2B4 Acts As a Non–Major Histocompatibility Complex Binding Inhibitory Receptor on Mouse Natural Killer Cells

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The Journal of Experimental Medicine

Abstract

Natural killer (NK) cells are critical in the immune response to tumor cells, virally infected cells, and bone marrow allografts. 2B4 (CD244) is expressed on all NK cells and the ligand for 2B4, CD48, is expressed on hematopoietic cells. Cross-linking 2B4 on NK cells with anti-2B4 monoclonal antibody leads to NK cell activation in vitro. Therefore, 2B4 is considered to be an activating receptor. Surprisingly, we have found, using antibody-blocking and 2B4-deficient NK cells, that NK lysis of $CD48^+$ tumor and allogeneic targets is inhibited by 2B4 ligation. Interferon γ production by NK cells is also inhibited. Using a peritoneal tumor clearance assay, it was found that $2B4^{-/-}$ mice have increased clearance of $CD48^+$ tumor cells in vivo. Retroviral transduction of 2B4 was sufficient to restore inhibition in $2B4^{-/-}$ primary NK cells. It was found that although mature NK cells express SH2D1A, in vitro–derived NK cells do not. However, both populations are inhibited by 2B4 ligation. This indicates that 2B4 inhibitory signaling occurs regardless of the presence of SH2D1A. These findings reveal a novel role for 2B4 as a non–major histocompatibility complex binding negative regulator of NK cells.

Key words: CD48 • CD150 • tumor • IFN-γ • innate immunity

Introduction

NK cells are innate lymphocytes named for their ability to lyse tumor cells. The functions of NK cells also include responding to viral infection, rejecting allogeneic bone marrow, and promoting type 1 helper T cell responses (1, 2). Activating and inhibiting surface receptors expressed on partially overlapping subsets of NK cells provide the specificity for NK cell responses. Positive signals lead to activation of cytokine production and cytotoxicity, but these signals can be negated by simultaneous engagement of inhibitory receptors. Murine lectin-like Ly49 receptors and human killer cell Ig-like receptors are two families that recognize polymorphic epitopes of MHC class I molecules (3). A third family, the CD94/NKG2 lectin-like heterodimer family, is conserved in mice and humans and recognizes nonclassical murine MHC class I $Qa\text{-}1^b$ or human HLA-E (4). The inhibitory members of these families, which are essential for protecting normal cells from NK-mediated lysis, contain intracellular immunoreceptor tyrosine-based inhibition motifs (ITIMs; I/VxYxxL/V). Activating receptors of these families lack an ITIM and are instead associated with immunoreceptor tyrosine-based activation motif (D/ExxYxxL/Ix[6–8]YxxL/I)–containing transmembrane adaptor molecules (5).

2B4 is an unusual NK receptor in that it is not regulated by MHC molecules, nor does it contain ITIM or immunoreceptor tyrosine-based activation motifs. It is a member of the CD150 (signaling lymphocyte activation molecule) subfamily of the CD2 family of receptors. The members of this subfamily are distinguished by the presence of at least

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Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; CFSE, carboxyl fluorescein succinimidyl ester; EAT-2, EWS-activated transcript 2; ITIM, immunoreceptor tyrosine-based inhibition motif; ITSM, immunoreceptor tyrosine-based switch motif; LAK, lymphokine-activated killer; XLP, X-linked lymphoproliferative disorder.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/05/1245/10 $8.00
Volume 199, Number 9, May 3, 2004 1245–1254
http://www.jem.org/cgi/doi/10.1084/jem.20031989

two cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSMs; TxYxxV/I) and include 2B4, CD84, CD229, NTB-A, and CS1 (6). Murine 2B4 has two isoforms derived from alternative splicing. The short form has one ITSM, and the long form has four (7, 8). Human 2B4 is only found as the long form (9). 2B4 is expressed by all NK cells, γδ T cells, basophils, monocytes, and a subset of $CD8^+$ αβ T cells (10–13). The ligand for 2B4, CD48, is a GPI-linked CD2 family member (14). CD48 is a low affinity ligand for CD2 and a high affinity ligand for 2B4 and is expressed on hematopoietic cells (14–16).

ITSMs bind SH2 domain–containing proteins including SH2D1A (signaling lymphocyte activation molecule–associated protein) and a similar protein, EWS-activated transcript 2 (EAT-2; 17, 18). Mutations in SH2D1A are found in patients with X-linked lymphoproliferative disorder (XLP), a progressive combined variable immunodeficiency often aggravated by EBV infection (17, 19–22). It is possible that altered signaling by ITSM-containing proteins, including 2B4, may contribute to the pathogenesis of XLP. One hypothesis is that in the absence of functional SH2D1A, 2B4 is unable to initiate an activating signal and instead recruits phosphatases, thus becoming inhibitory (23–29).

Murine 2B4 was originally identified as an activating receptor because treatment of NK cells with anti-2B4 mAb lead to increased IFN-γ production and killing of tumor cell lines in vitro (10). Human 2B4 was found to activate NK cells in a redirected lysis assay using FcR^+ target cells treated with anti-2B4 to cross-link 2B4 present on the effector cells (11). To elucidate the role of 2B4 in the regulation of murine NK cells in more physiological experiments, we have used $2B4^{-/-}$ mice and ligand positive or negative targets to investigate 2B4 function. Our data indicate that 2B4 acts as a non-MHC binding inhibitory receptor both in vitro and in vivo.

Materials and Methods

Mice. C57BL/6 (B6, WT) and BALB/c mice were purchased from NCI-Frederick. SCID mice were obtained from Jackson ImmunoResearch Laboratories. $2B4^{-/-}$ mice are described elsewhere (unpublished data). In brief, $2B4^{-/-}$ mice were generated by insertion of a pPNT construct encoding bacterial aminoglycoside phosphotransferase under a phosphoglycerate kinase promoter (PGK-neo^r) flanked by PCR-cloned 2B4 DNA. The vector replaced the extracellular region, exon 2 and part of exon 3, with the PGK-neo^r gene. Embryonic stem cells derived from C57BL/6 mice were used. 2B4-deficient mice expressed no 2B4 as determined by flow cytometry. All mice were used at 6–12 wk of age. B6 controls for $2B4^{-/-}$ mice were age matched.

