

Bone Marrow Allograft Rejection Mediated by a Novel Murine NK Receptor, NKG2I

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Abstract

Natural killer (NK) cells mediate bone marrow allograft rejection. However, the molecular mechanisms underlying such a rejection remain elusive. In previous analyses, it has been shown that NK cells recognize allogeneic target cells through Ly-49s and CD94/NKG2 heterodimers. Here, we describe identification and characterization of a novel murine NK receptor, NKG2I, belonging to the NKG2 family. NKG2I, which was composed of 226 amino acids, showed ~40% homology to the murine NKG2D and CD94 in the C-type lectin domain. Flow cytometric analysis with anti-NKG2I monoclonal antibody (mAb) revealed that expression of NKG2I was largely confined to NK and NKT cells, but was not seen in T cells. Furthermore, anti-NKG2I mAb inhibited NK cell-mediated cytotoxicity, whereas cross-linking of NKG2I enhanced interleukin 2- and interleukin 12-dependent interferon- γ production. Similarly, the injection of anti-NKG2I mAb before the allogeneic bone marrow transfer in vivo impinged on the function of NKG2I, resulting in the enhanced colony formation in the spleen. NKG2I is a novel activating receptor mediating recognition and rejection of allogeneic target cells.

Key words: C-type lectin family • NK cells • activating receptor • IFN- γ • NKT cells

Introduction

NK cells act as a first line of defense in innate immunity and also mediate rejection of allogeneic target cells, such as BM allografts (1, 2). NK cell function is exquisitely controlled by the orchestration of signals from stimulatory and inhibitory NK receptors, which recognize MHC class I-related molecules on target cells (3, 4). NK receptors have been categorized into three groups based on their structural and sequence homology. The first is the killer cell inhibitory receptors, belonging to the Ig superfamily, which are expressed in humans but not in rodents (5, 6). The second group is the C-type lectinlike Ly-49 receptors present solely in rodents (7). The third is the lectinlike homo- and

heterodimer receptors, consisting of CD94 and a member of NKG2 family; they are observed both in humans and in rodents (8–10). Killer cell inhibitory receptors and Ly-49s recognize classical MHC class I molecules, whereas NKG2 family receptors bind nonclassical MHC class I molecules (11–13).

Regarding the role of NK receptors in NK cell-mediated allojection, involvement of the NK receptors, such as CD94 and Ly-49, has been reported. Blocking the function of CD94 with anti-CD94 mAb enhances the in vitro cytotoxicity of C57BL/6 NK cells against BALB/c Con A lymphoblast target cells (14), implying that CD94, most likely together with NKG2A, may recognize the Qa-1 on the target cells. In addition, anti-Ly-49D mAb treatment results in eradication of a Ly-49D⁺ subset in lethally irradiated C57BL/6 mice that, in turn, abrogates the ability to reject H-2D^d BM graft (15). In the present paper, we report identification and characterization of a novel murine

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NKG2 family receptor, NKG2I. We show that NKG2I acts as an activating receptor and mediates allorecognition and subsequent cytotoxic activity.

Materials and Methods

Animals. C57BL/6, BALB/c, C3H/HeN, ICR nu/nu mice, and Fisher rats were obtained from Charles River Laboratories. DBA/2Cr, AKR/N, (BALB/c × C57BL/6) F1, and 129/svj mice were obtained from SLC and Jackson Laboratory, respectively. All experiments were performed in accordance with our institutional guidelines.

Identification of NKG2I through the Library Subtraction. PCR-Select cDNA subtraction kit (CLONTECH Laboratories, Inc.) was used to generate a library enriched for genes expressed in NKT cells over T cells. In brief, T cells were purified from the spleen of J α 281 knockout mice (16), and NKT cells were purified by FACSVantage™ cell sorter (Becton Dickinson) with α -galactosylceramide/CD1-d tetramer staining from the spleen of C57BL/6 mice. Purity of sorted cells was estimated to be >98%. In subtraction, T cell cDNA was used as a tester, whereas that of NKT cells was used as a driver. After subtraction, 1,000 clones were sequenced and analyzed by virtual Northern blot analysis.

