Two Structurally Related Rat Ly49 Receptors with Opposing Functions (Ly49 Stimulatory Receptor 5 and Ly49 Inhibitory Receptor 5) Recognize Nonclassical MHC Class Ib-Encoded Target Ligands


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Two Structurally Related Rat Ly49 Receptors with Opposing Functions (Ly49 Stimulatory Receptor 5 and Ly49 Inhibitory Receptor 5) Recognize Nonclassical MHC Class Ib-Encoded Target Ligands

Christian Naper,* Ke-Zheng Dai,* Lise Kveberg,‡ Bent Rolstad,‡ Érène C. Niemi,† John T. Vaage,2* and James C. Ryan‡

The Ly49 family of lectin-like receptors in rodents includes both stimulatory and inhibitory members. Although NK alloreactivity in mice is regulated primarily by inhibitory Ly49 receptors, in rats activating Ly49 receptors are equally important. Previous studies have suggested that activating rat Ly49 receptors are triggered by polymorphic ligands encoded within the nonclassical class Ib region of the rat MHC, RT1-CE/N/M, while inhibitory Ly49 receptors bind to widely expressed classical class Ia molecules encoded from the RT1-A region. To further investigate rat Ly49-mediated regulation of NK alloreactivity, we report in this study the identification and characterization of two novel paired Ly49 receptors that we have termed Ly49 inhibitory receptor 5 (Ly49i5) and Ly49 stimulatory receptor 5 (Ly49s5). Using a new mAb (mAb Fly5), we showed that Ly49i5 is an inhibitory receptor that recognizes ligands encoded within the class Ib region of the u and l haplotypes, while the structurally related Ly49s5 is an activating receptor that recognizes class Ib ligands of the u haplotype. Ly49s5 is functionally expressed in the high NK-alloresponder PVG strain, but not in the low alloresponder BN strain, in which it is a pseudogene. Ly49s5 is hence not responsible for the striking anti-u NK alloresponse previously described in BN rats (haplotype u), which results from repeated alloimmunizations with u haplotype cells. The present studies support the notion of a complex regulation of rat NK alloreactivity by activating and inhibitory Ly49 receptors, which may be highly homologous in the extracellular region and bind similar class Ib-encoded target ligands. The Journal of Immunology, 2005, 174: 2702–2711.

Natural killer lymphocytes are capable of killing neoplastic, virally infected, or allogeneic target cells without prior sensitization. Although early studies suggested that NK cells were not MHC restricted, more recent studies have shown that NK cells possess a myriad of MHC-binding receptors that control NK cell effector functions. In the presence of self MHC class I ligands, target cells are protected from NK cytolysis, consistent with the missing self hypothesis; i.e., that target cells are killed if they fail to express a full array of self class I ligands (1). Inhibitory Ly49 receptors were the first class I-binding receptors to be characterized in mouse NK cells (2). In rats, functional genetic studies predicted the existence of activating NK receptors directed against nonclassical class Ib MHC alleles, in addition to inhibitory receptors for classical class Ia ligands (3, 4). Mapping of the NK alloreponder locus nka to the Ly49 part of the rat NK gene complex (NKC),3 suggested that Ly49 alloreceptors were involved (5). Rat NK cells can mount strong alloreponses in vivo that bear superficial resemblance to adaptive immune reactions. Repeated alloimmunizations of BN strain rats with fully MHC-mismatched u haplotype cells result in a striking i.p. accumulation of alloreactive NK cells (6–8). These NK cells display a much more potent anti-u cytolytic activity than those recruited to the peritoneal cavity by the cytokine IL-2 alone (9).

Subsequent studies in mice have identified Ly49 receptors that activate, rather than inhibit, NK cell functions (10). These include Ly49D and Ly49H (11, 12). Although Ly49D can recognize one class Ia allele (H-2Dd) of mouse MHC (13, 14), Ly49H fails to recognize any known mouse MHC molecule, but rather recognizes a class I-like homologue encoded within the genome of mouse CMV (mCMV) (15, 16). Although the Ly49H expression by mouse NK cells is clearly associated with enhanced survival after mCMV challenge, the physiologic roles of activating NK receptors for MHC class I have not been firmly established. Indeed, several strains of recombinant inbred mice have no activating Ly49 receptors, yet appear to be healthy (17). It has been speculated that, in mice, the activating Ly49 receptors predominantly screen for specific pathogens and that their recognition of allogeneic MHC is a chance evolutionary vestige (18). Indeed, in vivo studies in mice have shown that overriding effects of inhibitory receptors largely

1 Institute of Immunology, Rikshospitalet University Hospital, University of Oslo, Rikshospitalet, Oslo, Norway; ‡Veterans Affairs Medical Center, Northern California Institute for Research and Education, and University of California, San Francisco, CA 94121; and †Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

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Address correspondence and reprint requests to Dr. John T. Vaage, Institute of Immunology, Rikshospitalet University Hospital, NO-0027 Oslo, Norway. E-mail address: j.t.vaage@labmed.uio.no

3 Abbreviations used in this paper: NKC, NK gene complex; CHO, Chinese hamster ovary; cRPMI, complete RPMI; HCV, hepatitis C virus; KIR, killer cell Ig-like receptor; Ly49, Ly49 inhibitory receptor; Ly49s, Ly49 stimulatory receptor; mCMV, murine CMV; ORF, open reading frame.
overshadow NK-activating effects mediated by MHC-binding receptors (19). In contrast, studies in rats have provided ample evidence for stimulatory NK allorecognition (3, 20–22). These studies have suggested that rat NK cells express a potent array of activating receptors for several distinct target cell allodeterminants encoded within the class Ib region of the rat MHC, termed RT1-CE/N/M (3).

