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Molecular Basis of the Dual Functions of 2B4 (CD244)†

Lukasz K. Chlewicki,‡ C. Alejandro Velikovsky,† Vamsi Balakrishnan,* Roy A. Mariuzza,† and Vinay Kumar*

2B4 belongs to the CD2 family of molecules and is expressed on all NK, γδ, and memory CD8+ (αβ) T cells. The murine NK receptor 2B4 exhibits both inhibitory and activating functions, whereas human 2B4 has been reported to be an activating molecule. How murine 2B4 can act both as an activating and inhibitory receptor and what distinguishes its function from human 2B4 have remained largely unknown. We use here a model system that allows the study of human and murine 2B4 under identical and controlled conditions. These studies reveal that both human and mouse 2B4 can activate or inhibit NK cells. We show here that the level of 2B4 expression and the degree of 2B4 cross-linking play a significant role in the regulation of signaling lymphocyte activation molecule-associated protein-mediated activation by 2B4. A high level of 2B4 expression, heavy cross-linking, and relative paucity of signaling lymphocyte activation molecule-associated protein promote inhibitory function. Our studies demonstrate how a single receptor can have opposing function depending on the degree of receptor expression, extent of its ligation, and the relative abundance of certain adaptor molecules. Because the levels of 2B4 and CD48 are dynamically regulated, these findings have implications for the regulation of NK cell function. The Journal of Immunology, 2008, 180: 8159–8167.

Natural killer cells are capable of killing target cells infected with viruses or that are transformed (1–3). They are believed to provide the first line of defense against infections and cancers. NK cells express both inhibitory and activating receptors and the balance of their function determines NK cell function (4). Of the inhibitory receptors, the most carefully studied are the Ly49 family in mice and the killer Ig-like inhibitory receptors in humans (1). These receptors are sensitive to the proper levels of expression of class I MHC proteins on the surface of cells. Inadequate self-MHC expression, or “missing self,” reduces inhibition and allows activating receptors to lyse vulnerable target cells (5). There are, however, several NK receptors that do not recognize MHC class I molecules, among these is 2B4 (CD244) (6).

2B4 belongs to the CD2 family of molecules and is expressed on all NK, γδ, and memory CD8+ (αβ) T cells (7, 8). The CD2 family of NK receptors are type 1 transmembrane proteins characterized by extracellular amino terminal Ig-like variable and Ig-like constant domains (9). Most members of this super family, such as the receptor signaling lymphocyte activation molecule (CD150) (10), serve as their own ligand on target cells (11). Engagement leads to regulation of cell proliferation and cytokine secretion. The ligand for 2B4 is CD48, which is expressed on all hematopoietic cells and is up-regulated on B cells during EBV infection (12–14). CD48, like 2B4, belongs to the CD2 family of receptors and has been shown to be a high-affinity ligand for 2B4 and a low-affinity ligand for CD2 (14). 2B4 was originally proposed to have an activating role in murine NK cells (15) because treatment of NK cells with anti-2B4 Abs caused increased cytotoxicity and IFN-γ secretion. More recently, this idea has been independently supported (16). Experiments in C57BL/6 2B4−/− or CD48−/− mice, however, indicate that absence of 2B4 on NK cells or CD48 on target cells enhances both cytoketory and secretory functions of NK cells, thus, firmly establishing that 2B4 can act as an inhibitory receptor (6, 17). Recently, it has been proposed that the more common murine I29 allele of 2B4 has an activating function (18). Not only can CD48 act as a ligand for 2B4, but also 2B4 itself can behave as a ligand for CD48 (19). Interactions between 2B4 and CD48 expressed on adjacent NK or T cells have been shown to increase cell proliferation (20), and this effect is mediated by activation through CD48. Thus, the interaction between 2B4 and CD48 is bi-directional.

CD2 family proteins, such as 2B4, have no enzymatic function. Human and murine 2B4 alleles of C57BL/6 (B6) origin have four immunotyrosine-based switch motifs (ITSMs)3 in their cytoplasmic tails. These motifs have been shown to interact with numerous small adaptor molecules through SH2 domains; examples include SAP, EAT-2A, EAT-2B (ERT), Csk, and the phosphatases SHP-1, SHP-2, and SHIP (21–24). The signaling lymphocyte activation molecule (SLAM)-associated protein (SAP) has been shown to bind all four ITSM regions of 2B4. Binding is enhanced when ITSMs are phosphorylated. In SAP-deficient humans (X-linked lymphoproliferative disease (XLP)), 2B4 stimulation leads to a decrease in NK cell lytic activity and IFN-γ production (25) and these patients are unable to control B cell proliferation (26–28). This result suggests that SAP is required for the activating function of human 2B4. The inhibitory activity of 2B4 is assumed to be mediated by other adaptor molecules, such as EAT-2A and EAT-2B that can also bind to 2B4 ITSMs (29).

