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*J Immunol* 2006; 176:4133-4140; doi: 10.4049/jimmunol.176.7.4133

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The Novel Inhibitory NKR-P1C Receptor and Ly49s3 Identify Two Complementary, Functionally Distinct NK Cell Subsets in Rats

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The proximal region of the NK gene complex encodes the NKR-P1 family of killer cell lectin-like receptors which in mice bind members of the genetically linked C-type lectin-related family, while the distal region encodes Ly49 receptors for polymorphic MHC class I molecules. Although certain members of the NKR-P1 family are expressed by all NK cells, we have identified a novel inhibitory rat NKR-P1 molecule termed NKR-P1C that is selectively expressed by a Ly49-negative NK subset with unique functional characteristics. NKR-P1C+ NK cells efficiently lyse certain tumor target cells, secrete cytokines upon stimulation, and functionally recognize a nonpolymorphic ligand on Con A-activated lymphoblasts. However, they specifically fail to kill MHC-mismatched lymphoblast target cells. The NKR-P1C+ NK cell subset also appears earlier during development and shows a tissue distribution distinction from its complementary Ly49s3* subset, which expresses a wide range of Ly49 receptors. These data suggest the existence of two major, functionally distinct populations of rat NK cells possessing very different killer cell lectin-like receptor repertoires. The Journal of Immunology, 2006, 176: 4133–4140.

A superfamily of killer cell lectin-like receptors (KLRs)3 was originally discovered in NK cells, but certain family members are also variably expressed by other cell types (1–3). In rats, the KLRs are encoded by the NK gene complex (NKC) located on chromosome 4 (4), and syntenic chromosomal regions have been identified in many other species. Several KLR gene families are clustered together in specific subregions of the NKC, and there is considerable species-to-species variation in the number, expression patterns, and putative functions of the genes encoded by these different KLR families. Some families, like Ly49 (Kira), are polygenic in rodents (4, 5) while there is only a single family member in humans (6). The Ly49 genes are located in the distal (telomeric) part of the rat NKC (7). They encode both activating and inhibitory Ly49 molecules which are expressed in subsets of rat NK cells. Although inhibitory Ly49 receptors react with both classical (RT1-A) and nonclassical (RT1-CE/N/M) MHC class I molecules, their activating counterparts mainly recognize nonclassical MHC (8–11). Furthermore, the activating Ly49 receptors can be instrumental in triggering rat NK alloreactivity (10, 11).

The distantly related KLRH1 molecule, which is encoded by a gene just centromeric of the Ly49 region, shows slightly greater amino acid homology with Ly49 than with other KLR family members (12). It is possible that KLRH1 also functions as a receptor for MHC-encoded molecules on the basis of its expression pattern in MHC congenic strains. A putative ortholog of KLRH1 exists in the mouse but nothing is known about its cellular expression or function (12). The Ctd69, Clec, Klre, Cdh9, Nkg2, and Klri gene families are all located centromeric of Klrh1, i.e., in the central region of the rat NKC (13), while the Nkr-p1 and C-type lectin-related (Cirlo1ci) (14, 15) genes map to its most centromeric region. This organization of the rat NKC is similar to that in the mouse (1).

As with many of the other KLR families, the NKR-P1 (KLRB) family consists of both activating and inhibitory members. The prototypical NKR-P1A molecule has been used as a marker for rat NK cells and is an activating receptor (16–18). The NKR-P1B receptor (4), in contrast, has inhibitory structural features but it has not been possible to prove NKR-P1B-specific inhibitory functions on primary cells because available anti-NKR-P1 mAb fail to distinguish NKR-P1A from NKR-P1B (our unpublished data and Ref. 19). The anti-NKR-P1A/B mAb 3.2.3 (16) reacts with NK cells from a broad range of rat strains, but not from the AUG strain where there is only a very faint staining (our unpublished data). This suggests some allelic variation of the NKR-P1 molecules.

Despite their putative roles in the recognition of certain tumor cells (20), the natural ligands of NKR-P1 molecules have long remained elusive. Recently, it was shown, however, that members of the Clr family function as specific ligands for mouse NKR-P1 molecules. The Clr genes are interspersed between the Nkr-p1 genes. Thus, specific receptor-ligand pairs are therefore not inherited separately, but rather on the Clr cluster (21, 22). The recent findings of Clr ligands have created a renewed interest in the NKR-P1 family, because these ligands and their receptors represent a form of self-nonself discrimination that is independent of MHC class I, a principle that can now be extended to other NK cell receptors (23). In

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the present paper, we have cloned and characterized a novel inhibitory NKR-P1 member in the rat, which we have termed NKR-P1C. It has an unusual cellular distribution, in that it is mainly expressed by a Ly49-negative NK cell subset with unique functional characteristics.

