

Biphasic response of NK cells expressing both activating and inhibitory killer Ig-like receptors

Hilary S. Warren, Anita J. Campbell, John C. Waldron and Lewis L. Lanier¹

Cancer Research Unit, The Canberra Hospital, Canberra, ACT 2606, Australia

¹Department of Microbiology and Immunology, and the Cancer Research Institute, University of California San Francisco, CA 94143-0414, USA

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Abstract

NK cells can co-express inhibitory and activating killer Ig-like receptors (KIR) recognizing the same HLA class I ligand. We present evidence from experiments with NK cells expressing both activating (KIR2DS2) and inhibitory (KIR2DL2 and KIR2DL3) receptors that the activating KIR can function without apparent interference from the inhibitory KIR. These studies used CD158b mAb that is equally reactive with KIR2DS2, KIR2DL2 and KIR2DL3. First, we show using plastic-immobilized CD158b mAb that the activating KIR2DS2 is stimulated, resulting in NK cell division and degranulation. Second, we show using soluble CD158b mAb and FcR11⁺ P815 cells that high concentrations of CD158b mAb trigger the inhibitory KIR, whereas low concentrations stimulate the activating KIR2DS2 resulting in NK cell division and cytotoxicity. These results demonstrate that the activating KIR2DS2 can function on cells co-expressing the inhibitory KIR2DL2 and/or KIR2DL3, indicating the potential for independent function of activating KIR with natural ligand.

Introduction

NK cells express receptors for MHC class I that either inhibit or activate NK cell effector function (recently reviewed in 1,2). There are two families of receptors, the killer Ig-like receptors (KIR) that recognize specific groups of HLA-A, -B, -C alleles, and the C-type lectin receptors (CD94/NKG2 family) that recognize HLA -A, -B, -C indirectly through recognition of HLA-E that is surface expressed when it binds the leader sequences of certain HLA class I molecules. The inhibitory receptors of both receptor families are characterized by the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail. The activating receptors of both families are characterized by a charged residue in the transmembrane domain allowing association with a 12-kDa membrane adaptor molecule DAP12/KARAP that contains an immunoreceptor tyrosine-based activation motif (ITAM). Typically, individual NK cells express inhibitory and/or activating receptors of both the KIR and CD94/NKG2 receptor families. With respect to the KIR, it was reported earlier that activating and inhibitory receptors on NK cell clones have different MHC class I specificities (3), but more recently a RT-PCR analysis of >100 NK cell clones from one individual

revealed that 38 clones co-expressed inhibitory and activating receptors for the same MHC class I ligand (4).

In this study, we assess the functional activity of KIR on NK cells that co-express the activating KIR2DS2 (two Ig-like domains, short cytoplasmic tail, p50) and the inhibitory KIR2DL2 and KIR2DL3 (two Ig-like domains, long cytoplasmic tail, p58). These KIR recognize the polymorphism S77, N80 in HLA-Cw1, 3, 7 and 8. The extracellular domain of KIR2DS2 differs from KIR2DL2 and KIR2DL3 by only 4 and 3 amino acids respectively (5). These three KIR are collectively termed CD158b (6) and CD158b mAb cannot distinguish these three receptors. We have asked the question under what conditions NK cells co-expressing activating and inhibitory KIR to the same HLA class I ligand are stimulated through the activating receptor. We show in two different culture systems, using either plastic-immobilized CD158b mAb or low concentrations of soluble CD158b mAb and FcR11⁺ P815 target cells to cross-link the receptors, that the activating KIR2DS2 can be stimulated without interference from the inhibitory KIR2DL2 and/or KIR2DL3. The potential for independent functioning of activating and inhibitory KIR with natural ligand, that is

dependent on the density of class I MHC on the target cell, is suggested from this study.

Methods

Antibodies

The CD158b mAb used in these studies were GL183 (IgG1) (kindly provided by the organizers of the Sixth International Workshop on Human Leukocyte Differentiation Antigens) and DX27 IgG2a (DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, CA). Other mAb used were B73.1 (CD16, IgG1) from the hybridoma cell line kindly provided by Dr G. Trinchieri (Wistar Institute, Philadelphia, PA), OKT3 (CD3, IgG2a) from the ATCC (Rockville, MD), FMC63 (CD19, IgG2a) a gift from Professor H. Zola (Child Health Research Institute, Adelaide, South Australia), CLB-FcGran1 (CD16, IgG2a) from the organizers of the Fifth International Workshop on Human Leukocyte Differentiation Antigens, and WV3 (CD56, IgG1), WV5 (CD56, IgM), WV2 (CD94, IgG1), WV4 (CD94, IgM) and WV6 (CD158b, IgM) prepared in our laboratory. IgG mAb were purified by Protein A-Sepharose CL-4B chromatography (Pharmacia Biotech, Boronia, Victoria, Australia) using procedures described by the manufacturer. IgM mAb were purified by precipitation following dialysis against water. FITC-conjugated sheep anti-mouse Ig (code DF) and affinity-purified sheep anti-mouse Ig (code DA) were purchased from AMRAD Pharmacia Biotech. Biotinylated rat anti-mouse IgM was purchased from PharMingen (San Diego, CA), and FITC-conjugated sheep anti-mouse IgG2a and streptavidin-phycoerythrin (PE) from Serotec (Oxford, UK).

Plastic tissue culture wells were coated with mAb as follows. Culture wells were first precoated with affinity-purified anti-mouse Ig (50 μ l of 10 μ g/ml in 0.05M Na₂CO₃/NaHCO₃ buffer, pH 9.6, overnight at 4°C), washed 4 times with PBS and then blocked for 30 min with medium containing 5% heat-inactivated FCS (HIFCS) prior to incubation with mAb for 2 h. Culture wells were then washed 4 times with PBS prior to addition of cells. The mAb concentrations for coating (in the range 3–10 μ g/ml) were 4 times those determined as optimum by immunofluorescence staining of cells and flow cytometry.