3 Abbreviations used in this paper: ITSM, Immunotyrosine-based switch motif; XLP, X-linked lymphoproliferative disease; SAP, signaling lymphocyte activation molecule (SLAM)-associated protein; LAK, lymphokine-activated killer; DC, dendritic cell; MFU, mean fluorescence unit.

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How can 2B4 act as both an activating and inhibitory receptor? Our experiments reveal that regulation of 2B4 function is complex and dependent on many variables that include the conditions in which 2B4 is ligated. To test this hypothesis, we constructed a model system in which factors that can influence 2B4 function can be tightly regulated. We discovered three codependent factors responsible for determining 2B4 function: surface expression of 2B4, the degree of cross-linking of 2B4, and levels of SAP expression. These rules applied to human 2B4 as well as the two known alleles of murine 2B4. We found that when expressed at low surface levels, 2B4 generated an inhibitory signal. This inhibition could be overcome if less 2B4 molecules were engaged or whether the amount of SAP adaptor protein was increased.

Materials and Methods

Cells

Cells were grown in complete media (RPMI 1640, 10% FBS, HEPES, Penicillin/Streptomycin/Ampicillin, Na Pyruvate, and nonessential amino acids). The mouse T cell hybridoma 58-7/8 was expressing the 2C TCR m67 was provided as a gift from Dr. David Kranz (University of Illinois, Urbana, IL) and was used to assay the activity of transfected 2B4 and CD48 alleles. CD48" and CD48" variants of EL4 were used as targets for primary lymphokine-activated killer (LAK) and 58-7/8 transfectants. EL4 cells expressing different levels of murine CD48 were generated as stable cell lines from a CD48" EL4 line. Primary splenic NK cells were made as LAK preparations (30) and used between days 4 and 7 postisolation.

cDNA constructs

The murine B6 2B4 long allele, human 2B4 allele, and murine allele of CD48 were PCR amplified from retroviral vectors and subcloned into the mammalian expression vector pE6F6A (Invitrogen). cDNAs encoding murine SAP and EAT-2A were obtained from Dr. Cox Terhorst (Harvard University, Boston, MA) and subcloned into pE6F6A. The 129 allele of 2B4 was isolated from a LAK preparation from the spleen of a 129 mouse (Jackson ImmunoResearch Laboratories).

CD48 protein expression and purification

The ligand-binding domain of CD48 from C57BL/6 mice was expressed by yeast-display technology was used to select stable CD48 mutants. Several CD48-expressing clones were selected for higher expression and heat stability, and a clone with the lowest number of mutations outside of the binding site was chosen for further study. The CD48 protein was obtained by inserting the CD48 mutant DNA sequence (residues 1–106) into the expression vector pIT7.7. Protein was expressed in E. coli as inclusion bodies and solubilized in 6 M guanidine-HCl and 100 mM Tris-HCl (pH 8.0). Protein was diluted in refolding buffer (0.8 M arginine, 100 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 1 mM DTT), concentrated, and purified by gel filtration and Mono S cation exchange.

Abs and flow cytometry

Unlabeled Abs to murine B6 2B4, CD48, NGK2D, and 2.4G2 were all purified from hybridoma supernatants. Purified Abs to human 2B4, 129 allele of 2B4, PE-labeled Abs to 2B4, CD48, NGK2D, CD3, allopolyca- nin-labeled NKL1.1, matched pair ELISA Abs, and SA:HRP were all obtained from BD Biosciences. IgG control Abs were obtained from Jackson ImmunoResearch Laboratories. Abs to murine SAP and EAT-2A were obtained from Dr. Cox Terhorst. TCR cross-linking Ab F23.2 (anti-Vβ8.2) was provided as a gift from Dr. David Kranz (University of Illinois, Urbana, IL), and SIYRYYGL peptide was obtained from Dr. Hans Schreiber (University of Chicago, Chicago, IL).

Production of stable transfectants and retroviral transductions

SAP or EAT-2A overexpressing cells. 58-7/8 transfectants were screened for TCR, 2B4, and/or CD48 expression by flow cytometry. Double-positive cells were screened for IL-2 production by cross-linking the TCR using either anti-Vβ8.2 or anti-CD3. Transfectant clones secreting IL-2 were used for further study. Murine SAP and EAT-2A were amplified from a mammalian expression vector and inserted into the retroviral plasmid pMIG. PlatE (32) cells were transduced to produce retroviral particles. 58-7/8 cells were transduced with retroviral particles and GFP-positive cells were sorted to obtain a pure population of SAP or EAT-2A overexpressing cells.