**Materials and Methods**

**Rats**

Rats were housed in compliance with institutional guidelines and sacrificed at 8–12 wk of age. The MHC congenic and intra-MHC recombinant strains PVG.1N (RT1n) or n, i.e., RT1-Aβ-Bd-CE/Nmp (class Ia-class Ib), abbreviated n-n-n, FCS, and antibiotics, and the RNK-16 line in complete RPMI (cRPMI; cells, cytotoxic assay, and cytokine measurement.

**Flow cytometry (FCM)**

A total of 2.5–10 × 10^6 IL-2-activated NK cells or mononuclear cells (depleted for IgG cells) from the indicated organs were labeled with different combinations of the following conjugated mAbs: FITC-conjugated 3.2.3 (anti-NKR-P1A/B; Ref. 16), DAR13 (anti-Ly49s3; Ref. 10), STOK2 (anti-Ly49i2; Ref. 24), or STOK27 (anti-NKR-P1C); PE-conjugated G418 (anti-CD3; Ref. 25), or 10/78 (anti-NKR-P1A/B; both from BD Pharminogen); and biotinylated STOK27, STOK9 (anti-KLRH1; Ref. 12), DAR13, or Fly5 (anti-Ly49i5/s5; Ref. 11) followed by RPE-Cy5-conjugated streptavidin.

**Generation of the anti-NKR-P1C hybridoma STOK27 and biochemical characterization**

One BN rat was immunized twice s.c. with IL-2-activated NK cells (10^6 and 5 × 10^5) from PVG.1N (RT1n) rats with an interval of ~2 mo, and boosted i.v. with 10 × 10^6 cells 3 days before fusion. Mononuclear splenocytes were fused with NS0 cells using Polyethylene Glycol 4000 and cultured in hypoxanthine/aminopterin/thymidine selection medium. Supernatants were screened for staining of subsets of IL-2-activated NK cells by FCM.

**Immunoprecipitation, deglycosylation, and Western blotting**

NK cells were surface biotinylated at 2.5 × 10^6 cells/ml in PBS (pH 8), with 0.5 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at room temperature and lysed in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, protease inhibitors, and 1% Triton X-100. Lysates were preclarified and immunoprecipitated with STOK27 precoupled Sepharose 4B beads (Amersham Biosciences) at 2 h at 4°C. For deglycosylation, the immunoprecipitates were solubilized in 40 mM sodium phosphate (pH 6), with 1% Triton X-100 and 0.1% SDS, digested overnight at 37°C with 1 U of PNGase F/N-Glycosidase F complemented with 20 mM EDTA, 1% 2-ME, 1 mM O-glycosidase, 50 mM Clostridium perfringens neuraminidase, or a combination of O-glycosidase and neuraminidase (all enzymes were from Roche Diagnostics). The samples were resolved by 10 or 12.5% Criterion Tris-HCl gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). After blocking with 5% dry milk in TBS with 0.05% Tween 20, the PVDF membranes were incubated with a streptavidin-HRP conjugate before detection by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate: Pierce). Lysates from 20 × 10^6 RKN-16.NKR-P1C transfectants, stimulated for 5 min at 37°C with pervanadate or left unstimulated, were preclarified before incubation for 2 h at 4°C with 2 μg of mAb STOK27 or STOK2 (isotype-matched control). Immunoprecipitations were performed with GammaBind G-Sepharose Beads (Amersham Biosciences) for 1 h at 4°C. After washing (0.1% Triton X-100), the immunoprecipitates were resolved by Criterion Tris-HCl gels as above. After transfer to PVDF membranes and blocking, membranes were incubated with a polyclonal rabbit anti-SH-PTP1 (C-19; Santa Cruz Biotechnology) for 2 h at room temperature, washed, and incubated with peroxidase-conjugated goat anti-rabbit IgG and developed with chemiluminescence.

**Cells, cytotoxicity assay, and cytokine measurement**

The YAC-1, 293T, and P388.D1 cell lines were grown in RPMI 1640, 10% FCS, and antibiotics, and the RNK-16 line in complete RPMI (crPME; RPMI 1640, 10% FCS, 5 × 10^{-3} M 2-ME, l-glutamine, and antibiotics). The generation of Con A lymphoblasts and 4-h 51Cr release assay was performed as described (26). Four micrograms per well of mAb STOK27 or the isotype control mAbs TIM2 (anti-mouse TIM2; a gift from Dr. M. Dawe, Oslo, Norway) or STOK2 was added to plated effector cells 20–30 min before the addition of target cells. Spontaneous release was usually below 10% of the total cpm in the cells. Results are presented as mean values from triplicates for each E:T ratio, error bars representing one SD. A two-sided unpaired ttest was used for statistical comparisons of triplicate values for each E:T ratio (by the built-in analysis package in the GraphPad Prism software).

