, which was neither augmented nor reduced when compared with cytotoxicity in the presence of control M1/75 Ab (Fig. 7A). To exclude that KLRH1 might function as an inhibitory receptor against a c haplotype-encoded (self) ligand, we tested the killing of PVG (c haplotype) targets by allogeneic KLRH1+ PVG.1U effectors (u haplotype). In this experiment, the PVG.1U effectors were depleted of Ly49i2+ cells before testing because the Ly49i2 receptor functions as an inhibitory receptor for the classical MHC-I molecule RT1-A1c and could thereby prevent killing of PVG targets irrespective of the effects of KLRH1. Depletion of Ly49i2+ cells had no effect on cytotoxicity by KLRH1+ NK cells (data not shown). KLRH1+ Ly49i2+ NK cells from PVG.1U (u) lysed both PVG (c) and PVG.1AV1 (av1) allotargets, but spared syngeneic PVG.1U control targets, and the addition of mAb STOK9 had no effect on the level of killing (Fig. 7B). Thus, despite the structural similarities between KLRH1 and known NK alloreceptors and despite the MHC-dependent regulation of KLRH1 expression by NK cells, these data failed to demonstrate a role for KLRH1 in the NK cell recognition of allogeneic targets.

Identification of a putative mouse homolog of Klrh1

Initial attempts to clone the mouse Klrh1 homolog by low-stringency homology screening of a high-quality cDNA library from C57BL/6 NK cells using a full-length radiolabeled KLRH1 probe yielded no cross-hybridizing cDNA clones. Within the mouse genome, however,

Table II. Influence of MHC haplotype on the development of KLRH1+ cells: control by the class II/nonclassical class I regions (RT1-B/D-C/E/M)

<table>
<thead>
<tr>
<th>Strain</th>
<th>RT1 Regions</th>
<th>KLRH1+ Cells (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B/D</td>
<td>C/E/M</td>
</tr>
<tr>
<td>PVG</td>
<td>c</td>
<td>c</td>
<td>1.9 (1.8-2.0)</td>
</tr>
<tr>
<td>PVG.1N</td>
<td>n</td>
<td>n</td>
<td>1.5 (1.2-1.9)</td>
</tr>
<tr>
<td>PVG.1LV1</td>
<td>l</td>
<td>l</td>
<td>2.5 (2.2-2.8)</td>
</tr>
<tr>
<td>PVG.1AV1</td>
<td>a</td>
<td>a</td>
<td>2.8 (2.2-2.9)</td>
</tr>
<tr>
<td>PVG.R23</td>
<td>u</td>
<td>a</td>
<td>3.5 (2.7-3.7)</td>
</tr>
<tr>
<td>PVG.R8</td>
<td>a</td>
<td>u</td>
<td>1.1 (1.1-1.2)</td>
</tr>
<tr>
<td>PVG.1U</td>
<td>u</td>
<td>u</td>
<td>1.1 (0.7-1.3)</td>
</tr>
</tbody>
</table>

* Mononuclear spleen cells, depleted of Ig+ cells, from a panel of MHC congenic and intra-MHC recombinant strains were analyzed by three-color flow cytometry for the percentage of KLRH1+ cells among CD3+ NK-P1+ NK cells. Spleens were obtained from age- and sex-matched 2-mo-old male rats. Values represent medians of two to three individual animals analyzed.

Statistically different (p < 0.01) from PVG.1U, PVG.R8, PVG.1N, or PVG (p = 0.02 between PVG.1AV1 and PVG) with a two-sample t test (two-sided). No statistical difference was observed against PVG.1LV1 or between PVG.1AV1 and PVG.R23.

Statistically different (p < 0.01) from PVG.1AV1, PVG.R23, PVG.1LV1, or PVG with a two-sample t test (two-sided). No statistical difference was observed against PVG.1N or between PVG.1U and PVG.R8.

