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The Murine NK Receptor 2B4 (CD244) Exhibits Inhibitory Function Independent of Signaling Lymphocytic Activation Molecule-Associated Protein Expression¹

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2B4 (CD244) is a receptor belonging to the CD2-signaling lymphocytic activation molecule family and is found on all murine NK cells and a subset of NKT and CD8⁺ T cells. Murine 2B4 is expressed as two isoforms (2B4 short and 2B4 long) that arise by alternative splicing. They differ only in their cytoplasmic domains and exhibit opposing function when expressed in the RNK-16 cell line. The ligand for 2B4, CD48, is expressed on all hemopoietic cells. Previous studies have shown that treatment of NK cells with a 2B4 mAb results in increased cytotoxicity and IFN- γ production. In this report, we used CD48^{+/-} variants of the P815 tumor cell line and 2B4 knockout mice to show that engagement of 2B4 by its counterreceptor, CD48, expressed on target cells leads to an inhibition in NK cytotoxicity. The addition of 2B4 or CD48 mAb relieves this inhibition resulting in enhanced target cell lysis. This 2B4-mediated inhibition acts independently of signaling lymphocytic activation molecule-associated protein expression. Imaging studies show that 2B4 preferentially accumulates at the interface between NK and target cells during nonlytic events also indicative of an inhibitory receptor. This predominant inhibitory function of murine 2B4 correlates with increased 2B4 long isoform level expression over 2B4 short. *The Journal of Immunology*, 2004, 173: 3953–3961.

Natural killer cells are large granular lymphocytes that mediate killing of tumors and virally infected cells (1, 2). They are also responsible for the acute rejection of MHC mismatched bone marrow transplants (3). In addition to their cytotoxic effector function, NK cells modulate the immune system via secreted cytokines, such as IFN- γ (4, 5). NK cell function is governed by a balance between inhibitory and activating receptors, where the inhibitory signal generally dominates the activating signal (6). These receptors can be divided into two families based on structure: the C-type lectin superfamily and the Ig superfamily (6, 7). The C-type lectin family, which contains type II membrane glycoproteins, includes the murine Ly49 family of receptors, and the NKG2/CD94 receptors found on human and murine NK cells (6). MHC class I molecules represent the majority of known ligands for the C-type lectin family. The Ig superfamily includes the killer cell inhibitory receptors (KIR)³ and natural cytotoxicity receptors (NCR) found on human NK cells, and the CD2/signaling lymphocytic activation molecule (SLAM) family of receptors

found on murine and human NK cells. Members of the Ig superfamily are type I membrane glycoproteins that contain two or more Ig domains (6). The known ligands for the KIRs include mostly MHC class I molecules; however, the ligands for the NCRs are still relatively undefined. The CD2/SLAM family of receptors exhibit homotypic binding or bind other family members and several members also serve as receptors for pathogens such as the case with SLAM and the measles virus (8). The CD2/SLAM family includes CD2, CD48, CD2F-10, CD58, 2B4 (CD244), Ly9 (CD229), Ly108, CD84, CS1, SLAM (CD150), and BLAME (9–19).

Murine 2B4 is expressed on subsets of $\gamma\delta$ T cells, mast cells, $\alpha\beta$ T cells, and all NK cells (13, 20, 21). In C57BL/6 mice the 2B4 gene encodes two different isoforms that arise by alternative splicing and differ only in their cytoplasmic tails (22). These isoforms have been shown to have opposing functions in transfected RNK-16 cells where the long form (2B4L) has an inhibitory function and the short form (2B4S) has an activating function (23). In contrast, the human 2B4 gene encodes a single transcript that is closely related to the long form of murine 2B4 and is expressed on NK cells, $\gamma\delta$ T cells, monocytes, and 50% of peripheral CD8⁺ T cells (24, 25). A role for human 2B4 in mediating cytotoxicity and IFN- γ secretion has been described by engagement with mAbs and the ligand for 2B4, CD48 (13, 24, 26–29). Studies have also shown that 2B4 may act as a coreceptor to the NCR Nkp46 (30). However, an inhibitory role for human 2B4 has also been described in NK cells isolated from patients with X-linked lymphoproliferative disease (XLP) (31–34). XLP is an inherited disease characterized by an inability to control EBV infection, which results in acute infectious mononucleosis or lymphoma. XLP is the result of mutations in the SAP gene; SAP associates with the immunoreceptor tyrosine-based switch motifs (ITSMs) in the cytoplasmic tail of 2B4 and other SLAM family members (35). In addition, immature human NK cells that also lack SAP expression demonstrate an inhibitory function of 2B4 (36). Thus lack of SAP

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³ Abbreviations used in this paper: KIR, killer cell inhibitory receptor; NCR, natural cytotoxicity receptor; XLP, x-linked lymphoproliferative disease; SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated protein; ITSM, immunoreceptor tyrosine-based switch motif; LAK, lymphokine-activated killer; SHP-1/2, Src homology 2 domain-containing tyrosine phosphatase 1 and 2.

correlates with inhibition of NK cytotoxicity after 2B4 engagement in human NK cells.

Our studies focus on examining the role of 2B4-CD48 interactions in murine NK cell cytotoxicity. We show that CD48 expression on target cells inhibits NK cytotoxicity in a 2B4-dependent manner. Surprisingly, unlike in human NK cells this inhibition is independent of SAP expression. This inhibitory phenotype correlates with the preferential expression of the 2B4L isoform, which has been shown to have inhibitory function in transfected cell lines.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in a conventional colony at the University of Texas Southwestern Medical Center (Dallas, TX). 2B4^{-/-} mice were generated by disruption of exon 2 and part of exon 3 with the neomycin-resistant gene. Embryonic stem cells derived from C57BL/6 mice were used for generation of the 2B4^{-/-} mice and a full description of these mice is provided elsewhere (37). SAP^{-/-} mice were generated by introducing a site mutation that truncated the SAP at the beginning of the third exon. The targeting vector was introduced into TC1 embryonic stem cells and then chimeric mice were bred to C57BL/6 mice (38). All mice used were 2–4 mo of age.

