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

Kyung-Mi Lee,1 Megan E. McNerney,2 Susan E. Stepp,4 Porunelloor A. Mathew,5 John D. Schatzle,6 Michael Bennett,6 and Vinay Kumar3

1Department of Biochemistry, Korea University College of Medicine, Seoul 136-705, Korea
2Committee on Immunology and 3Department of Pathology, University of Chicago, Chicago, IL 60637
4Pathology, University of Massachusetts Medical School, Worcester, MA 01655
5Molecular Biology and Immunology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX 76107
6Department of Pathology, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390

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-1b 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[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 K.-M. Lee and M.E. McNerney contributed equally to this work.

Address correspondence to Kyung-Mi Lee, Department of Biochemistry, Korea University College of Medicine, Sungbuk-Gu, Anam-Dong, Seoul 136-705, Korea. Phone: 011-822-920-6409; Fax: 011-822-928-4853; email: kyunglee@korea.ac.kr

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.
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 (complete RPMI) at 37°C-glutamine, supplemented with 10% FBS, 0.1 mM nonessential amino acids, and 50 μM M2-ME. 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. Cells were cultured in 0.5 ng/ml interleukin-2 (IL-2; BD Biosciences) followed by streptavidin 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 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-kit+ cells were positively selected by anti-c-kit-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.

Materials and Methods

Mice. C57BL/6 (B6, WT) and BALB/c mice were purchased from NCI-Frederick. SCID mice were obtained from Jackson 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) flanked by PCR-cloned 2B4 DNA. The vector replaced the extracellular region, exon 2 and part of exon 3, with the PGK-neo 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-2b low, thymoma cells) CD48+, RMA-S CD48−, and P815 (H-2b, mastocytoma cells) were cultured in RPMI 1640 with 1-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% CO2. 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-FcRγII, 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 IgG1A, mouse IgG2b, and rat IgG1k. 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 105 lymphokine-activated killer (LAK) cells with 105 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-kit+ cells were positively selected by anti-c-kit-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. 106 target cells were labeled with 100 μCi of sodium chromate ([51Cr] 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.
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-2b 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 pre-treated 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
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 cytoxicity 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-2d 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 restested 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 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
Lee et al.1249

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 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
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.
and absence of RT. Primers specific for GAPDH were used as a control.

RT-PCR was performed for (A) SH2D1A and (B) EAT-2 mRNA in the presence of day 5 and day 11 in vitro–derived NK cells, LAK cells, and thymocytes as a control. RT-PCR 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). Discrepan-
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 immuncompetent 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 lye 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

References


