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Review

2B4 (CD244) is a non-MHC binding receptor with multiple functions on natural killer cells and CD8$^+$ T cells

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Abstract

2B4 (CD244) is expressed by memory-phenotype CD8$^+$ T cells and all natural killer (NK) cells. The ligand for 2B4, CD48, is expressed on hematopoietic cells. 2B4 is conserved in humans and mice, and a number of reports have linked 2B4 with activation of lymphocytes. We have employed 2B4-deficient mice and antibody blocking to analyze 2B4 function both in vitro and in vivo and found that 2B4 is a receptor with multiple functions. 2B4 is required for optimal activation of CD8$^+$ T cells and NK cells – in this context 2B4 requires interaction with CD48 on neighboring lymphocytes, demonstrating that homotypic interaction within NK cell or T cell populations augments immunity. When 2B4 is engaged by CD48 on a target cell, 2B4 conversely inhibits NK effector function. As an inhibitory receptor, 2B4 is unconventional as it is not regulated by MHC class I molecules. In this review we will discuss the significance of these multiple functions and the events that may regulate differential 2B4 signaling outcome.
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1. CD150 subfamily

The CD2 receptor family is within the immunoglobulin (Ig) superfamily and consists of type 1 transmembrane proteins with an *N*-terminal variable Ig domain followed by a constant two Ig domain (Sidorenko and Clark, 2003). Within the CD2 family falls the CD150 (signaling lymphocyte activation molecule, SLAM) subfamily. The receptors of this subset are defined by two or more cytoplasmic immunoreceptor tyrosine-based switch motifs (ITSM; TxYxxV/I) and include 2B4 (CD244), CD84, CD229, NTB-A, and CS1. All of these molecules have been shown to modify lymphocyte function, and many of them, including CD150, CD84, CS1, and CD229, engage in homophilic interactions (Engel et al., 2003). CD150 is the best characterized, and has been shown to act as co-receptor – it engages in homophilic interactions that lead to enhanced activation of B cells and regulation of proliferation and cytokine production by T cells (Sidorenko and Clark, 2003; Veillette and Latour, 2003).

ITSM motifs bind a number of SH2 domain containing proteins, including both activating and inhibitory signaling molecules. The ITSM-interacting protein that has received the most attention is SLAM-associated protein (SAP, SH2D1A). SAP is expressed in B, T and NK cells, and has been found to be mutated in patients with X-linked lymphoproliferative disorder (XLP) (Coffey et al., 1998; Nichols et al., 1998; Sayos et al., 1998). XLP is a progressive combined variable immunodeficiency, characterized by lymphohistiocytosis often initiated by EBV infection (Purtilo et al., 1975, 1977). Patients with this disorder frequently have agammaglobulinemia, increased susceptibility to fatal infectious mononucleosis, and lymphoma. It is thought that the absence of functional SAP leads to abnormal signaling by

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ITSM-containing proteins, including 2B4, leading to immune insufficiency. The unique roles of individual CD150 family members, as well as the specific action of SAP in signaling pathways of XLP and non-disease states are still being characterized.

2. 2B4 (CD244)

2B4, which bears four ITSM motifs, is expressed by all NK cells, a subset of memory-phenotype $CD8^+$ αβ T cells, γδ T cells, basophils and monocytes (Garni-Wagner et al., 1993; Nakajima et al., 1999; Schuhmachers et al., 1995; Valiante and Trinchieri, 1993). In mice a "short" form of 2B4, containing only one ITSM also exists; it is generated by alternative splicing of 2B4 RNA (Stepp et al., 1999). Murine 2B4 was originally described as an activating receptor on NK cells since NK cells treated with whole anti-2B4 antibody or its Fab fragments lead to increased killing of several murine tumor cell lines, and whole anti-2B4 cross-linking lead to IFNγ production and granule exocytosis (Garni-Wagner et al., 1993). Human NK cells were found to be activated by anti-2B4 in a redirected lysis assay (Valiante and Trinchieri, 1993).