Cells and tissue culture

A P815 CD48⁻ FcR-positive variant cell line was transfected with CD48 and cultured in complete RPMI 1640 medium (10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES) supplemented with 1 mg/ml G418. The variant cell line was also transfected with empty pcDNA 3.1 vector and cultured under selection. Lymphokine-activated killer (LAK) cells were made from a single cell suspension of splenocytes devoid of RBC and cultured at 3 × 10⁶ cells/ml for 5 days in DMEM complete medium supplemented with 0.25 µM 2-ME and a total of 1000 U/ml IL-2 as described (39). A total of 300 ng/ml human rIL-15, or 1 ng/ml IL-12 and 100 ng/ml IL-18, were provided for IL-15 or IL-12/IL-18 LAK cultures, respectively.

Antibodies

Purified 2B4 mAb (mouse IgG2bκ), PE-conjugated 2B4 mAb, FITC-conjugated CD3ε (Hamster IgG), biotin-conjugated NK1.1 (mouse IgG2aκ), purified NK1.1, FITC-conjugated NK1.1, allophycocyanin-conjugated CD3ε and streptavidin-PerCp were obtained from BD Pharmingen (San Diego, CA). Undiluted cell-free 2.4G2 hybridoma supernatant and FITC-conjugated anti-rat secondary Abs were used to stain for FcR expression. CD48 and 2B4 Fab were prepared using the papain enzyme as described (40).

Cytotoxicity assays

Specific lysis of targets was determined by using a standard 4-h ⁵¹Cr release assay in 96-well flat-bottom plates as previously described (39). Redirected lysis assays using P815 CD48⁻ and P815 CD48⁺ targets were performed as previously described (23).

Generation of 2B4-GFP fusion protein and video fluorescence microscopy

The GFP was fused to the C terminus of murine 2B4 (the 2B4L isoform, as it is the major isoform present in LAK cells) with an 18 amino acid linker (AAAGGGGSGGGSGGGGS). Primers for amplification of 2B4 were GGATCCGTGGTGATGTTGGGGCAAGC for the 5' primer (start codon underlined) and CCTGCGGCCGCGGAGTAGACATCAAAGTTC (codon preceding original stop codon underlined) for the 3' primer (bold indicates added restriction sites for cloning, *Bam*HI for the 5' primer and *Not*I for the 3' primer). The PCR product for 2B4 was then cloned in frame (via the *Not*I site) with a GFP-18 amino acid linker construct obtained from the laboratory of C. Wülfing (Center for Immunology, University of Texas Southwestern Medical Center). The resulting fusion construct was confirmed by sequencing analysis. To confirm that the fusion protein retained structure of 2B4 and GFP, 293 cells were transfected with the expression construct and surface 2B4 expression and GFP fluorescence were detected by FACS analysis. The fusion protein was then expressed in wild-type IL-12/IL-18 LAK cells using a Moloney murine leukemia virus-based retroviral system as previously described (41, 42). FACS analysis of transduced and nontransduced LAKs demonstrated that the total surface expression of 2B4 was doubled by

transduction. Video fluorescence microscopy systems and procedures, including the identification of productive cell couples, have been previously described (41, 42). Briefly, 2B4-GFP accumulation was classified as follows. Average fluorescence intensity of the area of accumulation at a cellular interface was measured using Metamorph Software, Universal Imaging (Downington, PA). Strong accumulation at an interface was defined as >40% intensity over anywhere else in the cell. Partial accumulation was defined as >40% intensity of the background but <40% greater than other areas of the cell. Minimal accumulation was defined as >40% intensity of background but less than other areas of accumulation within the cell. No accumulation was defined as <40% intensity of the background. Incubation with jasplakinolide (0.5 µM pretreatment of NK cells and 0.1 µM in assay) was performed as previously described (43).

RT-PCR

2B4L and 2B4S isoforms, and actin transcripts were detected using total RNA isolated from NK cells cultured for 5 days with IL-2 and sorted for the NK1.1⁺ CD3⁻ population. RNA was converted to cDNA using SuperScript First-Strand Synthesis System for RT-PCR with *Taq* following the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA). The following actin- and isoform-specific primers were used: 2B4L (nt 1080) 5'-GTTGCCACAGCAGACTTTC; 2B4L (1250) 3'-TTCCAACCTCCTCGTACACGGTAC; 2B4S (1072) 5'-ATGTTTCAGCTCCCTTCTAG; 2B4S (1200) 3'-GAGTTCCTCCTGGCAGATC; actin 5'-GAGGC CCAGAGCAAGAGAG; actin 3'-GTCATCTTTTCACGGTTGG. Real-time PCR was then performed on the PerkinElmer GeneAmp 5700 sequence detection system using their SYBR green PCR assay. Standard curves for all three primer pairs showed similar slopes indicative of equivalent annealing efficiencies. In addition, cDNAs of each 2B4 isoform cloned into expression vectors were amplified with equal efficiency by their respective primers.

Immunoprecipitation and Western blot analysis

Approximately 5 × 10⁷ cells (RNK-16/2B4L, RNK-16/2B4S, or IL-2-generated LAKs) were surface labeled with biotin using the EZ-link sulfon-NHS-biotin reagent from Pierce (Rockford, IL). Cells were lysed and 2B4

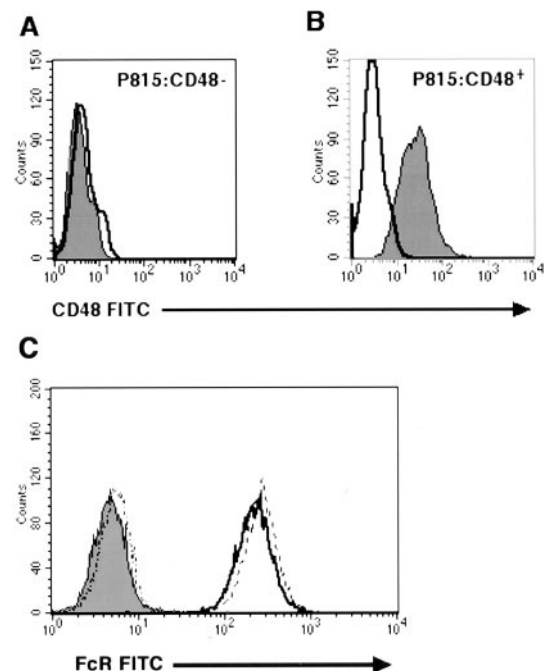


FIGURE 1. Isolation and characterization of CD48^{+/-} variants of the P815 cell line. *A* and *B*, The CD48⁻ variant of P815 cells transfected with empty vector (P815) or P815 CD48⁺ transfectants were stained with FITC-conjugated anti-CD48 mAb (shaded histogram) or FITC-conjugated isotype-matched control Ab (open histogram) and analyzed by flow cytometry. *C*, Empty vector P815 cells or P815 CD48⁺ transfectants were stained with anti-FcR (open solid line histogram and open dashed line histogram, respectively) and anti-rat FITC-conjugated secondary Ab or anti-rat FITC-conjugated Ab alone (shaded and open dotted line histograms, respectively) and analyzed by flow cytometry.

