HYBRIDOMA
Volume 18, Number 4, 1999
Mary Ann Liebert, Inc.

A New Monoclonal Antibody Reactive with Several Ly49 NK Cell Receptors Mediates Redirected Lysis of Target Cells

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ABSTRACT

We produced a novel hamster monoclonal antibody (MAb), 14B11, that recognizes the majority of mouse non-killer (NK) cells. Transfection studies demonstrated that 14B11 MAb binds a subset of Ly49 receptors, including three putative inhibitory receptors, Ly49F, I, and C. No binding to Ly49A, B, D, or G was detected. In addition, 14B11 was shown to bind the putative activating receptor Ly49H, which required co-transfection of the signaling molecule DAP12 for detectable cell surface expression. Thus, 14B11 is the first reported MAb to bind Ly49H and F. At the functional level, 14B11 MAb enhanced the lysis by IL-2 activated NK cells of an FcR$^+$ target cell line (Daudi), but not an FcR target cell (EL-4). Because F(ab')$_2$ fragments of 14B11 failed to enhance lytic activity, the enhancement of lysis by intact antibody is apparently due to "redirected lysis," in which stimulatory receptors on the NK cell are bridged by antibody to Fc receptors on the target cell. Cell separation experiments demonstrated that the 14B11-dependent redirected lysis was markedly increased using NK cell populations that had been depleted of Ly49F,$^+$ I,$^+$ or C$^+$ NK cells. Because such depletions are expected to enrich for Ly49H$^+$ NK cells, these results suggest that the enhancement of lysis mediated by 14B11 MAb may be due to stimulation of the activating Ly49H receptor. In conjunction with other anti-Ly49 MAbs, the 14B11 MAb will be useful in further studies of Ly49 receptor function and specificity.

INTRODUCTION

THE MURINE Ly49 GENE FAMILY encodes activating and inhibitory non-killer (NK) cell receptors. Consisting of at least nine members, these C-type lectins are homodimeric type II integral membrane proteins.[1] Functional and binding studies have established that the interaction of specific major histocompatibility complex (MHC) class I molecules with Ly49A, Ly49C, and Ly49G2 receptors inhibits the functional activities of NK cells.[2–5] Ly49B, E, F, and I are probably also inhibitory receptors, based upon the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic tails.[6–8] Stimulation of the inhibitory receptors results in the phosphorylation of the ITIM, leading to the recruitment of protein tyrosine phosphatases, such as SHP-1, which are thought to mediate downstream inhibitory signals.[6,9]

Two of the Ly49 receptors, Ly49D and Ly49H, lack the ITIM motif.[10,11] Antibodies specific for Ly49D stimulate target cell lysis by NK cells, suggesting that Ly49D is an activating receptor, rather than an inhibitory one.[10] Ly49D and Ly49H are thought to stimulate NK cell activation by virtue of a noncovalent association with a 12-kDa phosphoprotein called DAP12.[12,13] Activating isoforms of the human Ig-like receptors and CD94/NKG2 lectin-like receptors also interact with DAP12.[14,15] DAP12 possesses a tyrosine-based activation motif called an ITAM, similar to those found in CD3ζ and other activating subunits of lymphocyte receptors. Recent evidence demonstrated that Ly49D and Ly49H each form a complex with DAP12 in a B-cell line.[13]

In this report, we describe a novel monoclonal antibody, (MAb) 14B11, that binds several members of the Ly49 family, and as a result reacts with the majority of NK cells in most mouse strains tested. In addition to reacting with Ly49F, I, and C, 14B11 reacts with the stimulatory Ly49 receptor, Ly49H. 14B11 MAb stimulates redirected lysis by NK cells. The cells mediating redirected lysis were enriched among NK cells that had been depleted of Ly49C$^+$Ly49I$^+$ and Ly49F$^+$ cells. The results suggest that in addition to reacting with inhibitory Ly49

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receptors, 14B11 reacts with stimulatory receptors, including Ly49H, that trigger cytolytic activity.

MATERIALS AND METHODS

Animals

C57BL/6J (B6), B10.BR, B10.D2/nSnJ, 129/J, B10.M, B10.S, B10.RIII, B10.Q, C3H.SW, BALB.K, AKR/J, and SJL mice were purchased from Jackson Laboratory, (Bar Harbor, ME). BALB/cAnNCr and BALB/cByJ mice were purchased from the National Cancer Institute (Frederick, MD) and Jackson Laboratory, respectively. BALB.B mice, kindly provided by Dr. I. Weismann, Stanford University, were bred and maintained at UC, Berkeley. Syrian hamsters were purchased from Simonsen (Gilroy, CA).

Cells and cell lines

The EL-4 cell line was provided by Terry Potter (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). P3X63Ag8.653 myeloma and the Daudi cell lines were provided by James P. Allison (Cancer Research Laboratory, UC Berkeley). COS-7 cells were purchased from the ATCC (Rockville, MD). All cell lines were grown in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Sigma Corp., St. Louis, MO), 50 mM 2-ME, 0.2M HEPES, and antibiotics.