Table III. Relative over-representation of the inhibitory Ly49 receptor Ly49d2 in the KLRH1+ NK subset

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ly49d2+ Cells in Unselected NK Cells (%)</th>
<th>Ly49d2+ Cells in the KLRH1+ NK Subset (%)</th>
<th>Relative Ly49d2 Overexpression by KLRH1+ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVG</td>
<td>7.0</td>
<td>17.7</td>
<td>2.5</td>
</tr>
<tr>
<td>PVG.1LV1</td>
<td>10.9</td>
<td>31.1</td>
<td>2.9</td>
</tr>
<tr>
<td>PVG.1AV1</td>
<td>11.1</td>
<td>33.2</td>
<td>3.0</td>
</tr>
<tr>
<td>PVG.R23</td>
<td>12.5</td>
<td>30.0</td>
<td>2.4</td>
</tr>
<tr>
<td>PVG.R8</td>
<td>3.3</td>
<td>20.7</td>
<td>6.3</td>
</tr>
<tr>
<td>PVG.1U</td>
<td>4.2</td>
<td>26.9</td>
<td>6.4</td>
</tr>
</tbody>
</table>

* Mononuclear spleen cells, gated for NKr-P1hi cells, from a panel of MHC congenic and intra-MHC recombinant strains were analyzed by three-color flow cytometry for the percentage of KLRH1+ cells. Values represent medians of two to three individual animals analyzed.

Figure 7. The KLRH1+ NK subset lysed Con A blast targets from a broad range of MHC haplotypes, with no blocking effect of mAb STOK9. A, Alloreactivity of KLRH1+ NK cells from PVG (RT1+) or c rats against allotargets from PVG.1U (u), PVG.1AV1 (av1), and PVG.1LV1 (lv1), as well as against PVG control targets in the presence of mAb STOK9 (●) or the M1/75 isotype–matched control mAb (●). B, Alloreactivity of KLRH1+ Ly49i2+ NK cells from PVG.1U rats against PVG.1AV1, PVG, and PVG.1U in the presence of mAb STOK9 (●) or the M1/75 control (●).
many matching genomic trace sequences were obtained at either NCBI/EBI (sequences derived from the C57BL/6 strain) or Celera (DBA/2J, A/J, and 129 × 1/SvJ strains). Homologies distributed into seven distinct parts that covered the whole length of the KLRH1 cDNA, likely corresponding to the individual exons of the mouse gene. These sequences as well as those from the intervening introns were assembled and analyzed with the Wisconsin Genetics Computer Group program package (Madison, WI), with the resulting exon-intron organization of the mouse gene depicted in Fig. 8A. Three different allelic variants were discernable in the C57BL/6, 129 × 1/SvJ, DBA/2J, and A/J strains, of which DBA/2J and A/J were nearly identical. The putative exon-intron junctional sequences used to construct a hypothetical cDNA molecule in the C57BL/6 strain are shown in Fig. 8B and were partially confirmed by the few rat trace sequences available at NCBI/EBI. The hypothetical C57BL/6 cDNA contained an open reading frame that encoded a shorter protein of 223 aa that showed 76% identity with the rat protein (Fig. 4A). The 129 × 1/SvJ and DBA/2J, A/J variants differed by only a few single amino acid substitutions (Fig. 4A). As mentioned above, the first start codon present in rat KIRh1 was lacking in the mouse gene. This corresponded with the last 3 nt in exon 1, being ACT in the mouse (Fig. 8B) and ATG in the rat (data not shown). Despite the apparent presence of Klrh1 in the mouse genome, we failed to clone it by homology screening in the C57BL/6 strain. However, this may reflect strain-specific expression patterns for mouse KLRH1, in line with the expression data for KLRH1 in the rat (Table I), because the expression patterns for mouse KLRH1, in line with the expression pattern of P2X7 in human skin keratinocytes, may be a pseudogene or a nonfunctional pseudogene.