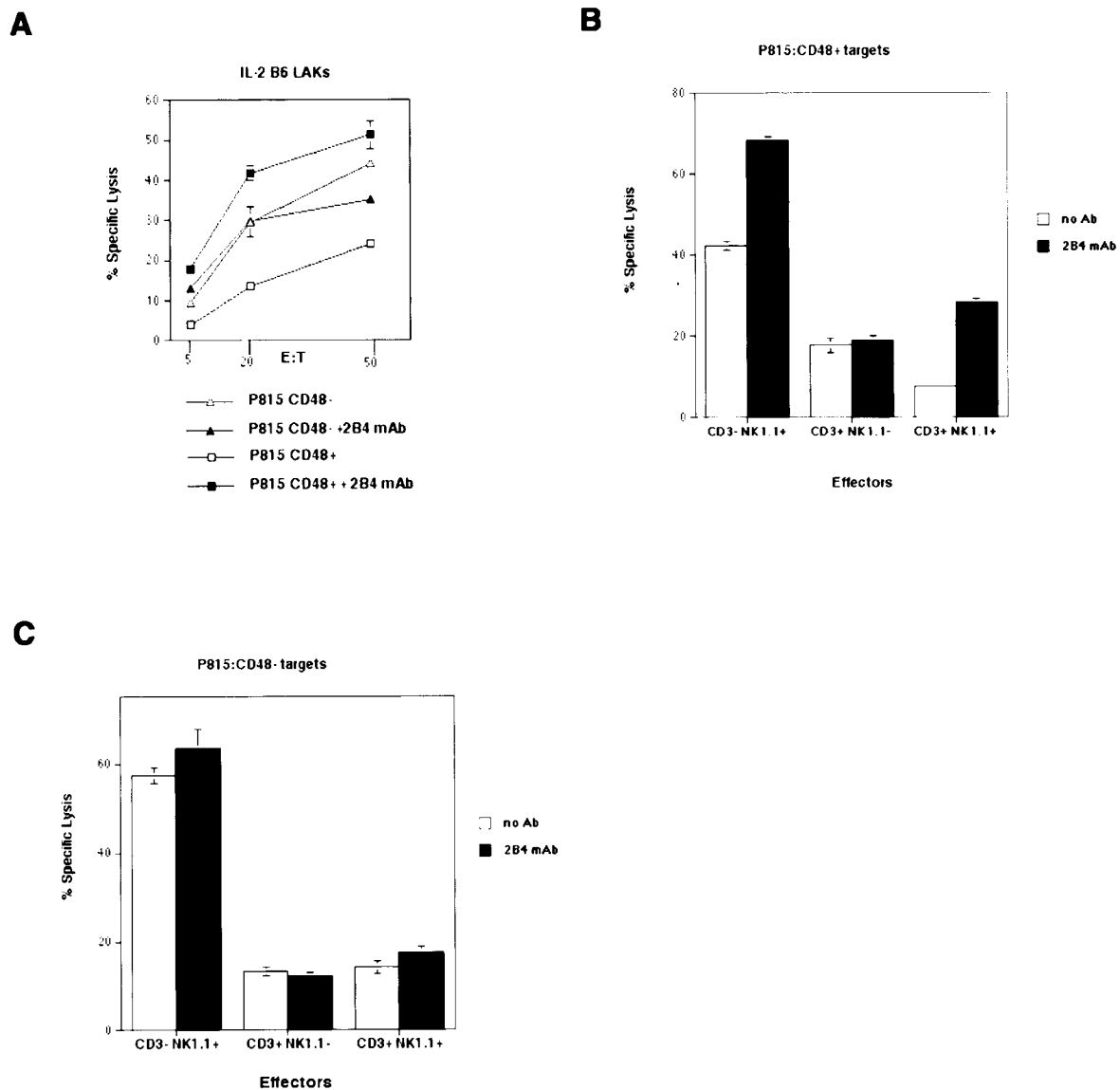


FIGURE 2. NK cytotoxicity is inhibited by CD48 expression on target cells. IL-2-stimulated C57BL/6 LAK cells were used as effectors in standard 4-h ^{51}Cr release assays with CD48⁻ and CD48⁺ P815 (FcR-positive) target cells at the indicated E:T ratios. Results are representative of three independent experiments. *A*, Anti-2B4 mAb was added at 10 $\mu\text{g}/\text{ml}$ where indicated. *B* and *C*, Sorted IL-2 LAK subsets were used as effectors in a Cr release assay against P815 CD48⁺ and P815 CD48⁻ targets at a 25:1 E:T ratio. A total of 10 $\mu\text{g}/\text{ml}$ anti-2B4 mAb was added to effectors where indicated.

was immunoprecipitated as previously described (44). Immunoprecipitates were treated with 1 U of *N*-glycosidase F from Roche Laboratories (Indianapolis, IN) overnight at 37°C according to manufacturer's instructions to remove *N*-linked carbohydrates. Samples were then resolved by 10% non-reducing SDS-PAGE analysis and subjected to Western blot analysis using streptavidin-HRP, obtained from Pierce, to detect biotinylated, immunoprecipitated proteins by ECL.

