

MURINE NATURAL KILLER CELLS EXPRESS FUNCTIONAL Fc γ RECEPTOR II ENCODED BY THE Fc γ R α GENE

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The surface phenotype of murine NK cells, freshly obtained from untreated mice or from mice in which spontaneous cytotoxicity has been boosted with IFN or IFN inducers such as poly(I:C) or acute virus infections, is well characterized (1-3). Although both freshly obtained splenic NK cells and in vitro cultured NK cells (1) do not express most markers present on the majority of B (sIg) or T cells (CD3; CD8, Lyt2; CD4, L3T4), they share with T and myelomonocytic cells other markers (asialo GM1, Thy-1, Qa-2, Qa-5, and CR3). NK cells from appropriate strains express a unique allospecific marker, NK-1.1 (4, 5), that is restricted to NK cells (reviewed in reference 6). The murine NK cell phenotype resembles that of human NK cells. In humans the most useful marker to distinguish both freshly isolated and cultured NK cells from other lymphocytes is a low affinity receptor for the Fc portion of immune complexed IgG (CD16 or human [hu]¹ Fc γ RIII) (7). This Fc γ R is shared with granulocytes and macrophages (8, 9), is antigenically and biochemically distinct from other known Fc γ R (10), and allows NK cells to mediate antibody-dependent cell-mediated cytotoxicity (ADCC) (11).

Indirect evidence that murine NK cells bear Fc γ R is provided by data showing that: (a) removal of Fc γ R-bearing cells from a spleen cell population, by absorption on monolayers of IgG-sensitized erythrocytes (EA7S), significantly reduces, although never completely abolishes, its spontaneous cytotoxicity against tumor cells (12); and (b) a relevant proportion of the splenic lymphocytes that form conjugates with the NK-sensitive target YAC-1 belong to the lymphocyte subset that forms rosettes with EAIG2b (13). However, no direct evidence exists for the presence of a defined Fc γ R on murine NK cells. Indeed, in one study, Fc γ R was not detected on cultured NK cell clones (14).

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¹ Abbreviations used in this paper: ADCC, antibody-dependent cell-mediated cytotoxicity; hu, human; LU, lytic units; mo, mouse; PE, phycoerythrin.

Murine macrophages express three distinct Fc γ R types (15, 16). All cell types expressing any of the three receptors form EA7S rosettes, and rosette formation using indicator systems sensitized with polyclonal IgG cannot distinguish among them (reviewed in reference 10). mAb 2.4G2 detects an Fc γ R expressed at high density on mouse macrophages and, at lower density, on B and T lymphocytes (mouse [mo] Fc γ R II) (17), which binds murine IgG2b, IgG1, and IgG2a with low affinity (17-19). Functional properties of the Fc γ R are inhibited by antibody 2.4G2 both in vitro and in vivo (20-22). The genes encoding moFc γ R II recognized by 2.4G2 have been cloned (23-25) and isolated. cDNA probes are derived from transcripts of two different genes, α and β ; α appears to be expressed solely in macrophages (23). Two β gene transcripts, β 1 and β 2, have been identified. β 1, and possibly β 2, is expressed in 2.4G2⁺ B and T cell lines, whereas only β 2 is expressed in macrophage-like cell lines (23).

In this paper we report direct evidence that both fresh and cultured murine NK cells express on their surface a functional Fc γ R recognized by antibody 2.4G2. In contrast to macrophages or B and T lymphocytes, murine NK cells express only Fc γ R α transcripts.

Materials and Methods

Cell Lines. The murine mastocytoma P815, thymoma YAC-1, B cell lymphoma BCL₁, T cell lymphomas EL-4, S49.1, and CTLL, the macrophage-like cells P388D₁ and J774a, and the fibroblast L cells were maintained in culture in RPMI 1640 (Flow Laboratories, Rockville, MD) medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY). All cell lines were mycoplasma-free on repeated testing.

Antibodies. mAbs 2.4G2 (anti-moFc RII) (17), PK136 (anti-NK-1.1) (5), and 3C7 (anti-IL-2R) were kindly provided by Drs. J. Unkeless (Mount Sinai Hospital, New York, NY), G. Koo (Merck, Sharp and Dohme Research Laboratories, Rahway, NJ), and T. Malek (Miami University, Miami, FL), respectively. Antibody F4/80 (26), recognizing a specific macrophage marker, was obtained from Dr. P. Witte (University of Texas, Dallas, TX). Antibody anti-Mac1 (anti-CD11b) was prepared from cells obtained from the American Type Culture Collection (American Type Culture Collection, Rockville, MD). B137.17 is a murine IgG2a with no known antigen specificity. Polyclonal sera anti-asialo GM1 and anti-NK-2.1 were purchased from Wako Chemicals (Dallas, TX) and kindly provided by Dr. R. Burton (University of Newcastle, Australia), respectively. The FITC-labeled mAbs anti-Thy-1, anti-Lyt-1, anti-Lyt-2, anti-L3T4 and anti-I-A were kindly provided by Dr. J. Cebra (University of Pennsylvania, Philadelphia, PA). When indicated, the antibodies were labeled with biotin or FITC according to standard procedures. The rabbit IgG anti-bovine E used to prepare EA7S and the polyclonal affinity-purified FITC-labeled goat F(ab')₂ anti-murine IgM and IgG antibodies were obtained from Cooper Laboratories (Malvern, PA). The goat anti-murine Ig used for indirect rosetting and the mouse C3H anti-DBA/2 polyclonal antibodies used for ADCC were produced in our laboratories (1, 7).