IL-2-activated NK cells were generated by positive selection of NKR-P1A/B-expressing splenocytes, as described (27). Purified NKR-P1C+ and Ly49s3+ NP cells were obtained either by positive or negative selection. For positive selection, splenocytes were stained with biotinylated mAb STOK27 or DAR13, washed, incubated with anti-biotin microbeads, separated with a SuperMACS magnetic cell separator (Miltenyi Biotec), and cultured for 1–2 wk with cRPMLI-2. For negative selection, IL-2-activated NK cells were generated by adherence to plastic (28). At the day of the experiment, NKR-P1C+ cells were depleted of Ly49s3-expressing cells using DAR13-precoated rat anti-mouse IgG1 Dynabeads (Dynal). Conversely, the Ly49s3+ cells were depleted of NKR-P1C-expressing cells using STOK27-precoated sheep-anti-rat Dynabeads. Purity was generally >80%.

Concentrations of cytokines were measured by Luminex using the Bio-Plex cytokine assay (Bio-Rad). NKR-P1C+ and Ly49s3+ NK cells were obtained by negative selection, as described above, to avoid mAb-mediated stimulatory effects before the assay. They were cultured at 1–2 × 10^6 cells/well at 37°C for 20 h in 24-well plates before the collection of supernatants. The wells were either left uncoated or were precoated with purified mAb 3.2.3 alone or in combination with a secondary rabbit anti-mouse IgG.

**Expression cloning and transfection**

cDNAs encoding NKR-P1C were isolated by eukaryotic expression cloning as previously described using mAb STOK27 and a cDNA library from KLH1+ PVG NK cells (12). After four rounds of immunoenrichment, the final sublibrary induced the transient expression of a molecule that stained with mAb STOK27 on ~20% of the 293T cells. Four positive clones of 384 A were analyzed and sequenced on both strands.

RNK-16 cells (3 × 10^6) were transfected with a full-length NKR-P1C cDNA subclone into the EMCV.Sre expression vector, as described (29). Electroporation with 20 μg of Scil-linearized construct was performed at 120 mV, 960 μF, and with capacitance extender (Bio-Rad Electroporator). The cells were grown for 24 h in cRPMLI plated out in 96-well plates at 10 000 cells/well in cRPMLI with 1 mg/ml active G418. Cells appeared in 10% of the wells and were tested for STOK27 staining by FCM after 2–3 wk.

**RT-PCR**

NKR-P1C+ and Ly49s3+ single-positive NK cells from the spleen were obtained by FACS sorting (FACSDivia; BD Biosciences), total RNA was isolated with TriReagent (Sigma-Aldrich), and cDNA was generated with Moloney murine leukemia virus reverse transcriptase (Promega). PCRs were typically performed on a GeneAmp PCR thermocycler (Applied Biosystems) using hot start for 3 min at 96°C. Pyrococcus woese polymerase was added at 80°C before running for 35 cycles at 95°C 20 s, 54°C 30 s, 72°C 30 s. The following upper and lower primers, respectively, were used: Ly49s3 L: 5’-AGGCATGATCCTTCTGCT-3’ and 5’TCAATCAGC GAATTCTCTCA-3’; Lyg9s2 L: 5’T-GTTGTTCTTCTCGTCTAGTAT-3’ and 5’-AGCCAGTGAAGATGGGGT-3’; Ly49s1 L: 5’T-GTGGAAATCTCCACCAT-3’ and 5’-CCAGTAATTGCTGGAATAAATG-3’; Ly49h8 L: 5’T-AGCACAGCGAGAACCTGGAC-3’ and 5’-GAAGTTGGGAGCGAAG-3’; Cd4 L: 5’T-CTCTCAGGGAGCTTATGAGG-3’ and 5’T-CCTTGACCTTCTGGTCATCA-3’; Nkg2a L: 5’T-GAAATGAGCCGAAACTCTGTT-3’ and 5’T-GTGAGTTGCTGGTCTCTGTA-3’; Nkg2d L: 5’T-CTCCTCAGGATGAGCAATAT-3’ and 5’T-CTCGTGGAGGAGCGAATGA-3’; Cd5 L: 5’T-CGCGGTTGTCTCTGCTGTGTC-3’ and 5’T-CTTGTCGTTCTCCTGGGCTTGTT-3’; PCR products were resolved by agarose gel electrophoresis (1% Tris-borate-EDTA).

**Results**

Generation of the monoclonal alloantibody STOK27 reacting with a major subset of rat NK cells

In an attempt to identify novel alloreactors on rat NK cells, we immunized low NK-alloresponder BN strain rats with alloreactive and MHC-matched PVG.1N NK cells. The supernatant of one hybridoma, STOK27, labeled a subpopulation of NKR-P1A/B+ NK
cells by FCM that differed from that seen when staining with available mAbs against the Ly49s3, Ly49i2, and KLRH1 receptors (Fig. 1A and our unpublished data). These data suggested that the STOK27" population constituted a novel subset of NK cells in PVG strain rats. Similar results were obtained in two other high NK alloresponder strains, AO and WAG. Cells from BN strain rats as well as from two other low NK-alloresponder strains, DA and LEW, failed to react with mAb STOK27 (our unpublished data).