Generation of Anti-NKG2I Monoclonal Antibodies. Fisher rats were injected with a fusion protein consisting of the extracellular portion of NKG2I and the Fc region of the human immunoglobulin. Spleen cells were fused to the SP2/O-Ag14 fusion partner. Hybridoma supernatants were screened for the staining ability of NKG2I-transfected COS7 cells by flow cytometric analysis. Five clones were established as follows: 3G7 (IgG1/ κ), 5C6 (IgG1/ κ), 7E8 (IgG2a/ κ), 9D5 (IgG2a/ κ), and 9F2 (IgG2a/ κ). The mAbs were purified from ascites with protein G-Sepharose 4B and conjugated with biotin, FITC, and Cy5 with commercial kits.

Antibodies and cDNA. Anti-NKG2D antibody, murine CD94, NKG2C, E, D, DAP12, and DAP10 cDNA were provided by D.H. Raulet (University of California, Berkeley, Berkeley, CA). All other antibodies were obtained from BD Biosciences.

NK Cell Preparation and Stimulation by Anti-NKG2I mAb. Splenic NK cells were enriched with anti-DX5 Ab using the AutoMACS™ (Miltenyi Biotec). For stimulation experiments, NK cells were further purified as NK1.1⁺CD3⁻ cells to >90% by depleting CD4⁺, CD8⁺, CD3⁺, Gr-1⁺, Ter-119⁺, and CD19⁺ cells. NK cells (10⁵ cells/well) were incubated for 24 h with or without IL-2 and IL-12 in 96-well plates precoated with 10 μ g/ml of mAb, and the concentration of IFN- γ was measured by ELISA.

Cytotoxic Assay against Con A Lymphoblasts. Con A lymphoblasts were generated by stimulation of spleen cells with 2 μ g/ml Con A (Sigma-Aldrich) for 48 h. Cytotoxic activities of IL-2-expanded NK cells (cultured for 1 wk) against Con A lymphoblasts were measured by a standard ⁵¹Cr release assay. The specific lysis was calculated as follows: percent-specific lysis = $^{51}\text{Cr cpm} [(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})] \times 100$.

Biochemistry. IL-2-expanded NK cells were surface labeled with ¹²⁵I (Amersham Biosciences) using lactoperoxidase and lysed with buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, and the protease inhibitor cocktail). The cell lysates were incubated with the 7E8-coated protein G-Sepharose 4 FF (Amersham Biosciences) and washed extensively. The samples were separated on an SDS-PAGE, and bands were visualized using the Bio Image Analyser (model BAS2500; Fuji Film).

Assay for BM Engraftment. Recipient C57BL/6 or (BALB/c × C57BL/6) F1 mice irradiated at 9.5 Gy were received intravenously with BALB/c or C57BL/6 BM cells. Spleen was removed and fixed with the Bouin's fixative on day 8, and the colony number was counted.

GenBank Accession No. The NKG2I sequence has been deposited in GenBank/EMBL/DDBJ under accession no. AF306663.

Online Supplemental Material. Table S1 summarizes the results of cDNA subtraction (NKT vs. T cells), and Figs. S1 and S2 show the control experiments using anti-asialo GM1 and anti-NKG2I. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20030851/DC1>.

Results and Discussion

A Novel NK Receptor Belonging to NKG2 Family. NKG2I was originally identified as a gene expressed preferentially in NKT cells over T cells by subtractive hybridization. Analysis of subtracted cDNA revealed that NK receptors, such as NKG2A/B, NKR1-C, and NKG2D, were preferentially expressed in NKT cells (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20030851/DC1>). A novel clone, preferentially expressed in NK and NKT cells, showed an extensive homology to the NK receptors and was subjected to further analysis (Figs. 1 and 2 A).