Cell Lines. RMA-S (H-2^b low, thymoma cells) $CD48^+$, RMA-S $CD48^-$, and P815 (H-2^d, mastocytoma cells) were cultured in RPMI 1640 with L-glutamine, supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μM 2-ME (complete RPMI) at 37°C and 5% CO_2. The $CD48^-$ RMA-S cell line arose spontaneously from the $CD48^+$ RMA-S during culture. To generate $CD48^+$ P815 cells, CD48 cDNA was cloned into the pcDNA3.1 expression vector that was then stably transfected into $CD48^-$ P815 cells. $CD48^-$ P815 cells stably transfected with an empty vector were used as the control $CD48^-$ cell line.

Antibodies and Flow Cytometry. Anti-CD16/32 (anti-FcRγIII, 2.4G2, anti-FcR) was produced from a hybridoma. Purified and fluorescently labeled mAbs purchased from BD Biosciences are: anti-FcR, anti-2B4 (2B4), anti-CD48 (HM48-1), anti-CD2 (RM2-5), anti-DX5 (DX5), hamster IgG1λ, mouse IgG2bκ, and rat IgG1κ. Anti–IFN-γ (XMG1.2), anti-NK1.1 (PK136), and anti-CD3 (145-2C11) were purchased from eBioscience. For immunofluorescence analysis, cells were suspended in PBS with 2% FBS after blocking with anti-FcR supernatant. Cells were incubated in the indicated antibodies for 30 min. Analysis was performed on a FACSCalibur™ flow cytometer with CELLQuest™ software (BD Biosciences). Detection of intracellular IFN-γ was performed by incubation of 10^5 lymphokine-activated killer (LAK) cells with 10^5 RMA-S cells for 6 h in the presence of GolgiStop (BD Biosciences). Cells were washed, surface stained as described above, and then permeabilized with Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's directions. Cells were then stained with anti–IFN-γ–PE or isotype control.

Ex Vivo–derived Cell Preparations. NK cells were enriched by passage of splenic cell suspensions through a nylon wool column for negative depletion of adherent cells followed by magnetic depletion of $CD3^+$ cells using anti-CD3 biotin and magnetic microbeads coated with streptavidin (Miltenyi Biotec). Cells bound to microbeads were depleted using a MACS separation column according to the manufacturer's instructions (Miltenyi Biotec). LAK cells were generated by culturing the remaining cells in complete RPMI with 1,000 U/ml human rIL-2 (National Institutes of Health [NIH]). On day 3 after the onset of culture, half the media was replaced with fresh media containing IL-2. LAK cells were used on days 6–10 for experiments and were >95% $NK1.1^+$ $CD3^-$. In vitro–derived NK cells were obtained from bone marrow cell suspensions as previously described (30). Bone marrow cells were released from femurs, tibias, sternum, and pelvic bones by mortar and pestle. $Lineage^+$ cells were depleted using biotin-conjugated anti-CD2, anti-B220 (RA3-6B2), anti–Gr-1 (RB6-8C5), anti-CD11b (M1/70), anti-NK1.1, and anti-Ter119 (Ly-76; BD Biosciences) followed by streptavidin microbeads and MACS columns. $c\text{-}kit^+$ cells were positively selected by anti–ckit-FITC (BD Biosciences) followed by anti-FITC microbeads and MACS columns. Cells were cultured in 0.5 ng/ml murine IL-7, 50 ng/ml murine stem cell factor, and 20 ng/ml human Flt3L (Biosource International) for 5 d followed by 6 d in 1,000 U/ml IL-2 and complete RPMI.

In Vitro Cytotoxicity Assay. 10^6 target cells were labeled with 100 μCi of sodium chromate (^{51}Cr) for 1 h at 37°C. Target cells were washed and 2,000/well were plated in 96-well plates. Effector cells at indicated E/T ratios were added. After 4 h of incubation, supernatants were collected and percent-specific lysis was calculated using standard methods. Cells pretreated with blocking antibodies were incubated at 5 μg/ml with the indicated antibody for 15 min on ice after similar blocking with anti-FcR where indicated. Con A–stimulated targets were generated by culturing either whole splenocytes or splenocytes depleted of nylon wool adherent cells for 24 or 48 h with 3 μg/ml Con A (Sigma-Aldrich). Con A targets were whole splenocytes stimulated for 48 h unless otherwise stated. Data are the mean ± standard deviation from triplicates from one representative experiment of at least three independent experiments.

RT-PCR. Total RNA was isolated from LAK cells, in vitro–derived NK cells, and thymocytes using RNeasy Mini Kit

(QIAGEN) and was reverse transcribed. The primers used for amplification of SH2D1A cDNA (394 bp) were 5′ CTA GGC ATG GAT GCA GTG ACT GTG and 3′ ACT CTT CTT CAT GGT GCA TTC AGG. The primers used for amplification of EAT-2 cDNA (313 bp) were 5′ GAC CAA GCG AGA GTG TGA and 3′ CTC CAT TCT TCT CCC TCT TTG GCA. Amplification was performed for 35 cycles: 30 s at 94°C, 60 s at 58°C, and 2 min at 72°C. PCR products were resolved on a 1% agarose gel.

Retroviral Transduction. Retroviral transduction was performed as previously described (31). In brief, murine 2B4 short or 2B4 long cDNA was inserted in the SAMEN CMV/SRα vector under a hybrid promoter consisting of a CMV enhancer and promoter fused to Moloney murine leukemia virus 5′ LTR (32), which allows maximum expression of transduced genes. Infectious retroviral supernatants were generated by transient transfection with 10 μg of either empty vector or vector containing 2B4 long or 2B4 short cDNA into 5×10^6 Plat-E ecotropic packaging cells (33) plated on 10-cm dishes 16 h before the transfection. Transfection was performed using a standard Hepes-buffered calcium phosphate coprecipitation method. The next day, the media was removed and replaced with 3 ml fresh media. After an additional 48 h, the supernatant was collected, centrifuged at 3,000 rpm for 10 min, and filtered with a 0.45-μm filter to remove cell debris, and aliquots were stored at −80°C. NK cells cultured in IL-2 for 5 d were plated at a density of 1.5×10^6 cells/well in 24-well plates and were transduced by adding 1 ml retroviral supernatants containing 2B4 short, 2B4 long, or empty vector in the presence of 10 μg/ml polybrene (Sigma-Aldrich). Plates were spun at 1,500 rpm at 4°C for 1.5 h and then incubated at 37°C for 18 h. This process was repeated and NK cells were used on the next day. $CD48^-$ RMA-S cells were transduced with either empty vector or SAMEN vector containing murine CD48 cDNA according to the same method, and selected with neomycin.