NK cell lines

Cultured human NK cell lines were established as reported previously (7). Briefly, peripheral blood mononuclear cells were depleted of monocytes by adherence on plastic for 1 h at 37°C, and the T cells and B cells labeled with OKT3 (CD3) and FMC63 (CD19) mAb followed by FITC-conjugated anti-mouse IgG2a. CD158b⁺ NK cells were labeled using WV6 (CD158b) mAb followed by biotinylated anti-mouse IgM and streptavidin-PE. The sorted FITC⁺, PE⁺ NK cells were cultured at 2.5×10^3 with 10^4 γ -irradiated (40 Gy, from a Cs¹³⁷ source) MM-170 malignant melanoma cells and rIL-2 in 0.2 ml volumes in 96-well round-bottom trays. The culture medium was MEM (41500-034; Gibco, Grand Island, NY) supplemented with antibiotics (100 μ g/ml penicillin, 100 μ g/ml streptomycin and 60 μ g/ml gentamycin), 24 mM NaHCO₃, 0.1 mM 2-mercaptoethanol, 10% HIFCs and 200 U/ml (87 ng/ml) rIL-2. Human rIL-2 was generously provided by Dr G. Zurawski and S. Menon (DNAX Research Institute, Palo Alto, CA). Cells generated

in these cultures were entirely NK cells (CD3⁺CD56⁺CD16⁺CD94⁺). NK cells were subcultured from day 8 as required, maintaining cell concentrations at 0.5×10^6 /ml. NK cells reached quiescence at about day 18 of culture. NK cells obtained at day 18–24 of these primary cultures were re-stimulated in secondary and subsequent cultures under similar conditions, including additional stimulation with plastic-bound CD16 mAb as required (8).

RT-PCR and immunoprecipitation

The presence of mRNA transcripts for the activating KIR2DS2, and inhibitory KIR2DL2 and KIR2DL3 was established by RT-PCR by using established primer sequences and conditions, essentially as described by Uhrberg *et al.* (9), except that the PCR amplification was carried out in a Corbett thermocycler (model 960C; Corbett Research, Mortlake, NSW, Australia), and the products were subject to electrophoresis on 1.8% agarose gels and then stained with ethidium bromide. Cell surface expression of KIR was confirmed by immunoprecipitation of surface biotinylated NK cells and analysis by SDS-PAGE using procedures described previously (10), except that the CD158b mAb (WV6, IgM) used for immunoprecipitation was bound to anti-mouse IgM (μ chain-specific) agarose beads (Sigma, St. Louis, MO; cat. no. A-4540) and the gels were run under reducing conditions using 0.1 M dithiothreitol.

Measurement of cytolytic function by ⁵¹Cr release

Cytotoxic activity of NK cells was measured in a 4-h ⁵¹Cr-release assay using 5000 targets at an E:T cell ratio of 1:1 or 2:1 unless otherwise stated, by methods described previously (7). Cytotoxicity is expressed as percent specific lysis from c.p.m. released into the supernatant from target cells: [(c.p.m. in test cultures – c.p.m. in control cultures)/(c.p.m. in 1% Triton X-100 lysates – c.p.m. in control cultures)] $\times$ 100.

For most experiments, percent specific lysis was then converted to cytotoxic units (CU) to linearize the data between 30 and 60% lysis (11) according to the formula: CU = $-\ln [(1 - \text{percent specific lysis})/100] \times 5000$. Data were then expressed as a percentage of the maximum lysis achieved using saturating concentrations of mAb to activating receptors. The dose of mAb to achieve 50% lysis (ED₅₀) was calculated from this data using Biolinx software.

Measurement of cytolytic function by degranulation

NK cells were cultured in a balanced salt solution containing 10% HIFCS at 400,000 cells per 0.1 ml in flat 96-well tissue culture plates (Linbro, 76-032-05; ICN Biomedicals, Sydney, Australia) that had been precoated with mAb. After 4 h at 37°C the supernatant was harvested, and granzyme A activity was assayed as *N*- α -carbobenzoxy-L-Lys-thiobenzyl ester (BLT)-esterase activity (12). Degranulation is recorded as OD₄₀₅ after subtraction of the value for the reagent controls.

Measuring NK cell division using 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE)

NK cells were labeled prior to culture with the intracellular fluorescent dye CFSE (Molecular Probes, Eugene, OR) (8). Briefly, quiescent NK cells from day 18–24 of primary culture were labeled directly without prior centrifugation in complete tissue culture medium by the addition of CFSE (final concentra-

tion 5 μ M) with rapid mixing. After 7 min at room temperature the cells were washed twice in PBS/5% HIFCS and treated for 5 min at 37°C with 0.5 mM EDTA in PBS to prevent aggregation prior to resuspension in complete tissue culture medium. Labeled NK cells were cultured in 0.2 ml volumes in flat 96-well tissue culture plates at 5×10^4 cells/well when cultured alone, or in triplicate cultures at 10^4 /well when cultured with 3×10^4 γ -irradiated (40 Gy) MM-170 human malignant melanoma cells or 5×10^4 γ -irradiated (40 Gy) FcRII⁺ mouse P815 cells. NK cells cultured with P815 cells also contained soluble IgG mAb. NK cells cultured alone or with MM-170 cells used plastic wells precoated with mAb as described above. Cultures were harvested at day 6 or 7, washed twice in PBS, treated with 0.5mM EDTA in PBS (5 min, 37°C) and resuspended in PBS/5% HIFCS for analysis by flow cytometry.

Flow cytometry

NK cell binding of mAb was detected by indirect immunofluorescence using FITC-conjugated anti-mouse Ig. Briefly, 2×10^4 NK cells were incubated in 96-well V-bottom plates with mAb at optimum concentrations or at the dilutions indicated for 30 min on ice. The plates were centrifuged and the cells washed 3 times in PBS/5% HIFCS containing 0.1% sodium azide. Optimum concentration of FITC-conjugated sheep anti-mouse Ig was added and following a further 30 min incubation on ice the cells were washed as above prior to fixation in 0.1% (w/v) paraformaldehyde. Immunofluorescence was measured using a FACScan (Becton Dickinson, San Jose, CA) calibrated using CaliBRITE beads (Becton Dickinson). Lymphocytes were gated using forward scatter (size) and side scatter (granularity) parameters. Data were analyzed using PC Lysys software.

CFSE-labeled NK cells were analyzed by flow cytometry and the percentage of cells entering cell division was calculated as described previously (7,8). In experiments using titrations of soluble mAb and P815 cells, data were expressed as a percentage of the maximum response. The concentration of mAb to achieve 50% (ED_{50}) of this value was calculated using Biolinx software.

Results

CD158b mAb and NK cell lines

The CD158b mAb GL183 (13) and DX27 (14) bind equally to the extracellular domains of the activating KIR2DS2, and the inhibitory KIR2DL2 and KIR2DL3. This was demonstrated using Ba/F3 transfectants expressing individual receptors (14). The mAb bind similarly to the transfectants (Fig. 1A) and show similar titration curves (Fig. 1B). The mAb do not bind to parent Ba/F3 cells (data not shown).