Putative proteins deduced from the nucleotide sequence consisted of 226 amino acids with four structural domains as follows: cytoplasmic (1-68), transmembrane (69-93), stalk (94-109), and C-type lectin domain (CTLD; 110-226) (Fig. 2 A). Sequence comparison indicated that NKG2I was a novel NK receptor belonging to the NKG2 family. In fact, the CTLD within NKG2I showed ~40% homology to that of murine NKG2D and CD94. Moreover, the residues in NKG2I, which may be engaged in disulfide bond formation (Fig. 2 A, closed circles) and in receptor dimerization (Fig. 2 A, closed circles and arrowheads), are readily inferred from the crystal structures of CD94 and NKG2D (17, 18). These data suggest that the CTLD in NKG2I possesses a higher order structure similar to CD94 and NKG2D. Nevertheless, NKG2I lacks any signaling motifs and positively charged amino acids in its putative transmembrane region, which are required for association with adaptor molecules such as Fc ϵ RI γ , CD3 ζ , DAP10, and DAP12 (Fig. 2 A; references 19, 20).

Ligands for other members of the murine NKG2 receptors are MHC class I-related molecules as follows: Qa-1 for

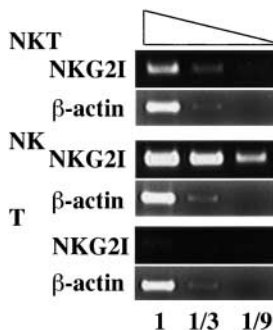


Figure 1. Preferential expression of NKG2I in NK and NKT cells over T cells. NKG2I expression was assessed by RT-PCR. β -actin serves as input cDNA control. Threefold serial dilution of cDNA was used.

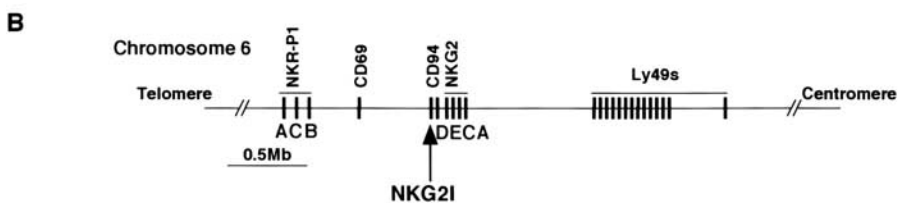
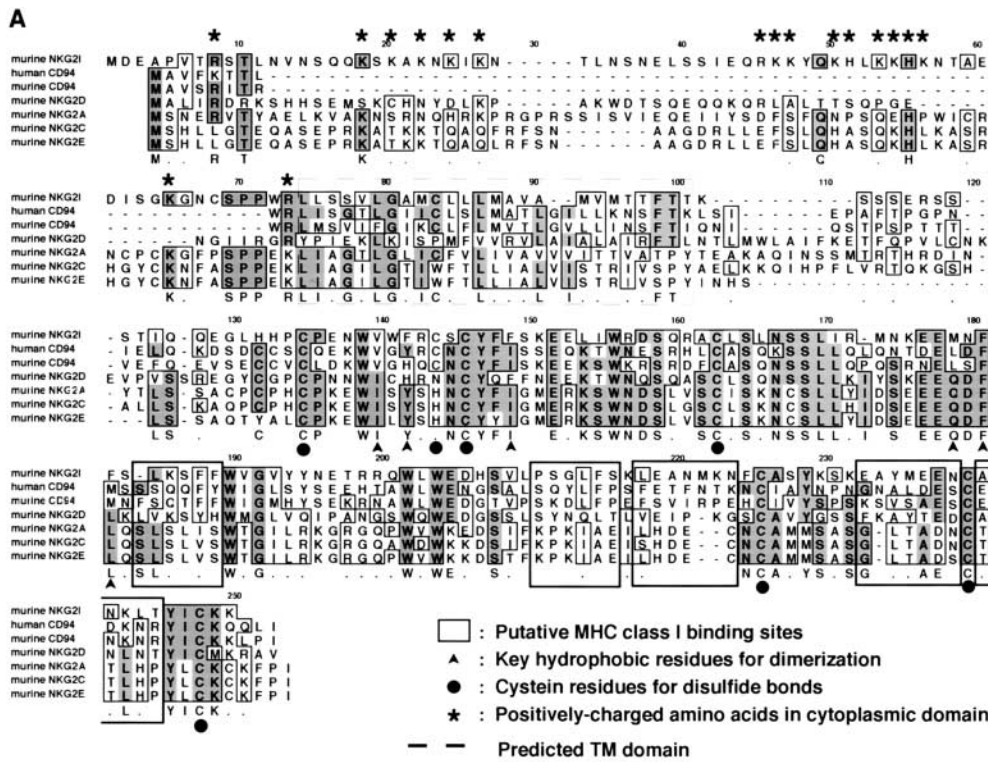