In Vivo Cytotoxicity Assay. RMA-S cells suspended in PBS were labeled with 5 μM carboxyl fluorescein succinimidyl ester (CFSE; Molecular Probes) at 37°C for 10 min and the reaction was quenched with an equal volume of FBS. Cells were washed in PBS, and 5×10^6 $CD48^+$ or 10×10^6 $CD48^-$ RMA-S cells were injected i.p. in 500 μl PBS. Titration of tumor cells was performed and cell numbers injected reflect the number necessary to detect residual tumor cells after 3 d (unpublished data). After 3 d, the mice were killed and peritoneal cells were recovered. Tumor cells were distinguished by forward versus side scatter and CFSE labeling. Mice that failed to engraft were excluded from analysis. For NK depletion controls, mice were injected i.p. with 100 μg anti-NK1.1 2 d before tumor injection and again on the day of the injection. Depletion of NK cells was complete as no $DX5^+$ $CD3^-$ cells remained in the peritoneum on day 3 as determined by flow cytometry (unpublished data).

Results

2B4 Inhibits NK Cell Lysis of $CD48^+$ Tumor Cells In Vitro. To investigate the role of 2B4 signaling in the regulation of NK cell cytotoxicity, we identified cell lines that were either $CD48^+$ or $CD48^-$ (Fig. 1 A). $CD48^+$ RMA-S ($H\text{-}2^b$ low, FcR^-) tumor cells, or a variant that is $CD48^-$, were then tested as NK targets in killing assays. Purified WT NK cells expanded in IL-2 (LAK cells) lyse $CD48^-$ RMA-S but surprisingly, lysed $CD48^+$ targets much less efficiently (Fig. 1 B). To determine if this inhibition was due to CD48 ligation of 2B4, $CD48^+$ RMA-S cells were pretreated with anti-CD48 to block 2B4–CD48 interaction. Blocking 2B4 ligation with anti-CD48 antibody restored NK lysis of $CD48^+$ RMA-S (Fig. 1 D). Although the NK cells were pretreated with anti-FcR antibody to block FcR binding of anti-CD48 and subsequent antibody-dependent cellular cytotoxicity (ADCC), it could not be ruled out that the lysis of $CD48^+$ targets was contributed to by ADCC. To exclude this possibility, NK cells were pretreated with anti-2B4 or isotype control antibody. Anti-2B4 treatment of NK cells also increased cytotoxicity against $CD48^+$, but not $CD48^-$ RMA-S (Fig. 1 C). This confirmed that 2B4 and CD48 interaction was inhibitory.

To test if expression of CD48 could protect $CD48^-$ cells from NK lysis, $CD48^-$ RMA-S cells were transduced with

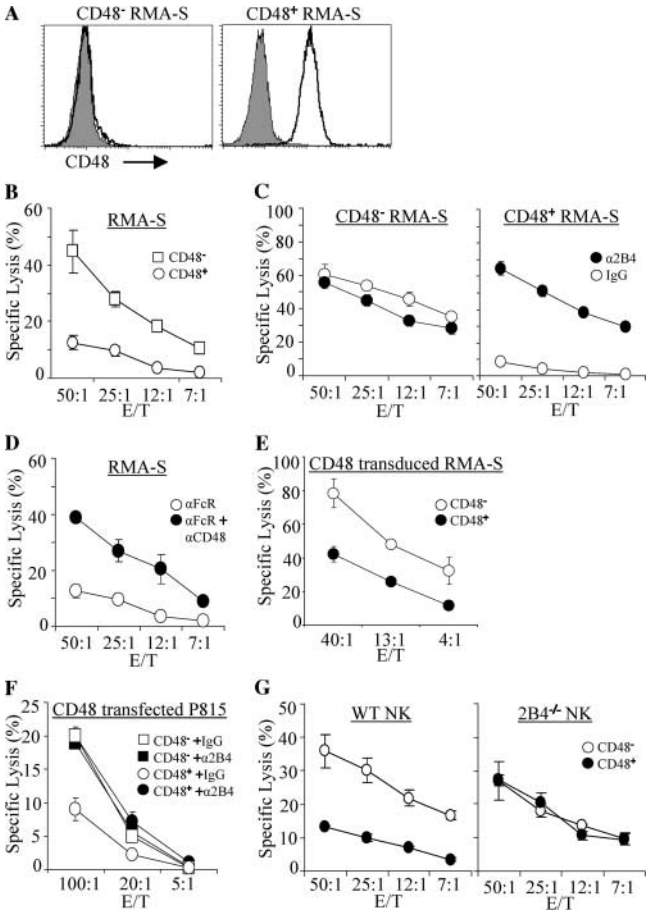

Figure 1. Ligation of 2B4 by CD48 expressed on tumor cells inhibits NK cytotoxicity. (A) $CD48^-$ and $CD48^+$ RMA-S cells were stained with anti-CD48 (open histogram) or isotype control (shaded histogram). (B) WT LAK cells were tested in a 4-h chromium release assay against $CD48^-$ or $CD48^+$ RMA-S cells at the indicated E/T ratios. (C) WT LAK cells were pretreated with anti-FcR and either anti-2B4 or isotype control and tested against $CD48^-$ or $CD48^+$ RMA-S. (D) WT LAK cells were tested against $CD48^+$ RMA-S. NK cells were pretreated with anti-FcR and targets were pretreated with or without anti-CD48. (E) $CD48^-$ RMA-S cells were transduced with CD48 or control empty vector and tested as targets of WT NK cells. (F) WT LAK cells were pretreated with anti-2B4 or isotype control antibody and tested for killing of $CD48^-$ P815 or P815 transfected with CD48. (G) WT and $2B4^{-/-}$ LAK cells were tested against $CD48^-$ and $CD48^+$ RMA-S targets.