Two CD158b⁺ NK cell lines, A and C, generated from different donors were used in this study (7). Figure 2 compares the characteristics of NK cell line A (Fig. 2A–C) and NK cell line C (Fig. 2D–F). Results presented are representative of between four and six different experiments, and in the case of line A were generated from three different bleeds of the same donor. The results show cell surface expression of CD158b (Fig. 2A and D), functional testing of KIR status by

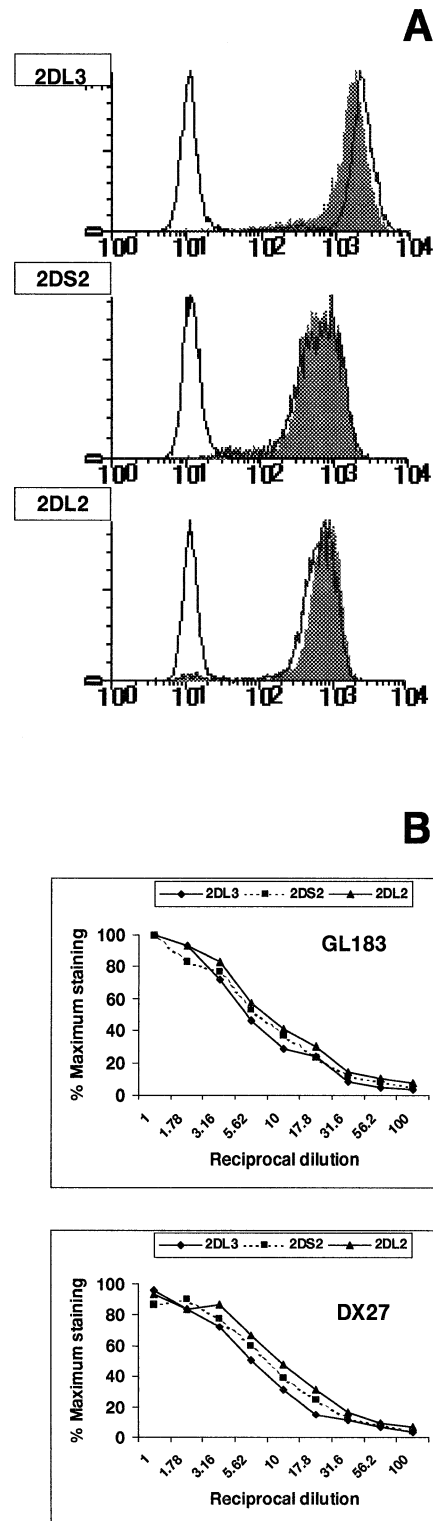


Fig. 1. Reactivity of CD158b mAb GL183 and DX27 on Ba/F3 transfectants expressing KIR2DL3, KIR2DS2 or KIR2DL2. (A) Flow cytometry profiles of transfectants stained with GL183 mAb (filled histogram) or DX27 mAb (line histogram). The histogram to the left is the anti-mouse Ig–FITC control. (B) Titration of mAb on the transfectants. Optimum concentration of mAb for staining is indicated as 1. The mAb dilutions are at 0.25 log₁₀ intervals.