Results

The 2B4-CD48 interaction inhibits target cell lysis

The studies defining a role for murine 2B4 as an NK activating receptor have relied upon engagement of the receptor by mAb (13). To study 2B4 function due to engagement by its ligand, CD48, we took advantage of a naturally occurring CD48-negative variant of the FcR-positive P815 tumor cell line. This variant was isolated by flow cytometric screening for CD48 expression on P815 cells. This variant was confirmed to be CD48-negative by flow cytometry (Fig. 1, *A* and *B*), Western blot analysis, and RT-PCR (data not shown). To establish cohort cell lines that differ

only in CD48 expression (both cell lines express similar levels of FcR, Fig. 1C), this variant was transfected with a CD48 expression construct. Multiple clonal isolates exhibiting CD48 expression profiles similar to the parental CD48-positive cell line were used in Cr release assays and showed the same susceptibility to lysis as the parental cell line (data not shown). As a control, an empty expression vector was also transfected into the CD48-negative variant and put under selection. The CD48-transfected cell line was used in all the following studies and is denoted P815 CD48⁺, whereas the empty vector transfected cell line is denoted P815 CD48⁻. These transfectants were then used in Cr release assays to determine the effect of CD48 expression on target cell lysis. If 2B4 were acting as an activating receptor as described in the human system, then CD48-positive targets should be more susceptible to lysis. However, as shown in Fig. 2A, P815 CD48⁺ targets were less susceptible to lysis than their CD48⁻ cohorts (this was true of the original CD48^{+/-} variants isolated as well as the CD48^{+/-} transfected cells). To determine whether the differential lysis of these

two targets was due to 2B4-CD48 interaction, we added anti-2B4 mAb to the Cr release assays. As shown in Fig. 2A, the addition of anti-2B4 mAb only increased lysis of the P815 CD48⁺ targets. If 2B4 were mediating redirected lysis, both targets should have shown increased killing in the presence of the anti-2B4 mAb because their FcR levels were similar.

Similar results were obtained with LAKs prepared from C57BL/6 SCID mice (90% NK cells), IL-12/IL-18 LAKs (90% NK cells), and IL-15 LAKs (60% NK cells) (data not shown). However, this effect is not restricted to NK cells alone. IL-2 LAKs are composed of three 2B4-positive populations NK1.1⁺ T cells (40%), NK cells (15%), and NK1.1⁻ CD3⁺ T cells (35%) (data not shown). These populations were sorted and used as effectors in Cr release assays. Only the NK1.1⁺ T cells and NK cells showed decreased baseline lysis of the P815 CD48⁺ targets that was abrogated in the presence of 2B4 mAb (Fig. 2, B and C). Decreased lysis of CD48⁺ targets is not restricted to P815 cell lines as similar results were observed with CD48^{high/low}-expressing variants of the FcR-negative tumor line, RMA/S (Fig. 3, A and C vs B and D). Addition of anti-2B4 mAb only increased the lysis of the CD48^{high}-expressing variant.

There are two possible explanations for the results we observed: 1) the 2B4-CD48 interaction actually inhibits target cell lysis, or 2) the two targets are inherently different in their ability to be killed by NK cells. Each of these is discussed below.

Blocking the 2B4-CD48 interaction relieves inhibition of NK cytotoxicity

As further confirmation that the anti-2B4 mAb blocked the 2B4-CD48 interaction and relieved inhibition of target cell lysis, Cr release assays were done in the presence of anti-2B4 or anti-CD48 Fab that lack the ability to induce redirected lysis. Thus, any difference in cytotoxicity observed would be due to an interruption of the 2B4-CD48 interaction. It has been shown that the anti-CD48 and anti-2B4 mAbs either completely or partially block the 2B4-CD48 interaction (Ref. 26 and C. Wülfing and J. D. Schatzle, unpublished observations). As shown in Fig. 3, A and C, the addition of anti-2B4 or anti-CD48 Fabs increased lysis of only the P815 CD48⁺ targets. Furthermore, lysis of CD48^{high}-expressing, FcR-negative RMA/S cells was increased in the presence of anti-2B4 or anti-CD48 Fabs, whereas CD48^{low}-expressing RMA/S cells showed no difference (Fig. 3, B and D). These data support an inhibitory role for the 2B4-CD48 interaction.

To confirm further that 2B4 is solely responsible for the decreased lysis of CD48-positive targets, that there are no inherent differences between these two P815 target cells, and that anti-CD48 Ab-directed cell cytotoxicity is not taking place, 2B4 knockout IL-2 LAK effector cells were generated and used in Cr release assays against both P815 tumor targets (Fig. 4, A and B). The 2B4 knockout mice have normal NK cell numbers and receptor