Lymphocyte Preparations. BALB/c, C3H/He, C57BL/6, and DBA/2 mice were obtained from The Jackson Laboratories (Bar Harbor, ME); the (C57BL/6 \times DBA/2)F₁ [(B6D2)F1] hybrid mice were produced as previously described (27). C.B-17 scid/scid (severe combined immunodeficiency, *scid*) mice were produced under specific pathogen free conditions at the University of Texas (Dallas, TX). When indicated, mice were injected with poly(I:C) (Pharmacia Inc., Piscataway, NJ), 50 μ g i.p., 24 h before they were killed. Spleens were teased and splenic lymphocytes were obtained by Ficoll/Hypaque (F/H) density gradient centrifugation (1.092 \pm 0.001 g/ml). When indicated, the total lymphocyte preparations were depleted of macrophages and B lymphocytes by adherence to plastic and/or direct rosetting with CrCl₃-treated goat anti-mouse Ig-coated sheep E and density-gradient centrifugation as previously described (7). 2.4G2⁺ and 2.4G2⁻ subsets were obtained from sIg⁻ lymphocytes by sorting on an Ortho

Cytofluorograf 50H, connected to a 2100 Data Analyzer (Ortho Instruments, Westwood, MA), after sensitization with biotin-labeled 2.4G2 and FITC-avidin (Vector Laboratories, Burlingame, CA) as previously described (28). In vitro propagated NK cells were prepared as described (29, 30). Briefly, NK-1.1⁺ cells were selected from nylon-wool passaged and Percoll-fractionated (B6D2)F₁ mouse splenocytes using indirect immunofluorescence and FACS (FACStar; Becton Dickinson & Co., Mountain View, CA). NK-1.1⁺ cells (95% pure, on FACS reanalysis) or unfractionated *scid* splenocytes were cultured (10⁵, 10⁶ cells/ml, respectively) at 37°C for 5–11 d in RPMI 1640 (Gibco Laboratories) supplemented with 10% FCS and 500 U/ml human rIL-2, generously provided by Cetus Corp. (Emeryville, CA).

Immunofluorescence and FACS. The surface phenotype of the cells was detected with mAbs using direct and indirect immunofluorescence (flow cytometry) using biotin-labeled antibodies and FITC-avidin or FITC-labeled antibodies, as indicated, according to previously published procedures (31). Fluorescence intensity was measured on either exponential or linear scales, as indicated. Two-color immunofluorescence was performed as previously described (31) using predetermined saturating concentrations of FITC-labeled antibodies and of biotin-labeled antibodies detected with phycoerythrin (PE)-labeled avidin (Becton Dickinson & Co.).

Cell-mediated Cytotoxicity. Spontaneous and antibody-dependent cytotoxicity were tested at several E/T cell ratios using ⁵¹Cr-labeled YAC-1 and antibody-sensitized P815 cells as target cells (10⁴/well) in either 18-h or 4-h assays, as indicated. To quantitate cytotoxicity, lytic units (LU) were calculated on the basis of linear regression analysis to a modified Von Grogh equation according to established methods (7) and referred to the absolute number of cells recovered. 1 LU is defined as the number of effector cells that kills 45% of the targets (LU [45%]) during the test period. When indicated, anti-moFcγRII and rat control isotype-matched antibodies (ascites, 1:200 dilution) were present in the E/T cell mixture.

FcγR Functional Assay. The ability of cell surface FcγR to bind immune complexes was tested using EA7S as indicator system in rosetting assays, as previously described (8).

FcγR mRNA Detection. To define the gene(s) encoding the FcγRII in murine NK cells, Northern blot analysis was performed on mRNA obtained from either (B6D2)F₁ NK1.1⁺ or C.B-17 *scid* NK2.1⁺ spleen cells that were propagated in 500 U/ml rIL-2 for 11 and 5 d, respectively. Methods for RNA preparation, fractionation in agarose gels, transfer to nylon membranes, hybridization with ³²P-labeled probes, and stripping of filters for rehybridization were as described previously (29). RNAs were also prepared from the BCL₁, EL4, and P388D₁ cell lines. The FcγRα + β probe is a 1.0-kb Nco I restriction fragment excised from the FcγRα cDNA clone (23). This probe detects FcγRα/β1/β2 mRNA. Specific probes were obtained from the 5' portion of the FcγRα and FcγRβ2 cDNA clones. The α-specific probe is a 125-bp Hinf I–Alu I fragment and the β-specific probe is a 115-bp Hinf I fragment. Each fragment contains 5'-untranslated sequences as well as the coding region for the signal peptide. cDNA probes for MHC class II I-Eβ (32) and β-actin (33) were used as controls. Integrity and quantity of mRNA loaded per lane (20 μg) were monitored by ethidium bromide staining of the gels and by hybridization with the β-actin probe.

RNase Protection. A cDNA probe encoding the mouse FcγRα gene (23) was inserted into the pGEM-3 vector and used to generate uniformly-labeled antisense RNA. A 920-bp run-off transcript was generated by linearizing the cloned cDNA at the Bgl II site. Labeling and protection were performed essentially as described by the manufacturer (Promega-Biotec, Madison, WI). Briefly, 10 μg of total RNA from P388D₁, J774a, S49.1, L cells and the in vitro propagated NK cell populations described above were hybridized (55°C, 16 h) to the probe in a reaction mixture that included 80% formamide, 0.4 M NaCl, and 0.04 M Pipes, pH 6.4. The unprotected RNA was digested with RNase A (34°C, 90 min) and the protected fragments were analyzed on a 5% acrylamide, 8M urea, Tris-Borate-EDTA (TBE) gel. The gel was dried and allowed to expose x-ray film for 6 h.