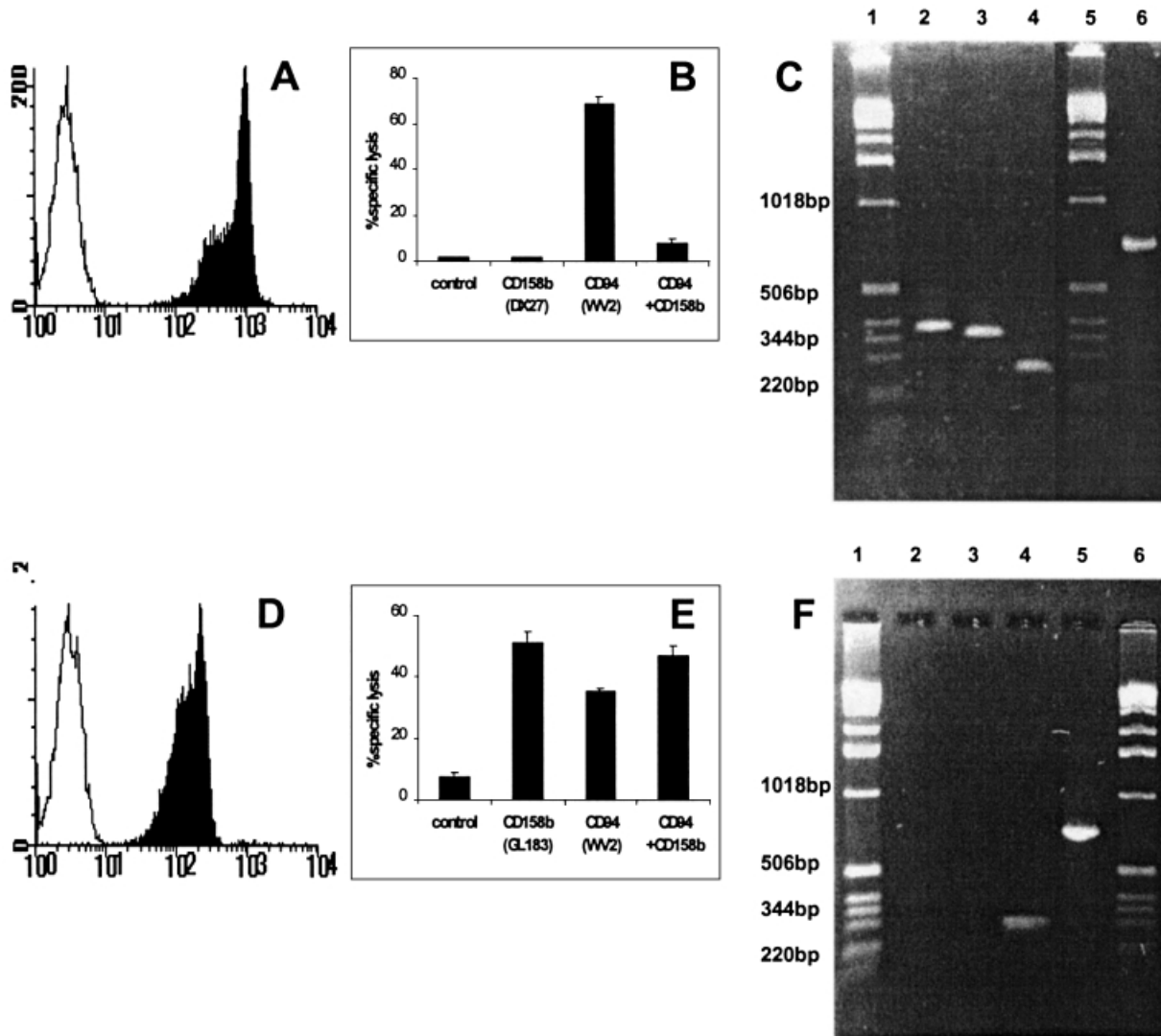


Fig. 2. Properties of NK cell line A (A–C) and NK cell line C (D–F). (A and D) Flow cytometry profiles of staining with CD158b mAb. (B and E) Cytotoxicity of NK cell lines on P815 cells in the presence of control CD56 mAb (WV3) or other mAb as indicated. (C and F) RT-PCR analysis of KIR expression on NK cell lines showing KIR2DL2 (370bp), KIR2DL3 (352bp) and KIR2DS2 (256 bp). Markers (1 kb DNA ladder; Life Technologies) and β -actin (734 bp) are shown.

cytotoxic activity using CD158b mAb and FcR11⁺ murine P815 target cells (Fig. 2B and E), and the presence of mRNA transcripts for KIR by RT-PCR (Fig. 2C and F).

For NK cell line A, cross-linking CD158b using mAb and FcR11⁺ P815 cells inhibited CD94 mAb-stimulated NK cell cytotoxicity (Fig. 2B). There was no stimulation of cytotoxicity in the presence of CD158b mAb alone. These results were obtained with both GL183 and DX27 mAb at concentrations that were optimum for binding to these cells. Although the functional read out for CD158b on NK cell line A indicated the presence of an inhibitory receptor, RT-PCR analysis of KIR mRNA expression revealed the presence of transcripts for both inhibitory (KIR2DL2 and KIR2DL3) and activating (KIR2DS2) receptors on NK cells of this line (Fig. 2C). Immunoprecipitation of cell surface CD158b from NK cell line A (Fig. 3) showed the presence of 58 (inhibitory)- and 50 (activating)-kDa receptors. It should be noted that the RT-PCR

data in Fig. 2(C) and the immunoprecipitation data in Fig. 3 are qualitative, and do not give a quantitative measure of expression of the different receptors on NK cells in line A.

For NK cell line C, functional testing (Fig. 2E) demonstrated that CD158b is an activating receptor on this cell line. Thus, CD158b mAb stimulated killing of FcR11⁺ P815 cells and there was no inhibitory effect of CD158b mAb on CD94 mAb stimulated killing. RT-PCR analysis of KIR mRNA expression for NK cell line C revealed the presence of the activating KIR2DS2 but no inhibitory KIR (Fig. 2F), consistent with the results in Fig. 2(E).

We next undertook cytotoxicity experiments to show that NK cells in line A that express the activating KIR2DS2 must also co-express inhibitory KIR2DL2 and/or KIR2DL3. P815 is killed in the presence of CD158b mAb by NK cells expressing only KIR2DS2 as shown for NK cell line C (Fig. 2E). The data in Fig. 4 show that there is no killing of P815 with NK cells

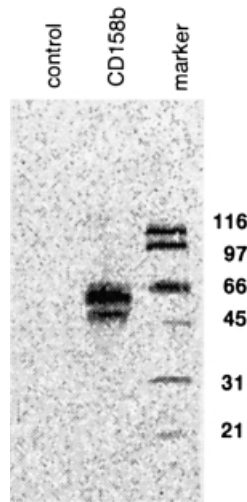


Fig. 3. Immunoprecipitation of KIR from Triton X-100 lysates of cell-surface biotinylated NK cell line A. The lysates were precleared with anti-mouse IgM (μ chain-specific) agarose beads (control) and then immunoprecipitated with agarose-coupled CD158b (WV6, IgM) mAb. The immunoprecipitates were analyzed by SDS-PAGE under reducing conditions on a gradient (4–20%) gel. The mol. wt markers are shown. WV6 mAb immunoprecipitates both the 58-kDa inhibitory KIR2DL2 and/or KIR2DL3, and the 50-kDa activating KIR2DS2.

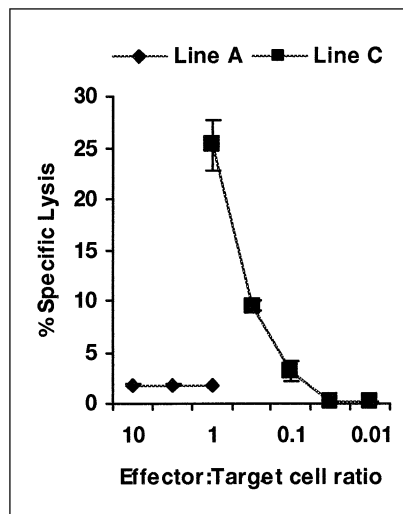


Fig. 4. Cytotoxicity stimulated using soluble CD158b mAb and FcR11⁺ P815 target cells. NK cell line A and NK cell line C were incubated with ⁵¹Cr-labeled P815 cells and CD158b mAb (DX27) at E:T cell ratios from 10:1 at 0.5 log₁₀ intervals. Cytotoxicity is expressed as percent specific lysis of the mean and SD of triplicate cultures.

from line A in the presence of CD158b mAb even at high E:T cell ratios (10:1), whereas killing of P815 by NK cell line C is detectable at 100-fold lower E:T cell ratios (0.1:1). This difference does not reflect different cytolytic abilities of the two lines since both NK cell lines killed P815 to similar extents when activated using CD94 mAb (Fig. 2B and E), a result confirmed in other experiments (not shown) over a range of different E:T cell ratios.

Activation of NK cell division using plastic-bound CD158b mAb

Cross-linking activating receptors such as CD16 using plastic-bound mAb stimulates NK cell division and this is readily monitored using cells prelabeled with CFSE (8), where division is recorded as the sequential decrease in fluorescence as cells divide (15). Because of the sensitivity of this assay we compared NK cell line A (KIR2DS2⁺, KIR2DL2⁺ and/or KIR2DL3⁺) with NK cell line C (KIR2DS2⁺) for their ability to divide when stimulated using plastic-bound CD158b mAb. The results in Fig. 5 compare NK cell line A (Fig. 5A and C) and NK cell line C (Fig. 5B and D) for their ability to divide following stimulation on plastic coated with control CD56 (WV3 or WV5) mAb, CD94 (WV2) mAb (as positive control) and CD158b (GL183 and DX27) mAb. The CFSE profiles are shown in Fig. 5(A and B) and the quantitative data from these profiles showing the percentage of cells that have entered division is shown in Fig. 5(C and D). Compared to control cultures, both NK cell lines divided when stimulated with plastic-bound CD94 mAb, consistent with their expression of the CD94 activating receptor (Fig. 2B and E). Importantly for NK cell line A, cell division was stimulated using plastic-coated CD158b mAb with 36% (GL-183) and 27% (DX27) of cells entering division compared to 12% in control cultures. Plastic-immobilized CD158b mAb stimulated division of NK cell line C with 50% of cells entering division, but did not stimulate division of an NK cell line lacking CD158b (data not shown).