A minor population (~5%) of freshly isolated mononuclear splenocytes from PVG.7B rats stained with the mAb STOK27 by single-color FCM (Fig. 1B). Lymph node (LN) T and B cells and thymocytes, in contrast, were mainly STOK27-negative (Fig. 1B and our unpublished data). Three-color FCM analysis of the splenocytes revealed that ~60% of the NKR-P1A/B CD3" NK cells were STOK27". There were also a few heterogeneous STOK27" cells among the NKR-P1A/B CD3" T cells (hereafter referred to as NK-T cells), whereas the "conventional" NKR-P1A/B CD3" T cells were negative (Fig. 1C).

As can be deduced from Fig. 1, A and C, the proportion of STOK27" NK cells was consistently higher among freshly isolated splenic NK cells than among IL-2-activated NK cells. Therefore, we considered whether expression of the STOK27-Ag could be labile, being switched off (or on) during culture. Purified STOK27" NK cells, however, maintained their positive phenotype (>80%) during the standard culture period of 2 wk. Furthermore, STOK27" cells failed to reappear within 1 wk after cellular depletion (our unpublished data). Therefore, we concluded that the STOK27-Ag most likely is a stable marker whose surface expression is fixed during culture. This further implied that the relative reduction of STOK27" cells among the IL-2-activated NK cells was likely caused by a preferential expansion of STOK27− (Ly49s3+) NK cells.

**Characterization of the STOK27-Ag as a novel inhibitory NKR-P1 receptor (NKR-P1C)**

Initial biochemical characterization suggested that the STOK27-Ag exists as a homodimeric membrane glycoprotein in IL-2-activated NK cells. Protein bands migrated at ~45 and 90 kDa under reducing and nonreducing conditions, respectively, after immunoprecipitation with mAb STOK27 (Fig. 1D). The 45-kDa band was reduced to ~28 kDa by PNGase F treatment (Fig. 1E), suggesting the existence of abundant N-linked carbohydrates. Treatment with neuraminidase or O-glycosidase was without any apparent effect.

Four cDNAs encoding the STOK27-Ag were isolated by expression cloning in eukaryotic 293T cells using a cDNA library from PVG NK cells (12). The four cDNAs were identical in the open reading frame (669 nt) and encoded a novel NKR-P1 protein of 223 aa that we have termed NKR-P1C (Fig. 2). At the amino acid level, this receptor is ~70 and 85% identical with the respective NKR-P1A and NKR-P1B receptors. In the lectin-like domain, NKR-P1C and NKR-P1A or NKR-P1B are 82 and 78% identical, respectively. Identity is much lower for NKR-P1D being only 48% (for the lectin-like domain 45%). As shown in Fig. 2B, transient transfection of 293T cells with an Nkr-p1c construct induced surface expression of the STOK27-Ag, whereas the Nkr-p1a control construct did not (the latter construct induced NKR-P1A expression in the same cells as judged by staining with mAb 3.2.3; data not shown). It should be noted that the Nkr-p1c designation has been used once previously, for an unpublished partial Nkr-p1 clone from the F344 strain (30). The corresponding gene is present in the BN strain genome and is most likely a pseudogene, and we have therefore adopted the Nkr-p1c name for the present sequence instead. A phylogenetic tree depicts how the full-length NKR-P1C protein relates to selected members of other rat KLR families (Fig. 2C).

The cytoplasmic domain of NKR-P1C contains an ITIM, VVY-ADL (motif-specific amino acids underlined), suggesting inhibitory function (Fig. 2A). It also lacks the positively charged amino acid in the transmembrane region required for the binding to activating signaling adapters (31, 32). Inhibitory signaling was confirmed by functional studies of the rat NK line, RNK-16, stably transfected with an Nkr-p1c construct (RNK-16.NKR-P1C transfectants; Fig. 3A). Four individual RNK-16.NKR-P1C clones were
A NOVEL NKR-P1C+, Ly49-NEGATIVE NK CELL SUBSET IN RATS

We have recently shown that some inhibitory and activating Ly49 receptors are coexpressed in certain subsets of rat NK cells (11). A striking finding in the present study is that NKR-P1C was expressed mainly by the Ly49-negative fraction of NK cells. Although 64% of splenic NK cells were STOK27+ (22), only 13% of the Ly49s3+ cells coexpressed NKR-P1C (Fig. 4A, upper left plot). The NKR-P1C+/Ly49s3+ double-positive fraction represented only 4.5% of the NK cells which is much less than would be expected from random expression of these two receptors (0.34 × 0.64 × 100 = 22% ). Similar results were obtained for the Ly49i2 and Ly49i5/s5 receptors, as well as for KLRH1 (Fig. 4A). The KLRH1 receptor, encoded by a gene located just proximal (centromeric) to the Ly49 region, is slightly more related to the Ly49 receptors than to other KLRs, as visualized by the phylogenetic tree in Fig. 2C.