Results

A Small Proportion of sIg⁻ Splenic Lymphocytes Bear FcγRII. Since treatment of mice with poly(I:C) induces a slightly increased number of NK cells without altering the

phenotype or function of other lymphocyte populations (2), mice were pretreated with poly (I:C) in most experiments. About 50% of murine splenic lymphocytes from all strains tested reacted with anti-Fc γ RII antibody 2.4G2. To analyze whether all 2.4G2⁺ cells were sIg⁺ B lymphocytes, additive staining experiments were performed. The proportion of cells detected using each mixture of two antibodies was compared with the sum of those obtained using each antibody separately. The percentage of BALB/c splenic lymphocytes detected with a combination of 2.4G2 and either anti-Lyt-2 or anti-L3T4 antibodies did not differ significantly from the sum of those detected with each antibody separately (Table I), indicating that the antibodies detect discrete lymphocyte populations. Combination of 2.4G2 with either anti-Ig or anti-I-A antibody detected an additional 7% of positive lymphocytes compared with the number of cells detected with either anti-Ig or anti-I-A alone, whereas combination of 2.4G2 and anti-Thy-1 antibodies detected a proportion of cells \sim 7% lower than the sum of those positive with 2.4G2 and anti-Thy-1 separately. These data indicate that \sim 7% of the splenic lymphocytes coexpress the 2.4G2 and Thy-1 antigens, but not sIg, I-A, Lyt-2, or L3T4.

Similar experiments were performed on lymphocytes from C57BL/6 spleens depleted of sIg⁺ cells by direct rosetting with CrCl₃-treated goat anti-mouse Ig-coated E. The proportion of sIg⁺, 2.4G2⁺ and NK-1.1⁺ cells in the original spleens were, on average, 43.4, 47.4, and 11.3%, respectively. The proportion of sIg⁺

TABLE I
Reactivity of Murine Splenic Lymphocytes with
Anti-Fc γ RII Antibody 2.4G2

Strain	Antibody	Added antibody	
		None	2.4G2
BALB/c (total cells)	None	0.5 \pm 0.1*	47.8 \pm 3.8
	Anti-Ig	50.3 \pm 8.6	57.9 \pm 5.2
	Anti-I-A	45.0 \pm 11.4	60.9 \pm 5.7
	Anti-Thy-1	51.5 \pm 6.6	91.7 \pm 2.0
	Anti-Lyt-2	15.1 \pm 5.7	64.3 \pm 9.4
	Anti-L3T4	31.4 \pm 2.6	77.2 \pm 2.4
C57BL/6 [†] (sIg ⁻ cells)	None	0.8	13.4
	Anti-Ig	4.1	17.2
	NK-1.1	8.4	12.1
	Anti-Ig + NK-1.1	11.9	13.5
	EA7S [§]	12.1	0.1
	EA7 + NK-1.1	12.4	0.1

BALB/c mice were injected with poly(I:C) 24 h before they were killed. Splenic lymphocytes were purified by density gradient centrifugation. Direct immunofluorescence was performed using FITC-labeled avidin to detect the different biotin-labeled antibodies, alone or in combination with 2.4G2.

* Percent positive cells, mean \pm SD (three experiments).

[†] The sIg⁻ population was purified from the total splenic lymphocytes of C57BL/6 mice by rosetting with CrCl₃-treated goat anti-mouse Ig-coated E. Direct immunofluorescence was performed, as for BALB/c mice. Experiment representative of three performed.

[§] Fc γ R were detected by rosetting lymphocytes with EA7S, as described in Materials and Methods, in the absence or presence of a 10⁻² dilution of the ascites of the indicated antibodies.

lymphocytes in the sIg⁻ population was reduced by >90% (Table I). Although the proportion of 2.4G2⁺ lymphocytes was also decreased, on average 10% of the sIg⁻ lymphocytes expressed FcγRII, as indicated by their reactivity with antibody 2.4G2 and binding of EA7S.

The proportion of NK-1.1⁺ cells was similar in the total splenocyte population and in the sIg⁻ lymphocytes. The percentage of cells positive with a combination of NK1.1 and anti-Ig was equal to the sum of those positive with each antibody separately, suggesting that the two reagents recognize two discrete subsets of lymphocytes, NK and B cells, respectively. The proportion of cells positive with a combination of NK-1.1 and 2.4G2 antibodies was almost identical to that of the cells positive with 2.4G2 antibody alone, an indication that these antibodies reacted with the same subset of cells. As is the case in total spleen cell preparations (data not shown), antibody 2.4G2, but not NK-1.1, completely inhibited EA7S rosette formation by sIg⁻ lymphocytes.

2.4G2 Reacts with Freshly Obtained Splenic NK Cells. To test directly whether moFcγRII is expressed on NK cells, two-color immunofluorescence experiments were performed, using 2.4G2 and NK-1.1 antibodies, with C57BL/6 splenic lymphocytes. In these lymphocyte preparations the average proportion of sIg⁺ and of 2.4G2⁺ cells were 53.5 and 58.2%, respectively. The vast majority (average 97.7%) of sIg⁺ lymphocytes (Fig. 1) coexpressed 2.4G2, but 8.3% of the lymphocytes were positive with 2.4G2 and not with anti-Ig. This proportion is similar to that of the NK-1.1⁺ cells (9.1% on average). Most NK-1.1⁺ cells (92.5%) coexpressed the 2.4G2 antigen, whereas only a minor proportion of the 2.4G2⁺ cells (15.4%) expressed NK-1.1 antigen.