Production of 14B11 MAb

Syrian hamsters were immunized five times at weekly intervals in the footpad with Day 9 or 10 IL-2 activated NK (A-LAK) cells from B6 mice. Three days after the last immunization, the draining lymph node cells were fused with P3X63Ag8.653 mouse myeloma cells. Following hypoxantine, aminopterine, and thymidine (HAT) selection, hybridoma supernatants were screened for reactivity with A-LAK cells and spleen cells by flow cytometry. The 14B11 hybridoma, chosen because its antibody stained primarily NK cells, was cloned five times in succession. 14B11 MAb was purified from culture supernatants by Protein G chromatography (Boehringer Mannheim, Indianapolis, IN) and was conjugated with biotin or fluorescein (FITC) using standard procedures. $F(ab')_2$ fragments of 14B11 MAb were prepared by digestion with pepsin as previously described.[16]

Antibodies and reagents

In addition to MAb 14B11, this study used three new anti-Ly49 MAbs, YLI-90 (anti-Ly49I), HBF 7-19 (anti-Ly49F), and SED-85 (anti-Ly49D), all conjugated with biotin. All three MAbs were derived from BALB/c mice immunized with cells transfected with the corresponding B6-derived Ly49 receptor (H.T. and D.H.R., in preparation). The MAbs were screened for staining of COS-7 cells expressing the immunizing Ly49 receptor, but not others (Ly49A-I were tested), in assays performed as in Fig. 2. 5E6 (anti-Ly49C/I,[17] 4D11 (anti-Ly49G2),[18] and JR9-318 (anti-Ly49A)[19] MAbs were conjugated with biotin. PK136 MAb (anti-NK1.1)[20] was conjugated with biotin or purchased as a PE-conjugated antibody from PharMingen (San Diego, CA). 500A2 MAb (anti-CD3ϵ) was conjugated to FITC. 500A2, H57-597 (anti-TCRβ), goat $F(ab')_2$ anti-mouse IgG(H + L), all MAbs conjugated to TRI-COLOR, goat anti-hamster IgG(H + L) MAb (adsorbed with mouse and rat IgG) conjugated to biotin and FITC, normal mouse IgG and streptavidin-TRICOLOR were purchased from Caltag (Burlingame, CA). Goat anti-mouse IgG(H + L) MAb (adsorbed with rat IgG) conjugated to PE was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Streptavidin-PE and streptavidin-613 were purchased from Becton Dickinson (Mountain View, CA) and Gibco (Grand Island, NY), respectively. 6xHis (anti-His) MAB was purchased from Clontech Laboratories, Inc. (Palto Alto, CA).

Transfection of COS-7 cells

Ly49A, Ly49I and Ly49G2 cDNAs, all from B6 mice, have been previously described.[21] Ly49B, Ly49C, Ly49D, Ly49E, Ly49F, and Ly49H cDNAs were isolated by RT-PCR from B6 mice. The Ly49D and Ly49H cDNAs corresponded to the longer splice variants.[11] Each of the DNAs was inserted into the pME18S vector and transfected into COS-7 cells with Lipofectamine Plus (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Control transfections used the empty pME18S vector. Mouse DAP12 cDNA[12] was obtained from Image Consortium and subcloned into the pME18S vector. Transfections employed either 4 μg of Ly49 expression vector alone, or 2 μg of Ly49 expression vector and 2 μg of DAP12 expression vector. Three days after the transfections, the cells were harvested and analyzed by flow cytometry.

A-LAK cell preparation

A-LAK cells were prepared as described,[22] with modifications. Nonadherent cells from nylon wool-passed B6 splenocytes were cultured at 2×10^6 cells/mL in medium containing from 100 to 200 ng/mL of recombinant IL-2 from Chiron Inc. (Emeryville, CA). After 3 days of culture, adherent cells were harvested and depleted of T cells.[22] The surviving cells were returned to culture at 2×10^5 cells/mL in fresh IL-2 containing media. Three days later, adherent and nonadherent cells were harvested and reseeded at the same concentration as on Day 3. Day 9 A-LAK cells were used for staining and as immunogen for preparing MAbs in hamsters.

LAK cell preparation

Spleen cells from B6 and B10.D2 mice were depleted of red blood cells and cultured in IL-2-containing medium at a concentration of 2×10^6 cells/mL for 4 days. For sorts, after 2 to 2.5 days in culture, the cells were depleted of T and B cells with a complement kill containing antibodies against anti-CD4, anti-CD8, anti-MHC class II, guinea pig complement and rabbit complement, as previously described.[16] The NK cells were sorted according to the protocol below and returned to culture for two additional days at a concentration of 2×10^5 cells/mL.