The ability of NK cell line A to divide when stimulated using plastic-immobilized CD158b mAb was confirmed in a separate series of experiments where CFSE-labeled NK cells were cultured with γ -irradiated MM-170 malignant melanoma cells and rIL-2 on plastic coated with mAb (Fig. 6). NK cells from line A are refractory to re-stimulation in secondary and subsequent cultures in the presence of MM-170 cells and rIL-2 unless there is additional stimulation by ligating activating receptors with mAb (8). In these experiments plastic-bound CD158b mAb provided the additional activation signal for MM-170-stimulated NK cell division with 36% (GL183) and 27% (DX27) of cells entering division compared to 14% in control cultures with plastic-immobilized CD56 mAb. Cultures with plastic-immobilized CD94 mAb were the positive control with 48% of cells stimulated to divide. The percentage of cells stimulated to divide was calculated from CFSE profiles as shown for Fig. 5. Results are presented as the mean and SEM of 10 different experiments.

Activation of NK cell degranulation using plastic-bound CD158b mAb

Plastic-immobilized CD158b mAb was also able to stimulate degranulation of NK cells from line A despite their co-expression of the activating KIR2DS2 and the inhibitory KIR2DL2 and/or KIR2DL3 (Fig. 7A). Degranulation was assayed by measuring BLT esterase activity released into the culture supernatant after 4 h. Results are presented as the mean OD₄₀₅ and SD of triplicate assay points. The results show that degranulation of NK cell line A is stimulated strongly by CD94 mAb and also by the CD158b mAb GL183. The CD158b mAb DX27 stimulated only a marginal response in this assay. Degranulation of control NK cell line C (KIR2DS2⁺)

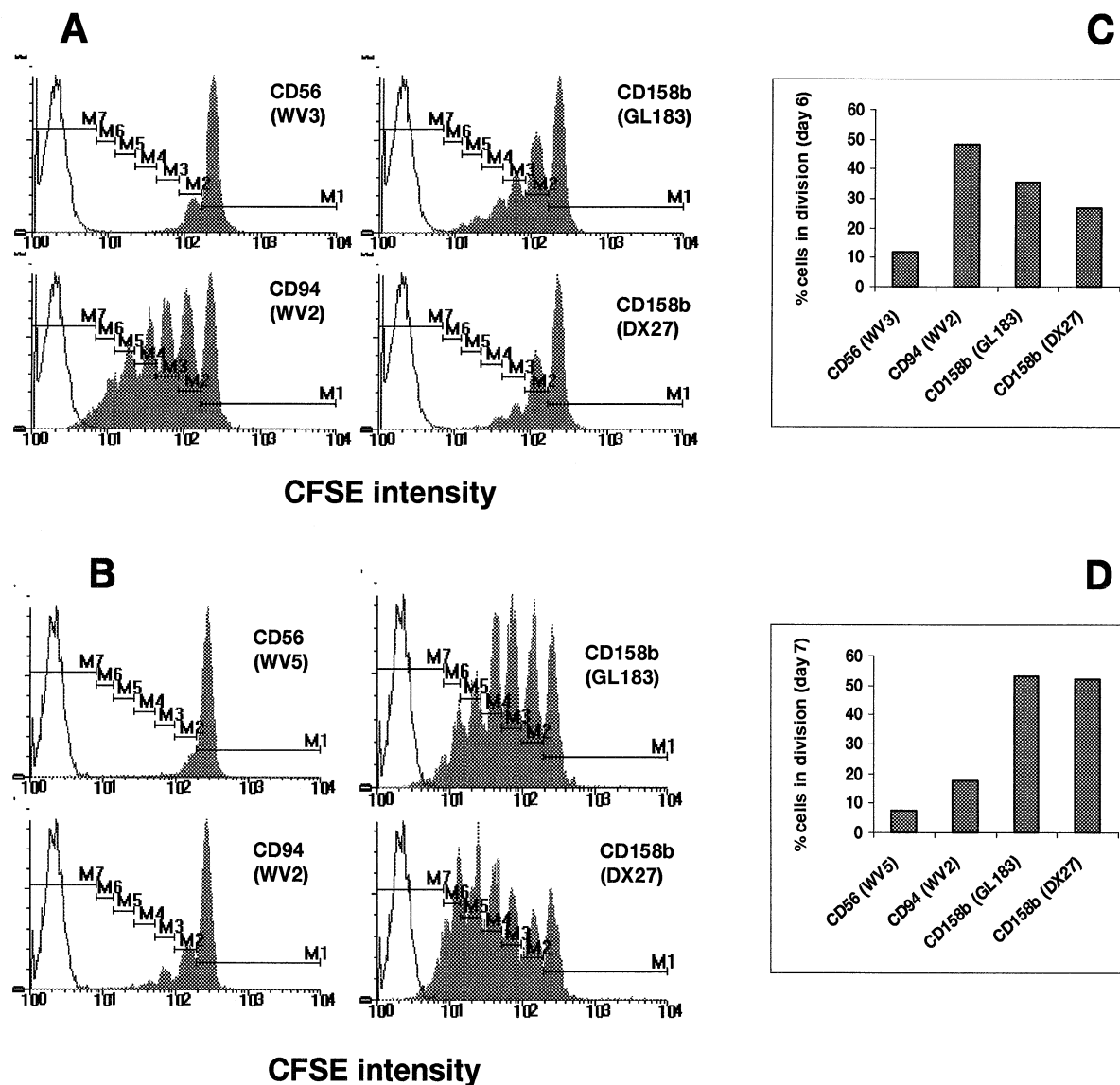


Fig. 5. NK cell division stimulated by plastic-immobilized mAb and rIL-2. Fifty thousand CFSE-labeled NK cells were cultured in 200 U/ml of rIL-2 on plastic coated with the mAb indicated. Data are shown for NK cell line A (A and C) and for NK cell line C (B and D). (A and B) Flow cytometry profiles of CFSE-labeled cells after 6 (A) or 7 (B) days cultured on plastic coated with CD56 (WV3 and WV5) mAb (control), CD94 (WV2) mAb or CD158b (GL183 and DX27) mAb. Markers indicate division numbers, undivided (M1), divisions 1 through 5 (M2 through M6) and division number exceeding 6 (M7). (C and D) Data are presented as the percent of cells in division calculated from the CFSE profiles in (A) and (B) respectively.

was stimulated strongly by CD94 mAb and by both CD158b mAb (Fig. 7B).