CD48 is a ligand of high-affinity for 2B4 and is a GPI-linked CD2 family member (Brown et al., 1998). Murine CD48 is also a low-affinity ligand for CD2 (Brown et al., 1998; Latchman et al., 1998). CD48 is expressed on lymphoid and myeloid cells, and its expression is upregulated in response to EBV-infection, PMA, IFN-γ, and IFN-α/β (Tissot et al., 1997; Yokoyama et al., 1991). The majority of studies on 2B4 used antibody-mediated crosslinking to assess 2B4 regulation of NK cells. In two studies that used CD48 transfected and non-transfected targets it was found that CD48 expression on targets activated primary human NK cell clones in one study (Tangye et al., 2000a) but not the other (Nakajima et al., 1999).

Since the initial discovery of 2B4, there has been increasing evidence that 2B4 also has inhibitory potential. 2B4 appears to be inhibitory in the absence of functional SAP, as seen in XLP patients and in normal developing human NK cells (Benoit et al., 2000; Nakajima et al., 2000; Parolini et al., 2000; Sivori et al., 2002; Tangye et al., 2000b). When the murine 2B4 isoforms were individually transfected into an NK cell line, the long form inhibited NK cytotoxicity (Schatzle et al., 1999). Now, results from experiments employing 2B4-deficient mice underscore the inhibitory role for murine 2B4 (Lee et al., 2004).

3. 2B4 inhibition of murine NK cells

Analysis of newly generated 2B4-deficient mice has provided the opportunity to study 2B4 physiology without relying on anti-2B4 antibodies that may be acting either as agonists or antagonists. 2B4-deficient mice are phenotypically normal (Vaidya et al., submitted). NK cell development in these mice appears to be normal, which is notable because 2B4 is expressed early during development, prior to acquisition of MHC binding inhibitory receptors, and has been suggested to have a role in maintenance of self-tolerance on immature NK cells (Rosmaraki et al., 2001; Sivori et al., 2000) (M. McNerney and V. Kumar, unpublished observations). Consistent with this hypothesis, 2B4-deficient immature NK cells have increased killing of syngeneic ConA blasts (Lee et al., 2004). However, this lack of self-tolerance is not overtly manifested in vivo since NK cell numbers and receptor expression are unaltered. Furthermore, hematopoiesis appears grossly normal as determined by lymphocyte subsets in various immune organs. It cannot be excluded as yet that there are subtle defects in hematopoiesis that are compensated for in steady-state conditions.

Surprisingly, when mature, splenic 2B4-deficient NK cells were cultured in IL-2 and tested in cytotoxicity assays, they demonstrated enhanced killing of several $CD48^+$ target cells as compared to wild-type NK cells, a finding that is consistent with 2B4 being an inhibitory receptor. NK cells lacking 2B4 demonstrated increased lysis of $CD48^+$ RMA-S tumors, allogeneic ConA blasts, and syngeneic ConA blasts, but not $CD48^-$ RMA-S. Consistent with the in vitro findings, 2B4-deficient mice had an increased capacity to clear $CD48^+$ tumor cells in vivo. Engaging 2B4 on wild-type NK cells inhibited not only cytotoxicity, but also production of IFNγ in response to tumor cells. These inhibitory effects could be reversed by antibody blockade with anti-2B4 and -CD48 (Lee et al., 2004).

To delineate the signaling pathway responsible for 2B4-mediated inhibition, the 2B4 short and long isoforms were retrovirally transduced into 2B4-deficient primary NK cells. Confirming a previous report using the RNK cell line, 2B4 long inhibited NK cell cytotoxicity (Schatzle et al., 1999). 2B4 short did not restore inhibition to 2B4-deficient cells, and furthermore, in contrast to the finding with the RNK cell line, 2B4 short did not activate primary NK cells. These data illustrate that the portion of 2B4 unique to the long isoform is necessary for inhibitory signaling. Supporting this finding, 2B4 long, but not short, associates with SHP-2 (Schatzle et al., 1999).

It was found that like immature human NK cells, immature murine NK cells do not express SAP transcripts. However mature splenic NK cells do express SAP transcripts and are known to express SAP protein (Sayos et al., 2000). Thus in mice, the differential expression of SAP does not necessarily dictate 2B4 signaling outcome – this inference is supported by the finding that 2B4 signaling is not dependent on SAP using SAP deficient mice (Czar et al., 2001) (Mooney et al., in press). Both immature and mature NK cells express EAT-2 transcripts, and EAT-2 protein has been found in NK cells and shown to bind 2B4 (Bouchon et al., 2001; Lee et al., 2004; Morra et al., 2001). It is still undetermined if different expression patterns of EAT-2, or other SAP-like molecules, influence 2B4 signaling.