Flow cytometry and cell sorting

For mouse strain analysis, nylon wool passed spleen cells were depleted of CD4 and CD8 T cells. The remaining cells were stained in four steps. First, goat $F(ab')_2$ anti-mouse

IgG(H + L) conjugated to TRICOLOR was added to stain B cells, then mouse IgG was added to block free arms, followed by biotinylated 14B11 MAb. In the last step, a mixture of CD3-TRICOLOR, H57-TRICOLOR, DX5-FITC and streptavidin-PE was added. For analysis of freshly isolated NK cell preparations, nylon wool passed cells were stained first with biotinylated 5E6 (anti-Ly49C/I) or a mixture of biotinylated 5E6 and HBF 7-19 (anti-Ly49F) followed by streptavidin-613, CD3-TRICOLOR, NK1.1-PE and 14B11-FITC or streptavidin-TRICOLOR, PK136-PE, and 14B11-FITC.

For cell sorting, after depletion of T and B cells, LAK cells were incubated in the first step with a mixture of biotinylated 14B11 MAb and FITC-conjugated 5E6 (anti-Ly49C/I) MAb or a mixture of 5E6, YLI-90 (anti-Ly49I), and HBF 7-19 (anti-Ly49F). In the second step, the cells were incubated and streptavidin-TRICOLOR and PK136PE or with goat anti-mouse IgG conjugated to PE and CD3-FITC. Either $14B11^+5E6^-$ and $14B11^+5E6^+$ populations (gated on $NK1.1^+$ cells) or $Ly49F/I/C^+$ and $Ly49F/I/C^-$ (gated on $CD3^-$ cells) were sorted on a Coulter EPICS Elite-ESP (Coulter Electronics, Hialeah, FL) flow cytometer and used in cytolysis experiments.

Cytolytic assay

Sorted and unsorted LAK cells were incubated for 4 h with ^{51}Cr-labeled target cells in triplicate as previously described.[23] To assay the effects of antibodies during the cytolytic assay, the effector cells were preincubated for 30–60 min at 37°C with intact antibody or $F(ab')_2$ fragments, after which target cells were added to the wells. Mouse IgG_{2a} (Caltag, Burlingame, CA) was used as an isotype control for 5E6 MAb. F536 (anti-Vγ3)[24] was used as a hamster isotype control for 14B11 MAb. All antibodies were added at a final concentration of 2 μg/mL for the duration of the cytotoxicity assay. The range of triplicate values was usually less than ±4% of specific lysis.

RESULTS

A new NK-specific MAb, 14B11, reacts with a large subset of NK cells

Hamster MAbs specific for adherent IL-2-activated NK cells (A-LAK cells) were screened for their capacity to specifically stain NK cells. One of the MAbs, 14B11, stained most (>70%) splenic $NK1.1^+$ cells in B6 mice (Fig. 1A). 14B11 MAb also stained the majority of $NK1.1^+$ IL-2-activated NK cells present in preparations of LAK cells and A-LAK cells (see the Materials and Methods section for details of cell preparations) (Figs. 1B and 1C). 14B11 MAb stained only a small percentage of $NK1.1^-$ cells in the spleen (Fig. 1A), and most of those cells were T cells (data not shown). The fraction of T cells that stained with 14B11 was approximately 3% (data not shown). A larger fraction of thymic $NK1.1^+$ T cells, around 50%, stained with 14B11 (data not shown). No reactivity with LPS-stimulated B cells was observed (data not shown). Furthermore, 14B11 did not stain any of the cell lines tested, including T lymphomas EL-4, S49.1, YAC-1, R1.1 and R1E.TL8x.1, a B lymphoma (C1498), an Abelson-transformed B cell line (R8.15), a dendritic epithelial T-cell line (717.A2), a mastocytoma (P815), and a macrophage cell line (P388D1) (data not shown).

Several Ly49 family members are recognized by 14B11 MAb

The fraction of NK cells that stained with 14B11 was substantially higher than the fraction that stained with any known anti-Ly49 MAb. To investigate the possible specificity of 14B11 MAb for Ly49 family members, we tested the reactivity of the MAb with COS-7 cells that had been transiently transfected with each of the Ly49 cDNAs. In each case, expression of the transfected Ly49 receptor was confirmed by staining the transfectants with relevant anti-Ly49 MAbs, including the new anti-Ly49I (YLI-90), anti-Ly49F (HBF 7-19), and anti-Ly49D (SED-85) MAbs (see the Materials and Methods section), or with antibodies specific for epitope tags that had been introduced at the C-termini of the molecules. As a negative control, COS-7 cells were transfected with empty vector. Flow cytometric analysis revealed that 14B11 MAb stained a significant fraction of COS-7 cells transiently transfected with Ly49C, Ly49E, and Ly49I cDNAs (Fig. 2A). In contrast, 14B11 MAb failed to react with COS-7 cells transfected with Ly49A, Ly49B, or Ly49G2 (Fig. 2B). Ly49E-transfected cells also were not stained (Fig. 2B), although in some experiments a small percentage (2–8%) of Ly49E transfected cells stained very dimly (data not shown). The possibility that the His tags inter-