Activation assays

Transfectant cells (5 × 105) were added to each well of a 96-well plate alone or containing EL4 (5 × 104) as targets that had been previously incubated with 1 μM SIYRYYGL peptide in complete media. Plates were incubated for 30 h at 37°C and 5% CO2. For assays requiring TCR and 2B4 cross-linking, EIA/RIA plates (Corning) were coated with 0.5 μg/ml anti-TCR Vβ8.2 and various concentrations of anti-2B4, or the appropriate quantity control IgG overnight at 4°C. To eliminate the variability that different levels of IgG per well may alter activity, each well was standardized to contain 4.5 μg/ml total IgG, and IgG control was used to make up the difference between dilutions. Plates were blocked in PBS-1% BSA for 1 h at room temperature and then washed in PBS-0.05% Tween 20 (PBST). For blocking experiments, Abs to 2B4 or CD48 was preincubated at 10 μg/ml with transfectants of 58-7/8, or for primary LAK cells in the presence of both blocking Ab and 2.4G2 to block Fc receptors for 1 h before addition to wells. For 58-7/8 transfectants, supernatants were harvested after 30 h, and assayed for IL-2 release using a sandwich ELISA format (BD Biosciences). For primary LAK cells, supernatants were harvested after an overnight incubation and assayed for IFN-γ secretion by ELISA.

IL-2 and IFN-γ ELISA

Corning EIA/RIA high-binding plates were coated with 2 μg/ml purified rat anti-mouse IL-2 (BD clone JES6-1A12) or purified rat anti-mouse IFN-γ (BD clone AN-18) for 1 h at room temperature. Plates were blocked and then washed in PBST, and harvested supernatants were added to the wells. Plates were incubated overnight at 4°C, washed three times with PBST, and then incubated with 2 μg/ml biotinylated rat anti-mouse IL-2 (BD clone JES6–5H4) or biotinylated rat anti-mouse IFN-γ (BD clone R4–6A2) Ab. After 1 h, plates were washed three times and incubated with 1 μg/ml streptavidin-HRP (BD Biosciences). After 1 h, plates were washed four times and incubated with 1× tetramethylbenzidine substrate (eBioscience). After ~5 min, the reaction was stopped by the addition of 3M sulfuric acid. Plates were read at 450 nm and values standardized to titrations of rIL-2 or IFN-γ.

RT-PCR

Total RNA was isolated from transfected or day 4–7 NK cells using Trizol/Chloroform extraction (33). Equal amounts of RNA were then subjected to reverse transcription using mLVM-RT (Fermentas) to generate cDNA. cDNAs were then amplified using SAP-, EAT-2-, or GAPDH-specific primers. Amplifications and annealing temperatures were optimized and reactions were resolved using 1.5% agarose gel electrophoresis.

Results

B6 alleles of 2B4 and CD48 can modulate TCR activity

To study 2B4 functions in a single model system that allows control of multiple variables, we introduced 2B4 into the T cell line 58-7/8 (34). The 58-7/8 cell line is a derivative of the DO-11.10.7 mouse T cell hybridoma (H-2k). It does not express TCR α- and β-chains and is negative for CD4, CD8, CD2, 2B4, CD48, FcR, and NGK2D but expresses CD3 (data not shown). Because NK cells express 2B4 and CD2, and both bind to CD48, it is not always clear whether results obtained by using primary NK cells are due to signaling from CD48, 2B4, CD2, or some combination thereof. In addition, it is also not clear whether 2B4 is ligated by CD48 from adjacent NK cells or target cells. Because this cell line does not naturally express CD2, CD48, or 2B4 but expresses SAP, we felt that this cell line may be an appropriate model system to study 2B4 function.

To validate this model we used a stable 58-7/8 transfectant line expressing a variant of the 2C TCR (m67) as a triggering receptor. The 2C TCR was chosen because more is known about the biochemical properties of this receptor (35–39) than any activating NK receptor, and because 2B4 is also expressed on T cells. Using 2C TCR also allows the use of EL4 target cells, which express the
class I MHC K b and CD48 and, therefore, can deliver both TCR and 2B4-CD48 signals simultaneously. The activation of the 2C TCR does not require CD8 costimulation for IL-2 secretion (38); however, activation occurs when the superagonist peptide SIYRYGGL (40) is loaded or expressed by EL4 or whether the TCR is ligated by anti-TCR Abs. In both instances, activation leads to IL-2 secretion.