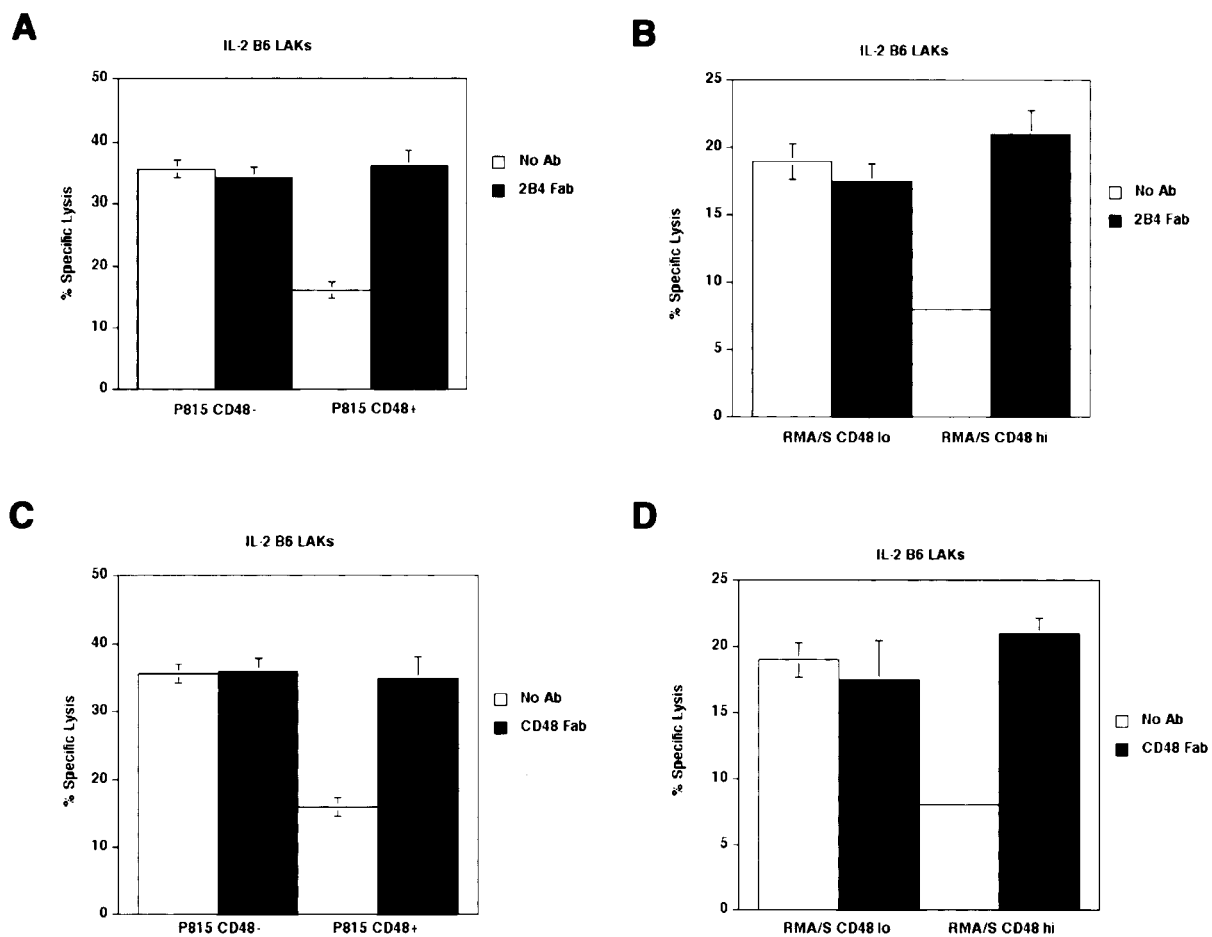


FIGURE 3. Blocking the 2B4/CD48 interaction results in enhanced target cell lysis. IL-2-stimulated C57BL/6 LAK cells were used in a standard 4-h ⁵¹Cr release assay as described for Fig. 1. Results are representative of three independent experiments. A, P815 CD48⁻ and P815 CD48⁺ target cells at an E:T ratio of 20:1 with the addition of anti-2B4 Fab at 10 μg/ml. B, RMA/S CD48^{low} and RMA/S CD48^{high} target cells at an E:T of 100:1 with the addition of anti-2B4 Fab at 10 μg/ml. C, P815 CD48⁻ and P815 CD48⁺ target cells at an E:T of 20:1 with the addition of anti-CD48 Fab at 10 μg/ml. D, RMA/S CD48^{low} and RMA/S CD48^{high} target cells at an E:T of 100:1 with the addition of anti-CD48 Fab at 10 μg/ml.

repertoires as described in detail by Lee et al. (37) and V. Kumar (unpublished observation). Unlike what we observed for wild-type LAKs (Fig. 2A), CD48 expression on target cells did not affect baseline lysis by 2B4 knockout IL-2 LAKs. The addition of anti-CD48 Fab also had no effect on lysis. The addition of anti-NK1.1 mAb resulted in both P815 targets being killed equally well indicating their equivalent susceptibility to lysis by engagement of a known activating receptor (Fig. 4, A and B). Taken together, these results confirm that the 2B4-CD48 interaction inhibits lysis of CD48-expressing target cells and that addition of blocking reagents that disrupt this interaction restores the susceptibility of these targets to NK lysis.

2B4 preferentially accumulates between NK and target cell interfaces of nonlytic interactions

Taking advantage of a retroviral transduction approach coupled with live cell imaging that we have recently described, we were able to define the pattern of 2B4 accumulation at interfaces between NK and target cells (41, 42). This system affords us the opportunity to observe accumulation of fluorescently tagged proteins and assign accumulation patterns to both lytic and nonlytic events. Based on observations with fixed cell couples examining MHC inhibitory receptors, one would predict that as an inhibitory receptor 2B4 accumulation would preferentially occur at those

NK-target cell interactions that represent nonlytic cell couples (45). To visualize the pattern of 2B4 accumulation in NK cell-target cell interactions, we fused GFP to the C terminus of the murine 2B4 receptor and this fusion construct was expressed by retroviral transduction of primary IL-12/IL-18 cultured NK cells. The interaction between transduced NK cells (expressing 2B4-GFP) and P815 CD48⁺ targets (labeled with the vital dye, SNARF-1) was then observed using video fluorescence microscopy as previously described (41). Using this approach, we observed 44 total productive cell couples of which 32 constituted nonlytic interactions and only 12 led to target cell lysis. Using a scoring system as defined in *Materials and Methods*, we determined that 56% of nonlytic events exhibited strong accumulation of 2B4-GFP at the NK-target cell interface compared with only 8% of lytic events (Fig. 5). In addition, 75% of lytic events showed no accumulation of 2B4-GFP compared with only 21% of nonlytic events. Addition of anti-CD48 or anti-2B4 Fab prevented the accumulation of 2B4-GFP at target cell interfaces further indicating that these are blocking reagents (data not shown). These data indicate that accumulation of 2B4 at target cell interfaces inversely correlates with the ability of NK cells to lyse that target, a feature that would be expected of an inhibitory receptor.

A trivial explanation for these observations may be that 2B4 accumulation at nonlytic interfaces is merely a passive diffusion process that occurs due to the prolonged interaction of nonlytic couples. To address this, NK cells were treated with a concentration of the inhibitor of actin depolymerization, jasplakinolide, which slows but does not completely block actin dynamics. We have previously determined that this treatment inhibits NK lysis but still allows for effective cell couple formation (41). We observed that jasplakinolide treatment inhibited 2B4-GFP accumulation at nonlytic interfaces (Fig. 5). This shows that 2B4 accumulation at the nonlytic interfaces is an active process and not due to passive diffusion. The fact that strong 2B4 accumulation only occurs during nonlytic NK-target cell interactions, and 2B4 accumulation is minimal or absent during lytic NK-target cell interactions is consistent with an inhibitory role of 2B4-CD48 engagement.