Inhibition and activation of NK cell division using soluble CD158b mAb and FcR11⁺ P815 cells

FcR11⁺ P815 cells and IgG mAb provide a convenient experimental system to test the function of inhibitory and activating NK cell receptors in cell division (7). The results in Fig. 8(A) are of a representative experiment showing cell division by NK cell line A stimulated by CD94 mAb and the ability of different concentrations of CD158b mAb to inhibit this response. Saturating concentrations of CD158b mAb totally inhibited cell division and at low concentrations inhibition

became progressively less. CD94 mAb alone stimulated 82.4% of NK cells to enter division, compared to 9.3% in control cultures. The effect of CD158b mAb is presented as a percentage of the CD94 mAb stimulated response. For five different experiments the reciprocal dilution of CD158b mAb giving 50% inhibition of cell division was on average 698 ± 188 (SEM) of the optimum concentration for binding to these cells. Importantly, we observed in five of eight experiments that low concentrations of CD158b mAb were able to stimulate cell division in the absence of CD94 mAb. Results from one of these five experiments is shown in Fig. 8(B). For the five experiments in which cell division was stimulated by low concentrations of CD158b mAb, on average $12.4 \pm 3.9\%$ of

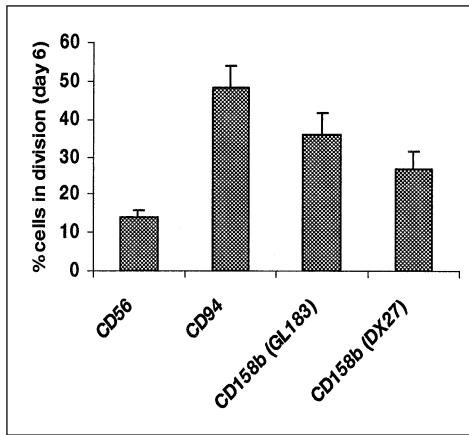


Fig. 6. Cell division of NK cell line A stimulated by plastic-immobilized mAb in the presence of MM-170 cells and rIL-2. Ten thousand CFSE-labeled NK cells were cultured in triplicate in 200 U/ml rIL-2 with 3×10^4 γ -irradiated MM-170 cells on plastic coated with CD56 (WV3 or WV5) mAb (control), CD94 (WV2 or WV4) mAb or CD158b (GL183 or DX27) mAb. Cultures were harvested on day 6 or 7 and data from CFSE profiles recorded as percent of cells in division. Results presented are the mean and SEM of 10 different experiments.

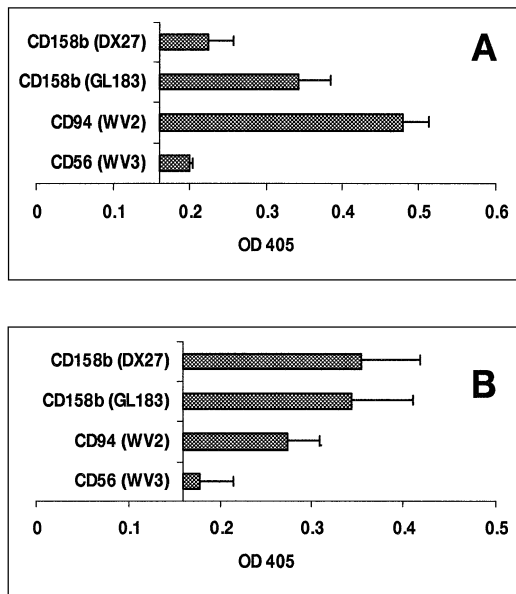


Fig. 7. Degranulation of NK cells using plastic-immobilized mAb. Four hundred thousand NK cells in 200 μ l volumes were cultured for 4 h on plastic coated with CD56 (WV3) mAb (control), CD94 (WV2) mAb or CD158b (GL183 or DX27) mAb. Culture supernatants were harvested and BLT-esterase activity measured (OD₄₀₅). The mean and SD of triplicate assays are recorded. (A) NK cell line A. (B) NK cell line C.

NK cells were stimulated to divide, compared to $5.4 \pm 2.0\%$ in control cultures without mAb, a difference of 2.3-fold. For comparison, the data in Fig. 8(C) show the effect of different concentrations of CD158b mAb on cell division for NK cells from line C (KIR2052⁺). Comparison of data from six different experiments with NK cell line C showed that the reciprocal dilution of CD158b mAb required for 50% activation of cell

division was on average 1490 ± 349 of the optimum concentration for binding to these cells.

Inhibition and activation of NK cell cytotoxicity using soluble CD158b mAb and FcR11⁺ P815 cells

The results in Fig. 9(A) are of a representative experiment with NK cell line A showing the dose-dependent inhibition of CD94 mAb-stimulated cytotoxicity by CD158b mAb. Similar results were obtained using either GL183 or DX27 CD158b mAb to inhibit the response and either CD16 or CD94 mAb to stimulate cytotoxicity. For seven different experiments the reciprocal dilution of CD158b mAb required to give 50% inhibition of cytotoxicity was on average 167 ± 17 (SEM) of the optimum concentration for binding to these cells. Consistent with our observation showing that low concentrations of CD158b mAb stimulated proliferation of NK cell line A, we observed that CD158b mAb also stimulated cytotoxicity at low concentrations (Fig. 9B). The results from seven different experiments showed that there was on average $17.3 \pm 2.7\%$ specific lysis by NK cell line A in the presence of low concentrations of CD158b mAb compared to $3.4 \pm 0.7\%$ in control cultures, a difference of 5-fold. For comparison, CD158b mAb-stimulated killing by NK cell line C (KIR2DS2⁺) also was measurable at low mAb concentrations (Fig. 9C). In five different experiments, the reciprocal dilution of CD158b mAb required to give 50% maximum response for NK cell line C was on average 1301 ± 133 of the optimum concentration for binding to these cells. These experiments show that CD158b mAb stimulates the activating KIR2DS2 at concentrations lower than that effective for inhibition.