The RT1-CE/N/M region is located telomeric to the class II/III regions, in the same position as H2-D,L,Q/T/M in mouse, and it has recently been fully mapped and sequenced in the BN rat strain (23, 24). The whole region spans roughly 2 Mb and harbors several clusters of class I genes that are separated by framework genes conserved between mouse, rat, and human. The first or the most centromeric class Ib cluster, termed the RT1-CE cluster, harbors 16 class I genes in the BN strain, of which 13 may be functional (24). Analysis of an RT1-CE-deleted mutant rat strain (LEW.ILM1), which has lost ∼100 kb of chromosomal DNA from this cluster, indicated that it encodes target ligands triggering alloreactive rat NK cells (3, 25). This contention has received support from studies showing that target cells transfected with selected RT1-CE genes, i.e., RT1-C (LW2) and RT1-E, are rendered more sensitive to allospecific NK lysis (7, 26).

Previously, we have identified a variety of MHC-binding Ly49 receptors, including Ly49 inhibitory receptor 2 (Ly49i2), which inhibits NK killing of targets expressing the class Ia MHC ligand; RT1-A1 (27, 28); as well as the activating Ly49 stimulatory receptor 3 (Ly49s3) (29). Unlike the prototypical activating mouse Ly49D receptor from mice, which is specific for a solitary class Ia allele, the rat Ly49s3 receptor recognizes a broad array of nonclassical MHC-encoded determinants from rat strains of the c, av1, lv1, l, and n, but not of the u or b haplotypes (29) (our unpublished data). These data support the previous genetic data predicting that activating rat Ly49 receptors bind public class Ib epitopes that may be shared between selected RT1-I haplotypes (4). It is likely that the Ly49s3 ligand(s) is encoded from the first class Ib cluster, i.e., RT1-CE. To more fully analyze the phenomenon of NK allorecognition in rats, we have used a series of gene discovery techniques, including homology screening and complementation cloning, to identify Ly49i5 and Ly49s5, novel paired Ly49 receptors with opposing NK cell signaling functions and overlapping MHC class Ib ligand repertoires in rats.

Materials and Methods

Animals

Four to 6-wk-old female BALB/c mice and BALB/c nu/nu mice were from Simonsen Laboratories and were reared at the Veterans Affairs Medical Center under conventional conditions in accordance with institutional guidelines. The rat strains used and their MHC haplotypes are listed in Table I. The following strains were reared at the Institute of Basic Medical Sciences: PVG, PVG.R23, PVG.1N, PVG.1L, and PVG.1LV1. AO, DA, PVG.1AV, PVG.1U, and PVG.R8 rats were obtained from Harlan; WAG rats from Harlan Netherlands; and F344, LEW, and BN rats from Mollegaard. Rats were regularly screened for common pathogens and housed in compliance with guidelines set by the Experimental Animal Board under the Ministry of Agriculture of Norway. They were sacrificed at 8–16 wk of age.

Cloning of Ly49i5 cDNA

Ly49i5 was cloned using the ZAP-Express system (Stratagene) and a previously described cDNA library from KLRH1 NK cells (30), except that the primary cDNA was ligated into the ZAP-Express vector instead of into pME7. Complexity of the primary phage library comprised >2 × 106 clones. Ten plates, each containing 50,000 clones, were screened with a mixture of 12P-labeled cDNAs, rat Ly49i2 (28), rat Ly49i2 (formerly Ly49.12), and rat Ly49i1 (formerly Ly49.29) (5), and mouse Ly49H1 (12). Hybridization of nitrocellulose filters was performed overnight at 42°C in 6× SSC, 5× Denhart solution, 0.5% SDS, 200 μg/ml sonicated salmon sperm DNA, and 50% formamide. The filters were washed twice with 2× SSC, 0.1% SDS and twice with 0.5× SSC, 0.1% SDS, and films were developed after 3 days of exposure at ∼80°C. Cross-hybridizing plaques were subjected to additional rounds of screening, and the resultant clonal phages were converted into pBK-CMV phagemids by in vivo excision, according to the manufacturer’s instructions (Stratagene), and sequenced on both strands. One Ly49i5 clone was obtained that was fully sequenced on both strands.

Transfection of cells

Cell lines were routinely grown in complete RPMI (cRPMI; RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, and penicillin/streptomycin). Chinese hamster ovary (CHO) cells were from the American Type Culture Collection, and were transfected by electroporation in cRPMI supplemented with 1 mg/ml G418. Cell clones surviving selection were examined for transfection by a transcellular FLAG epitope (FLAG/DAP12) were a gift from L. Lanier (University of California, San Francisco, CA) and were transiently transfected using FuGene transfection reagent according to the manufacturer’s instructions (Roche).