4. 2B4 homotypic stimulation of lymphocytes

As many of the CD150 family members engage in homophilic interaction, there is the opportunity for cells expressing one of these receptors to engage the same receptors on neighboring lymphocytes in the absence of APC or target cells. Despite this possibility, there is little or no precedent for such homotypic modulation of lymphocytes occurring, or having functional consequences. It was therefore of interest to find that 2B4 and CD48, which can both be expressed in the same NK cell or memory-phenotype T cell population, engage in such homotypic interactions, thereby providing maximal effector function.

Initial experiments demonstrating this requirement were performed with purified T cells expressing the 2C transgenic TCR (Lee et al., 2003). These cells were primed in vitro and transduced with the 2B4 short or long isoforms. It was found that T cells expressing 2B4 had greater killing of P815 tumor cells, which express H-2L^{d}, the ligand for the 2C TCR. This enhancement of lytic activity occurred regardless of whether the target cells expressed CD48, indicating that 2B4-CD48 engagement was occurring between T cells. This hypothesis was confirmed by blocking with anti-2B4 or -CD48 during the experiment – the consequences of 2B4/CD48 interaction were evident mainly during T cell priming and less so during the killing assay. 2B4-expressing T cells did not have enhanced lysis of targets that lacked the TCR ligand, indicating that costimulation resulting from 2B4 signaling was ultimately TCR restricted. Stimulation occurred with either the short or long 2B4 isoform.

This finding was complimented by another study that demonstrated the role of homotypic 2B4/CD48 interaction in the proliferation of T cells (Kambayashi et al., 2001). In this report, blocking 2B4/CD48 interaction impaired CD48^{+} 2B4^{+} T cell proliferation in response to activation. Intriguingly, the authors demonstrate that 2B4 was acting as a ligand for CD48 on neighboring T cells.

As discussed above, when CD48 is present on a potential target, the engagement of 2B4 inhibits murine NK cell responses. However, when the target is CD48^{-} a costimulatory function for 2B4 is unveiled. 2B4- or CD48-deficient murine NK cells, or NK cells cultured in the presence of anti-2B4 or -CD48, are compromised in their capacity for mounting inflammatory responses (K. Lee, J. Forman, M. McNerney, A. Sharpe and V. Kumar, unpublished data). In the absence of 2B4, NK cells activated in vitro or in vivo exhibit impaired lytic activity and resist elaborating IFNγ in response to CD48^{-} tumor cells. Like T cells, NK cells demonstrate a dependence on 2B4 for optimal proliferation. Hence 2B4 in murine NK cells, like human 2B4, is involved in activating interactions. In mice, however, the activating function of 2B4 seems restricted to homotypic interactions between lymphocytes.

In summary, these studies demonstrate a novel role for 2B4/CD48 receptors in fraternal stimulation of T cells and NK cells. In vivo costimulation between neighboring lymphocytes may be a mechanism to maintain innate lymphocytes or long-lived memory T cells. It may also provide a mechanism for amplifying an early immune response. Accordingly, it is known that immune cells, although dispersed throughout the body, can expand and contract as necessary to escalate or terminate an immune response. While the role for factors expressed by stroma or APCs is well known in homeostasis, it can be envisaged that lymphocytes accumulating in an immune organ or site of inflammation can also regulate each other via 2B4/CD48. This type of quorum sensing would be useful in rapidly activating immune cells at the time of, or prior to, antigen exposure; likewise, it may be that this interaction can be pathological in an immune disorder.

5. 2B4 signaling

An important question regarding 2B4, and other CD150 family members, is the mechanism by which ITSM-containing receptors signal. Upon ligation, the ITSM motifs of 2B4 are phosphorylated by the Src family kinases Fyn and Lck (Nakajima et al., 2000; Sayos et al., 2000). Subsequently, 2B4 has been shown to interact with several SH2 domain containing proteins, SHP-1, -2, SAP, and EAT-2, and this is the point at which the activating and inhibitory pathways diverge (Morra et al., 2001; Parolini et al., 2000; Sayos et al., 2000; Schatzle et al., 1999; Tangye et al., 1999). In the activating pathway (Fig. 1A), SAP appears to function by re-