Discussion

This study demonstrates, using CD158b mAb, the potential for the activating KIR2DS2 to function independently of co-expressed inhibitory KIR2DL2 and/or KIR2DL3. For the NK cell line A used in these studies we established by testing cytotoxic activity over a wide range of E:T cell ratios that when the activating KIR2DS2 is expressed so also is KIR2DL2 and/or KIR2DL3. Although inhibitory KIR are dominant in cytotoxicity assays when mAb is used at optimum concentrations, when CD158b mAb is plastic-immobilized a significant proportion of NK cells in line A are stimulated to divide and to degranulate. Re-investigating the ability of soluble CD158b mAb and P815 cells to stimulate cytotoxicity revealed that NK cells in line A consistently kill P815 at CD158b mAb concentrations below that stimulating the inhibitory KIR. Similar results were obtained measuring NK cell division, although the differences were not as marked. This is the first functional data demonstrating that NK cells can co-express activating and inhibitory KIR for the same HLA specificity, and that experimentally these receptors can function independently.

One hypothesis to explain the significance of co-expression of activating and inhibitory KIR with the same HLA specificity is that the activating receptor maintains activity of the inhibitory receptor via protein tyrosine kinase activity and phosphorylation of the ITIM (16). An alternative hypothesis, favored by our studies, is that the activating and inhibitory KIR have different threshold levels of occupancy for stimulation.

Specifically, that the activating KIR2DS2 can function at CD158b mAb concentrations below that effective for stimulation of the inhibitory KIR. Importantly, the CD158b mAb used in these studies bind equally to the activating and inhibitory KIR. Vales-Gomez (17) showed by plasmon surface resonance measurements that the CD158b (GL183) mAb binds with similar kinetics to surface immobilized activating KIR2DS2 and inhibitory KIR2DL3 receptors. Winter (5) showed in an ELISA assay that CD158b (GL183) mAb binds comparably to Ig fusion proteins of KIR2DS2, KIR2DL3 and KIR2DL2. These results agree with our analysis by immunofluorescence

staining and flow cytometry of the binding of GL183 and DX27 mAb to Ba/F3 transfectants that express the different KIR (Fig. 1). Therefore, the results with CD158b mAb reflect different threshold levels for activation of the KIR2DS2 compared to KIR2DL2 and KIR2DL3.

Activation of KIR2DS2 on NK cells from line A co-expressing inhibitory KIR was shown using plastic-immobilized CD158b mAb, and using low concentrations of soluble CD158b mAb and cross-linking the receptors with FcR11⁺ P815 cells. The two culture systems were equally effective in stimulating cytotoxic activity. By contrast, plastic-immobilized mAb is a more effective stimulus for cell division than is soluble mAb and P815 cells. These observations may reflect differences in the level of sustained signaling afforded by the two culture systems and that are required for the different functional responses. In the case of T cell activation, plastic-immobilized CD3 mAb gave more sustained signaling than cross-linking with soluble mAb, resulting in different activation outcomes (18).

The ability of activating KIR2DS2 to be stimulated without interference from the co-expressed inhibitory KIR using plastic-immobilized CD158b mAb or using low concentrations of soluble mAb with P815 presumably reflects differences in the intracellular signaling pathways for the activating and inhibitory KIR. As reviewed by Tomasello *et al.* (19), the activating KIR2DS2 associates with the ITAM-containing DAP12 (KARAP). It is likely that ligation results in activation of src family protein kinases and phosphorylation of the ITAM on DAP12 and ZAP-70 and Syk, with subsequent involvement of key adaptor proteins in downstream signaling. The inhibitory KIR2DL2 and KIR2DL3 have ITIM in their cytoplasmic domains. Following ligation there is activation of src family protein tyrosine kinases resulting in phosphorylation of the ITIM, and recruitment of the intracellular protein tyrosine phosphatases SHP-1 and/or SHP-2. SHP-1 may be responsible for inhibition of NK cell function through dephosphorylation of ZAP-70, Syk and the adaptor proteins involved in downstream signaling. There is evidence that engagement of

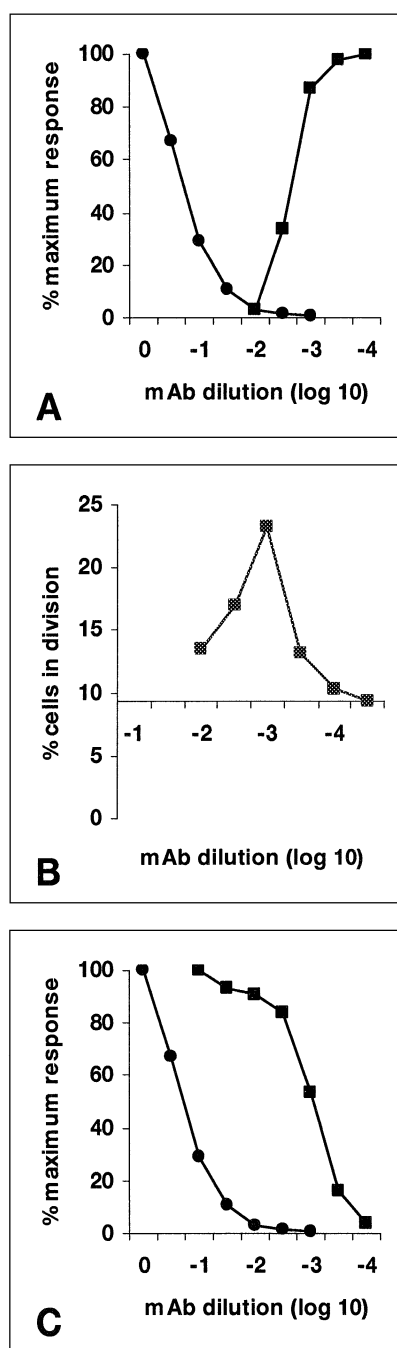


Fig. 8. NK cell division at different dilutions of CD158b mAb. The optimum concentration of mAb for binding was arbitrarily given the number 1.0 ($\log_{10} 1 = 0$). The mAb dilutions are at 0.5 $\log_{10}$ intervals. Binding of CD158b mAb to NK cells was determined by immunofluorescence flow cytometry in median fluorescence units and is recorded on the y-axis as percent of the maximum response (●, A and C). Triplicate cultures of 10^4 CFSE-labeled NK cells contained soluble mAb, 200 U/ml rIL-2 and 5×10^4 γ -irradiated P815 cells. After 6 days, the CFSE profiles of the cultured cells were analyzed and the percentage of cells in division was calculated. (A) NK cell line A was stimulated with optimum concentrations of CD94 mAb and dilutions of CD158b (GL183) mAb were added to inhibit the response. The percentage of cells in division is presented as percent of the maximum response (■). (B) Cell division of NK cell line A was stimulated using CD158b (GL183) mAb at the dilutions indicated. Results are presented as the percent of cells in division. The background cell division of unstimulated NK cells in control cultures lacking mAb was 9.3%, which is the point of intersection of the x-axis with the y-axis. (C) NK cell line C was stimulated with dilutions of CD158b (GL183) mAb and the number of cells in division is recorded as percent of the maximum response (■).

inhibitory KIR prevents raft formation (20) that is presumed to be a prerequisite for triggering through activating receptors.