We first determined the basal function of 2B4 in our system. The murine B6 allele of 2B4 was transfected into the 58/H11002 cell line that expresses the 2C TCR. Because no CD48 is present on the surface of these effector cells, the contribution of CD48 molecules from other effectors is completely eliminated. We isolated over two dozen transfectant clones expressing various surface levels of B6 2B4 ranging over several orders of magnitude. Expression levels were compared with B6 wild-type NK cells created as LAKs from spleens. The 4-day cultured B6 LAK cells typically express 500–1100 mean fluorescence units (MFU) of 2B4 (data not shown). Transfectants matching these levels were used in the initial set of experiments.

Activity of 2B4 was assayed by ligation of TCR with a fixed concentration of anti-TCR Abs and with various concentrations of cross-linking anti-2B4 Abs. A clear concentration-dependent inhibition (decrease in TCR-mediated IL-2 secretion) was seen in all clones of B6 2B4 that were tested (Fig. 1A). These results were then compared with day 4 LAK cells isolated from B6 mice. IFN-γ secretion by these cells was also inhibited upon cross-linking with anti-2B4 Abs with kinetics almost identical with those seen with the 58/H11002 cells (Fig. 1B). As a control, there was no effect in LAK cells from 2B4/H11002 mice. These results confirm earlier reports with primary NK cells that 2B4 ligation leads to inhibition of NK function and, thus, we felt confident using this system to test various factors influencing 2B4 function.

We next determined whether 2B4 ligation leads to inhibition of NK function and, thus, we felt confident using this system to test various factors influencing 2B4 function. When 2B4 was ligated by CD48 expressed on EL4 target cells (rather than cross-linking Abs), a similar decline in IL-2 secretion was noted; this inhibition was reversed when blocking anti-CD48 Abs were added (Fig. 1C). Because both EL4 cells expressing CD48 and anti-2B4 cross-linking Ab stimulated 2B4 effectively, in most of the studies we used a target-cell-free system. This eliminates variances due to level of expression of CD48 on EL4 or other NK targets as well as interactions between 2B4 and CD48 on adjacent cells, as would occur with primary NK cells.

FIGURE 1. B6 2B4 and CD48 can modulate TCR activity on TCR+/58−/− transfectant clones. A, TCR+/58−/− transfecteds of 2B4 are stimulated with 0.5 μg/ml anti-TCR Ab and increasing concentrations of anti-2B4 cross-linking Ab and quantitated for IL-2 release by ELISA. Production of IL-2 is normalized to IL-2 secretion based on TCR stimulation using anti-TCR cross-linking Abs along with IgG control and is shown as percentage of change of control. Inhibitory activity is reported henceforth as negative percentage of change and activating activity is reported as positive percentage of change. All data points are averages of three or more wells with error bars indicating SE. Data is representative of at least three separate experiments. B, Day 4 B6 or 2B4+/− LAK cells are cross-linked using increasing concentrations of anti-2B4 Ab. IFN-γ secretion is assayed by ELISA in the same manner as IL-2 for 58−/−. C, TCR+/58−/− or control TCR−, 2B4+/58−/− cells are stimulated overnight with EL4 target cells loaded with SIYRYGGL peptide. The EL4 cells were either CD48− or CD48+. EL4 cells were incubated with IgG control or blocking anti-CD48 Ab and assayed for IL-2 secretion. D, CD48+/58−/− or 2B4+/58−/− transfectants of TCR−, 58−/− cells are stimulated using 0.5 μg/ml anti-TCR along with increasing concentrations of anti-CD48 Ab and assayed for changes in IL-2 secretion. E, CD48+/58−/− or 2B4+/58−/− transfectants of 58−/− are stimulated with SIYRYGGL peptide-loaded CD48+/+, CD48− or 2B4+/ EL4 cells with IgG control or blocking anti-CD48 Ab and assayed for IL-2 secretion.
Because all NK cells also express CD48 and ligation of CD48 has been demonstrated to cause NK and T cell activation, we stably transfected both CD48 and TCR in 58\(^{-/-}\) cells, without the presence of 2B4 (analogous to NK cells from 2B4 knockout animals) and tested them for function. CD48 engagement on this cell line (by anti-CD48 antibodies) led to a dramatic increase in IL-2 secretion of \(\sim 900\%\) (Fig. 1D). Because 2B4 itself has been shown to behave as the ligand for CD48-mediated signaling (19), we constructed a CD48 negative EL4 cell line expressing only 2B4. Using these cells as targets for CD48 expressing 58\(^{-/-}\) effector cells also led to a \(\sim 1000\%\) increase in IL-2 secretion, which could be effectively reversed using anti-CD48 blocking Abs (Fig. 1E). These results confirmed that CD48 can activate cells using 2B4 as ligand. This is consistent with the reported role of CD48 in potentiating T cell responses (41). Thus, we see that this system can be used to study receptors that can both increase (activate) and decrease (inhibit) TCR-mediated IL-2 secretion. The magnitude of the activation by CD48, from +900-1000\%, shows why CD48-deficient “effector cells” are required to separate the function of 2B4 and CD48. In addition, this cell line is CD2 negative (data not shown). CD2 can be used by CD48 as a ligand and itself has been shown to help activate T cell function (42).