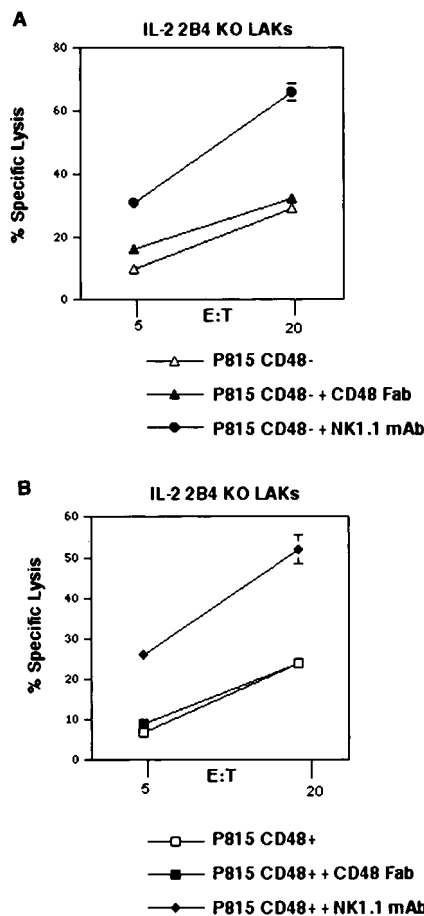


FIGURE 4. 2B4 is required for the decreased lysis of CD48-expressing target cells. IL-2-stimulated 2B4 knockout LAK cells were used in a Cr release assay as described for Fig. 1. Results are representative of three independent experiments. A, P815 CD48⁻ targets with anti-CD48 Fab or anti-NK1.1 mAb at 10 μg/ml included where indicated. B, P815 CD48⁺ targets with anti-CD48 Fab or anti-NK1.1 mAb at 10 μg/ml included where indicated.

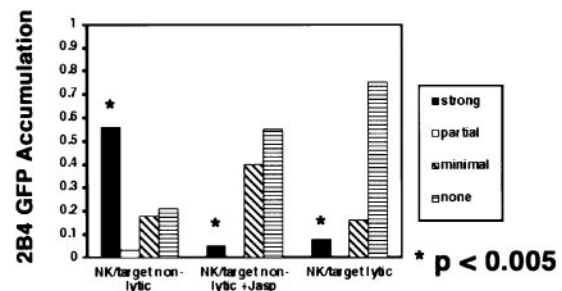


FIGURE 5. 2B4 preferentially accumulates at the interface of NK/Target cell nonlytic interactions. A 2B4-GFP construct was transduced into primary NK cells and GFP⁺ cells were sorted by flow cytometry and mixed with P815 CD48⁺ target cells. 2B4-GFP accumulation was classified as follows. Strong accumulation at an interface was defined as >40% intensity over anywhere else in the cell. Partial accumulation was defined as >40% intensity of the background but <40% greater than other areas of the cell. Minimal accumulation was defined as >40% intensity of background but less than other areas of accumulation within the cell. No accumulation is <40% intensity of background. Where indicated, NK cells were pretreated for 30 min with 0.5 μm jasplakinolide and 0.1 μm jasplakinolide was included for the duration of the assay. *, *p* < 0.005 statistically significant difference between strong accumulation of nonlytic and lytic target cell interactions.

The 2B4-CD48 interaction inhibits target cell lysis independent of SAP expression

Human and murine 2B4 have been shown to associate with SAP through their cytoplasmic ITSMs (31, 34, 46). In XLP patients who lack functional SAP, 2B4 is unable to promote cytotoxicity of NK cells when engaged by either the natural ligand for 2B4, CD48, or by Abs to 2B4 (31–34). The LAK cultures used in our studies all express SAP as determined by RT-PCR and Western blot analysis (data not shown). To assess whether SAP levels can modulate the cytotoxic function of 2B4 in murine NK cells, IL-2 LAKs were generated from SAP knockout mice and used in Cr release assays with P815 CD48⁻ and P815 CD48⁺ targets in the presence and absence of anti-2B4 mAb (Fig. 6). SAP knockout NK cells have no gross NK defects and no change in NK function (P. Schwartzberg, unpublished observation). To assess whether SAP knockout IL-2 LAKs have aberrant 2B4 expression, flow cytometry staining for NK1.1, 2B4, and CD3 expression after culture for 5 days was done. The NK1.1⁺ and NK1.1⁺ T cell populations, which composes 17–40% of LAK cultures, were all positive for 2B4 with expression levels similar to that of wild-type LAKs (data not shown). Fig. 6 shows that SAP knockout LAKs behave like wild-type LAKs in that a decrease in lysis is observed for only P815 CD48⁺ targets, which is abrogated by the addition of anti-2B4 mAb. The addition of anti-NK1.1 mAb showed increased killing of both CD48⁻ and CD48⁺ targets similar to wild-type (data not shown). These results suggest that blocking the 2B4-CD48 interaction with anti-2B4 mAb relieves NK cytotoxicity, and unlike for human NK cells, 2B4 inhibition is independent of SAP expression.

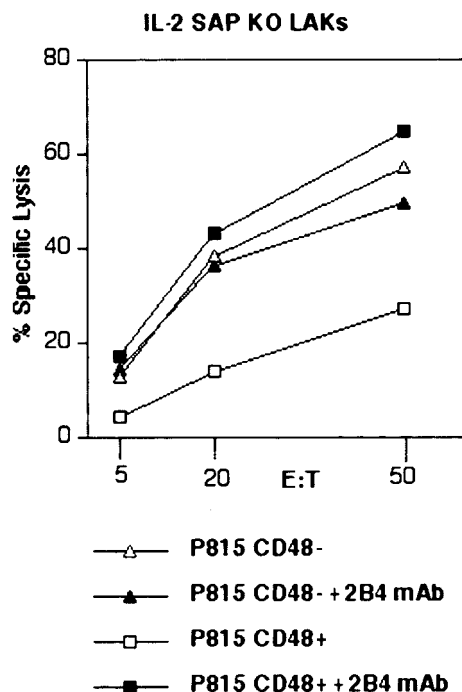


FIGURE 6. 2B4-mediated inhibition of NK cytotoxicity is independent of SAP expression. IL-2-stimulated SAP knockout (KO) LAK cells were used as effectors in a standard 4-h ⁵¹Cr release assay with CD48⁻ and CD48⁺ P815 (FcR-positive) target cells at the indicated E:T ratios. Results are representative of two independent experiments. Anti-2B4 mAb was added at 10 μg/ml where indicated.