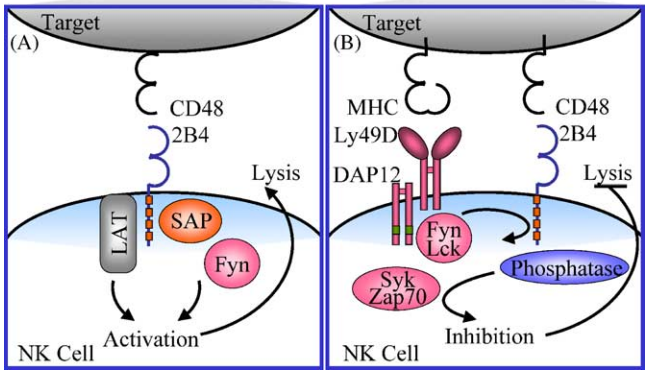

Fig. 1. Models of 2B4 activating and inhibitory signaling. (A) 2B4-mediated activation is initiated by binding CD48 on a target and phosphorylation of ITSM sequences by Src family kinases. 2B4 associates with both LAT and SAP directly. LAT activates PLC-γ and Grb2, while SAP binds to the SH3 domain of Fyn, all of which promotes activation of downstream effector molecules (Bottino et al., 2000; Latour et al., 2003). (B) In mice, or human immature or XLP NK cells, ligation of an ITAM-containing receptor on an NK cell by the cognate ligand on a potential target cell leads to activation of Src family kinases, Lck and Fyn. Recruitment of Syk family kinases such as Syk and Zap-70 follows, leading to downstream signaling events that provoke degranulation and cytokine production. When 2B4 is co-ligated by CD48 expressed on the target, 2B4 is phosphorylated by a Src family kinase. This phosphorylation event may not be regulated by SAP in mice. An inhibitory SH2-domain containing protein is recruited to the ITSM sequence of the 2B4 long isoform. The phosphatase then acts on proximal kinases or adaptor molecules to inhibit NK cell inflammatory functions.

cruiting other signaling molecules, specifically Fyn (Latour et al., 2001). Fyn may amplify the activating signal in part by further phosphorylating 2B4. 2B4 also activates linker for activation of T cells (LAT) and Vav-1, leading to PI3K activation, map kinase activation, and calcium flux (Bottino et al., 2000; Klem et al., 2002; Watzl et al., 2000), culminating in activation of lysis and cytokine production. This pathway best fits with the data for activating signals from human 2B4 upon interaction with a target cell. Although murine NK cells express the 2B4 long isoform found in humans, these forms are only 70% identical in sequence, and the murine form has an additional 33 amino acids in the cytoplasmic tail (Boles et al., 1999). These dissimilarities may account for differential influences of SAP and subsequent signaling in human and mouse. The homotypic costimulatory pathway may have commonalities with human 2B4 activation in response to a target, but there is little data at this point to support this.

2B4 has also been reported to bind inhibitory SH2 domain containing proteins SHP-1 and -2. In the inhibitory pathway, these phosphatases presumably inhibit other signaling pathways in a manner similar to that of inhibitory Ly49 receptors (Anderson et al., 2001). We propose the following inhibitory model for murine 2B4 (Fig. 1B). When an NK cell recognizes a potential target, ligation of an ITAM-containing activating receptor, e.g. Ly49D,leads to activation and recruitment of the Src family kinases Lck and/or Fyn. If 2B4 is co-engaged by CD48 on the target, the ITSM sequences of 2B4 become phosphorylated by the locally activated Src family kinases. Lck can directly bind and phosphorylate 2B4, thus this mechanism can account for the potential for 2B4 to be inhibitory independently of the presence or absence of SAP (Nakajima et al., 2000). Upon phosphorylation, a phosphatase is recruited to the cytoplasmic domain of 2B4 long. The phosphatase then inactivates proximal kinases or adaptor molecules of the activating receptor to terminate the signaling cascade prior to NK activation of killing machinery or cytokine production. Which protein tyrosine phosphatase is necessary in the inhibitory pathway is an unanswered question. Alternatively, the lipid phosphatase SHIP may associate with 2B4, dephosphorylate PI(3,4,5)P3, canceling the recruitment of PH-domain containing proteins and thereby decreasing PLCγ activity. In either the activating or inhibitory pathways, XLP patients have defective ITSM signaling that results in either lack of signaling, or more likely, alternate signaling routes.