**B6 and 129 alleles of 2B4 bind CD48 at similar affinities**

The 129 allele of 2B4 has been reported to have an activating function (18, 31). It differs from the more commonly studied B6 allele at 10 of 18 residues that contact CD48 and shares 84% similarity to the B6 allele. Both the human (43) and 129 allele of 2B4 contain a small deletion in the cytoplasmic domain not present in the B6 allele. Human 2B4, which is activating, has been reported to bind CD48 at 8 \(\mu\)M, whereas the B6 allele of 2B4 has been previously measured to bind CD48 at 16 \(\mu\)M (14). Affinity has been shown to play a significant role in TCR (44–47) and NK receptor activity (48). Given the fact that over 50% of the residues contacting CD48 differ between 129 and B6 alleles, we wanted to determine whether differences in affinities of 2B4-CD48 between B6 and 129 alleles could account for the reported differences in function of these two alleles.

Equilibrium binding data showed that both B6 and 129 alleles bind CD48 with similar affinities of around 4 \(\mu\)M (Fig. 2). These numbers have also been independently confirmed using surface plasmon resonance (31). The similarity of affinity between 129 and B6 alleles indicates that binding affinity likely plays no role in the functional difference reported for these two alleles of 2B4.

**The functions of human, 129, and B6 alleles of 2B4 correlate with surface expression**

To test the idea that 2B4 function is determined by the specific 2B4 allele a cell expresses and not other factors arising from strain or species differences, stable transfectants were generated for human and the 129 allele of 2B4 in 58\(^{-/-}\) cells expressing 2C TCR as the triggering receptor. We isolated over 20 clones expressing human 2B4. When we stimulated these transfectants using anti-human 2B4 cross-linking Abs along with TCR stimulation, we found two types of clones: those in which ligation of 2B4 decreased TCR-mediated IL-2 secretion (inhibition) and those in which 2B4 ligation increased TCR-mediated IL-2 secretion (activation) (Fig. 3A). More clones exhibited an inhibitory phenotype. This is contrary to what has been demonstrated in most studies in the literature for human 2B4, where an activating phenotype seems to be favored (49). When various parameters of the clones transfected with human 2B4 were analyzed, we noted a striking correlation between surface densities of 2B4 expression and function. Lower surface
expression of human 2B4 favored activation and higher surface expression favored inhibition (Fig. 3A). Experiments with cells containing the 129 allele also showed clones that exhibited activating or inhibitory function and this activity also seemed to correlate with surface expression (Fig. 3B).

In view of these findings, we tested a large panel of clones transfected with the B6 allele of 2B4 that expressed a broad range of 2B4. In keeping with data obtained with human and 129 transfecants, we found that clones that expressed lower surface levels of 2B4 generally exhibited an activating phenotype, whereas clones with high surface expression generally exhibited an inhibitory phenotype (Fig. 3C). Although there is a general correlation between surface levels and 2B4 function, we also noted that several clones with similar surface levels performed opposite functions (an explanation for these exceptions is discussed later). Clones that match the density of day 4–6 LAK cells fell into both activating and inhibitory categories. These results suggest that the B6 allele of 2B4 can both activate and inhibit, and that this function seems to be regulated in general by the level of surface expression of 2B4, but exceptions to the correlation with surface expression suggest that other factors are also involved (see later). These studies also imply that differences in function reported for alternative 2B4 alleles may not be based on gene structure, and most likely other factors, such as surface expression levels of 2B4, are involved.