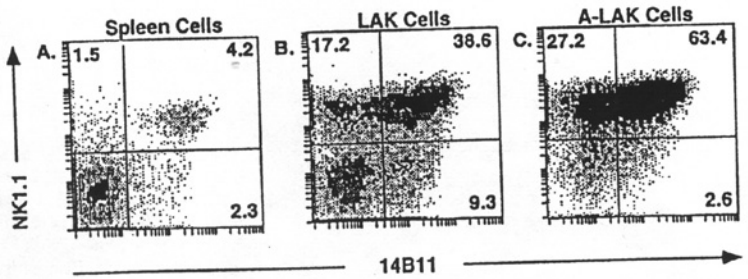
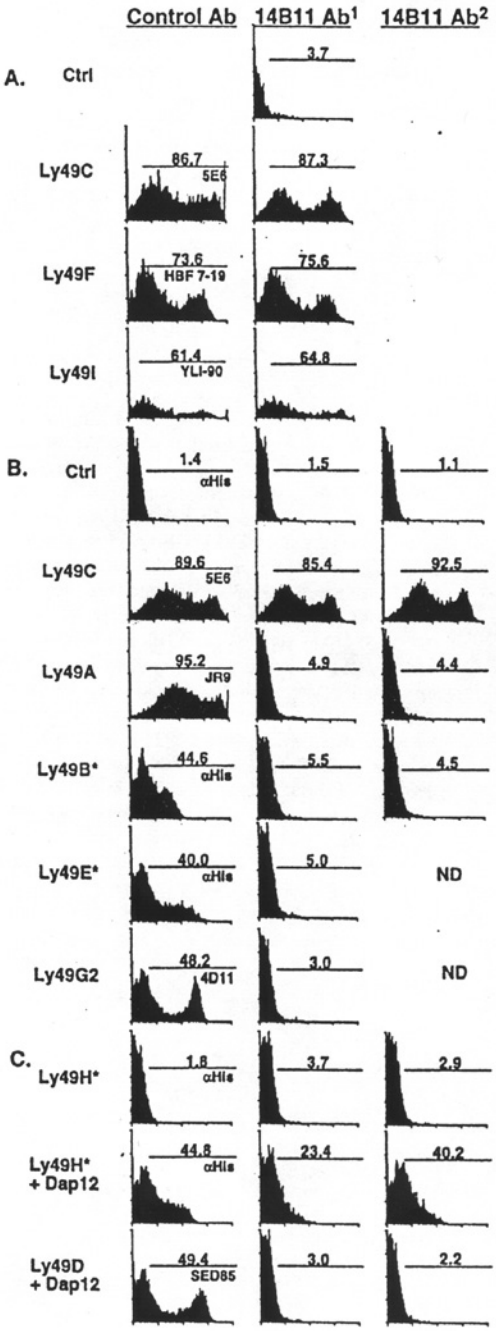


FIG. 1. 14B11 MAb stains the majority of freshly isolated $NK1.1^+$ spleen cells as well as the majority of IL-2-activated $NK1.1^+$ cells present in LAK cell and A-LAK cell preparations (see the Materials and Methods section). Cell preparations were stained with PK136-PE and 14B11 MAb, followed by goat anti-hamster IgG-FITC.

fered with the binding of Ly49B or Ly49E to 14B11 is unlikely because His tagged Ly49H bound to 14B11 (see below). We conclude that 14B11 binds well to Ly49F, I and C, does not bind detectably to Ly49G, A and B, and binds weakly or not at all with Ly49E.



We also tested reactivity of 14B11 with Ly49D and Ly49H, the putative activating Ly49 receptors. As reported previously, cell surface expression of these receptors is enhanced by co-expression of a cDNA encoding the DAP12 signaling molecule.[13] The cell surface level of Ly49H was substantially elevated when the cDNA was co-transfected with a DAP12 cDNA, as shown by staining with antibody specific for a His tag on the *C*-terminus of Ly49H (Fig. 2B). Importantly, 14B11 MAb stained a significant fraction of COS-7 cells that had been co-transfected with Ly49H and DAP12 (Fig 2B, middle column). Although 14B11 stained Ly49H/DAP12 co-transfected cells reproducibly in four experiments, the percentage of positive cells and the staining intensity was less than observed with cells transfected with Ly49F, I, or C. The use of a three-step staining procedure (Fig. 2, right column) enhanced the percentage of positive cells, which approached the percentage that stained with the anti-His tag antibody. Nevertheless, the staining intensity was clearly less than observed for the other 14B11-reactive receptors. Significantly, 14B11 failed to stain COS-7 cells that had been co-transfected with Ly49D and DAP12 cDNAs, although the latter cells clearly expressed Ly49D on the cell surface (Fig. 2B). To summarize the Ly49 specificity of 14B11, the MAb reacts with Ly49F, Ly49I, Ly49C, and Ly49H and poorly if at all with Ly49E. No reactivity with Ly49G, Ly49A, Ly49D, or Ly49B was observed.