6. Models for 2B4 signaling differences

It is perplexing to learn that murine 2B4 can participate in both costimulatory and inhibitory signaling pathways and begs the question as to how, or under what circumstances, 2B4 switches from one to another function. There are a few likely explanations. The first is that in the case of T–T, or NK–NK homotypic signaling, 2B4 is not actually signaling but is acting as a ligand for CD48. Several pieces of evidence support this, including the finding that CD48 can potentially signal as it binds both Lck, Fyn, and heterotrimeric G proteins subunits (Garnett et al., 1993; Solomon et al., 1996). Secondly, data on T–T homotypic costimulation indicate that the requirement for 2B4 is not on the proliferating cell, but on the neighboring, or by-stander cell (Kambayashi et al., 2001). This model could also explain the finding that both 2B4 long and short are activating on T cells, which is consistent with the paradigm wherein 2B4 is acting as a ligand (Lee et al., 2003). Consequently, this model would predict that 2B4 could stimulate T cells or NK cells in the absence of a cytoplasmic tail. One might speculate that if CD48 was signaling through a G protein, then 2B4 co-engaged on that same cell would not inhibit it, as the inhibitory pathway downstream of 2B4 is specific for phosphotyrosine-containing molecules.

It is also likely that 2B4 signals are dependent on the context in which they occur. 2B4 is known to be a modulator of NK cells – in human NK cells only those clones that were co-engaged by an activating receptor were activated by anti-2B4 in a redirected lysis assay (Sivori et al., 2000). Likewise in the presence of particular adhesion molecules at an NK–NK or T–T interface, 2B4 may preferentially recruit activating signaling molecules. In murine NK cells, 2B4 at an NK-target interface may preferentially recruit inhibitory signaling molecules in a fashion that is influenced by the content of lipid rafts or the presence of other NK receptors. Thus in this scenario, even murine 2B4 may be activating depending on the target and other receptors that are co-engaged. This versatility is likely afforded by the unique switching ability of ITSMs.

7. The evolution of non-MHC binding inhibitory receptors

The definition of self and non-self for NK cells is continuing to become more sophisticated. Initially, NK cells were characterized by their vigilance for missing- or non-self MHC class I ligands (Karre, 2002). Work on NKG2D has placed “altered- or stressed-self” within NK cell surveillance. Finally, the finding that 2B4 acts as an inhibitory receptor indicates that there is another tier of regulation. This regulation is non-MHC dependent and portrayed by other newly characterized NKR-P1 family members as well (Carlyle et al., 2004; Iizuka et al., 2003). This type of regulation may be more primitive than the others. CD2 family members are conserved evolutionarily – mouse and human CD2 have structural homology to genes encoded in chickens, yeast, and barley (Astwood and Hill, 1996; Grigorescu et al., 2000; Vainio et al., 1991). Conceivably, CD2 family members may have evolved from homophilic Ig-superfamily adhesion molecules regulating cell growth in invertebrates (Williams and Barclay, 1988). MHC molecules, on the other hand, are not found in invertebrates (Kumanovics et al., 2003). NK cells may have co-opted the invertebrate system prior to, or in conjunction with the evolution of MHC class I and adaptive immunity. For instance, in the absence of MHC class I, self-recognition

may have relied on a non-MHC binding inhibitory receptor such as 2B4. CD150 family members are postulated to have ancestral inhibitory relatives, as supported by the finding of novel immune-type receptors in puffer fish that contain inhibitory cytoplasmic motifs and the same V domain, C2 domain and transmembrane organization as CD2 family members, including 2B4 (Barclay, 1999; Strong et al., 1999).

As pathogens are known to manipulate immune responses, the upregulation of CD48 in response to EBV infection may have been an early mechanism of immune evasion. NK cell receptors appear to co-evolve with pathogens (Carayannopoulos and Yokoyama, 2004), and in the case of Herpes viruses, there may have been selective pressure to lose CD48-mediated inhibition. This may have occurred by altering the cytoplasmic domain of 2B4, or via the evolution of SH2 domain containing proteins, such as SAP. In this scenario, it would not be surprising that 2B4 may have retained both inhibitory and stimulatory capacities in the mouse, as both may have been necessary at one point. Clearly, to understand the functional role of 2B4 on NK cells, it will be important to better delineate the circumstances under which 2B4 has positive or negative effects on NK cells.

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