Table I. MHC constitution of the rat strains used

<table>
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<tr>
<th>Strain</th>
<th>RT1 (Rat MHC) Haplotype</th>
<th>RT1 Regions</th>
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The Journal of Immunology

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Production of the mAb Fly5

A cDNA construct was engineered in the EMCV-SRa vector to express Ly49s as a fusion protein tagged with an extracellular FLAG epitope (Ly49s/FLAG). After stable transfection and selection in cRPMM with G418, CHO cells that stained with the anti-FLAG mAb M2 were sorted by flow cytometry and expanded. A total of 10^7 transfected cells was injected i.p. into 6-wk-old female BALB/c mice. After 3 biweekly immunizations, mice were boosted i.p. 72 h before fusion of splenocytes with SP2/0 myeloma cells using Hybrimax PEG/DMSO solution (Sigma-Aldrich) and tions, mice were boosted i.p. into 6-wk-old female BALB/c mice. After three biweekly immunizations, mice were boosted i.p. 72 h before fusion of splenocytes with SP2/0 myeloma cells using Hybrimax PEG/DMSO solution (Sigma-Aldrich) and 2704 MHC CLASS Ib RECOGNITION BY TWO PAIRED RAT Ly49 RECEPTORS

primary antibody (Goat anti-mouse FITC) preincubated with mAb Fly5-biotin. Selected Fly5
tions were screened by flow cytometry for the presence of anti-Ly49i5

Primary cells, flow cytometry, and cytotoxicity assay

Peritoneal cells from alloimmunized BN rats were retrieved as described (7). A total of 10^7 mononuclear splenocytes from PVG.1U rats, obtained by Lymphoprep centrifugation, was injected i.p. once weekly for 4 wk. The peritoneal cells were retrieved 3–4 days after the last immunization by injection of 30 ml of PBS i.p. and aspiration after gentle massage of the abdomen, followed by separation by Lymphoprep centrifugation.

Expression levels of Ly49i5 and Ly49s5 were determined by RT-PCR in FACSSorted NK subsets, as indicated. A total of 10^6 cells was lysed in 250 µl of bacterial growth from each clone was blotted onto nitrocellulose

Cloning of Ly49s5 cDNA

Ly49s5 cDNA was cloned by a variation of the complementation cloning approach previously described for Ly49s3 (29). In short, a monolayer of DT381 cells was transiently transfected with the Ly49s5 cDNA expression library (AT-3) clone, with the previously described method (31), by positive selection of mononuclear splenocytes with streptavidin-coated M280 magnetic Dynabeads (Dynal Biotech) preincubated with mAb Fly5-biotin. Selected Fly5
cultures were selected by a modification of a previously described method (31), by positive selection of mononuclear splenocytes with streptavidin-coated M280 magnetic Dynabeads (Dynal Biotech) preincubated with mAb Fly5-biotin. Selected Fly5
cell lines were lysed in Hirt solution (0.6% SDS, 10 mM EDTA), and NaCl was added to a final concentration of 1 M. The reaction mixture was precipitated overnight at 4°C and centrifuged at 15,000 × g for 30 min. The primary cDNA sublibrary, obtained from the Hirt supernatant, was amplified in ElectroMAX DH10B

Results

Molecular cloning of Ly49s5 cDNA by sequence homology screening

Previous studies have suggested that NK cells from the high NK allosreactive PVG rat strain express a broad panel of Ly49 receptors (5, 28, 29). To expand our knowledge of rat Ly49 receptors, we performed homology screening using a cDNA library generated from a PVG NK cell subset (KLRH1+) with heightened allogeneic reactivity (30). Hybridization was performed at low stringency using a mixture of broadly divergent Ly49 genes from the rat and mouse. These included our previously published rat genes Ly49i2, Ly49s1, and Ly49s2, and the mouse NK receptor for mCMV-infected cells, Ly49H. Among other receptors, we identified a novel inhibitory Ly49 molecule that we have termed Ly49i5. This receptor is ~80% identical with Ly49i2 at the amino acid level (90% at the cDNA level in the open reading frame (ORF)) (Fig. 1A and data not shown). Identity is somewhat lower for Ly49s3, at the amino acid level being 70% (but is more homologous at the cDNA level; 86% in the ORF). In the extracellular region corresponding to the stalk (exons 4) and the lectin-like domain (exons 5–7), Ly49i5 and Ly49i2 or Ly49s3 are 76 and 75% identical, respectively. Hence, Ly49i5 and Ly49s3 differ mostly in their primary cDNA library, obtained from the Hirt supernatant, was amplified in ElectroMAX DH10B

Cloning of DT381 cells were transfected with the Ly49s5 cDNA expression library referred to above in the pMET7 vector (30). After 2 days, cells were stimulated with mAb Fly5, washed, and panned on petri dishes precoated with rabbit anti-mouse IgG (Valeant Pharmaceuticals/Cappel). Adherent cells were lysed in Hirt solution (0.6% SDS, 10 mM EDTA), and NaCl was added to a final concentration of 1 M. The reaction mixture was precipitated

staining on DT381 cells, were obtained by screening of 480 individual bacterial colonies by dot blotting with a Ly49s5 cDNA probe. A total of 50 µl of bacterial growth from each clone was blotted onto nitrocellulose filters, lysed, and DNA denatured using 0.4 M NaOH for 10 min. The filters were neutralized for 10 min in 6× SSC, 0.5 M Tris-HCl (pH 7), washed for 10 min with 6× SSC, baked at 80°C for 2 h, and probed with a 32P-labeled full-length Ly49s5 probe at 42°C for 1 h in 6× SSC, 5× Denhart solution, 0.5% SDS, 200 µg/ml sonicated salmon sperm DNA, and 50% Formamide. Filters were washed with 0.1% SSC, 1% SDS, and exposed to films.