Function of 2B4 depends on level of receptor stimulation

Because low surface expression yielded activation, we hypothesized that this could be due to lower levels of ligand exposure of the receptor by its ligand. To determine whether the function of 2B4 is dependent on the degree of ligand stimulation, we tested B6 2B4 transfectants of various surface densities to different levels of stimulation. We chose three groups of transfectants that represented high, low, and medium surface densities as measured by flow cytometry, and titrated increasing concentrations of anti-2B4 cross-linking Ab, along with a fixed level of anti-TCR Abs. In other words, we kept the activation signal constant and varied the signal generated by 2B4. Clones with very low surface expression showed a concentration-dependent activation (increase in IL-2 secretion) that increased as the level of cross-linking Ab was increased (Fig. 4A). Clones with very high surface expression exhibited a concentration-dependent inhibition (decrease in IL-2 secretion) that increased with higher concentrations of cross-linking Ab (Fig. 4B). Transfectants of intermediate surface density exhibited both activating and inhibitory function in the same assay. At low levels of cross-linking, an activating response was favored, but as the concentration of anti-2B4 Ab increased, the activation turned to inhibition, eventually giving a degree of inhibition similar to clones with much higher surface densities of 2B4 (Fig. 4C).

Low surface expression and stimulation can also be mimicked using soluble blocking anti-2B4 Abs that may reduce available receptors or mask ligands. We tested this on clones of medium surface density, which normally show a switching phenotype, and converted one such clone to a phenotype similar to that of a clone of low surface expression (Fig. 4D). This was then repeated on day 4 LAK cells. With increasing anti-2B4 cross-linking, we saw a shift in the kinetics of IFN-γ secretion that approaches kinetics observed for IL-2 secretion by 58–/– clones with low to medium surface densities (Fig. 4E). These results indicate that surface expression and the level of 2B4 engagement both influence the function of 2B4.
Function of 2B4 depends on SAP but not EAT-2 adaptor molecule expression

The 58/− cell line was screened for adaptor protein expression by RT-PCR, and we found that the levels of SAP expressed in these cells is similar to that seen in day 4 B6 LAK cells. However, 58/− cells lacked expression of EAT-2A and EAT-2B (ERT), which were both detected in B6 LAK cells (Fig. 5A). Because 2B4-mediated inhibition was observed in our transfectants, EAT-2A and EAT2-B may not be required for inhibition, contrary to some published reports (29, 50) and supported by a recent study (51). Perhaps yet to be discovered members of this family of adaptor proteins can play the same role.

As reported in Fig. 4, we found some clones with identical cell surface expression of 2B4 that differed in their response to 2B4 ligation; some were activating and others inhibitory. We hypothesized that such differences may be due to varying levels of SAP in these clones. To test this, we did RT-PCR for SAP in these clones and indeed found differences in SAP levels that correlated with function (data not shown). Low SAP levels favored inhibition and vice-versa. Because 2B4 has four ITSMs in its cytoplasmic domain (each capable of binding SAP), we hypothesized that SAP adapter levels, essential for transmitting activation signals from 2B4, may become limiting in cells expressing higher levels of 2B4. To test the idea that SAP levels may become limiting when large numbers of 2B4 molecules are ligated, we over-expressed SAP in various clones. No change in IL-2 secretion was seen in clones with low surface expression of 2B4 when SAP was over-expressed, because these clones were already activating (Fig. 5B).

Over-expression of SAP in transfectants with high surface levels of 2B4 changed their function from inhibiting IL-2 secretion to enhancing IL-2 secretion (Fig. 5C). Over-expression of SAP in clones that could be switched from activation to inhibition by different degrees of 2B4 cross-linking shifted the curve more to activation (Fig. 5D). Similar results were obtained with the human allele of 2B4 (Fig. 5, E and F).