Because we only have mAbs to a group of related Ly49 receptors encoded from the same block of Ly49 genes (block 2, 3′UTR type 4 ) (7), we extended the analysis to more distant Ly49 members by RT-PCR. In these studies, we analyzed Ly49s3+ and NKR-P1C+ single-positive splenocytes obtained by FACS sorting, which together account for ~90% of the splenic NK cells (Fig. 4A, upper left plot). mRNA levels of the Ly49s3 (block 1), Ly49s2 (block 2, 3′UTR type 2), and Ly49s1/s7 (block 3) genes were much higher, by a factor of 100 or more, in the Ly49s3+ than the NKR-P1C+ single-positive NK subset (Fig. 4B). The same was also the case for Ly49i8 gene, which is located in the distal (telomeric) end of the Ly49 region. Ly49i8 is phylogenetically the most distant Ly49 member, and the only one with a putative homolog in the mouse genome (Ly49B) (7, 33). The expression of Cd94, Nkg2a, and Nkg2d was comparable in the two NK subsets (Nkg2a message was somewhat increased in the NKR-P1C+ subset, Fig. 4B). These studies show that the NKR-P1C+ NK subset expresses little Ly49 (and KLRH1) receptors, suggesting the presence of a common regulatory mechanism for gene expression within the Ly49 region of the rat NKc. Furthermore, most Ly49-expressing NK cells apparently coexpress the Ly49s3 receptor which identifies a complementary subset of rat NK cells.

FIGURE 2. The STOK27-Ag is a novel NKR-P1 molecule, termed NKR-P1C. A, Deduced amino acid sequence of NKR-P1C. The three known rat NKR-P1 molecules (NKR-P1A, -B, and -D) are shown as a comparison. A putative ITIM (VYVYD; motif-specific amino acids underlined) is marked with a dotted line and is present in NKR-P1C and NKR-P1B. The predicted transmembrane region is underlined; note the lack of a basic amino acid (Arginine/R), marked with an asterisk (*), in the transmembrane region of the two ITIM-containing NKR-P1 molecules. B, mAb STOK27 stains 293T cells transiently transfected with an NKR-P1C expression construct, but not with NKR-P1A as a control. mAb STOK27 associates with the 72-kDa tyrosine phosphatase (SHP-1) in pervanadate-treated (Stim.) RNK-16(NKR-P1C transfectants, but not in untreated or control transfectants (Ctrl.) mAb STOK2. C, NKR-P1C associates with the 72-kDa tyrosine phosphatase SHP-1 in pervanadate-treated RNK-16 transfectants, but not in untreated or control transfectants (Fig. 2C).

RNK-16 transfectants, but not in untreated or control transfectants (Fig. 3C).

The NKR-P1C+ NK cell subset is predominantly Ly49 negative and is complementary to the Ly49s3+ NK subset

obtained which killed the FcR+ P388.D1 mouse macrophage line (our unpublished data). Addition of purified mAb STOK27 to the killing assay resulted in a variable reduction of cytotoxicity, i.e., redirected inhibition, as shown in Fig. 3B. Accordingly, immunoprecipitation experiments showed an association of NKR-P1C with the SHP-1 phosphatase (~72 kDa) in pervanadate-treated

FIGURE 3. NKR-P1C is an inhibitory receptor associating with the tyrosine phosphatase SHP-1. A, mAb STOK27 stains brightly RNK-16 cells stably transfected with NKR-P1C (RNK-16.NKR-P1C cells) as detected by single-color FCM. B, Lysis of FcR+ P388 target cells by the NKR-P1C RNK-16 transfectants is reduced by the addition of mAb STOK27 (redirected inhibition), as compared with the isotype control (Ctr.) mAb STOK2. C, NKR-P1C associates with the 72-kDa tyrosine phosphatase SHP-1 in pervanadate-treated (Stim.) RNK-16.NKR-P1C transfectants following immunoprecipitation with mAb STOK2, and not with the control (Ctrl.) mAb STOK2. The last lane shows detection of SHP-1 in whole cell lysate (Wcl).
Ly49s3 A lymphoblasts. In accordance with previous findings (10), selection were tested for their ability to lyse MHC-allogeneic Con equally well as the Ly49s3 general nonresponsiveness as they lysed YAC-1 tumor target cells 5. Their inability to lyse the allotargets was not caused by a A geneic PVG. By contrast, neither allogeneic nor PVG.R23 ( targets, while sparing syn- u-a-av1 a-a-av1, and DA (a-a-av1)), and DA (a-a-av1)) rats, obtained by negative selection in the upper left FACS plot in A. Ten-fold dilutions (-1, -2, -3, and -4) of cDNA were analyzed, with CD45 used as a cDNA loading control.