Figure 2. Characterization of NKG2I. (A) Alignment of the murine NKG2 family NK receptors and human CD94. The amino acid sequences of the murine NKG2 family receptors and human CD94 are aligned using the ClustalW algorithm. Highly conserved residues are displayed in the dark shaded boxes and depicted as consensus amino acids below the alignment. Similar amino acids are shown in light shaded boxes and are indicated with dots. The dotted line indicates the predicted transmembrane region using the TMpred program in EMBnet (<http://www.ch.embnet.org>). The squares indicate the putative ligand-binding site based on the crystal structure of the human CD94. The arrowheads show the hydrophobic residues responsible for dimerization. The closed circles indicate the cysteins involved in inter- and/or intramolecular disulfide bonding. The asterisks exhibit the positively charged amino acids in the cytoplasmic domain. (B) Genomic map of the murine NK gene complex. The map is presented schematically based on the mouse genome sequence from the National Center for Biotechnology Information. The relative location of the NKG2I is indicated by the arrow.

CD94/NKG2 heterodimers (21, 22) and Raes, H60, and MULT1 for NKG2D homodimers (23–25). As NKG2I exhibits little similarity in a putative ligand binding domain to other NK receptors (Fig. 2 A, squares), it may recognize ligands distinct from those aforementioned (17, 18). An abundance of positively charged amino acids in the cytoplasmic domain of NKG2I points to the possibility that it may associate with novel adaptors responsible for signal transduction (Fig. 2 A, asterisks).

DNA sequence analysis of NKG2I in the National Center for Biotechnology Information mouse genome database revealed that NKG2I maps adjacent to the CD94/NKG2 gene cluster and between CD69 and CD94 in NK receptor gene complex on murine chromosome 6 (Fig. 2 B). Curiously, no human orthologue of NKG2I was found, suggesting that this receptor may be unique to rodents.

Characterization of NKG2I by the mAbs. To characterize NKG2I, we established several mAbs specific for NKG2I (Fig. 3 A). These mAbs stained NKG2I-transfected COS7 cells specifically, and did not cross react with CD94, NKG2A/C/E, or NKG2D molecules (Fig. 3 A). Staining with 3G7 mAb revealed that most NK (NK1.1⁺CD3⁻) cells

in the spleen (84%), BM (74%), and liver (77%) expressed NKG2I (Fig. 3 B). NKG2I was also expressed on a subset of NKT (NK1.1⁺CD3⁺) cells, the expression levels of which varied significantly among tissues (ranging from 13 to 54%), but was barely detected on T (NK1.1⁻CD3⁺) cells (Fig. 3 B).

Because the expression of NKG2 varies depending on mouse strains (14), we examined whether this is also the case for NKG2I. Flow cytometric analysis showed that NKG2I as well as CD94 and NKG2A/C/E expressions were found regardless of strains (Fig. 3 C, C57BL/6, BALB/c, C3H/HeN, DBA/2Cr, AKR/N, and 129/svJ). However, the expression level of NKG2I differed among the strains, implying the “calibration” of this receptor (26). Moreover, the cDNA sequence for NKG2I from these strains revealed that they encoded an identical amino acid, suggesting that NKG2I is an invariant molecule on NK cells and exhibits an expression profile distinct from that of Ly-49s, which carry allelic polymorphisms (27).