2B4−, CD48− double transfectants exhibit inhibition from adjacent effector cells

Unlike the transfectants used in our studies thus far, NK cells express both 2B4 and CD48. Because this would allow 2B4-CD48 interactions among NK cells in addition to 2B4-CD48 engagement with target cells, we decided to extend our findings to cells that express both receptors, analogous to wild-type B6 NK cells activated by IL-2. We constructed transfectants expressing 2B4 and CD48 concurrently in 58/− cells along with 2C as the triggering receptor. When several of these clones were screened for IL-2 secretion using only anti-TCR Abs, very low levels of IL-2 were produced in transfectants with higher surface levels of 2B4 (Fig. 6). In fact, the basal levels of IL-2 secretion upon ligation of 2C TCR varied inversely with the surface levels of 2B4 in these clones. IL-2 secretion was restored in high 2B4 expressing clones only when 2B4 blocking Abs were added. Therefore, the CD48-2B4 interaction on adjacent effector cells was enough to inhibit TCR-mediated IL-2 secretion. These studies indicate that 2B4-mediated inhibition is favored as surface levels of 2B4 on the effector cells increase.
adjacent effector cells. 2B4 TCR) were stimulated with 0.5/\mu g/ml H9262 mutations in the SAP molecules. NK cells from XLP patients can—binding studies and by studies in humans with XLP who have that deliver an activation signal (54). This has been proven both by previous studies have revealed that SAP is recruited entirely clear. Previous studies have revealed that SAP is recruited function. But how the threshold is converted into a signal is not the surface level and degree of cross-linking that determine 2B4 indicate that there is a threshold of engagement, determined both by for 2B4 was titrated, low surface expressers were always activating clones of intermediate level of 2B4 (within range of LAK cells) were very instructive because at a fixed level of cell surface 2B4, the function of the clone could be modulated from activation to inhibition by altering the degree of cross-linking. The biphasic response of clones expressing intermediate density of 2B4 could be skewed to activation by over-expression of SAP. That such bi-functionality can be extrapolated to results with primary culture of NK cells is shown by the fact that IFN-\gamma secretion by LAK cells can be similarly modulated by the degree of 2B4 cross-linking. These cells behave as clones of intermediate density of 2B4.

The regulation of 2B4 function by levels of expression and cross-linking has important implications in vivo because 2B4 and CD48 expression is dynamically regulated in vivo. The level of 2B4 is up-regulated in vitro with IL-2 and also in vivo by injection of CpG (R. Taniguchi and V. Kumar, submitted). Concomitant with activation of NK cells by IL-2, the inhibitory function of 2B4 was also seen to increase (R. Taniguchi and V. Kumar, submitted). CD48 was discovered initially as a molecule whose expression is up-regulated on B cells infected by EBV (12). Subsequently it has been learned that a variety of signals can increase the expression of CD48. Dendritic cells (DC) express CD48, and the expression is increased upon DC activation. The lysis of syngeneic DC by activated NK cells is inhibited by the ligation of 2B4 by CD48 on DCs (M. McNerney and V. Kumar, unpublished observations). Thus, 2B4-CD48 interactions can influence NK-DC interactions, a subject of intense interest because it is at the interface of innate and adaptive immune responses. Viruses, such as EBV, may have evolved to dampen the NK responses by altering the 2B4-CD48 regulatory pathways. CD48 expression is up-regulated during EBV infection (52), and this may serve to inhibit the function of highly activated NK cells and, thus, prevent the lysis of EBV-infected B cells. HIV-1 has been recently shown to down-regulate CD48 expression (55); this could render HIV-infected T cells susceptible to lysis by activated NK cells.

It has been reported that the SAP-related adaptor molecules EAT-2A/B can negatively influence NK cell function (29). This is thought to occur by EAT-2 molecules binding 2B4 in the absence of SAP. We observed inhibition mediated through 2B4 in 58/ cells, which did not have detectable levels of transcripts for either EAT-2A or EAT-2B, suggesting that EAT-2 is not obligatory for inhibition. At this time, it is unclear which adaptor molecules transmit a 2B4-mediated inhibitory signal, but perhaps SAP-related molecules that have yet to be identified are involved. Although the simple model system we have developed and analyzed is useful to dissect various factors that can regulate 2B4 inhibited by engaging CD48 (52). Thus, the role of SAP in 2B4-mediated activation is firmly established. Our studies now unravel a novel aspect of 2B4-SAP interactions. We propose that the SAP-dependent activating function of 2B4 is regulated not only by the presence or absence of SAP, but also by the amount of SAP relative to the number of 2B4 molecules and the extent of their cross-linking.

2B4 has four ITSM motifs, and each ITSM is capable of binding one SAP molecule; thus, when 2B4 is expressed at high level, the availability of SAP for binding to 2B4 molecules can become limiting. Because SAP recruits activating molecules, such as Fyn tyrosine kinases, such a limitation of SAP may allow other adaptors to bind to ITSMs and, thus, shift the function of 2B4 to an inhibitory molecule. This notion is supported by the finding that when SAP was over-expressed in clones with high 2B4 surface densities, the function switched from inhibition to activation. At low 2B4 surface densities, there are probably enough SAP molecules for binding to ITSM motifs and, hence, activation is unaffected by addition of more SAP. The studies of clones that expressed an intermediate level of 2B4 (within range of LAK cells) were very instructive because at a fixed level of cell surface 2B4, the function of the clone could be modulated from activation to inhibition by altering the degree of cross-linking. The biphasic response of clones expressing intermediate density of 2B4 could be skewed to activation by over-expression of SAP. That such bi-functionality can be extrapolated to results with primary culture of NK cells is shown by the fact that IFN-\gamma secretion by LAK cells can be similarly modulated by the degree of 2B4 cross-linking. These cells behave as clones of intermediate density of 2B4.