FcγRII-bearing Lymphocytes Mediate Spontaneous Cytotoxicity. Splenocytes from all strains of mice are spontaneously cytotoxic to YAC-1 target cells. To determine the phenotype of the YAC-1-responsive cells, FcγRII⁺ and FcγRII⁻ lymphocytes were

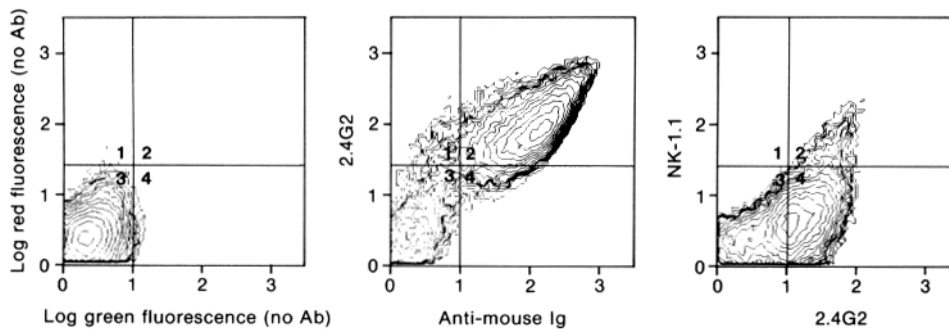


FIGURE 1. NK-1.1⁺ splenic NK cells express FcγRII. Total splenic lymphocytes were obtained from poly(I:C) treated C57BL/6 mice (experiment representative of five performed). Two-color immunofluorescence was performed using FITC-labeled anti-Ig or 2.4G2 (green fluorescence) and 2.4G2 or NK-1.1 mAbs labeled with biotin and detected with PE-avidin (red fluorescence). Correlate measurements of green (*x*-axis, log scale) and red (*y*-axis, log scale) fluorescence are displayed as two-dimensional contour plots. Based on the negative control samples in the presence of PE- and FITC-avidin only, the contour plots were divided into quadrants indicating: (1) cells with red fluorescence (binding PE-avidin only); (2) double-positive cells; (3) double-negative cells; (4) cells with green fluorescence (binding FITC-labeled antibody only). The numbers on the scales refer to arbitrary units of fluorescence intensity.

separated by FACS from sIg⁻ spleen cell preparations and the cytotoxic activity of each population was tested (Fig. 2). Cytotoxicity mediated by the sIg⁺ cells from BALB/c and C3H/HeN mice was 10- and 20-fold lower, respectively, than that mediated by the sIg⁻ cells (43 and 20 LU [45%] compared with 527 and 411 LU [45%]). The sIg⁻/2.4G2⁺ cells mediated levels of cytotoxicity similar to (BALB/c) or slightly higher (C3H/HeN) than those mediated by the total sIg⁻ cells. On a per cell basis, spontaneous cytotoxicity mediated by sIg⁻/2.4G2⁻ cells was ~50-70% lower than that mediated by either total or 2.4G2⁺/sIg⁻ cells.

In Vitro Propagated NK Cells Express Functional Fc γ RII. NK cells, obtained from (B6D2)F₁ mice and propagated in vitro with rIL-2, were tested for expression of the 2.4G2 antigen. The majority of these cells maintained a cell-surface phenotype similar to that of fresh NK cells (34), i.e., Lyt-2 and L3T4⁻, sIg⁻ (34), F4/80⁻, IL-2R(3C7)⁻, but low-density Thy-1⁺ (29 and data not shown), asialo GM1⁺ and NK-1.1⁺ (Fig. 3). The TCR molecules (α , β , and γ chains) and CD3 are not expressed in these cells (29, 30, and data not shown). The observation that in vitro propagated NK cell populations are F4/80⁻ rules out the possibility that they are contaminated with macrophages expressing the 2.4G2 antigen. Fc γ RII, as detected using antibody 2.4G2, was expressed on the majority of these cells (Fig. 3) at a density higher than that on fresh NK cells, but reproducibly lower than that on murine macrophages or B cells (not shown). NK cells propagated in vitro were unable to bind monomeric murine IgG2a, as detected by binding of FITC- or biotin-labeled murine IgG2a B137.17 (Fig. 3). These NK cells propagated in vitro are potent effectors of spontaneous cytotoxicity (27, and data not shown) and ADCC (Fig. 4). Antibody 2.4G2, but not other isotype-matched control antibodies, present during the ADCC