Strain distribution of 14B11 antigens

The expression of 14B11 antigens on splenic NK cells from a cross section of laboratory mouse strains was analyzed by flow cytometry. NK cells were co-stained with DX5, a pan NK MAb, and T and B cells were gated out of the analysis. Nearly 90% of splenic NK cells from B6 mice stained with 14B11 MAb (Fig. 3). This percentage is higher than was observed in Fig. 1 because TCR^+ cells had not been gated out of the earlier experiment. A similar fraction of NK cells from each of six B10 MHC congenic strains also stained with 14B11 MAb, indicating that MHC differences do not appreciably affect the frequency of $14B11^+$ cells (Fig. 3). In BALB/c mice and two BALB MHC congenic strains, the percentages of $14B11^+$ cells

FIG. 2. Specificity of 14B11 for Ly49 receptors revealed by staining COS-7 cell transfectants. COS-7 cells were transfected with the indicated cDNA expression constructs, or with empty pME18S vector as a control (Ctrl). The transfected cells were stained with 14B11 MAb (left histograms) or an antibody known to react with the transfected receptor (right histograms, antibody indicated). In the case of receptors for which no specific antibodies were available (Ly49B, E, and H, indicated by asterisks), we inserted epitope (His) tags at the *C*-terminus of the molecules, to allow detection of the molecules by staining with anti-His antibodies. (A and B) Transfections with inhibitory Ly49 receptors. The transfected cells in (A) and (B) were stained by adding the biotinylated primary antibody followed by streptavidin-PE. (C) Transfection with putative activating Ly49 receptors. The transfected cells in this panel were stained with 14B11 in a three step procedure, first with unconjugated 14B11 antibody, then with biotinylated goat anti-hamster IgG followed by streptavidin-PE. The data in panels (B) and (C) were from the same experiment.

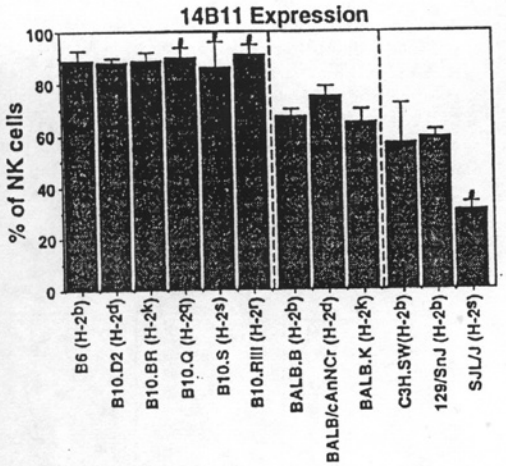


FIG. 3. 14B11 MAb stains a majority of NK1.1$^+$CD3$^-$ splenocytes in most mouse strains tested, with little or no effect of MHC expression on the percentage of 14B11$^+$ cells. TCR$^+$ and IgG$^+$ cells were gated out of the analysis. The data represent means of triplicate determinations ± S.D., except for the samples designated with #, which represent duplicate determinations.

were reduced to approximately 70–80% of NK cells. The lower percentage may be due in part to the absence of Ly49I and possibly other Ly49 receptors in BALB mice in comparison to B6/B10 mice (see Discussion). As was seen for the B10 congenic strains, there was little effect of MHC on the percentage of 14B11$^+$ cells in the BALB congenic strains. In the other mouse strains, the percentage of 14B11$^+$NK cells was in the same range as BALB congenics with the exception of the SJL strain, which had significantly fewer 14B11$^+$ NK cells.

14B11 MAb mediates redirected lysis of target cells

14B11 MAb was examined for its ability to modulate the lytic function of IL-2 activated NK cells (LAK cells). As shown in Fig. 4A, the addition of 14B11 MAb resulted in significantly enhanced lysis of Daudi (FcR$^+$) target cells by LAK cells. The enhancement by 14B11 was observed with LAK cells from B6 and B10.D2 mice (Fig. 4A), indicating that the effect was not dependent on a particular MHC genotype of the LAK cell donor. The isotype control (anti-Vγ3) MAb had no effect on lysis compared with the control without antibody. Removal of residual T cells from the LAK cell preparations did not reduce the extent of redirected lysis (data not shown, see also Fig. 6). 14B11 enhanced lysis of Daudi cells to a similar extent as the PK136 MAb, which has been previously shown to enhance NK-mediated lysis.[25] PK136 recognizes the activating NK1.1 receptor, also known as NKR-P1C.

Two lines of evidence indicate that the enhanced lysis of Daudi cells mediated by 14B11 MAb involves direct bridging of the NK cell to the target cell via Fc receptors on the latter cells, a process known as "redirected lysis" or "reverse ADCC." First, the 14B11 MAb enhanced lysis of FcR$^+$ Daudi (Fig. 4A) and P815 (data not shown) target cells, but did not enhance lysis of the FcR$^-$ cell line EL-4 (Fig. 4). Second, F(ab')$_2$ fragments of 14B11 MAb, which should not bind Fc receptors, failed to enhance lysis of Daudi cells (Fig. 4B). In control experiments, 14B11 F(ab')$_2$ fragments stained LAK cells efficiently, demonstrating that they retained their binding activity (data not shown).