Increased 2B4L over 2B4S isoform expression correlates with inhibition of cytotoxicity

The C57BL/6 gene of 2B4 is known to encode two isoforms (22), a long isoform, which contains four ITSMs, and a short isoform that contains one ITSM. These isoforms have been shown to have opposing functions, the long inhibitory and the short activating when expressed in RNK-16 cells (23). Northern blot analysis using 3'- and 5'-specific probes for hybridization showed multiple 2B4 transcripts in C57BL/6 LAKs (21). The most prominent transcript corresponds to the 2B4L isoform (21). Thus, skewed isoform expression favoring 2B4L could explain the observation of inhibited cytotoxicity when 2B4 is engaged by CD48 on target cells. To determine the relative levels of 2B4 isoform expression in C57BL/6 IL-2 LAK cultures, RNA was isolated from sorted NK

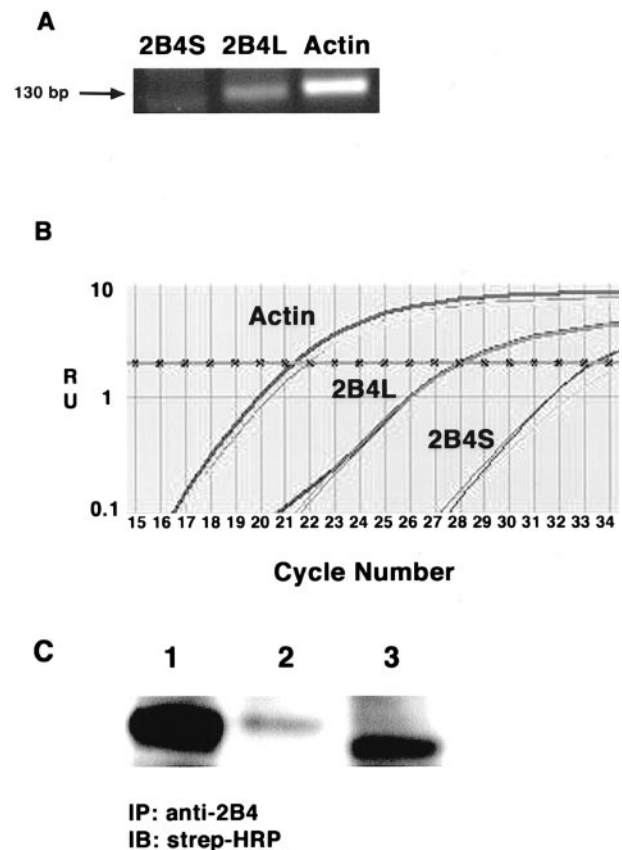


FIGURE 7. Relative expression of 2B4L and 2B4S isoforms in IL-2 C57BL/6 LAKs. RNA was isolated from sorted NK1.1⁺ CD3⁻ IL-2 C57BL/6 LAKs and used to synthesize cDNA. **A**, Standard PCR was run at 30 cycles with isoform-specific and actin primers where indicated. 2B4S migrates at 170 bp; 2B4L migrates at 128 bp. **B**, An amplification plot representative of three real-time PCR experiments with isoform-specific primers or actin primers is shown. All samples were run in triplicate. **C**, Approximately 5 × 10⁷ cells RNK-16/2B4L (lane 1), IL-2 murine LAKs (lane 2), or RNK-16/2B4S (lane 3) were incubated with sulfo-NHS-biotin to biotinylate surface proteins, and cell lysates were subjected to immunoprecipitation with anti-2B4 mAb or isotype control Ab. The resulting immunoprecipitates were treated with *N*-glycosidase F and resolved by 10% SDS-PAGE followed by Western blotting with streptavidin-HRP. This resulted in the detection of biotinylated proteins of the correct size 43 kDa for the 2B4L isoform and 37 kDa for the 2B4S isoform. The predominant species of biotinylated surface protein immunoprecipitated from LAK cultures comigrates with the 2B4L isoform. Immunoprecipitation with isotype control Ab failed to generate any detectable bands following blotting with streptavidin-HRP.

cells and converted to cDNA, which was used in PCR and real-time PCR analysis. 2B4 isoform and actin-specific primers showed an amplification of 2B4L and actin transcripts whereas 2B4S transcripts are undetectable at 30 cycles (Fig. 7A). A representative amplification plot of real-time PCR analysis confirms this observation as shown in Fig. 7B. Transcripts of 2B4L are amplified in earlier cycles compared with 2B4S indicating increased amounts of 2B4L over 2B4S. In fact, 2B4S transcripts only become detectable after 2B4L amplification is out of the range of linear amplification. Differences in primer efficiency do not account for this skewed isoform expression because both primer pairs amplified 2B4 isoform cDNA in expression vectors equally well and have similar standard curve slopes. Both freshly isolated and cultured NK cells showed increased 2B4L levels over 2B4S (data not shown).

To confirm that these differences in mRNA expression levels were also reflected by differential protein expression, surface proteins were biotinylated and cell lysates were immunoprecipitated with anti-2B4 mAb. Lysates were prepared from C57BL/6 IL-2 cultured LAK cells and lysates from RNK-16 cells transfected with each 2B4 isoform served as controls. To control for differential glycosylation patterns between RNK-16 cell lines and primary, murine NK cells, the immunoprecipitates were subjected to treatment with *N*-glycosidase F to remove *N*-linked carbohydrates and resolved by nonreducing SDS-PAGE analysis followed by Western blotting with streptavidin-HRP. As seen in Fig. 7C, immunoprecipitation and blotting of lysates from RNK-16/2B4L and RNK-16/2B4S detected biotinylated proteins of the predicted size (43 and 37 kD, respectively). The predominant surface protein immunoprecipitated by the anti-2B4 mAb from LAK cells comigrated with the deglycosylated 2B4L isoform from RNK-16/2B4L. Immunoprecipitation with isotype control Abs failed to result in any detectable biotinylated proteins (data not shown). Therefore, it appears that the major isoform expressed on the surface of these C57BL/6 IL-2 LAKs is the 2B4L isoform consistent with the RT-PCR data previously described. The preferential expression of this isoform may account for the inhibitory role of CD48–2B4 engagement in LAK cells. Recent studies also support the predominant role of the 2B4L isoform in inhibition of murine NK cell function (37).