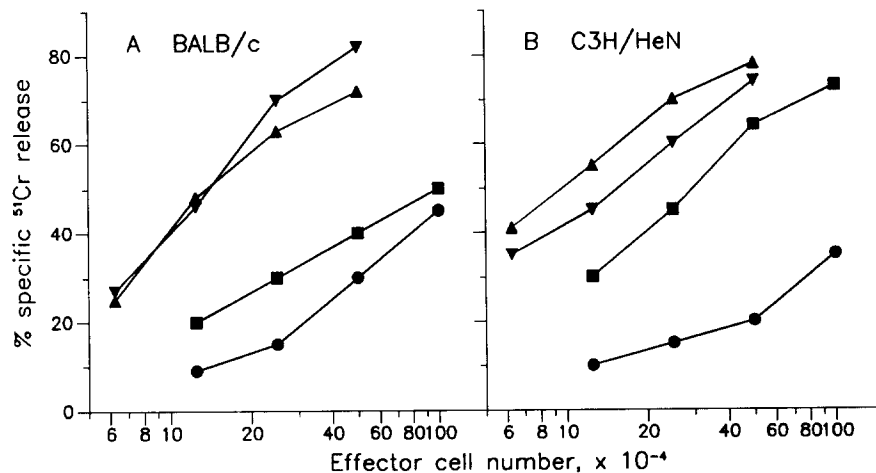


FIGURE 2. 2.4G2⁺/sIg⁻ lymphocytes mediate spontaneous cytotoxicity. Splenic lymphocytes were purified by density gradient centrifugation from poly(I:C)-treated BALB/c (A) or C3H/HeN (B) mice (experiments representative of three performed with each mouse strain). sIg⁺ (●) and sIg⁻ cells (▼) were separated from the lymphocyte preparations by rosetting with CrCl₃-treated goat anti-mouse Ig coated E and density gradient centrifugation. 2.4G2⁺ (▲) and 2.4G2⁻ (■) cells were obtained from the sIg⁻ populations by indirect rosetting after sensitization with 2.4G2. Spontaneous cytotoxicity of each population was tested against ⁵¹Cr-labeled YAC-1 target cells (10⁴/well) in an 18-h assay.

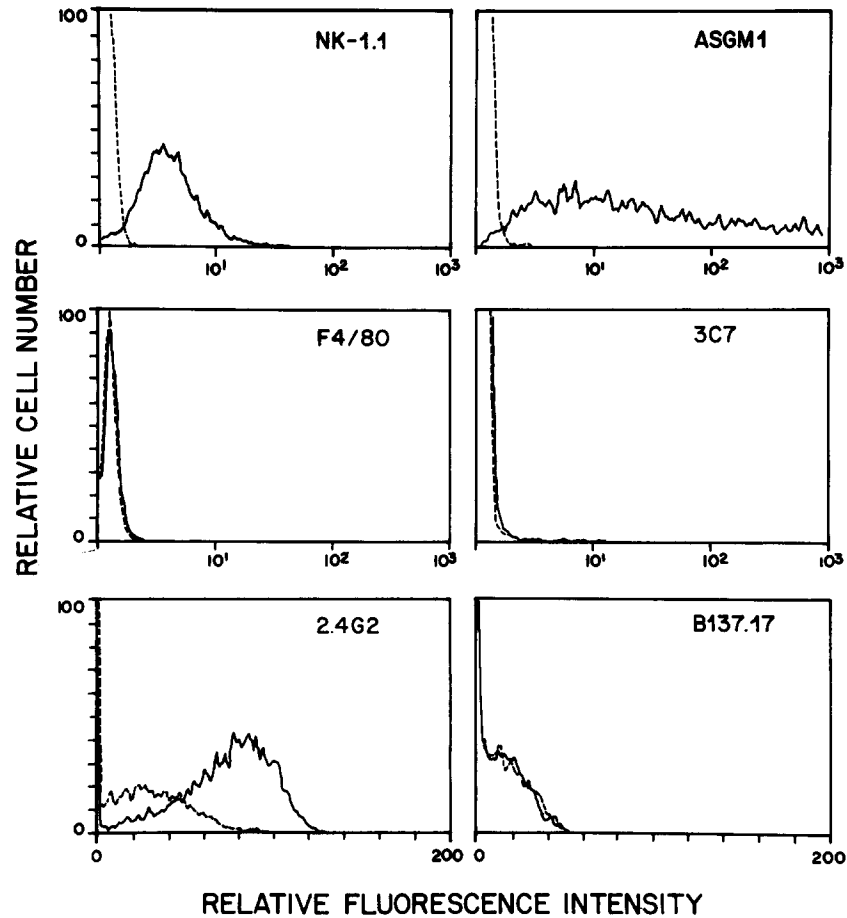


FIGURE 3. Antibody 2.4G2 reacts with in vitro propagated murine NK cells. NK-1.1⁺ cells, purified by FACS from spleens of (B6D2)F₁ mice and propagated in culture in the presence of rIL-2 (500 U/ml) were tested for expression of the indicated cell surface markers by indirect immunofluorescence after staining with the indicated antibody (*solid line*). Background fluorescence with the second antibody alone (*dotted line*). *x*-axis, intensity of fluorescence (log scale, top and middle panels; linear scale, bottom panels); *y*-axis, number of cells.

assay, resulted in inhibition of the antibody-dependent killing to levels of cytotoxicity similar to those observed on non-Ab-sensitized target cells.

The FcγRII Expressed on NK Cells Is Encoded by the FcγRα Gene. To determine which of the FcγR genes encode the FcγRII polypeptide(s) expressed on the surface of NK cells, FcγR cDNA fragments were used to probe mRNA isolated from NK1.1⁺ cells from (B6D2)F₁ and from NK-2.1⁺ cells from *scid* mice propagated in vitro. The mRNA transcripts in cultured (B6D2)F₁ splenic NK cells detected by the FcγRα + β probe, i.e., the complete FcγRα cDNA that hybridizes to transcripts of both α and β genes (23), appear as one single band migrating faster than those in cell lines EL4 and BCL₁ (Fig. 5 A). No hybridization was observed with the control CTLL cell line. EL4 and BCL₁ express only the FcγRβ1 gene, and both FcγRα and FcγRβ2