CD48. Although vector-transduced CD48$^-$ RMA-S remained sensitive to NK cells, CD48 expression shielded RMA-S from lysis (Fig. 1 E). Another tumor cell line, P815, was also tested with similar results. CD48$^-$ P815 cells were killed by NK cells, but the lysis of CD48$^-$ P815 cells was inhibited by transfection with CD48 (Fig. 1 F). This inhibition was specific to 2B4–CD48 interaction, as it was completely relieved by anti-2B4 blocking (Fig. 1 F). Thus, CD48 engagement of 2B4 inhibits NK cells, as blocking this interaction restores NK activity, whereas introducing CD48 imposes inhibition.

To further investigate the function of 2B4 on NK cells, 2B4-deficient mice were generated. 2B4$^{-/-}$ mice were phenotypically normal as were the lymphocyte populations, including NK cell numbers and the NK receptor repertoire (unpublished data). We hypothesized that if CD48 interaction with 2B4 inhibits NK lysis, then 2B4$^{-/-}$ NK cells will not be inhibited by CD48 expressed on target cells. To test this hypothesis, IL-2–activated NK cells generated from 2B4$^{-/-}$ and WT mice were assayed for cytotoxicity against RMA-S cells. 2B4$^{-/-}$ and WT NK cells both lysed CD48$^-$ RMA-S cells but as predicted, only 2B4$^{-/-}$ NK cells lysed CD48$^+$ targets, and to an extent equivalent to that of the lysis of CD48$^-$ targets (Fig. 1 G). This confirms that 2B4 ligation on WT NK cells inhibits NK cytotoxicity because these data are not prone to any artifacts (such as ADCC or FcR triggering) that are inherent in antibody-blocking experiments.

2B4 Ligation Inhibits NK Cell Lysis of Nontransformed Targets. NK cells readily lyse allogeneic cells as allogeneic cells lack the protection of self–MHC class I alleles (34). As all lymphocytes express CD48 (16), we used Con A–stimulated allogeneic BALB/c splenocytes as targets for B6 NK effector cells to determine if nontransformed targets also inhibit NK cells via 2B4 signaling. As previously reported, B6 LAK cells lyse H-2^d Con A blasts via the Ly49D receptor (35). Such lysis of allogeneic Con A blasts was enhanced by blocking 2B4–CD48 interaction using anti-CD48–treated targets or anti-2B4–treated NK cells (Fig. 2 A). This effect was not T cell mediated, as SCID LAK cells also showed enhanced killing when CD48 is blocked (Fig. 2 B). Confirming these results, 2B4$^{-/-}$ NK cells showed enhanced killing of allogeneic Con A blasts as compared with WT NK cells (Fig. 2 C). WT and 2B4$^{-/-}$ NK lysis of whole B6 splenocytes activated for 2 d did not appear different (Fig. 2 C). A recent report suggested that Con A blasts made from B cell–depleted spleen cells activated for 1 d are more sensitive to lysis, therefore, 2B4$^{-/-}$ NK cells were retested according to this protocol (36). Under these conditions, 2B4$^{-/-}$ NK cells did have increased killing of B6 splenocytes (Fig. 2 D). These data demonstrate that 2B4 engagement by allogeneic as well as syngeneic, nontransformed cells leads to inhibition of NK cytotoxicity.

In Vitro–derived NK Cells Are Inhibited by 2B4. It has been proposed that 2B4 is inhibitory on immature human NK cells until they have matured and acquired MHC class I inhibitory receptors (24). Ly49$^-$ murine NK cells can be

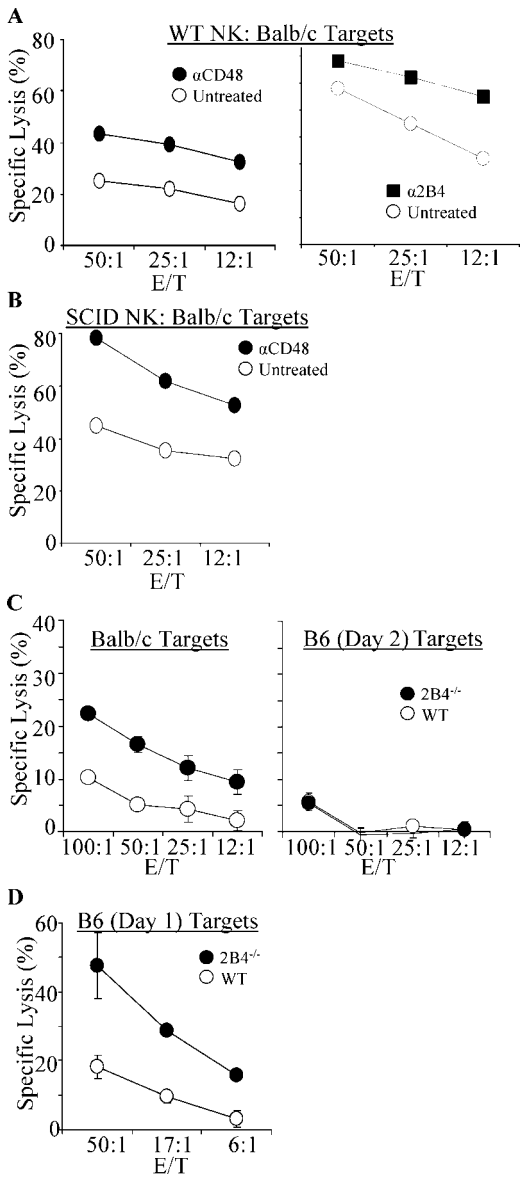

Figure 2. Ligation of 2B4 by CD48 expressed on allogeneic and syngeneic targets inhibits NK cytotoxicity. B6 LAK cells were tested in a cytotoxicity assay against Con A–stimulated splenic cells at the indicated E/T ratios. (A) On the left, WT LAK cells were tested against BALB/c targets either pretreated with anti-CD48 or not treated. On the right, WT LAK cells pretreated with anti-2B4 or not treated were tested against BALB/c targets. (B) SCID LAK cells were tested against BALB/c targets either pretreated with anti-CD48 or untreated. (C) WT and 2B4$^{-/-}$ LAK cells were assayed against BALB/c or B6 Con A blasts. (D) WT and 2B4$^{-/-}$ LAK cells were tested against B cell–depleted B6 splenocytes stimulated with Con A for 1 d only.