It should be noted that we obtained evidence for two distinct genes in the mouse with homology to rat Klrh1, one of which we have described above and that has been tentatively termed mouse Klrh1. The second gene, which we have tentatively termed mouse Klrh2, contained sequences that showed close homology with exons 1, 2, 3, and 6 in that order, but that apparently lacked exons 4, 5, and 7. It is not possible to conclude whether Klrh2 may be a pseudogene or whether distant relatives without significant sequence homology have substituted the missing exons. In any event, the notion that there are two distinct Klrh genes in the mouse genome is supported by the finding of one large Celera contig (GA_x58877W59GN) containing both variants of the gene ~10–20 kb apart. On this contig, mouse Klrh1 and Klrh2 were situated between a Ly49 gene and Nkg2c, in accordance with the localization in the rat (Fig. 6). It is also possible that there is a Klrh1 homolog in the human. We obtained significant homology between exon 5 and a contig from human chromosome 12 (AC068775), but because we could not identify homologies with the other six exons, this conclusion remains uncertain.

**Discussion**

The mammalian nonadaptive immune system employs a number of different strategies to identify foreign or altered cells, but recognition of MHC-I molecules is often essential. In the current study, we attempted to identify novel receptors involved in the recognition of allogeenic targets by exploiting differences between the two rat strains PVG and DA. We immunized the poorly alloreactive DA rat with NK cells from the highly alloreactive, MHC-matched PVG.1AV1 rat strain. The mAb STOK9, generated in this way, reacted with a small subpopulation of NKR-P1− T cells and NK cells (1–4%). After expansion in IL-2, ~5–30% of the NK cells expressed the KLRH1 molecule, suggesting either that KLRH1+ NK cells have a selective growth advantage in IL-2 or that KLRH1 expression can be induced on KLRH1-negative NK cells after activation. The last possibility was countered by the finding that freshly isolated KLRH1-depleted NK cells and T cells failed to express KLRH1 even after 10 days in IL-2-culture (data not shown). In addition, neither KLRH1 nor NKR-P1 expression could be induced on NKR-P1-depleted T cells after mitogen stimulation for 3 days in vitro with Con A (data not shown).

Expression cloning of the KLRH1 molecule showed it to be a type II protein containing an extracellular domain with homology to C-type lectins. In its cytoplasmic domain, KLRH1 contains a putative ITIM (LT[AYAEI]; conserved amino acid is underlined), which predicts inhibitory receptor function. The predicted KLRH1 peptide sequence is most closely related to the Ly49/KLRA receptor family, although it shows ~5% amino acid identity with all known KL/R receptor families, thus classifying it as a novel branch of the lectin receptor superfamily (Fig. 4).

It is likely that KLRH1 is encoded by a single gene with low homology to other genes because 1) hybridization of the KLRH1 probe with Southern blots under high stringency gave simple band patterns with one to three bands, depending on the restriction enzyme used (data not shown), 2) the KLRH1 expression cloning yielded eight bacterial clones predicting identical coding regions, and 3) homology screening using the cloned KLRH1 cDNA as a probe on rat NK cDNA libraries yielded 10 essentially identical cDNAs from PVG rats, but no cross-hybridizing clones from the KLRH1− F344 strain. This latter finding suggested that KLRH1-negative rat strains might fail to express KLRH1 mRNA transcripts, a finding that is supported by studies of the KLRH1− DA rat strain. A cross-hybridizing Klrh gene is present in the genome of DA rats, but DA NK cells express no KLRH1 mRNA transcripts by Northern blot analysis. Thus, the deficiency in cell surface KLRH1 expression in this strain is not due to a genomic deletion. Rather, it is likely caused by strain-specific differences in transcriptional control or in other regulatory events.