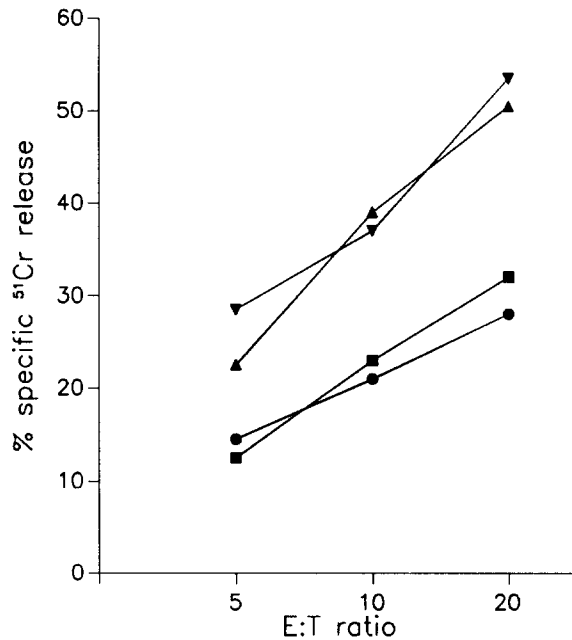


FIGURE 4. Anti-Fc γ RII antibody 2.4G2 inhibits NK cell-mediated ADCC. Cytotoxicity of in vitro propagated NK-1.1⁺ cells was tested against P815 targets, sensitized or not with C3H anti-DBA/2 immune serum, in a 4-h ⁵¹Cr release assay. (■) Nonsensitized targets; (▼) antibody-sensitized targets (ADCC); (●) ADCC in the presence of 2.4G2 (ascites, 10⁻² dilution); (▲) ADCC in the presence of isotype-matched control antibody Mac-1.

RNA have higher mobility than Fc γ R β 1 RNA (23). Fc γ R α - and β -specific cDNA fragments hybridize with RNA from the macrophage line P388D₁, producing two bands of sizes compatible with those of Fc γ R α and Fc γ R β 2 transcripts, respectively (reference 23 and Fig. 5 A). To distinguish between expression of Fc γ R α and Fc γ R β RNA in NK cells, the specific probes were hybridized to RNA from cultured C.B-17 *scid* splenic NK cells (Fig. 5 B). NK cell RNA hybridized only to the common Fc γ R

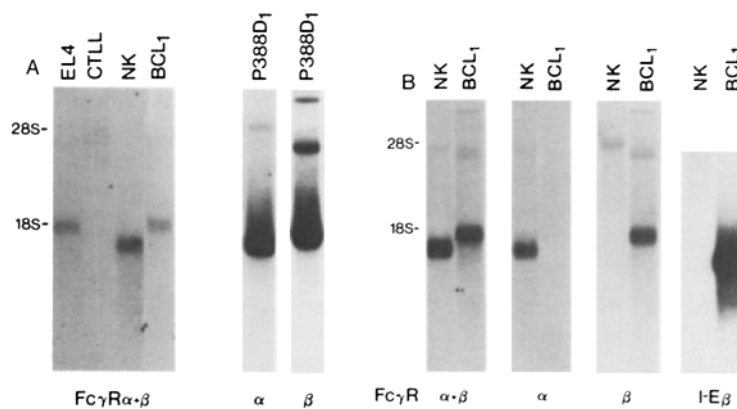


FIGURE 5. Specificity of Fc γ R transcripts in NK cells. RNA was isolated from splenic NK-1.1⁺ and NK-2.1⁺ NK cells, originally purified from normal (B6D2)F₁ (A) or C.B-17 *scid* mice (B), and cultured (11 and 5 d, respectively) in the presence of 500 U/ml rIL-2. Fc γ R RNA expression in these cultured NK cells was analyzed relative to reference cell lines EL4, BCL₁, and P388D₁. Migration of the 18S and 28S ribosomal RNA markers is indicated.

probe and to the $Fc\gamma R\alpha$ -specific probe. As expected, BCL_1 RNA hybridized only to the common $Fc\gamma R$ probe and to the $Fc\gamma R\beta$ -specific probe. To rule out the possibility that the propagated NK cells contained macrophages expressing $Fc\gamma R\alpha$ transcripts, the same filter was hybridized with a cDNA probe specific for MHC class II I-E β ; no detectable class II RNA was present in the NK cell preparation (Fig. 5 B).

RNase protection assay indicates that the $Fc\gamma R\alpha$ transcript in NK cells is identical to that in the macrophage-like lines J774a (not shown) and P388D₁ (Fig. 6). An antisense probe that corresponds to the α transcript, extending from the Bgl II site within the region encoding the EC2 domain (23) to the polyadenylation site, was protected as a full-length 920-bp fragment by RNA derived from P388D₁ and J774a cells (lane 3, and not shown) and from in vitro propagated NK cells (lane 4), indicating that this region of the $Fc\gamma R\alpha$ transcript is identical in NK cells and macrophages. $Fc\gamma R\alpha$ and β transcripts share >95% homology in their extracellular domains. Crosshybridization of the 920-bp α -antisense probe to $\beta 1$ and $\beta 2$ tran-