derived by sequential culture of lineage$^-$ ckit$^+$ multipotent bone marrow progenitors in early acting cytokines followed by IL-2 (30). To determine the role for 2B4 on these "immature" NK cells, they were assayed for lysis of CD48$^-$ and CD48$^+$ RMA-S cells. Ly49$^-$ NK cells derived from bone marrow progenitors lysed CD48$^-$ targets, but were inhibited by the expression of CD48 on the target cells (Fig. 3 A). Anti-CD48 blocking restored lysis of

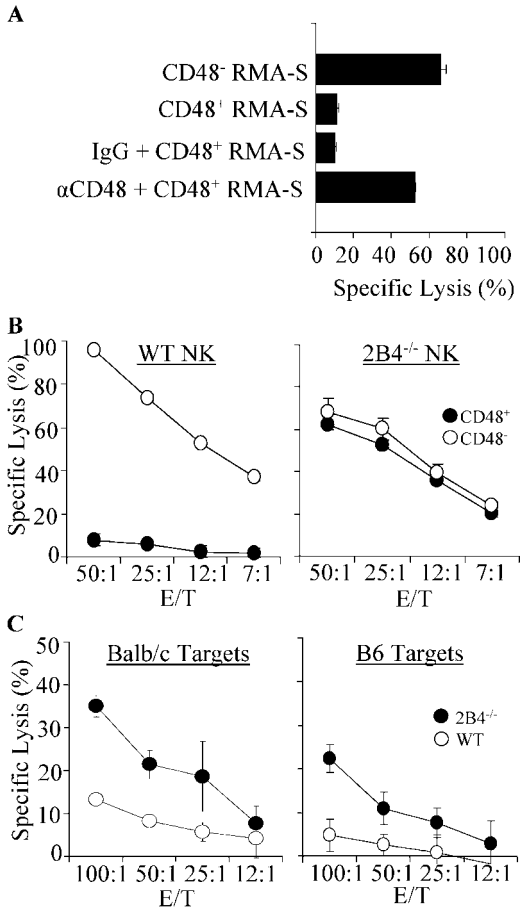

Figure 3. Ligation of 2B4 on in vitro–derived Ly49$^-$ NK cells by CD48 inhibits cytotoxicity. (A) WT in vitro–derived NK cells were tested in a 4-h chromium release assay against CD48$^-$ and CD48$^+$ RMA-S in the presence of anti-CD48, isotype control, or no antibody at a 50:1 E/T ratio. (B) WT and 2B4$^{-/-}$ in vitro–derived NK cells were tested against CD48$^-$ or CD48$^+$ RMA-S at the indicated E/T ratios. (C) WT and 2B4$^{-/-}$ in vitro–derived NK cells were tested against Con A–stimulated BALB/c or B6 splenic cells at the indicated E/T ratios.

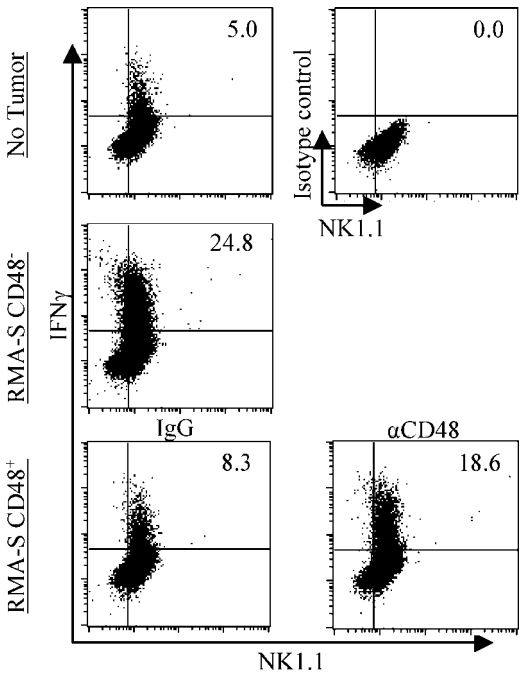

Figure 4. CD48 on target cells inhibits NK cell IFN-γ production. WT LAK cells pretreated with anti-FcR were incubated at a 1:1 ratio with CD48$^-$ or CD48$^+$ RMA-S cells for 6 h. CD48$^+$ RMA-S were pretreated with anti-CD48 or isotype control. NK cells incubated alone were used as controls. Cells were gated on the basis of forward and size scatter to exclude RMA-S and were gated on CD3$^-$ NK1.1$^+$ cells. Numbers indicate the percent of NK cells that were IFN-γ$^+$.

CD48$^+$ targets. 2B4$^{-/-}$ in vitro–derived NK cells were not inhibited by CD48 expressed on RMA-S targets (Fig. 3 B). Similarly, 2B4$^{-/-}$ in vitro–derived NK cells exhibited greater killing of allogeneic, as well as syngeneic, Con A blasts as compared with WT (Fig. 3 C). Thus, as for mature Ly49$^+$ NK cells, in vitro–derived Ly49$^-$ NK cells are also inhibited by 2B4 engagement. The finding that 2B4 is inhibitory on developing murine NK cells confirms a previous report for developing human NK cells (24). However, in contrast to this previous report, murine 2B4 is inhibitory even in mature NK cells.