The differences in function for all three forms of 2B4 tested seemed to be partly based on 2B4 surface density. By screening clones bearing the B6 allele with surface densities spanning several orders of magnitude we showed that low surface density favors an activating response that gradually shifts toward inhibition as surface density increases. Cells expressing high surface levels of 2B4 were almost always inhibitory. When the concentration of ligand for 2B4 was titrated, low surface expressers were always activating and high surface expressers were always inhibitory. Clones of intermediate 2B4 density exhibited activation at low concentrations of ligand and inhibition at high concentrations. These results indicate that there is a threshold of engagement, determined both by the surface level and degree of cross-linking that determine 2B4 function. But how the threshold is converted into a signal is not entirely clear. Previous studies have revealed that SAP is recruited to the ITSM sequences in the cytoplasmic portion of 2B4 and this then recruits additional molecules, such as Fyn tyrosine kinases, that deliver an activation signal (54). This has been proven both by binding studies and by studies in humans with XLP who have mutations in the SAP molecules. NK cells from XLP patients cannot be activated by CD48 ligation (27) and indeed appear to be

![FIGURE 6. 2B4⁺, CD48⁺ double transfectants exhibit inhibition from adjacent effector cells. 2B4⁺, CD48⁺ transfectants of 58⁻ expressing TCR were stimulated with 0.5 \mu g/ml anti-TCR Ab, in the presence of soluble control IgG or anti-2B4 blocking Ab, and assayed for IL-2 secretion. Clones are listed in order of increasing 2B4 surface expression indicated on the x-axis. Bars are the average of at least three points and SE indicated by error bars. The data is representative of at least three separate experiments.](http://www.jimmunol.org/)
function, one has to be careful in extrapolating the data to the in vivo situation. Whereas in most experiments we used 58−/− cells transfected with 2B4 (or in some cases CD48), in reality, NK cells express both 2B4 and CD48. This is true for mice and humans. The implication of this is that even when not confronted with a CD48-bearing target, NK cells in proximity to each other are very likely to incur bi-directional signaling between 2B4 and CD48 among NK cells. In fact, 2B4-CD48 clustering can be demonstrated in IL-2-cultured NK cells in vitro (56). What is the significance of this? We speculate that such NK-NK interactions involving 2B4 and CD48 also regulate NK function. As a first step in testing this hypothesis, we created 58−/− cells whose phenotype is more akin to NK cells, by cotransfecting 2B4 and CD48. The results are remarkable. When such 2B4+, CD48+ cells were activated by TCR ligation, they revealed 2B4-CD48-dependent inhibition of IL-2 secretion. This inhibition was inversely related to the degree of 2B4 expression in the transfectants. Blocking 2B4 in such cells enhances IL-2 secretion. Because, in this system, 2B4-CD48 interactions could have occurred only among 58−/− cells, this demonstrates a mechanism of regulation of basal cellular functions by 2B4. Indeed, we have recently demonstrated that killing of highly activated murine NK cells by other NK cells is inhibited by 2B4-CD48 interactions both in vitro and in vivo (57). Because, upon activation in vivo, both 2B4 and CD48 are up-regulated, it would seem that extensive engagement of 2B4-CD48 on adjacent NK cells or NK cells and other hematopoietic cells (all of which express CD48) is likely to polarize NK cells toward an “inhibitory” phenotype. That this is the case is supported by experiments in which the ability of wild-type and 2B4−/− mice were compared in their abilities to clear CD48− and CD48+ tumor cells in the absence of any external stimulation of NK cells (17). Under these in vivo conditions, 2B4−/− mice cleared the CD48+ tumor cells much more effectively than wild-type mice, clearly documenting an inhibitory function of 2B4 in this in vivo model system. We hesitate to make generalizations about the in vivo function of 2B4 on the basis of a single in vivo model of tumor clearance, because the regulation of 2B4 and CD48 and SAP-related molecules in vivo may vary in each specific circumstance (viral infections and bone marrow transplants). Hence, 2B4 function may also vary from activation to inhibition.

In addition to the significance of this work in understanding and possible manipulation of NK function, we believe there are broader implications. Multi-functionality is not unique to the 2B4-CD48 interaction. There are other well-defined molecules, such as family surface protein, is a ligand for CD48. Identification of the 2B4 molecule as a counter-receptor for CD48. J. Immunol. 161: 5809–5812.