RT-PCR

Expression levels of Ly49i5 and Ly49s5 were determined by RT-PCR in FACSsorted NK subsets, as indicated. A total of 10^6 cells was lysed in 250 µl of TRIREAGENT (Sigma-Aldrich) and supplemented with 5 µg of carrier RNA. RT-PCR were performed from total RNA, according to the manufacturer’s recommendations (Sigma-Aldrich). The following upper and lower primers, respectively, were used: Ly49i5 (PVG and BN), 5′-TTCCTGCAATTCCTCTTGAG-3′ and 5′-GACCCTATCCTCTCTATCTCG-3′; Ly49s5 (PVG), 5′-CCGGGAAAGTCTAAGCTCT-3′ and 5′-GAAGCGCATGCTGGAAG-3′. Primers for rat CD45 were included as a control. Typically, PCR were performed on a GeneAmp PCR thermocycler (Applied Biosystems) using hot start for 3 min at 90°C. Pyrococcus woesei DNA polymerase was added at 80°C before running for 35 cycles at 95°C, 20 s; 54°C, 30 s; and 72°C, 30 s. PCR products were resolved by agarose gel electrophoresis (2% Tris acetate EDTA buffer).

The coding region of the Ly49i5 and Ly49s5 alleles in BN strain rats was identified by RT-PCR with the following upper and lower primers: Ly49i5, 5′-TCGG CAT CGG AGA GAC-3′ and 5′-CCT GTG GAC CTC ATC TCT CT-3′; Ly49s5, 5′-AGA CAC AGA AAA CAC TCA AT-3′ and 5′-ATC GAG TTC TCC ATG TGG TC-3′. PCR conditions were as above, except that the extension time was 90 s at 72°C. The PCR products were cloned into a plasmid vector (pCR2.1-TOPO, TOPO- TA cloning system; Invitrogen Life Technologies) and transfected into TOP10 E. coli by electroporation. Single bacterial colonies were selected by blue/ white screening, followed by PCR with vector-specific primers. Five positive clones of the BN alleles of Ly49i5 and Ly49s5 (a pseudogene) were fully sequenced on both strands.
mAb Fly5 staining of NK and NKT cells: marked coexpression with the Ly49i2 and Ly49s3 receptors in distinct subsets of NK cells

As shown in Fig. 2A, mAb Fly5 stained a small, but distinct population (2–3%) of mononuclear splenocytes from PVG strain rats, while reactivity with mesenteric lymph node cells and thymocytes was close to background, suggesting that it does not react with the vast majority of T and B lymphocytes. To further evaluate its reactivity pattern, we analyzed freshly isolated splenocytes by three-color flow cytometry. As shown in Fig. 2B, Fly5 reacted with subsets of CD3⁺ NKR-P1A/B⁺ NKT cells and CD3⁺ NKR-P1A/B⁻ T cells (hereafter referred to as NKT cells), but not with the major population of CD3⁺ NKR-P1A/B⁻ T cells in PVG.1L and LEW strain rats. This reaction pattern mirrored previous results for Ly49i2 (31), as well as for the distantly related KLRH1 inhibitory receptor (30).

As can be deduced from Table II, mAb Fly5 reacted with a minor subset of NK cells in all rat strains tested, but there were strain-specific differences in the number of Fly5⁺ cells. The percentage of mAb Fly5⁺ NK cells in PVG, F344, and LEW was higher than that seen in the other strains. Among CD3⁺ NKR-P1A/B⁺ NKT cells, the Fly5⁺ subset was larger in DA, F344, LEW, and BN strain rats. One interesting observation was that while the Fly5-staining pattern was homogeneous among NK cells from most strains tested, including in the LEW strain, it was markedly heterogeneous in NK cells from MHC congenic strains on the PVG background, as shown in Fig. 2B for PVG.1L. The PVG.1L mAb Fly5 staining of NK and NKT cells: marked coexpression with the Ly49i2 and Ly49s3 receptors in distinct subsets of NK cells

A)

Nevertheless, Ly49i5 is much more homologous to both Ly49i2 and Ly49s3 than with the previously published receptors Ly49i1, Ly49i1, and Ly49s2, which have all been isolated from the low NK alloresponder F344 rat strain (Fig. 1B).

Generation of mAb Fly5 by immunization with a Ly49i5/FLAG chimeric protein

To functionally characterize the Ly49i5 receptor, we generated a mAb against Ly49i5. CHO cells were stably transfected with a FLAG-tagged Ly49i5 expression construct (Ly49i5/FLAG). These transfectants expressed the Ly49i5/FLAG fusion protein on their surface, as determined by flow cytometry using the anti-FLAG mAb M2 (data not shown). Following immunization of BALB/c mice and fusion with SP2/0 myeloma cells, culture supernatants were screened for specific staining of 293T cells transiently transfected with the Ly49i5 and Ly49s5 cDNA, but not with mock-transfected control (CTR.) cells. Accession numbers of novel cDNA sequences: Ly49i5 (PVG), AY649836 (protein identification = AAV74506); Ly49s5 (PVG), AY649832 (protein identification = AAV74506); Ly49i5 (BN), AY846264.

A

FIGURE 1. The Ly49i5 and Ly49s5 receptors are highly related in the ligand-binding extracellular region, but diverge in the transmembrane and intracellular parts. A, Although Ly49i5 contains an intracellular ITIM (VTY1TV), Ly49s5 possesses the DAP12-associating transmembrane residue, arginine. B, A dendrogram shows that Ly49i5 and Ly49s5 have a high degree of homology to each other, as well as to the Ly49i2 and Ly49s3 receptors previously cloned from highly alloreactive PVG NK cells (PVG), AY649832 (protein identification = AAV74506); Ly49i5 (BN), AY846264.
Rather, it appears to concentrate to certain rat NK subsets. Of Ly49 receptors may not be evenly distributed among NK cells. Expression cloning of Ly49s5 might be public for more than one NK receptor in PVG strain rats.