CD48 Expressed on Target Cells Inhibits NK Cell Production of IFN-γ. NK cell IFN-γ production and lytic activity are not always coordinated by the same signaling pathway, a phenomenon previously ascribed to 2B4 (37, 38). Therefore, to determine if 2B4 ligation by tumor cells regulates NK cell inflammatory cytokine production, WT NK cells were coincubated with RMA-S cells and intracellular IFN-γ accumulation was measured. 24.8% of NK cells produced IFN-γ in response to CD48$^-$ RMA-S, whereas only 8.3% produced IFN-γ in response to CD48$^+$ RMA-S (Fig. 4). Blocking CD48–2B4 interaction with anti-CD48 restored NK cell IFN-γ production. These data indicate that like lytic activity, NK IFN-γ elaboration is inhibited by CD48–2B4 interaction.

2B4 Inhibits NK-mediated Clearance of CD48$^+$ Tumor Cells In Vivo. To investigate the role of 2B4 on NK cells not activated with exogenous IL-2 and in the host environment, an in vivo peritoneal tumor clearance assay was performed. 2B4$^{-/-}$ and WT mice were injected i.p. with CFSE-labeled CD48$^+$ or CD48$^-$ RMA-S cells. After 3 d, the number of CD48$^+$ RMA-S cells in peritoneal lavage fluid of WT mice was significantly higher than the number recovered in 2B4$^{-/-}$ mice (Fig. 5). WT mice did reject CD48$^-$ tumor cells, indicating that in the absence of 2B4 ligation, WT mice clear tumor cells efficiently. RMA-S cells are class I low, thus are primarily NK cell targets. However, we confirmed that clearance in vivo was NK cell mediated, using NK-depleted WT and 2B4$^{-/-}$ mice. In the absence of NK cells, 2B4$^{-/-}$ and WT mice had similar accumulation of tumor cells, demonstrating that 2B4$^{-/-}$ NK cells are required for tumor clearance. These data demonstrate that 2B4 inhibits NK cell clearance of tumor cells in vivo.

2B4 Long Inhibits Primary NK Cell Cytotoxicity. In murine NK cells, 2B4 exists as short and long isoforms generated by alternative splicing that differs only in the cytoplasmic tail (7, 8). To determine if exogenous expression of

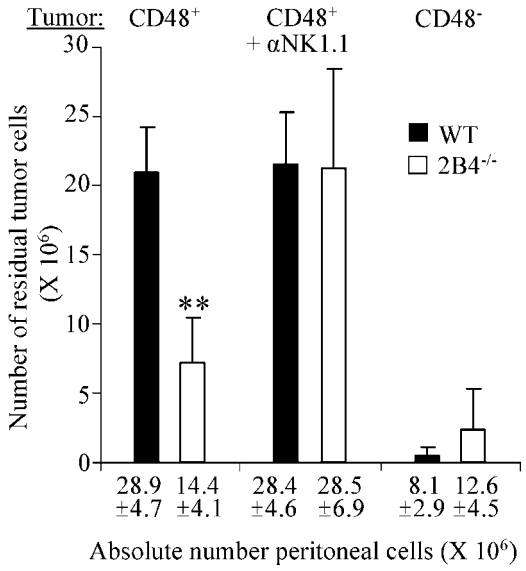

Figure 5. 2B4 engagement inhibits NK rejection of tumor cells in vivo. WT and $2B4^{-/-}$ mice were injected i.p. with CFSE-labeled tumor cells. The number of tumor cells recovered from the peritoneum after 3 d (depicted on y axis) was calculated on the basis of absolute number of peritoneal exudate cells (listed on x axis) multiplied by the percentage that were tumor cells as determined by flow cytometry. NK-depleted mice were treated with 100 μg anti-NK1.1 on days −2 and 0. Five mice per group were analyzed per experiment. Data are representative of three independent experiments. **, Student's *t* test for WT versus $2B4^{-/-}$; P = 0.0004.

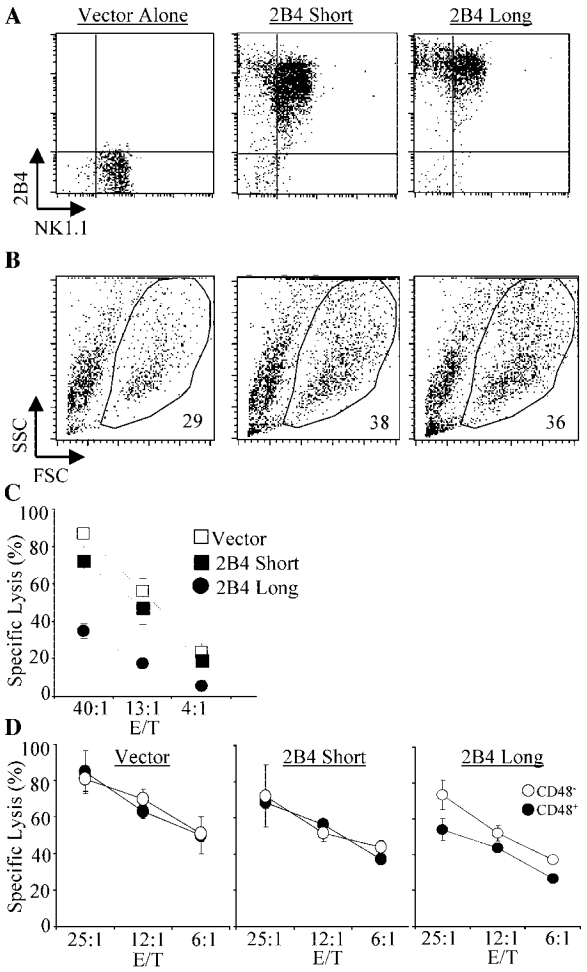

Figure 6. Retroviral transduction of 2B4 long into $2B4^{-/-}$ NK cells restores inhibition. $2B4^{-/-}$ LAK cells were retrovirally transduced with a vector expressing 2B4 short, 2B4 long, or empty vector control. (A) 24 h after transduction, cells were analyzed for 2B4 expression (B) and for live cells by forward and side scatter. Numbers indicate the percent of cells in the live gate. (C) Transduced cells were tested for killing of $CD48^+$ RMA-S. (D) In a separate experiment, transduced cells were tested for lysis of $CD48^-$ or $CD48^+$ RMA-S. Data are representative of at least three independent experiments.