The relative number of KLRH1+ ex vivo-isolated NK cells is influenced by the MHC haplotype, suggesting that this receptor might recognize a ligand encoded within the MHC. The gene(s) mediating this effect on the selection of KLRH1+ NK cells does not map to the classical MHC-I RTI-A region, but rather to the RTI-B/D region encoding MHC class II molecules or to the RTI-C/E/M region encoding nonclassical MHC-I-like molecules. Based on the importance of the RTI-C/E/M region in controlling alloreactive NK responses in the rat (20), it seems reasonable to speculate that the KLRH1 receptor might

**FIGURE 8.** Exon-intron organization of a putative mouse homolog of Klrh1. Individual genomic trace sequences from the C57BL/6 (NCBI/EBI), DBA/2J, A/J, and 129 × 1/SvJ strains (Celera) were assembled and analyzed with the Wisconsin Genetics Computer Group program package. A, The exon-intron organization of the mouse Klrh1 gene. The first exon in the mouse is silent due to ATG→ACT point mutations of the first ATG present in the rat cDNA sequence. The last 3 nt of exon 1 comprise the first start codon in the rat. B, Exon-intron junctional sequences in the C57BL/6 strain.
recognize a nonclassical class I-like protein encoded within this region. Despite extensive studies, however, we have been unable to directly identify a functionally relevant MHC-encoded ligand for KLRH1. We could discern no reproducible differences in the killing of MHC-disparate Con A lymphoblast targets by KLRH1<sup>+</sup> NK cells (Fig. 7) compared with unselected or KLRH1<sup>-</sup> NK cells (data not shown). The addition of blocking quantities of mAb STOK9 had no effect on Con A blast killing by KLRH1<sup>+</sup> NK cells from PVG or from PVG.1U rats. It should be noted that unlike classical RT1-A-encoded molecules, which are expressed at high levels on target cells, nonclassical RT1-C/E/M class I molecules are routinely expressed at very low levels on targets (21, 22). As such, the inhibitory effects of weakly expressed RT1-C/E/M-encoded ligands might be so minimal that they cannot be easily detected by the available in vitro cytotoxicity assays. Failure to identify KLRH1-specific effects in cytotoxicity assays might also result from at least three additional technical concerns: 1) possible contributions from redundant NK allorrecipients on KLRH1<sup>-</sup> cells that exert their effects despite mAb blockade of KLRH1, 2) inefficient inhibitory signaling through the KLRH1 receptor, or 3) inefficient mAb blockade of the KLRH1 receptor.

Attempts to examine inhibitory signals generated through the KLRH1 receptor were inconclusive. The cytoplasmic motif in the KLRH1 receptor (ITYAEI) meets the minimum criterion for a classical ITIM and is nearly identical with the C-terminal ITIM (ITYAEI) of NKGA2A, which has been demonstrated to bind directly to SHP-1 and SHP-2 in BlaRexA experiments (23). Despite the presence of a putative ITIM, however, we have not been able to demonstrate a physical association between KLRH1 and SHP-1 in lysates of peripandate-treated cells, and we have not been able to demonstrate an inhibitory function for KLRH1 in Ab-dependent redirected inhibition assays. However, we were able to show tyrosine phosphorylation of KLRH1 as a result of the peripandate stimulus, which suggested that the ITIM might be tyrosine phosphorylated in vivo. The negative results do not necessarily preclude inhibitory function, however, because some classical alloinhibitory killer cell Ig-like receptors fail to recruit SHP-1 in immunoprecipitation assays, and some Ly49 receptors fail to function in redirected inhibition assays (J. C. Ryan, unpublished observations) (24). The classical inhibitory allorrecceptor Ly49A fails to trigger Ab-dependent redirected inhibition in intact NK cells or in RNK-16 transfectants (J. C. Ryan, unpublished observations) (13). The inability of Ly49A to mediate redirected inhibition, however, because some classical alloinhibitory killer cell Ig-like receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. In conclusion, we have identified a novel rat NK cell surface molecule with a putative immunological function. Our experimental data so far have not allowed us to define the functions and physiologic ligands for this receptor, but we speculate that KLRH1 may be a candidate NK inhibitory receptor for nonclassical MHC-I-like molecules encoded by the RT1-C/E/M region in rats. The physiologic role of this receptor in the recognition mechanisms of NK cells still remains elusive and is the focus of further study.

References


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