Biochemical analysis with ¹²⁵I-labeled NK cells followed by immunoprecipitation with anti-NKG2I mAb (7E8) revealed that NKG2I migrated predominantly as a ~96-kD protein under nonreducing conditions, whereas a prominent

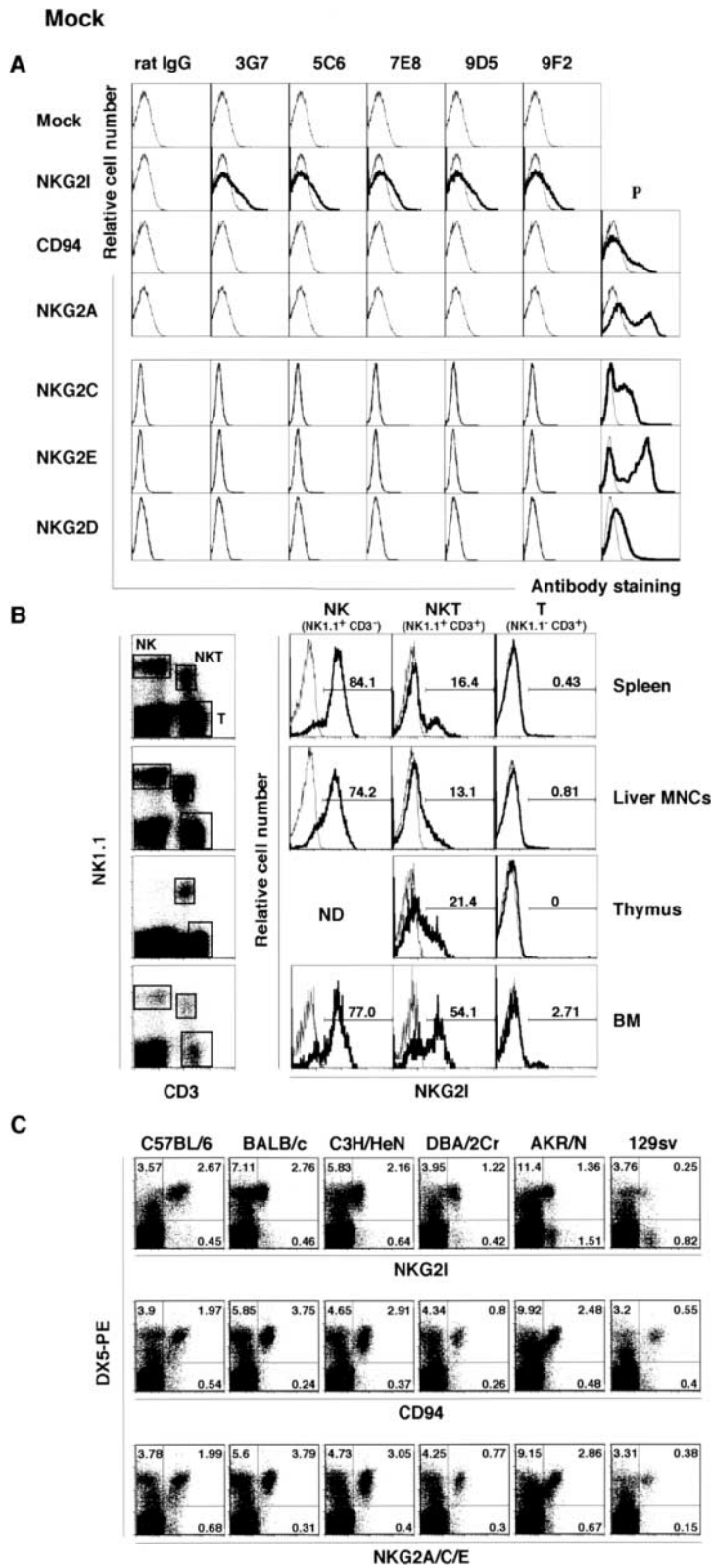


Figure 3. Characterization of anti-NKG2I monoclonal antibodies. (A) Establishment of the mAbs against NKG2I. COS7 cells were transiently transfected with mock, NKG2I, murine CD94, NKG2A/C with CD94, NKG2E with DAP12, and NKG2D with DAP10 cDNA expression vector. Cells were harvested 48 h later and incubated with the biotin-conjugated 3G7, 5C6, 7E8, 9D5, 9F2, or control rat IgG, followed by staining with the FITC-streptavidin (thick histogram). For CD94, NKG2A/C/E, and NKG2D transfectants, cells were incubated with the biotin-conjugated anti-CD94 mAb or with the anti-NKG2A/C/E or anti-NKG2D mAb as positive control (P, thick histogram). Background staining is shown as thin histograms. (B) Expression of NKG2I in the various lymphoid subsets. Spleen, liver mononuclear cells, thymocytes, and BM cells from C57BL/6 mice were stained with the FITC-anti-NKG2I (3G7), PE-NK1.1, and Cy5-anti-CD3. The expression of NKG2I in the gated lymphoid subsets for NK (NK1.1⁺CD3⁻), NKT (NK1.1⁺CD3⁺), or T (NK1.1⁻CD3⁺) cells is shown as histogram (thick lines) overlaid with the background staining (thin lines). The percentage of NKG2I⁺ cells in each lymphoid subset is shown. (C) Expression of the NKG2I, CD94, and NKG2A/C/E in the various strains of mice. Spleen cells from the different mouse strains were stained with PE-DX5 together with biotin-anti-NKG2I (3G7), anti-CD94, or anti-NKG2A/C/E mAb followed by FITC-streptavidin. Percentage of cells in each subset is shown. (D) NKG2I exists as a dimer with the disulfide bond. C57BL/6 NK cells expanded with IL-2 were surface labeled with ¹²⁵I and immunoprecipitated with the anti-NKG2I mAb (7E8) or rat IgG2a. Immunoprecipitated proteins were separated on an SDS-PAGE under nonreducing (in the absence of 2-mercaptoethanol [-2ME]) or reducing (in the presence of 2-mercaptoethanol [+2ME]) conditions, and visualized with BAS2500.

~48-kD band emerged under reducing conditions on an SDS-PAGE (Fig. 3 D, arrowheads). This suggests that NKG2I forms homo- or heterodimer-like other NKG2

family members (21, 23, 24). Because its predicted molecular weight is 26,265, it is conceivable that NKG2I is covalently modified, though its precise nature has yet to be examined.