FIGURE 4. The NKR-P1C + NK subset expresses little Ly49 receptors and is complementary to the Ly49s3 − NK subset. A. NK cells (NKR-P1A/ B + bright cells) from the spleen of PVG.7B rats were analyzed by three-color FCM for coexpression of NKR-P1C, Ly49 molecules, and KLRH1. B, NKR-P1C + and Ly49s3 − single-positive splenic NK cells were analyzed for relative expression levels of the indicated mRNAs by semiquantitative RT-PCR. The two complementary subpopulations account for almost 90% of the NK cells and were obtained by FACS-sorting using the gates indicated in the upper left FACS plot in A. Ten-fold dilutions (-1, -2, -3, and -4) of cDNA were analyzed, with CD45 used as a cDNA loading control.

NKR-P1C + NK cells secrete cytokines and are cytolytic, but fail to kill allogeneic Con A lymphoblasts
IL-2-activated NKR-P1C + and Ly49s3 + NK cells from PVG.7B (RT1 haplotype A'B1/B'D1-C1 or c-c-c) rats, obtained by negative selection were tested for their ability to lyse MHC-allogeneic Con A lymphoblasts. In accordance with previous findings (10), Ly49s3 + cells effectively lysed the allogeneic PVG.1N (n-n-n), PVG.R23 (a-a-av1), and DA (a-a-av1) targets, while sparing syngeneic PVG.7B control cells. By contrast, neither allogeneic nor syngeneic lymphoblasts were killed by NKR-P1C + NK cells (Fig. 5A). Their inability to lyse the allotargets was not caused by a general nonresponsiveness as they lysed YAC-1 tumor target cells equally well as the Ly49s3 − NK cells (Fig. 5B). Also, they showed a brisk secretory response of the TNF-α, GM-CSF, and INF-γ cytokines upon receptor stimulation with mAb 3.2.3 (anti-NKR-P1A/B; Fig. 5C). Therefore, it is likely that the general lack of activating Ly49 receptors (see above) explains why they do not have alloreactive specificities.

FIGURE 5. The NKR-P1C + NK cells are not alloreactive, but kill YAC-1 tumor cells and secrete cytokines upon stimulation. A and B, Cytolytic activity of the complementary NKR-P1C + ( ■) and Ly49s3 + ( ○) PVG.7B NK populations against MHC-mismatched (PVG.R23, PVG.1N, and DA) Con A lymphoblasts, syngeneic (PVG.7B) control (Ctr.) lymphoblasts, and YAC-1 tumor targets. The NKR-P1C + subset fails to lyse the allotargets but effectively lyse YAC-1 tumor cells. Results presented are representative of two to six experiments. The cytolytic activity of NKR-P1C + vs Ly49s3 − NK cells is statistically different (p < 0.01) against PVG.R23, PVG.1N, and DA targets, but not against PVG.7B or YAC-1. C, Cytokine production by NKR-P1C + and Ly49s3 − NK cells. Cells were stimulated with mAb 3.2.3, either alone (mAb 3.2.3) or cross-linked with a secondary anti-mouse Ab (3.2.3 + anti-mlg), or they were kept in medium as a control (Ctr.). Concentration (picograms per milliliter) of the indicated cytokines was determined in the supernatant, which was harvested after 20 h culture and analyzed by a multiplex bead assay (Luminex). Values above scale are indicated with an asterisk (*).

NKR-P1C + NK cells bind a conserved ligand on Con A lymphoblasts
In mice, the Clr ligands for mouse NKR-P1 receptors are present on normal hemopoietic cells (21, 22). Therefore, we tested normal lymphoblast targets for a functional inhibitory ligand for NKR-P1C. The NKR-P1C + NK cells did not kill syngeneic (PVG.7B) or MHC-allogeneic (PVG.1U, PVG.1L, PVG.R23, PVG.1N, and DA) lymphoblasts. Cytotoxicity against all the targets was induced by addition of mAb STOK27 irrespective of the target MHC haplotype (Fig. 6A and our unpublished data). By contrast, mAb STOK27 had no effect on the cytolytic activity of Ly49s3 + NK cells (Fig. 6B). These results suggested that NKR-P1C binds a monomorphic ligand, possibly a Clr ligand, on the Con A blasts.