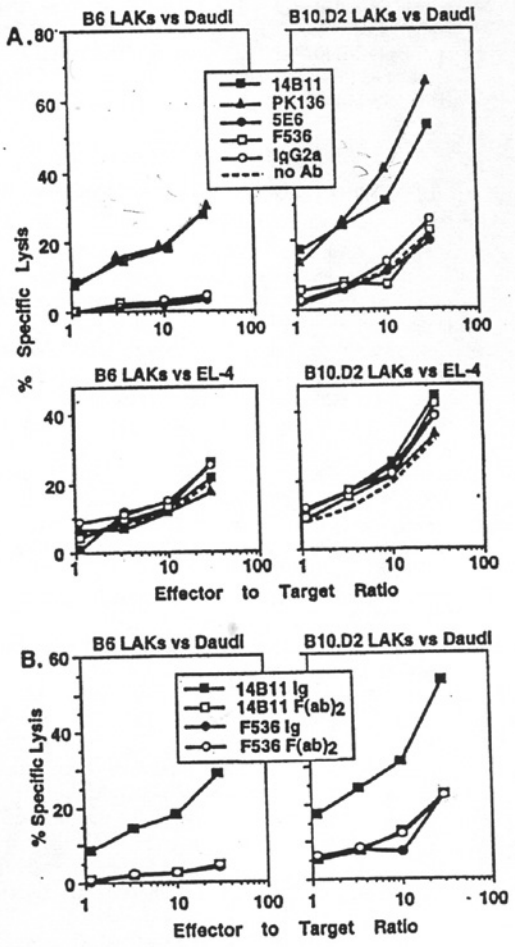


FIG. 4. Redirected lysis mediated by 14B11 MAb. (A) 14B11 MAb enhanced lysis by LAK cells of FcR$^+$ Daudi cells, but not FcR$^-$ EL-4 cells. PK136 (anti-NK1.1) MAb served as a positive control for redirected lysis. The irrelevant hamster mAb F536 (anti-Vγ3) served as an isotype control for 14B11 MAb, while mouse IgG2a served as the isotype control for 5E6. Similar results were obtained in four additional experiments. (B) Lysis of Daudi cells by IL-2 activated NK cells is enhanced by intact 14B11 MAb, but not by F(ab')$_2$ fragments of 14B11 MAb. Similar results were obtained in two additional experiments.

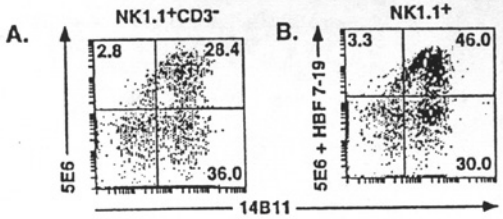


FIG. 5. Flow cytometric analysis of freshly isolated NK cells reveals a population of 14B11$^+$Ly49F/I/C$^-$ cells. (A) Co-staining of gated NK1.1$^+$CD3$^-$ splenocytes with 14B11 and 5E6 (anti-Ly49C/I) MAbs. (B) Co-staining of NK cells with 14B11 and Ly49C/I and F-specific antibodies (a mixture of 5E6 and HBF 7-19 MAbs).

An NK subset mediates 14B11-dependent redirected lysis

14B11 MAb binds to three Ly49 family members that are expected to be inhibitory based on the presence of an ITIM in their cytoplasmic domains (Ly49C, I, and F) and one believed to be an activating receptor (Ly49H). These receptors, like other Ly49 receptors, are expected to be distributed to overlapping NK cell subsets. Analysis of NK cells co-stained with 14B11 and anti-Ly49C/I (5E6) MAbs revealed that approximately 36% of NK cells from B6 mice are 14B11$^+$Ly49C/I, a population that is expected to include Ly49F$^+$ and Ly49H$^+$ NK cells (Fig. 5A). When NK cells were co-stained with 14B11 versus a mixture of anti-Ly49C/I and anti-Ly49F MAbs, a slightly smaller (~30%) 14B11$^+$Ly49F/I/C$^-$ cell population was observed (Fig. 5B). This population should include Ly49H$^+$ NK cells.

To examine whether 14B11-dependent redirected lysis segregates with a specific NK cell subset, LAK cells were first depleted of T cells followed by sorting NK.1.1$^+$ cells into 14B11$^+$5E6$^-$ and 14B11$^+$5E6$^+$ subsets. The sorted populations were cultured for an additional two days and tested for redirected lysis of target cells. As shown in Fig. 6A, the 14B11$^+$5E6$^-$ population was enriched in cells that mediate 14B11-dependent redirected lysis, while the 14B11$^+$5E6$^+$ population was partly depleted of these cells. These data suggest that the receptor responsible for 14B11-dependent redirected lysis is not Ly49C or Ly49I. Consistent with this conclusion, 5E6 MAb failed to enhance lysis of Daudi cells with any effector population we tested. In fact, in the case of the 5E6$^+$ effector cells, the 5E6 MAb had the opposite effect, consistently inhibiting lysis of Daudi cells below the level of lysis observed in the presence of control Ig or no antibody (Fig. 6A). The reduction in lysis may represent "redirected inhibition," wherein bridging of an inhibitory receptor, Ly49C and/or I in this case, with Fc receptors on target cells can inhibit NK cell activation.[26] Consistent with this possibility, 5E6 MAb did not inhibit lysis of FcR$^-$ EL4 cells by 5E6$^+$ effector cells. In addition to demarcating the Ly49 subsets involved in 14B11-mediated redirected lysis, the data in Fig. 6 demonstrates that the effector cells are NK1.1$^+$CD3$^-$ cells.