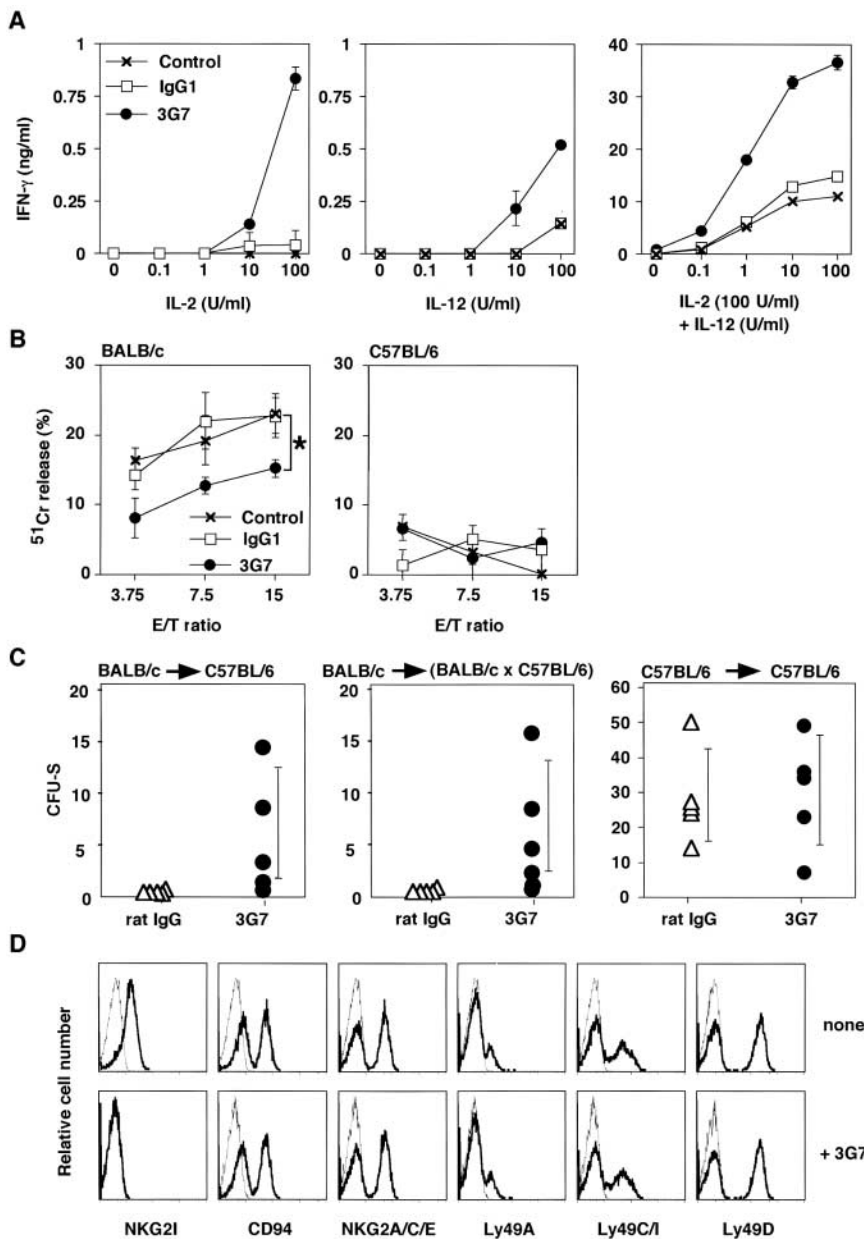


Figure 4. NKG2I functions as an activating NK receptor in allograft rejection. (A) IFN- γ production upon cross-linking of NKG2I in NK cells. NK cells from C57BL/6 spleen were incubated in the presence of IL-2, IL-12, or IL-2/IL-12 in the plate immobilized with 3G7 or rat IgG1. Control experiments were done without any antibody. The concentrations of IFN- γ in the culture supernatant are shown. Data are indicated as mean \pm SD from three independent experiments. (B) 3G7 mAb inhibits the cytotoxicity of NK cells against the allogeneic target cells. The cytolytic activity of C57BL/6 NK cells expanded with IL-2 was determined in the presence of 10 $\mu\text{g}/\text{ml}$ of 3G7 mAb or control rat IgG1. Control experiments were done without any antibody. Con A lymphoblasts prepared from BALB/c (left) or C57BL/6 (right) mice were used as target cells. Data are shown as mean \pm SD from three independent experiments ($n = 6/\text{experiment}$). *, $P < 0.05$ as compared 3G7 mAb versus rat IgG1 at the indicated E/T ratios. (C) Blocking the NKG2I function compromises rejection of the allogeneic BM cell transplantation. Responder C57BL/6 or (BALB/c \times C57BL/6) F1 mice were treated with 100 μg of 3G7 mAb or rat IgG before BM transplantation with 3×10^6 cells (for C57BL/6 recipients) or 0.1×10^6 cells (for F1 recipients) from BALB/c or C57BL/6, and received the same amount of each of the Abs on days 3 and 6. The number of colonies in the spleen of the recipient mice ($n = 5$) was counted 8 d after BM transfer. The error bars represent the standard deviation, and representative data from three independent experiments are shown. (D) Blocking the function of NKG2I does not interfere with the expression of other NK receptors. The effect of 3G7 mAb on the expression of other NK receptors relevant to the allograft rejection was evaluated by flow cytometric analysis on NK cells. C57BL/6 mice were left untreated (top) or treated with 100 $\mu\text{g}/\text{body}$ of 3G7 (bottom) for 1 h. NK (NK1.1 $^+$ CD3 $^-$) cells were stained with the biotin-anti-NKG2I (7E8), biotin-anti-CD94, biotin-anti-NKG2A/C/E, biotin-anti-Ly49A, biotin-anti-Ly49C/I, or FITC-anti-Ly49D, respectively. Biotin-conjugated Ab was visualized with FITC-streptavidin. Expression of each NK receptor is shown as histogram (thick lines) overlaid with background staining (thin lines).