NKR-P1C + NK cells appear early during ontogeny and show a distinct in vivo tissue distribution as compared with Ly49-expressing NK cells
Previous studies in mice have shown that NK cells acquire Ly49 receptors relatively late in ontogeny (34). This also appears to be the case in rats. As visualized in Fig. 7A, the percentage of Ly49s3 + cells was low (6.3%) among freshly isolated splenic NK cells from 3-day-old rats. It increased to 28% at 4 wk of age, similar to the levels seen in young adult (8-wk-old) rats (Fig. 7B). By contrast, the proportion of NKR-P1C + NK cells was normal,
i.e., with more than two-thirds being positive in neonatal rats, and remained at comparable levels until they reached the adult stage at 8 wk of age. At all ages, there were few Ly49s3/H11001/NKR-P1C/H11001 double-positive cells (Fig. 7, A and B). These data suggest that maturation of the NKR-P1C/H11001 NK subset precedes that of the Ly49 subset(s) during ontogeny.

In addition, in vivo distribution of the two complementary NK cell subsets differed markedly. In BM, the proportion of Ly49s3/H11001 NK cells was consistently increased compared with the spleen (41 vs 30%) and that of the NKR-P1C/H11001 NK cells was comparably reduced (53 vs 65%). The opposite pattern was observed in mesenteric LNs where there was a striking reduction of the Ly49s3/H11001 NK subset (14 vs 30%) as well as a marked increase of the NKR-P1C/H11001 NK subset vs the spleen (85 vs 65%). In cervical LNs, the composition was approximately the same as that seen in the spleen (Fig. 7C). The distribution of the Ly49i2 and Ly49i5/s5 NK subsets followed that of the Ly49s3/H11001 cells (our unpublished data).

There were also associated differences in receptor expression levels at the different locations. Ly49s3/H11001 cells from the spleen and BM expressed higher levels of Ly49s3 than from mesenteric and cervical LNs (x-mean intensity 680 vs 360). Expression level of NKR-P1C, in contrast, was higher in mesenteric LNs than in the other three tissues (y-mean intensity 126 vs 55–71; Fig. 7C). Together, with the results presented above, these data suggest that the two complementary NK cell subsets may perform different functions in vivo.

Discussion
We have identified an inhibitory NKR-P1 molecule (NKR-P1C) with a new monoclonal alloantibody generated in BN rats. This strain was chosen for immunization because it has a limited NK allorecognition repertoire compared with PVG strain rats. Thus, while BN strain NK cells only kill allogeneic lymphoblasts expressing the u-u-u rat MHC haplotype (35), PVG.1N NK cells lyse targets from haplotypes u-u-u, l-l-lv1, l-l-l, and a-a-av1 (our unpublished data). This difference could not be attributed to an MHC effect during NK maturation as the two strains are MHC identical. Rather, we suspected that it was due to the lack of certain activating alloreceptors, reminiscent of the functionally described Nka alloresponder gene(s) in the Ly49 region of PVG but not DA rats (4). Although the immunization protocol was aimed at identifying...
these activating alloreceptors, we instead obtained the anti-NKR-P1C mAb STOK27, reactive against a unique subset of nonalloreactive, Ly49-negative NK cells.

The NKR-P1C⁺ and Ly49s3⁺ NK cell subsets are largely complementary, i.e., with rare cells (<5%) being NKR-P1C/Ly49s3 double positive. Together, the two subsets account for >90% of the NK cell pool in PVG strain rats. They also express very different KLR repertoires, which may explain their observed functional differences, such as the inability of the NKR-P1C⁺ subset to kill allogeneic cells. It is unlikely that the NKR-P1C receptor specifically inhibits alloactivation of this subset, because blockade with mAb STOK27 equally affects killing of syngeneic and allogeneic targets, and fails to restore allokilling to levels seen for Ly49s3⁺ NK cells. Rather, the defect in killing of allogeneic targets by the NKR-P1C⁺ subset is likely related to the generally limited expression of Ly49 receptors in this subset.

We have recently estimated the number of functional, nonallelic Ly49 genes in the rat to be ~25 (7). In the present study, we tested for 12 of them by FCM or RT-PCR, and they were all preferentially expressed in the Ly49s3⁺ NK subset. In aggregate, our available mAbs react with all block 2 Ly49 molecules (7), i.e., Ly49h2/i3/i4/i5 and Ly49h3/i4/i5, except for Ly49h2, which was assayed by RT-PCR together with selected genes from block 1 (Ly49h3), block 3 (Ly49h1/6/7), and with Ly49h8 (orthologous to mouse Ly49b). It can be concluded from these studies that NKR-P1C⁺ NK cells express little Ly49 receptors and that Ly49s3 is a good overall marker for Ly49-expressing NK cells, at least in high NK alloresponder rat strains as well.