Discussion

These studies show that 2B4 engagement, by its counterreceptor CD48, inhibits NK cytotoxicity independent of SAP expression. The original work describing murine 2B4 function suggested that 2B4 was an activating receptor found on NK cells; however, in light of our studies the original work may need to be reinterpreted (13, 44, 47). At the time of this characterization, the ligand for 2B4, CD48, was not known. The original Cr release assays were done with CD48 expressing target cells making it difficult to determine whether the anti-2B4 mAb was engaging the 2B4 receptor or blocking the 2B4–CD48 interaction leading to increased target cell lysis. Interestingly, many of these targets were FcR negative yet addition of anti-2B4 mAb resulted in their increased lysis consistent with a blocking role of the anti-2B4 mAb. Subsequent studies failed to exhibit direct signaling either biochemically or through calcium flux by anti-2B4 mAb engagement arguing against a cross-linking role for this Ab (J. D. Schatzle, unpublished observation and B. A. Garmi-Wagner, unpublished observation). Similarly, comparing human and murine 2B4 studies is complicated by the variability between human NK clones, Abs used, and target cell lines (24, 29, 31–34).

To address some of these concerns, our studies use a defined system with CD48^{+/-} targets derived from the same parental cell line as well as 2B4 knockout and SAP knockout mice to determine the role of 2B4–CD48 engagement on NK cytotoxicity. We show that this engagement inhibits NK cytotoxicity independent of SAP expression and this inhibition can be blocked by the addition of anti-2B4 or anti-CD48 Abs. During the completion of these studies a similar conclusion regarding the inhibitory role of 2B4 in NK function was published. In this study, 2B4 engagement also inhibited cytokine production (IFN- γ) as well as cytotoxicity (37). There is precedence for an inhibitory role of 2B4 in human NK cells as well. Expression of 2B4 in human NK cells before the acquisition of KIR and NCR receptors prevents killing of normal autologous cells (36). Additionally, NK cells from XLP patients exhibit an inhibitory phenotype where blocking the 2B4–CD48 interaction between NK and target cells increases cytotoxicity (34). A common theme in both these systems is the absence of SAP expression correlating with the inhibitory function of 2B4. Previously, studies of the 2B4 signaling pathway have shown that SAP binds the ITSMs in the cytoplasmic domain of 2B4. It has been hypothesized that SAP and Src homology 2 domain-containing tyrosine phosphatases (SHP-1/SHP-2) compete for mutually exclusive binding to these ITSMs, which dictates 2B4 function; where binding of SAP leads to an activating signal possibly through Fyn recruitment, and binding of SHP-1/SHP-2 leads to an inhibitory function (35, 48–50). Surprisingly, the cytotoxic inhibition by 2B4 engagement observed in the murine system works independently of SAP expression (cf Figs. 2A and 6).

Although we have characterized 2B4 inhibition of NK cytotoxicity, the mechanism by which 2B4 inhibits cytotoxicity still remains largely undefined. If SAP levels do not dictate 2B4 function in the murine system, how might 2B4 inhibition be explained? Studies with RNK-16, a rat NK cell line, have shown that overexpression of the 2B4L isoform inhibits cytotoxicity despite SAP being expressed in these cells and coimmunoprecipitating with 2B4 (46). When looking at isoform expression via RT-PCR and Western blot analysis in C57BL/6 IL-2 LAKs, the 2B4L isoform is the major expressed version of this receptor and this could account for the predominant inhibitory function of 2B4 (Fig. 7). In support of this hypothesis, a recent study has shown that reconstitution of 2B4L but not 2B4S expression in 2B4 knockout NK cells is able to restore the inhibitory phenotype (37). Interestingly, these studies revealed some differences in the function of each isoform of 2B4 when exogenously expressed in murine NK cells compared with results obtained from RNK-16 transfectants. Whereas the inhibitory function of 2B4L was observed in both cell types, the activating role of 2B4S was only observed in RNK-16 transfectants. We do not know the reason for this discrepancy but murine 2B4 function may be due to differential recruitment of downstream signaling molecules to the cytoplasmic ITSMs of these 2B4 isoforms. Alternatively, the signaling milieu inherent to each cell type may dictate receptor function. This could explain differential receptor function in human and murine NK cells despite the fact that both human and murine 2B4 proteins are similar structurally in their cytoplasmic domains. Future studies should be aimed at defining the mechanisms to account for this difference and determine whether this is due to structural differences between the murine and human 2B4 receptors or differences in the signal transduction pathways predominant in murine vs human NK cells.

The ability of 2B4 to inhibit NK cytotoxicity may have important biological consequences by preventing lysis of hemopoietic cells in the absence of MHC inhibitory receptors. As mentioned, studies of human NK development have shown that 2B4 is expressed before the NCRs and MHC-restricted inhibitory receptors

(36). Interestingly, NK cells acquired lytic activity with NCR expression, which occurs before inhibitory receptors are expressed. Blocking the 2B4-CD48 interaction between these NK cells and autologous myelomonocytic precursors led to autologous cell lysis (36). Thus, 2B4 expression may be responsible for preventing lysis of normal autologous cells by immature but lytic NK cells. Similarly, using an in vitro murine NK cell differentiation system we observed that 2B4 is expressed before the NKp46 murine homologue Mar-1, a NCR, as determined by RT-PCR; (J. D. Schatzle, unpublished observations). As both of these receptors are expressed before Ly49 inhibitory receptors, this may be a mechanism to prevent NK cell autoreactivity. In addition, a recent study in the murine system has shown that the 2B4-CD48 interaction among NK cells is critical for the generation of maximal NK effector function (K. M. Lee and V. Kumar, manuscript in preparation). In this context, 2B4 serves as a ligand for CD48 on neighboring cells, which is required for the generation of fully lytic NK cells. Thus, it is beneficial for 2B4 to have an inhibitory function so that it is able to serve as a ligand for CD48 on adjacent NK cells, but prevent fratricide. 2B4 may have multiple functions that are regulated by the context of 2B4 engagement. When 2B4 serves as a ligand for CD48 it may increase signaling thresholds required for development of full NK function but engagement of CD48 alone is not sufficient to mediate lytic granule release. Instead 2B4 inhibition is either overcome by engagement of bona fide activating receptors or by lack or reduced expression of CD48 on target cells.

In summary, we show that 2B4 engagement on NK cells by CD48 expressing target cells inhibits NK cytotoxicity independent of the presence of SAP, and that 2B4 isoform expression may dictate 2B4 function in the murine system. It remains to be determined how isoform expression is regulated. And more importantly, defining the molecular mechanisms of 2B4 mediated inhibition of murine NK function is warranted.

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