Proportion and phenotype of Fly5 positive NK and NKT cells is influenced by the MHC haplotype of the host strain. Notably, the Fly5 NK cell subset is decreased from 16% of PVG NK cells to 4–4.5% of NK cells in MHC congenic PVG rats that are haplotype u at the RT1-CE/N/M class Ib region (i.e., in PVG.1U and PVG.R8). It should be noted that calculation of Fly5 NK cells in these two strains was based on detection of Fly5 bright cells only, as there was no clear distinction between Fly5 dim and negative cells; see below. By contrast, the number of Fly5 NK cells was largely unaffected in PVG rats congenic for MHC haplotypes av1, r23, l, lv1, or n (Table III).

When examined more closely, however, these expression data provided additional information. The MHC constitution of the host strain affected not only the number of Fly5 NK cells, but also the intensity of surface staining. As shown in Fig. 3, each PVG congenic rat strain expressed a Fly5 bright and Fly5 dim positive population. The expression levels of both the Fly5 bright and Fly5 dim NK cells were similar to those seen in wild-type PVG (c-c-c) in haplotypes a-a-av1, u-a-av1, l-l-lv1, and n-n-n rats, while they were down-regulated in PVG.1U (a-u-u) and PVG.R8 (a-a-u). These results suggest that the mAb Fly5-reactive receptors on both the Fly5 bright and Fly5 dim NK cells might selectively recognize class Ib ligands encoded within the RT1-CE/N/M region of haplotype u. Analysis of NK cells from haplotypes l-l-l and l-l-lv1, being disparate in the RT1-CE/N/M region only, was particularly intriguing. The Fly5 dim population, but not the Fly5 bright population, was selectively down-regulated in PVG.1L (l-l-l), but not in PVG.1L.V1 (l-l-lv1), suggesting that a class Ib-encoded structure unique to PVG.1L (RT1-CE/N/M) might serve as a ligand for a Fly5-reactive receptor. Together these data not only suggested possible MHC ligands for Fly5-binding receptors, but also that mAb Fly5 might be public for more than one NK receptor in PVG strain rats.

Expression cloning of Ly49s5

Initial attempts to block Ly49s5 function on IL-2-activated PVG NK cells using mAb Fly5 failed to reveal the expected functional effect, i.e., increased allospecific natural killing as a result of blockade of Ly49s5. Instead, we observed a reduction of lysis of u haplotype target cells (data not shown). Considering the biphasic staining pattern of PVG NK cells by mAb Fly5, we speculated that active cell numbers may also be reduced (33, 34). In the rat, such MHC-modulating effects have been observed both for the activating Ly49s3 receptor, and for the inhibitory Ly49i2 receptor (27, 29, 31). To glean clues as to potential Ly49i5 ligands, we examined the MHC influence on the mAb Fly5 reactivity in a set of congenic and intra-MHC recombinant strains on the PVG background (refer to Table I). These data reveal that the proportion of Fly5+ NK and NKT cells is influenced by the MHC haplotype of the host strain. Notably, the Fly5 NK cell subset is decreased from 16% of PVG NK cells to 4–4.5% of NK cells in MHC congenic PVG rats that are haplotype u at the RT1-CE/N/M class Ib region (i.e., in PVG.1U and PVG.R8). It should be noted that calculation of Fly5 NK cells in these two strains was based on detection of Fly5 bright cells only, as there was no clear distinction between Fly5 dim and negative cells; see below. By contrast, the number of Fly5 NK cells was largely unaffected in PVG rats congenic for MHC haplotypes av1, r23, l, lv1, or n (Table III).

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Initial attempts to block Ly49s5 function on IL-2-activated PVG NK cells using mAb Fly5 failed to reveal the expected functional effect, i.e., increased allospecific natural killing as a result of blockade of Ly49s5. Instead, we observed a reduction of lysis of u haplotype target cells (data not shown). Considering the biphasic staining pattern of PVG NK cells by mAb Fly5, we speculated that...
pressing the MHC haplotype. Both populations exhibit less staining in host strains ex-

Fly5bright and the Fly5dim NK populations. To examine the Ly49 to the obvious question as to the identities of the Fly5 Ag in the

The demonstration of two paired Ly49i5 and Ly49s5 receptors led

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Preferential expression of Ly49i5 and Ly49s5 in the respective

Fly5bright and Fly5dim NK populations

The demonstration of two paired Ly49i5 and Ly49s5 receptors led to the obvious question as to the identities of the Fly5 Ag in the

Fly5bright and the Fly5dim NK populations. To examine the Ly49 receptors in these subsets, we sorted the bright and dim NK pop-

ulations from MHC congenic PVG rat strains and assessed their

Ly49i5 and Ly49s5 mRNA expression using receptor-specific oli-
gonucleotides to prime the PCR. With oligonucleotides described in Materials and Methods, we were able to show that the Fly5bright population contained primarily Ly49i5 mRNA, while the Fly5dim population contained primarily Ly49s5 mRNA species (Fig. 4). Although these results were clear-cut for PVG.1AV1 and PVG.1U rats, the Fly5bright NK cells from PVG.1L had appreciable amounts of both Ly49i5 and Ly49s5, although a trend toward more Ly49i5 transcript was observed. The Fly5dim NK cells mainly expressed the Ly49s5 transcript (Fig. 4).