These studies represent the first step in understanding the response of the activating and inhibitory KIR to natural ligand. The mAb studies show the potential for the activating KIR to function in the presence of co-expressed inhibitory KIR. However, it is generally agreed that binding of HLA ligands to activating KIR is considerably weaker than binding to inhibitory KIR. Winter *et al.* (5) could not detect binding of KIR2DS2-Ig fusion protein to HLA-Cw3 transfectants, whereas binding by KIR2DL2 and KIR2DL3 fusion proteins was measurable. These studies are supported by plasmon surface resonance measurements using soluble recombinant KIR and

HLA-Cw7 ligand (17). Studies by Biassoni *et al.* (21) showed that the binding of soluble KIR2DS1 (p50.1) Ig-fusion protein to HLA-Cw4 is very weak compared to the KIR2DL1 (p58.1) Ig-fusion protein. Interestingly, a single amino acid substitution at position 70 from lysine to threonine increases the binding affinity of the activating KIR2DS1 receptor. Since this position is critical for the interaction of KIR2DS1 with the peptide-binding groove, it is suggested that binding of particular peptides may influence binding of the activating receptor (22). A dimension yet to be fully appreciated in the context of binding of activating KIR to natural ligand is the importance of raft formation and the generation of immunological synapses. Their importance has been shown dramatically in the case of T cell activation (23–26) and there is evidence that similar processes are likely to occur with NK cell receptors interacting with their cellular ligands (20,27). It remains to be determined whether in the context of an immunological synapse the activating KIR2DS2 is more readily activated by natural ligand through mechanisms appreciated for T cell activation, such as serial triggering (28), co-stimulatory thresholds (29) and avidity (29,30).

In conclusion, these studies show that the activating KIR2DS2 can function in the presence of co-expressed inhibitory KIR2DL2 and/or KIR2DL3. It will now be important to determine if these results using CD158b mAb are applicable to responses to a natural ligand such as HLA-Cw3. A possible rationale for activating and inhibitory KIR recognizing the same HLA ligand to be co-expressed on the same NK cell is that when HLA concentrations on target cells are below that capable of inhibiting NK cell function there is a fail-safe mechanism for activating NK cells if no other NK cell-activating receptors or target cell ligands to activate those receptors are present.

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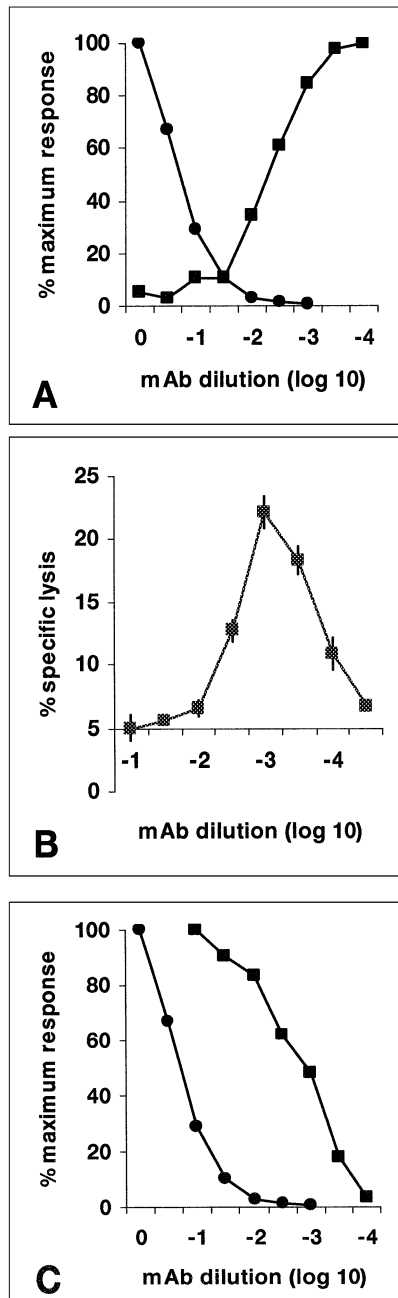


Fig. 9. NK cell cytotoxicity at different dilutions of CD158b mAb. The optimum concentration of mAb for binding was arbitrarily given the number 1.0 ($\log_{10} 1 = 0$). The mAb dilutions are at 0.5 $\log_{10}$ intervals. Binding of CD158b mAb to NK cells was determined by immunofluorescence flow cytometry in median fluorescence units and is recorded on the y-axis as percent of the maximum response (●, A and C). Triplicate cultures were incubated for 4 h with soluble mAb and ^{51}Cr -labeled P815 cells. (A) NK cells from line A were stimulated with optimum concentrations of CD94 (WV2) mAb and dilutions of CD158b (GL183) mAb added to inhibit this response (■). Data are presented as percent maximum response, calculated from cytotoxic units as described in Methods. The ratio of effector to P815 target cells was 1:1. (B) Cytotoxicity of NK cell line A was stimulated by CD158b (DX27) mAb at the dilutions indicated. Cytotoxicity is expressed as percent specific lysis, and the mean and SD of triplicate cultures is given. The background lysis of NK cells without mAb was 5%, which is the point of intersection of the x-axis with the y-axis. The ratio of effector to P815 target cells was 2:1. (C) NK cells from line C were stimulated with dilutions of CD158b (GL183) mAb and cytotoxicity calculated as cytotoxic units is presented as percent maximum response (■). The ratio of effector to P815 target cells was 2:1.

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Abbreviations

BLT	N- α -carbobenzoxyl-L-Lys-thiobenzyl ester
CFSE	5-(and -6)-carboxyfluorescein diacetate succinimidyl ester
HIFCS	heat-inactivated FCS
ITIM	immunoreceptor tyrosine-based inhibitory motif
PE	phycoerythrin
KIR	killer Ig-like receptor

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