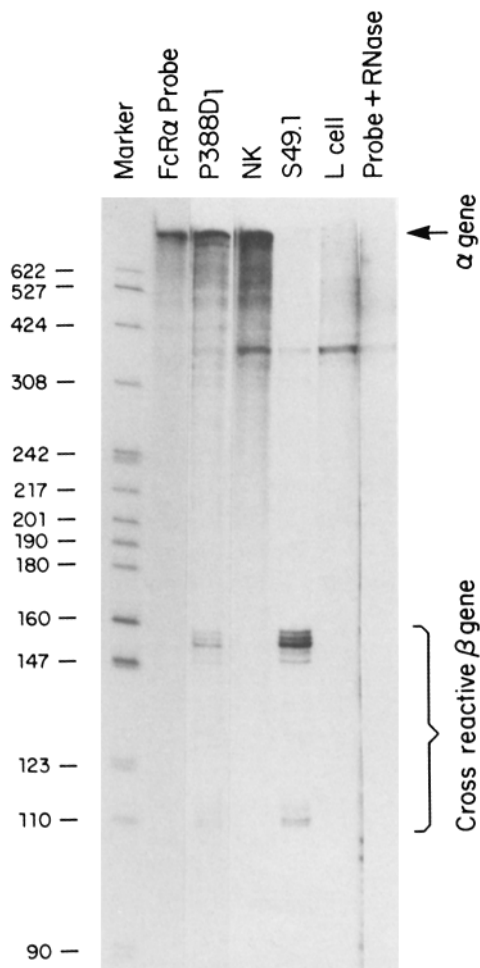


FIGURE 6. RNase protection of $Fc\gamma R\alpha$ transcripts in NK cells. RNA from α -expressing P388D₁ and NK cells protect a full-length, 920-bp fragment (lanes 3 and 4); RNA from $\beta 2$ - or $\beta 1$ -expressing cells crosshybridize with the probe and results in protection of 160- and 95-bp fragments (lanes 3 and 5, respectively). No bands were observed when RNA from the $Fc\gamma R$ negative L cells was used (lane 6); control sense RNA protects the full-length probe (lane 7).

scripts should generate fragments of 160 and 95 bp, respectively. The β 2-expressing P388D₁ cell line (lane 3) and the β 1-expressing T cell line S49.1 (lane 5) demonstrate the expected bands. The Fc γ R α is not expressed in T cells, accounting for the absence of the 920-bp band, while β was not expressed in the J774a variant (19, and not shown).

Discussion

We present evidence that murine NK cells, like human NK cells, bear a functional Fc γ R that triggers ADCC. The moFc γ RII α gene is the only Fc γ RII gene expressed in murine NK cells and presumably encodes this Fc γ R.

mAb 2.4G2 recognizes murine Fc γ RII, a highly glycosylated protein migrating in SDS-PAGE as a broad band of 50–70 kD (17), expressed on macrophages and B cells. The affinity (2.4G2)-purified Fc γ RII binds murine IgG1/IgG2a/IgG2b (17, 18) and has biochemical characteristics very similar to those of the Fc γ R(CD16) expressed on human NK cells, neutrophilic granulocytes and macrophages (8, 9). Thus, this antibody was used to test the hypothesis that murine NK cells bear an Fc γ R equivalent to the human Fc γ R (CD16). A small population of Thy-1⁺ cells that bear 2.4G2 but neither sIg or I-A determinants are found in splenic lymphocytes from a variety of mouse strains. Murine NK cells express Thy-1 antigen at low density but neither CD4 (L3T4) nor CD8 (Lyt-2) (27, 29). Our data, indicating no overlap between the 2.4G2⁺/Thy-1⁺ and the CD4 or CD8⁺ populations, are consistent with the hypothesis that the 2.4G2⁺/Thy-1⁺ cells are not T cells. The existence of this 2.4G2⁺/sIg⁻ lymphocyte subset was confirmed using B cell-depleted spleen lymphocytes. The observation that CrCl₃-treated anti-mouse Ig-coated E do not bind to lymphocyte populations depleted of sIg⁺ cells by nylon wool adherence (not shown) makes it unlikely that a significant proportion of NK cells are lost, using this procedure, in the B cell rosetting population. The 2.4G2⁺/sIg⁻ subset purified in this way is unlikely to represent sIg⁻ B cells or macrophages, because it lacks expression of MHC class II determinants and it is present in spleen cell preparations depleted of macrophages by adherence to plastic. The identification of the sIg⁻/2.4G2⁺ cells as NK cells is supported by the fact that, in C57BL/6 spleen, the 2.4G2⁺/sIg⁻ cell subset coexpresses the NK-specific antigen NK-1.1. Nonspecific binding of antibody NK-1.1 to the Fc γ R of 2.4G2⁺ cells through its Fc portion is excluded because antibody NK-1.1 does not inhibit EA7S rosette formation by either total or sIg⁻ cells and does not bind to spleen lymphocyte preparations from BALB/c mice that do not express NK-1.1 alloantigen but express Fc γ R and 2.4G2 determinant (data not shown). Although we did not use F(ab')₂ fragments of antibody 2.4G2, it is unlikely that the antibody binds to FcR on the positive cells through its Fc portion because all experiments have been performed in the presence of excess human IgG, previously shown to block nonspecific Fc binding.

The 2.4G2⁺/sIg⁻ cells mediate higher levels of spontaneous cytotoxicity, on a per cell basis, than the sIg⁻/2.4G2⁻ cells. However, the absolute number of LU recovered in the two populations is not significantly different. Several possibilities may explain these results. Similar functional consequences occur in human NK cells upon Fc γ R(CD16) interaction with anti-Fc γ R(CD16) antibodies and immune complexes (35). Low spontaneous cytotoxicity in murine 2.4G2⁺ cells may reflect down-modulation of this activity induced by the interaction of Fc γ R with anti-Fc γ R anti-

bodies, similar to that previously reported with both human (36) and mouse cells (12). Also, 2.4G2⁻ NK cells might exist, in analogy with the small proportion of human NK cells that reportedly do not express FcγR(CD16) (37). However, our inability to recover all spontaneous cytotoxicity in 2.4G2⁺ cells is most likely due to the low density with which this epitope is expressed and the insufficient sensitivity of available separation techniques to completely deplete the cells expressing the antigen at the lowest density.