Recognition of class Ib ligands encoded from the u and 1 rat MHC haplotypes by Ly49i5 and Ly49s5

The RT-PCR data in Fig. 4 combined with the data presented in Fig. 3 suggested that Ly49i5 (Fly5bright) and Ly49s5 (Fly5dim) both recognized a structure encoded by the nonclassical RT1-CE/NM region of rat haplotype u. In addition, we predicted that an RT1-CE/NM-encoded structure from haplotype l specifically binds and down-regulates Ly49s5 on the Fly5dim NK cell population in PVG.1L strain rats. To investigate these possibilities, we functionally analyzed purified Fly5 + NK effectors from several of the MHC congenic and intra-MHC recombinant PVG strains. Fly5 + NK cells from the PVG.R23 strain (haplotype u-a-av1) efficiently lysed lymphoblast targets expressing the u haplotype in the nonclassical RT1-CE/NM region, i.e., PVG.1U (a-a-u) and PVG.R8 (a-a-u), while they spared targets expressing the av1 haplotype in the same region, i.e., PVG.1AV1 (a-a-av1) and PVG.R23 (a-a-av1; Fig. 5A, first four panels). Killing of PVG.1U (a-a-u) and PVG.R8 (a-a-u) targets was markedly reduced by addition of blocking quantities of mAb Fly5, suggesting that this anti-u NK cytolytic response was triggered by the Ly49s5 receptor (Fig. 5A, first two panels). In contrast, Fly5 + NK cells from PVG.R8 rats (a-a-u) failed to lyse RT1-CE/NM-matched PVG.1U and PVG.R8

mAb Fly5 might recognize an activating receptor in addition to Ly49i5. Among the Ly49 receptors identified by our homology screen and by other methods, only Ly49i5 was recognized by the mAb Fly5 (data not shown).

We therefore used a variation of the complementation cloning approach previously used for Ly49s3 (29), which is based on the detection of an N-terminal FLAG epitope on DT381 cells (293T cells stably transfected with a FLAG/DAP12 chimeric molecule). The FLAG/DAP12 fusion protein is only stabilized on the cell surface in the presence of a paired DAP12-coupled activating receptor, and its presence can be inferred by staining with the anti-FLAG mAb M2. To select for Ly49i5-related receptors that couple to DAP12, we transfected DT381 cells with the cDNA library (30), and performed positive selection with both the Fly5 and anti-FLAG M2 mAb. Using this system, we expression cloned Ly49s5, a novel receptor that stabilized FLAG/DAP12 on the surface of DT381 cells (data not shown) and stained with mAb Fly5 (Fig. 1 C). The Ly49i5 and Ly49s5 receptors are highly homologous in the extracellular region (90% amino acid identity in the stalk and lectin-like domain; 95% identity in the lectin-like domain). In the cytoplasmic region, Ly49s5 lacks a cytoplasmic ITIM seen in Ly49i5, but it contains the DAP12-associating trans-

membrane residue, arginine, which is lacking in Ly49i5 (Fig. 1A).
FIGURE 5. Identification of class Ib ligands for the Ly49s5 and Ly49i5 receptors in the u and l haplotypes. Natural killing of purified Fly5⁺ NK cells from PVG.R23 (A) and PVG.R8 (B) against Con A lymphoblast target cells in the presence (■) and absence (□) of blocking quantities (5 μg/well) of mAb Fly5. The Con A blasts were from the PVG.1U (RT1-A⁺-B⁺-CE/N/M⁺ or u-u-u), PVG.R8 (a-u-u), PVG.R23 (a-a-av1), PVG.1AV1 (a-a-av1), PVG.IL (l-l-l), and PVG.ILV1 (l-l-l-lv1) strains. These data suggest that mAb Fly5 primarily blocks an activating receptor (Ly49s5) for a u-encoded class Ib ligand in PVG.R23 NK cells, and an inhibitory receptor (Ly49i5) directed against class Ib ligands from the u and l haplotypes in PVG.R8 NK cells (for details, see text).

targets, but killing was augmented by the addition of mAb Fly5 (Fig. 5B, first two panels). Minimal effects were seen on the killing of PVG.R23 (u-a-av1) and PVG.1AV1 (a-a-av1) targets (Fig. 5B, middle two panels). These data suggested that Ly49i5 functions as an inhibitory self receptor for a u haplotype-encoded RT1-CE molecule in PVG.R8 NK cells. It could be noted that one reason the inhibitory effects of Ly49i5 were so easily discernible using the Fly5 subset undergoes ligand-induced down-regulation of expression in l, but not in lv1 haplotype rats, which differ only in the RT1-CE/N/M class Ib region. These data suggest that the l haplotype contains a ligand for Ly49s5 (on the Fly5⁺ population), but not for Ly49i5 (Fly5bri ght population). Unfortunately, the Fly5⁺ effectors from PVG.R23 (Fig. 5A, fifth panel) or other strains available for study (data not shown) were not informative concerning Ly49s5/haplotype l functional interactions, as they failed to kill l targets. It is possible that the l targets were protected against allospecific natural killing by some uncharacterized inhibitory receptor that counteracted Ly49s5-dependent allovitiation. In contrast, NK cells from PVG.R8 effectors, which were preferentially Fly5bri ght (mainly expressed Ly49i5), failed to lyse l targets, while they efficiently killed the lv1 targets (Fig. 5B, last two panels). This suggested that an l-encoded class Ib ligand protected against natural killing by the Fly5⁺ NK cells. In accordance with this notion, lysis of l targets was induced by the addition of mAb Fly5, while killing of lv1 targets was unaffected (Fig. 5B, last two panels).

Taken together, these cytotoxicity data indicate that Ly49i5 recognizes a RT1-CE/N/M-encoded class Ib ligand from haplotypes u and l, while Ly49s5 minimally recognizes an RT1-CE/N/M-encoded ligand from haplotype u.