The Ly49 receptors in rodents and the killer cell Ig-like receptors (KIRs) in humans are functional analogs and bind polymorphic MHC class I epitopes. Similar to the marked haplotypic variation of the KIRs, the Ly49 repertoire shows marked differences both in gene and allele content between inbred strains of rats and mice (7, 36, 37). Such polymorphisms are a prerequisite for NK alloreactivity. Previous studies have shown a marked enrichment of NK alloreactivity in selected Ly49 NK subsets both in rats (10, 24) and mice (38, 39). Similarly, NK alloreactivity in humans is associated with the individually specific repertoire of KIR receptors (40). The precise contribution of inhibitory and activating Ly49 and KIR receptors to NK-mediated alloreactivity remains to be determined. Studies in rats have pointed toward a central role for the activating receptors (10, 11). Therefore, it is possible that transplant patients lacking activating KIRs have a reduced risk of rejection of NK-incompatible (KIR/HLA) stem cell grafts in the clinical setting, especially under nonmyeloablative regimens. This is relevant as the activating Ly49 and KIR receptors are often expressed in a stochastic fashion (43, 44). Other mechanisms must be sought, however, to explain why the NKR-P1C⁺ NK cells show such limited expression of Ly49 (and KLRH1) receptors spanning ~1.8 Mb of the rat genome, i.e., from the telomeric (Ly49h8) to the centromeric end (KLRH1) of the Ly49 genomic region. This suggests the presence of some dominant regulatory mechanism affecting gene expression of this region of the rat NKC. This could be a suppressor molecule binding to a common regulatory element or some sort of epigenetic process inhibiting access of transcription factors to the regulatory regions, e.g., DNA methylation, histone modification, or changes in higher-order chromatin structure (45).

The NKR-P1C-encoding gene is lacking in the genome from the BN rat strain, explaining why we were able to generate a mAb to NKR-P1C in BN rats. The BN strain harbors the three previously published Nkr-p1 genes encoding the NKR-P1A (activating; Refs. 17 and 18), NKR-P1B (inhibitory; Refs. 4 and 19), and NKR-P1D (presumably activating; Ref. 46) molecules, as well as a novel fourth Nkr-p1 gene with inhibitory structural features (our unpublished data). Hence, there are at least five different Nkr-p1 genes in rats, but little is known about their cellular distribution and function. As shown in the present study, NKR-P1C appears to be restricted to NK cells, and mainly to a Ly49-negative fraction with unique functions. The two available anti-NKR-P1 mAbs (3.2.3 and 10/78) do not distinguish between the NKR-P1A and NKR-P1B molecules. They are generally used as pan-NK cell reagents, but they also stain a significant proportion of CD8⁺ T cells. Generation of locus-specific reagents will aid the characterization of the rat NKR-P1 family.

The identity of the NKR-P1 ligands has long remained elusive. Candidates have been both histocompatibility Ags (20) and simple saccharide moieties, but the natural ligands for NKR-P1 receptors were recently shown to be the Clr molecules, which are encoded within the NKR-P1 genomic region. Although the ligand for NKR-P1C has not yet been identified, the functional data suggest it will be one of the 5–10 uncharacterized Clr molecules present in the BN strain genome. Blocking of NKR-P1C with mAb STOK27 induced modest lysis of allogeneic and syngeneic lymphoblasts irrespective of their MHC haplotype, pointing to the recognition of a monomorphic target cell ligand. This is compatible with NKR-P1C functioning as a “missing self” receptor for a non-MHC ligand, likely a conserved Clr molecule. Assuming that this is the case, its negative regulation of NK cytotoxicity must operate independently of any MHC-dependent inhibitory receptors present on the same cells. Comparable levels of CD94 and Nkg2a message were observed in the NKR-P1C⁺ and Ly49s3⁺ NK subsets, suggesting that many of the NKR-P1C⁺ cells coexpress a conserved inhibitory CD94/NKG2A receptor which is likely to bind the rat ortholog of mouse Qal (i.e., RT-BM1). NKR-P1C may be important for the maintenance of self-tolerance in the NKR-P1C⁺ subset, and the ontogenetic data suggest that it may be important already at an early stage of development.

It is likely that the observed functional differences between the NKR-P1C⁺ and Ly49s3⁺ NK cells are a reflection of their functions in vivo. This is compatible with their distinct tissue distribution. The marked predominance of the NKR-P1C⁺ subset in the mesenteric LNs is striking, but it can only be speculated as to whether this is due to preferential recruitment of these cells, exclusion of the Ly49s3⁺ cells, or enhanced in situ development. The involvement of various chemokines and their receptors in the trafficking of these NK subsets remains to be determined. It is also tempting to draw a comparison with subsets in other species, notably the CD56bright and CD56dim cells in humans (47). The CD56bright cells lack KIR expression, and are preferentially located in the LNs, where they apparently can develop from precursors (48). Future studies will have to be performed to further clarify the functional and phenotypic differences between the two unique subsets in rats.

Acknowledgments

We thank Bente Kahrs Omdal for excellent technical assistance, Dennis Buurman (Bio-Rad) for help with multiplex cytokine measurement, and Dr. Michael Daws (Oslo, Norway) for valuable discussions and critical comments of the manuscript.
Disclosures

The authors have no financial conflict of interest.

References