The low density expression of the FcγR detected by 2.4G2 on murine NK cells was confirmed using highly homogeneous NK-1.1⁺ cells purified from (B6D2)F₁ spleen cells. Like the FcγR on fresh NK cells, the FcγRII detected by 2.4G2 on cultured NK cells are functional, and endow NK cells with ADCC activity. The ability of antibody 2.4G2 at high concentrations to completely inhibit ADCC supports the hypothesis that FcγRII is the major, if not the only, FcγR type expressed on NK cells. Lack of binding of monomeric IgG2a to NK cells excludes the presence of the high affinity FcγRI. However, we cannot exclude the possibility that other, as yet unidentified FcγR type(s) are also present on NK cells.

Our data provide the first direct evidence that a single FcγRII RNA transcript is expressed in murine NK cells and that the FcγRIIα transcripts present in NK cells are identical to those present in macrophages. These data suggest that the FcγR expressed in murine NK cells is encoded by the FcγRIIα gene.

Antibody 2.4G2 reacts with the extracellular domain of any of the proteins encoded by FcγRII genes (19) and our data show that the epitope detected by 2.4G2 is responsible for FcγR-dependent functions also on these cells, as previously reported in macrophages (19). Although FcγRII molecules encoded by the different genes appear to bind IgG with the same specificity, a correlation exists, within the 2.4G2⁺ cell types tested, between expression of the FcγRIIα gene and ability to mediate ADCC. Recent data (Kuziel, W., and P. W. Tucker, manuscript in preparation) indicate that FcγRIIα transcripts are also uniquely expressed in a TCR-γ/δ⁺ subset of murine dendritic epidermal cells (38) able to mediate ADCC. Although the extracellular domains of the FcγR are similar, the transmembrane and cytoplasmic domains of the FcγRα-encoded protein bear no homology with the respective domains of the FcγRβ1 or β2-encoded proteins (23). The apparent requirement for an FcγRα-encoded receptor for ADCC activity suggests that features of these two domains may be responsible for this FcγR-mediated function, possibly via a role in signal transduction. The observation that only FcγRα transcripts are detectable in NK cells, as opposed to β1 transcripts in lymphocyte tumor lines and both the α and β2 transcripts in macrophages, may also bear on the lineage to which these cells belong.

A gene encoding huFcγR(CD16), the only type of FcγR expressed on human NK cells, has been cloned recently from a human placenta cDNA library (39) and reported to have the greatest sequence homology with the moFcγRα cDNA. The homology between the two genes extends throughout the transmembrane portion of the molecule, bearing no or minimal homology, in this portion of the molecule, to the other FcγR types defined at present. In human neutrophils, FcγR(CD16) lacks an intracytoplasmic domain and is anchored to the membrane through glycosylphosphatidyl inositol (GPI) (39, 40). In contrast to the GPI-linked form in granulocytes, a transmembrane form of FcγR(CD16) exists: macrophages from patients

affected with paroxysmal nocturnal hemoglobinuria, who have an impaired ability to form GPI anchors (41), express FcγR(CD16). Furthermore, FcγR(CD16) on both fresh and cultured human NK cells and FcγRII on murine NK cells are insensitive to doses of PI-specific phospholipase C (PI-PLC), which cleaves 90% of the FcγR(CD16) on both fresh and cultured granulocytes, the CD14 on monocytes, and the Thy-1 on murine T cells (reference 42 and data not shown). Two nearly identical, linked genes for huFcγRIII(CD16) have been cloned and sequenced (42). These genes (III-1 and III-2) are transcribed in a cell type-specific fashion to generate the two alternatively anchored forms of the receptor on neutrophils and NK cells. The FcγRIII(CD16) sequence for the NK cells molecule encodes a transmembrane protein and demonstrates homology with the moFcγRIIα molecule, not only in its transmembrane domain but in its cytoplasmic domain as well. Therefore, the moFcγRII encoded by the FcγRIIα gene in murine NK cells is the equivalent of the FcγR(CD16) on human NK cells.

Summary

We report evidence that murine NK cells express a functional FcγRII encoded by the FcγRIIα gene. Several lines of indirect evidence indicate that freshly obtained NK cells from mice of several strains bear a functional FcγRII: (a) anti-FcγRII antibody 2.4G2 detects a small but significant proportion of sIg⁻ cells and a small proportion of the 2.4G2⁺ cells are included in the Thy-1⁺ population; (b) sIg⁻ lymphocytes contain 2.4G2⁺ and FcγR-bearing cells in similar proportions; (c) binding of particulate immune complexes by sIg⁻ lymphocytes is completely inhibited by 2.4G2; (d) 2.4G2⁺ cells mediate >50% of the spontaneous cytotoxicity in sIg⁻ splenic lymphocytes. Direct evidence for the presence of FcγRII on murine NK cells is provided by the results of two-color immunofluorescence studies performed on splenic lymphocytes from C57BL/6 mice showing coexpression of NK-1.1 and 2.4G2. Studies of *in vitro* propagated homogeneous NK cell populations confirm that murine NK cells express only FcγRII and that this FcγR is functional, as shown in experiments of inhibition of ADCC by the anti-FcγRII antibody 2.4G2. The results of studies at the molecular level show that an FcγRIIα transcript identical to that expressed in macrophages is the only molecule encoding FcγRII in murine NK cells.

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