Ly49s5 is nonfunctional in BN strain rats

Our experiments showed that Ly49s5 from PVG rats recognizes a class Ib ligand encoded by the u MHC haplotype, but the exact molecular ligand for Ly49s5 remains unknown. Previous data from Petersson et al. (7) suggested that the class Ib allele, RT1-E⁺, codes for an activating ligand for NK cells from BN strain rats. They showed that BN rats (haplotype n) immunized with allogeneic (haplotype u) cells increased the number of NK cells in the peritoneum, and that these NK cells demonstrated enhanced lysis of targets transfected with RT1-E⁺. It was presumed that the presence of the RT1-E⁺ ligand in the peritoneum resulted in a selective recruitment or expansion of the subset of NK cells expressing an activating receptor for RT1-E⁺. These data suggested that the RT1-E⁺ molecule might be a candidate ligand for Ly49s5.

As in PVG strain rats, a subset of splenic NK cells from BN rats stained with the mAb Fly5, indicating that these cells express the BN allele of Ly49i5 and/or Ly49s5 (Table II). In agreement with previous studies (6), there were essentially no NK cells in the peritoneal cavity of nonimmunized BN rats, but many appeared as a result of repeated immunizations i.p. with u haplotype cells (splenocytes from PVG.1U rats; Fig. 6A). Only a very small proportion (1.7%) of these peritoneal NK cells, however, stained with mAb Fly5, a percentage that is lower than that in the spleens of the same rats (5.7%; Fig. 6B). These data suggested that i.p. immunization of BN rats with haplotype u cells does not select for mAb Fly5⁺ cells, but selects for other NK cell subsets.

Accordingly, sequence analysis of the Ly49s5 allele obtained by RT-PCR from BN NK cells indicated that Ly49s5 is a pseudogene in the BN strain. It contains a stop codon in exon 5 (in the first part of the lectin-like domain), and this stop codon is also present in the RT-PCR from BN NK cells indicated that Ly49s5 is a pseudogene in the BN strain. It contains a stop codon in exon 5 (in the first part of the lectin-like domain), and this stop codon is also present in the 3'-untranslated region (data not shown). That the Fly5⁺ NK cell
subset in BN rats indeed expresses Ly49i5 was confirmed by RT-PCR (Fig. 6C). These data do not preclude RT1-Eu as a Ly49i5 ligand; failure to be recruited to the peritoneal cavity of BN rats (u haplotype) following repeated u allogeneic stimulations. A, Peritoneal exudate cells from immunized and nonimmunized control BN rats were analyzed for the presence of NK cells, NKT cells, and T cells. B, Fly5 staining of peritoneal and splenic NK cells from an immunized BN rat. Only 1.7% of the CD3− NKR-P1A/Bbright peritoneal NK cells were Fly5−, which was less than among splenic NK cells from the same animal (5.2%). C, Detection of Ly49i5 mRNA in Fly5−, but not Fly5+ BN peritoneal NK cells by RT-PCR.

Discussion

In the last decade, it has become clear that NK cells are restricted by target cell MHC class I molecules (17). The NK cell receptors involved in this recognition include the rodent Ly49 receptors, the human KIR, and the CD94/NKG2 receptors in humans and rodents (35). Most of these receptors are specific for classical MHC class Ia molecules. The physiological functions of the nonclassical class Ib molecules often remain unknown. Class Ib molecules typically have a limited tissue distribution and low surface expression and do not in general function as restriction elements for conventional T cells (26). Some class Ib molecules, such as the nonpolymorphic molecules Qa-1 in mice and HLA-E in humans, bind the paired activating and inhibitory CD94/NKG2 lectin-like receptors (36). The MIC-A and MIC-B molecules serve as targets for the activating innate immune receptor NKG2D (37), whereas HLA-G is recognized by the conserved KIR2DL4 receptor in humans. In addition, Qa-2 molecules have been implicated as inhibitory ligands for mouse NK cells (38).

Early studies in rats clearly implicated nonclassical class Ib molecules in the triggering of NK cell allogeneic responses. Studies of the LEW.1LM1 mutant rat strain, containing a 100-kb chromosomal deletion within the first cluster of class Ib genes, the RT1-CE cluster (23), suggested that PVG strain NK cells express triggering alloreceptors for ligands encoded within this deletion (3), as well as inhibitory receptors for the same set of class Ib molecules (25). The present study shows that both Ly49s5 and Ly49i5 recognize ligands encoded within the class Ib RT1-CE/N/M region of the u and l haplotypes, and is therefore in good accordance with our previous genetic studies. Although we have not yet been able to fine map the genes for the specific Ly49s5/Ly49i5 ligands within the nonclassical RT1-CE/N/M region, the lm1 data strongly suggest that some NK cell allogeneic ligands reside within the first RT1-CE cluster. It could be noted that the content of class I RT1-CE genes varies remarkably between different haplotypes (39), and it can hence prove difficult to generalize findings obtained in a single rat haplotype.

The rat MHC for the BN haplotype has recently been fully sequenced and the genes mapped (24). This study shows that the entire nonclassical region in BN rats is comprised of >45 class I genes, located in clusters that are separated by conserved framework genes. A more detailed analysis shows that the first and most centromeric class Ib cluster, the RT1-CE cluster, encompasses 16 genes encoded between the framework genes Bat1 and Pouf5f1 in the BN rat, with 13 of them predicted to be functional. The orthologous region in the mouse genome contains the H2-D, L, and Q (Qa2) loci, but not Qa1, which is in the second cluster. The corresponding human region encodes the HLA-B, HLA-C,
MIC-A, and MIC-B molecules (24). The MIC genes are absent in the mouse and rat genomes. We are currently in the process of cloning and characterizing rat RT1-CE molecules from the various rat strains to further characterize them as potential NK cell receptor ligands.