To address the role of Ly49F (as well as Ly49 C and I), another sorting experiment was performed in which Ly49F$^+$ cells, as well as Ly49C/I$^+$cells were enriched or depleted from a LAK cell population. For this experiment, we depleted the T cells from the LAK cell population. We used the novel anti-Ly49F and anti-Ly49I MAbs (see the Materials and Methods section, H.T. and D.H.R., in preparation). These antibodies were combined with 5E6 MAb to stain all Ly49F/I/C$^+$ cells. Gating on CD3$^-$ cells, Ly49F/I/C$^+$ and Ly49F/I/C$^-$ effector populations were sorted and tested for lysis of Daudi cells in the presence or absence of 14B11 MAbs. As shown in Fig. 6B, 14B11 MAb significantly enhanced lysis mediated by the Ly49F/I/C$^-$ pop-

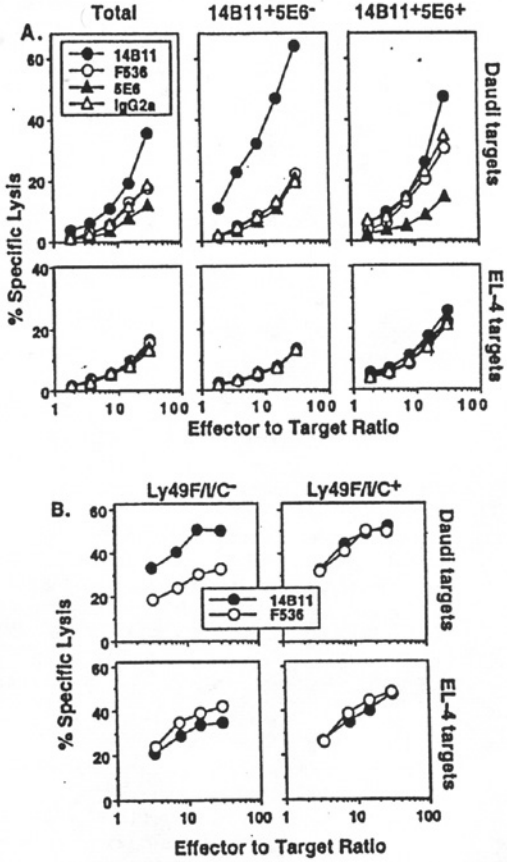


FIG. 6. Cells mediating 14B11-dependent redirected lysis are enriched in the Ly49F/I/C$^-$ population. (A) Analysis of 14B11-dependent redirected lysis by separated 14B11$^+$5E6$^-$ and 14B11$^+$5E6$^+$ NK cells. The sorted 14B11$^+$5E6$^-$ population contained 4.6% 5E6$^+$ cells on the day of assay, compared to 86.6% of the 14B11$^+$5E6$^+$ population. The results are typical of three experiments. (B) Analysis of 14B11-dependent redirected lysis by sorted Ly49F/I/C$^-$ and Ly49F/I/C$^+$ NK cell populations. At the time of assay, 5.4% of the sorted Ly49F/I/C$^-$ cells exhibited dim staining with a mixture of antibodies specific for Ly49F/I/C, while 87.6% of the sorted Ly49F/I/C$^+$ population cells were Ly49F/I/C$^+$. The Ly49F/I/C$^+$ population included 25.5% 14B11$^+$ cells at the time of assay. Similar results were obtained in a repetition of the experiment.

ulation, but had no effect on lysis mediated by the Ly49F/I/C$^+$ population. These data suggest that Ly49F, like Ly49C and Ly49I, is not responsible for redirected lysis mediated by 14B11 MAb. The data indicate that redirected lysis is mediated by a non-Ly49F/I/C receptor that is reactive with 14B11 MAb, possibly Ly49H.

DISCUSSION

Our results demonstrate that MAb 14B11 binds to four of the known Ly49 receptors in B6 mice. Of the inhibitory receptors, Ly49F, I, and C are efficiently bound by the 14B11 MAb. Of the putative activating receptors, Ly49H is bound by 14B11 and Ly49D is not bound detectably. Interestingly, the pattern of 14B11 reactivity corresponds to most members of a "subfamily" of Ly49 receptors defined by sequence relatedness. When arrayed in a tree dendrogram based on sequence similarity, Ly49F, I, C, H, and E fall into a related subfamily, the "FICHE" family, whereas Ly49G, A, and D define subfamily, "GAD," and Ly49B represents a relatively distinct third subfamily.[27] Thus, 14B11 MAb is reactive with at least four members of the "FICHE" family of Ly49 receptors. It is notable that 14B11 is the first MAb to be reported that reacts with Ly49H and Ly49F, although we have subsequently prepared a Ly49F-specific MAb (H.T. and D.H.R., in preparation). The reactivity of 14B11 with a small subset of T cells is consistent with the fact that a small fraction of T cells express various Ly49 receptors[28]; data not shown). The 14B11 MAb should be useful for a variety of studies of NK cell function and specificity.