NKG2I Acts as an Activating Receptor in Allograft Rejection. Given that NKG2I is expressed predominantly in NK cells (Fig. 3 B) and that CD94, which is quite similar to NKG2I, is implicated in recognition of allogeneic target cells (14), we hypothesized that NKG2I also plays a role in NK cell-mediated allograft rejection and subsequent cytotoxic activity. Because activating signals elicited from NK receptors often lead to cytokine production and/or activation of cytotoxicity (28), the function of NKG2I was assessed by measuring IFN- γ production (Fig. 4 A). Cross-linking of NKG2I with 3G7 mAb led to induction of IFN- γ in the presence of either IL-2 or IL-12 in a cytokine dose-dependent manner and their synergistic effects were observed (Fig. 4 A, right). These results indicate that NKG2I cross-linking en-

hances the extent of IL-2- and IL-12-elicited signaling leading to IFN- γ production. Thus, NKG2I may serve as an activating NK receptor.

We explored the function of NKG2I through an *in vitro* cytolytic assay with 3G7 mAb (Fig. 4 B). C57BL/6 NK cells lysed allogeneic BALB/c but not syngeneic C57BL/6 Con A lymphoblast target cells (Fig. 4 B). In contrast, treatment of C57BL/6 NK cells with 3G7 mAb inhibited cytolytic activity against BALB/c target cells significantly, whereas control Ab showed little effect (Fig. 4 B, left).

Allogeneic BM cell transplantation experiments further confirmed these results (Fig. 4 C). BALB/c BM cells administered into lethally irradiated C57BL/6 mice (BALB/c into C57BL/6) were rejected in a manner dependent on

NK cells, and no colony derived from the transplanted cells appeared in the spleen of recipient mice as reported (Fig. 4 C, left; reference 29). In contrast, administration of 3G7 mAb but not the control anti-rat IgG Ab into the recipient C57BL/6 mice before BALB/c BM transfer suppressed the rejection of BALB/c BM grafts and resulted in a significant number of colony formations in the spleen (Fig. 4 C, left). Similarly, (BALB/c × C57BL/6) F1 mice as a recipient, whose T cells are tolerant to the parent BALB/c, showed significantly impaired rejection of BALB/c BM cells (BALB/c into F1) in the presence of 3G7 mAb (Fig. 4 C, middle). In contrast, no effects were observed in the syngeneic BM transplantation (Fig. 4 C, right, C57BL/6 into C57BL/6). These results indicate that NKG2I on NK cells recognizes putative ligands present on allogeneic BM cells and induces signals leading to the rejection of allografts.

It should be mentioned that administration of anti-NK1.1, anti-asialo GM1, or anti-Ly-49D mAb also abrogated the rejection of allogeneic BM grafts (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20030851/DC1>), but these effects were primarily due to the depletion of NK cells expressing these molecules (15, 29). On the other hand, 3G7 mAb treatment did not change the number of NK cells in vivo (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20030851/DC1>). More importantly, 3G7 mAb treatment did not alter the expression of other NK receptors, such as CD94, NKG2A/C/E, Ly-49A, Ly-49C/I, and Ly-49D, under the conditions that NKG2I expression was rapidly down-regulated in vivo (Fig. 4 D). These results suggest that allogeneic recognition and subsequent activation of NK cells leading to cytotoxic activity against allogeneic BM cells is attributed to NKG2I but not to modulation of other NK receptor expression.

In summary, we have identified and characterized a novel activating NK receptor that plays a crucial role in allograft rejection. Identification of the cognate ligands and elucidation of the signaling pathway of NKG2I will shed light on mechanisms underlying allogeneic recognition and rejection.

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