The Ly49i5 and Ly49s5 receptors are highly similar in the extracellular region, and functional studies suggest that they recognize ligands encoded from the nonclassical class I region of the u and/or l MHC haplotypes. This is the first pair of activating and inhibitory Ly49 receptors that has been functionally characterized in the rat, although the Ly49s1 and Ly49i1 receptors derived from the F344 rat strain constitute another set of highly homologous receptors at the structural level (5). Analysis of the recently available rat genomic sequence in the BN strain has revealed a surprisingly large rat Ly49 gene cluster predicted to contain >30 genes, considerably more than in mice (40–42). Most of the rat Ly49 genes can be subdivided into three major blocks based on sequence homology, and this subdivision corresponds with their respective physical positions within the NKC (42). The Ly49i5 and Ly49s5 genes are located adjacent to each other in block II, while Ly49i1 and Ly49s1 cluster together in block III. Such related pairs of Ly49 receptors may have evolved by unequal crossing over and/or by gene conversion events. Pairs of NK receptors have previously been described both in the human and in the mouse system (43–45). The human inhibitory KIR2DL1 and activating KIR2DS1 receptors recognize HLA-C alleles, while CD94/NKG2A (inhibitory) and CD94/NKG2C (activating) recognize HLA-E. In the mouse, pairs of Ly49 receptors such as Ly49A/P, Ly49O/D, and Ly49O/W have been identified, and their MHC ligands have been characterized (46, 47).

In two previous analyses of the available BN genomic sequence (40, 41), the Ly49i5 sequence predicts a pseudogene. However, as we have shown in this study, the BN allele of Ly49i5 is complete and is functionally expressed in NK cells (Figs. 1 and 6). The previously described faulty categorization of Ly49i5 as a pseudogene was most likely caused by gaps and an incomplete genomic sequence. The Ly49i5 cDNA sequence presented in this study, in contrast, is in accordance with the genomic assembly and confirms that Ly49i5 is a pseudogene in BN rats. The Ly49s3 gene, which encodes an important NK alloactivating receptor in the PVG strain, is complete (39). It is likely that the functional absence of several NK stimulatory Ly49 receptors may contribute to the limited allorepertoire observed for BN NK cells (9).

Why should such opposing NK receptors be present in the same rat? If present on the same cell, such coexpression could possibly enable a more sophisticated regulation of cellular activation against a common ligand. It could also render target cells insensitive to NK cells expressing the activating receptor, because an inhibitory receptor might negate signals from an activating receptor (4, 48). Our studies using the PCR suggest that in PVG rats, most of the Ly49i5 and Ly49s5 receptors are expressed on separate NK cell subpopulations (Fig. 4). Greater insights into the physiologic functions of paired activating and inhibitory receptors in rat NK cells await our identification of the molecular ligands for Ly49i5 and Ly49i5. Although these receptors recognize shared MHC haplotypes, the exact ligands recognized by Ly49i5 and Ly49s5 may be structurally distinct. Moreover, the ligands for Ly49i5 and Ly49s5 may be expressed on different cell types and might be differentially regulated during states of inflammation and disease, allowing for the selective activation of NK cells despite the presence of inhibitory receptors.

Insights into innate immune receptor evolution can be gleaned from comparisons of NK cell functions in primates and rodents. Allele-specific inhibitory NK functions are mediated primarily by KIR in primates and lectin-like Ly49 receptors in rodents (34, 35). Humans only have a solitary Ly49 pseudogene, and rodents express a limited number of functionally uncharacterized KIR genes on the X chromosome (49, 50). Recently, it was demonstrated that the resistance to mCMV is mediated by the activating Ly49H receptor, which directly recognizes the virally encoded protein m157 on the surface of infected target cells (15, 16). Other data suggest that innate immune antiviral effects may be modulated by inhibitory innate immune receptors. Clearance of hepatitis C virus (HCV) in humans is associated with constitutive weak inhibitory KIR-HLA interactions, suggesting that HCV is more readily cleared in individuals whose NK cells have less tonic inhibition, and thus have a lower threshold for innate immune activation during HCV infection (51). Thus, it appears that innate receptors can alter host immune responses through the direct recognition of pathogens and through indirect mechanisms on host responsiveness.

Similarities between rodent and primate NK receptor systems are obvious. Still, some have speculated that innate immune receptors may serve distinct functions in even closely related species. Significant differences between the genomic organization of chimpanszee and human KIR clusters have manifested themselves within the relatively short evolutionary time span (4 million years) that separates humans and chimpanzees. The extreme diversity in innate immune receptor use has led some to speculate that innate immune receptors are subjected to powerful evolutionary pressures as new species occupy specific environmental niches (52). This hypothesis presupposes that different species might use these receptor systems in similar, yet distinct roles, in host defense against divergent pathogens. A direct comparison of the mouse and rat MHC genomic structure suggests that the nonclassical RT1-CE/N/M region of the rat MHC contains genomic features that strongly support independent evolution following the speciation of mouse and rat (24), and the same is true for the Ly49 family of receptors in these two closely related species (40–42). Although the physiologic functions of RT1-CE molecules in rats have not been clearly defined, we anticipate that they might serve to regulate innate immune responses to environmental agents through interactions with activating and inhibitory Ly49 receptors.

Disclosures

The authors have no financial conflict of interest.

References