these isoforms could restore inhibition in 2B4-deficient NK cells, retroviral transduction of primary $2B4^{-/-}$ NK cells was performed. Greater than 95% of LAK cells transduced with short or long expressed 2B4 on the cell surface 24 h after infection as compared with cells infected with vector alone (Fig. 6 A). Viability of transduced cells was similar to control cells as analyzed by forward versus side scatter discrimination of live cells (Fig. 6 B). When the transduced cells were tested for lysis of $CD48^+$ RMA-S cells, control vector–transduced $2B4^{-/-}$ cells lysed the targets as expected (Fig. 6 C). Transduction of 2B4 long restored inhibition of 2B4-deficient NK cells, whereas the short isoform did not. Comparison of $CD48^+$ and $CD48^-$ targets revealed that although the vector control– and 2B4 short–transduced cells had equivalent lysis of $CD48^+$ and $CD48^-$ target cells, the lysis of $CD48^+$ targets was lower than that of $CD48^-$ when NK cells were transduced with 2B4 long (Fig. 6 D). The finding that 2B4 long is inhibitory in primary NK cells is in agreement with a previous report on an NK cell line (39). However, this data does not confirm the activating role previously ascribed to 2B4 short.

SH2D1A Expression Does Not Correlate with 2B4-mediated Inhibition. In the absence of functional SH2D1A, as occurs in XLP patients, 2B4 is not activating and in some reports is in fact inhibitory (24, 26–29). Similarly, immature human NK cells lack SH2D1A and are inhibited by 2B4 ligation (24), so it could be argued that SH2D1A is necessary for 2B4-activating signals. In agreement with this hypothesis, we found that in vitro–derived, immature phenotype NK cells lacked SH2D1A as determined by RT-PCR (Fig. 7 A). However, LAK cells did express SH2D1A transcripts (Fig. 7 A) and have previously been reported to express SH2D1A protein (25). The discovery that 2B4 is inhibitory in both in vitro–derived and mature LAK cells is interesting in light of the finding that only one of these populations expresses SH2D1A. These findings are at variance with those reported previously for human NK cells inasmuch as 2B4 acts as an inhibitory receptor regardless of the expression, or lack thereof, of SH2D1A (24). EAT-2, a protein homologous to SH2D1A, was expressed at the mRNA level in both immature and mature NK cells (Fig. 7 B). Thymocytes were used as a control, as they express SH2D1A but lack EAT-2 mRNA (18). EAT-2 protein is expressed in NK cells (40) and binds to murine 2B4 (18), and therefore might be important for 2B4 inhibitory signaling.

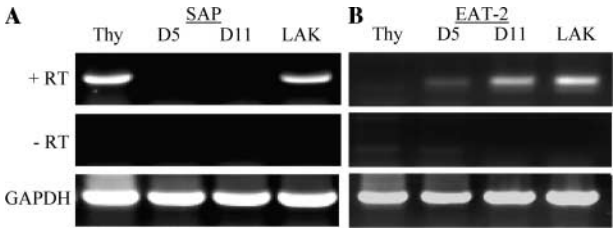

Figure 7. SH2D1A is differentially expressed in LAK and in vitro–derived NK cells. Total RNA was isolated from day 5 and day 11 in vitro–derived NK cells, LAK cells, and thymocytes as a control. RT-PCR was performed for (A) SH2D1A and (B) EAT-2 mRNA in the presence and absence of RT. Primers specific for GAPDH were used as a control.

Discussion

In this study we have investigated the regulation of NK cells by 2B4 and demonstrated that 2B4 engagement by CD48 expressed on target cells inhibits NK effector function. This was demonstrated in multiple ways: reduced lysis of $CD48^+$ targets compared with $CD48^-$ targets, reversal of this inhibition with anti-2B4 and anti-CD48 antibodies, and loss of inhibition in 2B4-deficient NK cells. The role of CD48 in delivery of an inhibitory signal to 2B4 on NK cells was confirmed by transfection of CD48 into two separate $CD48^-$ tumor cell lines. 2B4-mediated inhibition of killing was not restricted to $CD48^+$ tumor cells and also occurred in response to nontransformed allogeneic and syngeneic cells. CD48 expression on target cells also resulted in decreased inflammatory cytokine production by NK cells. 2B4 was found to inhibit both immature and mature NK cells, and these data were further confirmed in vivo by the increased clearance of tumor cells in $2B4^{-/-}$ mice.

Previous in vitro data suggested that murine 2B4 activates NK cells. The results of previous studies are not inconsistent with our findings. For example, in the original study looking at 2B4 function, the finding that anti-2B4 Fab fragments also increased lysis of target cells indicated that cross-linking of 2B4 was not necessary for activation (10). After the ligand for 2B4 was discovered, it was appreciated that the targets in that experiment were $CD48^+$. Therefore, the Fab fragments were actually blocking 2B4 engagement with the target, which means blocking inhibition and thereby permitting NK activation.

Most of the work analyzing murine 2B4 has used antibody-mediated cross-linking in vitro, such as redirected killing and plate-coated antibodies. These approaches may provide less physiological conditions for studying 2B4 function as opposed to using ligand-expressing targets in vitro or genetically modified NK cells in vivo. A similar phenomenon occurred with the receptor CD69. Cross-linking CD69 with antibody lead to NK cell activation in vitro, however, subsequent in vivo studies with CD69-deficient mice indicated that CD69 is a negative regulator of NK cells (41, 42).

Studies on human 2B4 using $CD48^+$ and $CD48^-$ cell lines found that CD48 on target cells activated primary NK cells in one study, but not in another (13, 43). Discrepancies may stem from other receptors that are coengaged by the target cell, likely altering the outcome of 2B4 ligation. This was found to be true in human NK cell clones. Only those clones that were coengaged by an activating receptor were regulated by anti-2B4 cross-linking in a redirected lysis assay (44). It is feasible, therefore, that under different conditions or with different targets, 2B4 signaling can be negative or positive. The NK receptor CD158d has also been shown to have both activating and inhibitory capabilities (45). 2B4 may derive this capability from the unique signaling versatility of ITSM motifs.