14B11 MAb stains nearly 90% of freshly isolated NK cells from B6 background mice, indicating that FICHE subfamily members are expressed by the large majority of NK cells. Many of these cells also express Ly49G, A, or D (data not shown) consistent with the overlapping pattern of receptor expression previously described in the case of other Ly49 molecules.

In comparing different mouse strains, we observed significantly fewer 14B11$^+$ NK cells in BALB, C3H.SW, 129, and AKR/J strains than in B6 mice, and even fewer in the SJL strain. Most of these strains have not been well studied for their content of Ly49 receptors or genes. Interestingly, however, evidence suggests that BALB/c NK cells express the Ly49C receptor, but lack the Ly49I receptor.[29] Furthermore, Southern hybridization studies with a Ly49C probe have demonstrated several bands in B6 genomic DNA, presumably corresponding to various FICHE subfamily members, but only one or two major bands in BALB genomic DNA.[4,30] These results suggest that BALB mice may lack two or more members of the FICHE family, consistent with the lower fraction of 14B11$^+$ NK cells in these mice. We have not been able to use our new Ly49F- and Ly49I-specific antibodies to aid in testing this hypothesis because they were prepared in BALB/c mice and therefore do not react with BALB alleles of the corresponding receptors, should they exist. The even lower percentage of 14B11$^+$ NK cells in SJL mice raises the possibility that these mice may express even fewer FICHE subfamily members. Alternatively, SJL NK cells may include fewer NK cells expressing the remaining members, or the 14B11 MAb may fail to react with some of the SJL allomorphs of the relevant receptors. The results are consistent with the emerging picture of the Ly49 genes as an allelically plastic gene family.

Considering the broad reactivity of 14B11 with inhibitory receptors, it was initially surprising that the antibody-mediated redirected lysis of target cells by polyclonal NK cell populations, an activity normally ascribed to antibodies specific for activating receptors. A possible explanation is that some of the Ly49 receptors can either inhibit or activate NK cells depending on the extent of aggregation of the receptor by ligands. Alternatively, 14B11 MAb may activate NK cells through only one of its target receptors, a noninhibitory, activating receptor. The cell separation experiments argue strongly for the latter conclusion. The redirected lysis activity was strongly enhanced in NK cell populations that had been depleted of Ly49C/I/F$^+$ NK cells. Conversely, redirected lysis activity was diminished to near background levels with populations that were enriched for Ly49C/I or F expression.

Because Ly49H binds to 14B11 MAb, it is an obvious candidate for the activating receptor that mediates redirected lysis. It was recently implicated as an activating receptor based on transfection studies in B cell lines,[13] although its role in NK cells has not been addressed. Ly49H exhibits several other features consistent with its assignment as an activating receptor, including the absence of a recognizable ITIM, and the requirement of DAP12 for efficient surface expression[13] (Fig. 2). However, we have no direct evidence that Ly49H mediates 14B11-induced redirected lysis. Recent studies have identified another *Ly49h*-like gene (*Ly49n*), which lacks an ITIM and may therefore also represent an activating receptor.[30] However, the *Ly49n* sequence has only been identified at the genomic level. It is unknown whether the gene is expressed, and whether the corresponding protein is reactive with 14B11 MAb.

14B11 MAb had no discernible functional effect on Ly49C/I/F$^+$ NK cells. In contrast, 5E6 MAb consistently caused inhibition of lysis with Daudi target cells, suggesting that the 5E6 antibody provided inhibitory signals to these NK cells. The failure of 14B11 MAb to inhibit lysis by Ly49C/I/F$^+$ NK cells could reflect differences in MAbs in the potential to initiate inhibition. An alternative explanation is that some of the Ly49C/I/F$^+$ NK cells co-express stimulatory receptors like Ly49H, to which 14B11 also binds. 14B11 MAb may therefore interact with activating and inhibitory receptors on these cells, with the two effects more or less canceling each other out. These effects, while speculative, fit with the idea that NK cell triggering is normally regulated by a balance between activating and inhibitory signaling, controlled by distinct receptors.[10,31,32]

ACKNOWLEDGMENTS

The authors thank Dr. Chris McMahon and Russell Vance for comments on the manuscript, Peter Schow for expert assistance with flow cytometry, and Chern-Sing Goh and Ann Lazar for technical assistance, and Chiron Pharmaceutical Corp. for the gift of IL-2. This work was supported by NIH grant RO1 AI35021 to D. H. R. T. H. was a recipient of a Research Fellowship from the Deutsche Forschungsgemeinschaft. The authors thank Otsuka Pharmaceutical Corp. for sponsoring H.T.

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Received for publication April 22, 1999. Accepted for publication May 20, 1999.