We have found that 2B4 ligation inhibits the putative NK receptor activated by several targets including RMA-S, T cell blasts, and P815 (Figs. 1 and 2). In the case of P815 and RMA-S, the activating receptor is unknown, but is likely not NKG2D (46). As P815 and BALB/c targets are $H\text{-}2^d$, then Ly49D might be activating the NK cells in both cases (35). NK lysis of activated T cells may also be regulated by NKG2D (36). The finding that 2B4 is inhibitory in the context of these various interactions argues that murine 2B4 is generally inhibitory when engaged by a target cell. It has previously been shown that the lysis of $H\text{-}2^d$ targets by B6 NK cells in vitro is mediated by $Ly49D^+$ NK cells that lack Ly49C/I/G2 expression (35). It is interesting to note, therefore, that the Ly49D-mediated lysis of $H\text{-}2^d$ Con A blasts (Fig. 2) was augmented by disrupting 2B4–CD48 interaction. This indicates that the lytic function of triggering Ly49 receptors can be regulated not only by inhibitory Ly49 receptors that bind MHC class I, but also by non-MHC–recognizing receptors such as 2B4.

Unlike Ly49 and CD94/NKG2 receptors, 2B4 does not monitor MHC expression. Several observations, particularly the presence of NK cell self-tolerance in MHC class I–deficient mice and humans, predicted that non-MHC–recognizing inhibitory receptors exist on NK cells (47, 48). This notion is further supported by the recent discovery of inhibitory forms of NKRP1 family members in mice (49). In this study, NKRP1d, like 2B4, was noted to be expressed on all splenic NK cells, and it inhibited cytolytic activity by recognition of the Clrb molecule. Unlike CD48 though, Clrb is expressed mainly on dendritic cells and macrophages.

2B4 and CD48 appear to act in parallel to the "missing self" system to safeguard against inappropriate NK aggression against hematopoietic cells. It might be that these two systems coevolved and are now functionally redundant. Neither 2B4-deficient mice nor MHC class I–deficient mice exhibit overt autoimmunity, suggesting this to be the case. On the other hand, even in the presence of self-MHC molecules, CD48 is providing additional protection from NK cells, indicating that MHC and CD48 act nonredundantly (Fig. 2 D). There may also be instances when CD48 alone is protective in the absence of MHC. Developing hematopoietic cells may go through MHC class I low stages where CD48 expression preserves them from NK cells. For example, $CD4^+$ $CD8^+$ double positive thymocytes transiently down-regulate MHC class I expression, yet CD48 is expressed early in thymic development (50, 51). Con-

versely, developing NK cells acquire lytic activity before acquisition of MHC receptors. Early expression of 2B4 may prevent NK cell lysis of neighboring hematopoietic cells before NK cell acquisition of self-tolerance (24). Indeed, 2B4 is expressed on developing NK cells before acquisition of Ly49 molecules in vitro (unpublished data). In agreement with this hypothesis, we found that Ly49$^-$ immature NK cells derived from 2B4$^{-/-}$ bone marrow cultures readily lyse syngeneic targets (Fig. 3 C). Likewise, it has also not been ruled out that 2B4 is necessary for self-tolerance of a potential population of Ly49$^-$ mature NK cells in the periphery.

The mechanism by which 2B4 inhibits is unknown. It has been proposed that 2B4 is inhibitory in the absence of SH2D1A. Yet, we have confirmed that mature NK cells express SH2D1A transcripts, whereas in vitro–derived NK cells do not (Fig. 7 A). As both of these cell types are inhibited by 2B4, this finding indicates that the presence or absence of SH2D1A does not dictate the nature of the 2B4 signal. Corroborating this result is the report that 2B4 in SH2D1A-deficient mice is not dysfunctional (52), and is capable of inhibitory signaling (unpublished data). The discrepancy between these data and previous reports might be due to differences between human and mouse 2B4 interaction with SH2D1A. EAT-2 is a similar protein, the transcripts of which were found in both immature, in vitro–derived NK cells as well as IL-2–activated NK cells (Fig. 7 B). Although it has been shown to bind 2B4, whether or not EAT-2 is necessary for 2B4 inhibitory signaling is unknown (18). 2B4 has also been shown to associate with SH2 domain–containing tyrosine phosphatases, SHP-1 and SHP-2, thus either of these molecules might be necessary for conducting 2B4 signals (23, 29, 39). We have excluded the requirement for SHP-1, as 2B4 inhibitory signaling is intact in motheaten viable mice (unpublished data). SH2 domain–containing inositol-5′ phosphatase might be involved, as it can be recruited to the 2B4 family member, CD150 (53).

In an NK cell line, RNK, exogenous expression of 2B4 long inhibited the NK cells (39). We have found that 2B4 long also inhibits primary NK cells (Fig. 6). As bulk LAK cultures express both forms (8) but are inhibited by 2B4 ligation, this suggests that 2B4 long provides the dominant signal. 2B4 short was not activating in primary NK cells, but it has been shown to provide a positive signal in T cells and RNK cells (31, 39). It is not clear what the biochemical difference is between the cell types, but the most likely explanation is the differential expression or recruitment of local SH2 domain–containing proteins to the ITSM motifs.

NK cells have been shown to interact with and lyse syngeneic activated immune cells, including T cells, macrophages, and dendritic cells, conceivably to help control or terminate an immune response (36, 54–56). Intriguingly, the expression of CD48 is up-regulated on several immune cell types in response to PMA, IFN-γ, and IFN-α/β (16, 57). Perhaps ligation of 2B4 by enhanced expression of CD48 is a means to protect activated immune cells from lysis by NK cells in the early stages of an immune response. The finding that 2B4 has an inhibitory effect on NK cells yields a reevaluation of how 2B4 and CD48 regulate NK interactions with target cells as well as other hematopoietic cells.

IL-2 was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH.

This work was supported by the NIH grant AI020451 (to V. Kumar) and the Medical Scientist Training Program GM07281 (to M.E. McNerney).

Submitted: 18 November 2003
Accepted: 29 March 2004

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