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2B4 Utilizes ITAM-Containing Receptor Complexes to Initiate Intracellular Signaling and Cytolysis¹

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Abstract

2B4 is a member of the SLAM receptor family capable of activating NK cell cytotoxicity in the context of EBV infection. SAP (SLAM Associated Protein) deficiency causes defective signaling downstream of SLAM family receptors and high susceptibility to EBV. 2B4 costimulates natural cytotoxicity receptor (NCR) and TCR initiated signals to induce cellular cytotoxicity and cytokine release. The 2B4-SAP signal transduction pathway is not predicted to overlap with the TCR-ITAM pathway, although SAP is required for some TCR-induced signals. We therefore examined the functional relationship between SLAM family receptor 2B4 and ITAM-containing adaptor complexes. Removal of FcεRIγ or CD3ζ-containing complexes, using genetically manipulated cell lines or siRNA specific suppression, significantly reduces 2B4-initiated functions in T and NK cells, respectively. Consistent with this relationship, Syk and ZAP-70 are capable of transducing 2B4 signals for calcium mobilization and cytolysis. Furthermore, ITAM-containing molecules constitutively associate with SAP. These results suggest a potential physical association between 2B4 and the ITAM receptor complexes that is required for 2B4-initiated signaling and cell-mediated killing.

Keywords

Natural Killer Cells; Cell Activation; Signal Transduction; 2B4; SAP

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1. Introduction

SAP (SLAM associated protein, also known as SH2 domain containing protein 1A)³ deficiency is the cause of X-linked lymphoproliferative disease (XLPD) (Morra et al., 2001) in which patients have a specific susceptibility to EBV. EBV-infected cells have enhanced expression of CD48 (Parolini et al., 2000), the ligand for SLAM family receptor 2B4, which is highly expressed on NK cells and used in killing of EBV infected cells (Roda-Navarro et al., 2004). Impaired 2B4 signaling in NK cells likely contributes to the decrease in survival seen in XLP patients following EBV infection (Nakajima et al., 2000). However, the mechanism by which SAP regulates 2B4-initiated signal transduction has not been fully elucidated.

Upon 2B4 ligation by CD48, SAP is recruited to the 2B4 intracellular domain (Sayos et al., 2000; Tangye et al., 1999). 2B4 contains four ITSMs (Immunoreceptor Tyrosine-based Switch Motifs), which mediate the majority of known signal transduction events upon 2B4 ligation. ITSMs have the unique ability to “switch” between activating and inhibitory signals, in contrast to ITAMs (Immunoreceptor Tyrosine-based Activation Motifs), which consistently provide activating signals upon ligation. SAP inducibly binds all four ITSMs on the intracellular domain of 2B4 (Chen et al., 2004) through its SH2 domain. SAP specifically recruits SH3 domain containing proteins (Li et al., 2009) including the Src family kinase FYN (Bloch-Queyrat et al., 2005), and FYN recruitment mediates 2B4 signal transduction, including PLC γ activation, (Kim et al.; Tassi and Colonna, 2005; Wang et al., 2000), calcium mobilization (Gu et al., 2006), and cytotoxicity (Bloch-Queyrat et al., 2005). In the absence of SAP, such as in XLP patient derived NK cells, inhibitory phosphatases (SHP1, 2 (Eissmann et al., 2005; Tangye et al., 1999) and SHIP (Eissmann et al., 2005)) or SAP-related molecules (Eat-2 (Roncagalli et al., 2005) and Ert (Chlewicki et al., 2008; Roncagalli et al., 2005)) are bound to the 2B4 ITSMs. SAP-mediated recruitment of activating molecules and displacement of inhibitory molecules likely determines the positive or negative outcome of 2B4 ligation.

2B4 ligation occurs when NK cells encounter normal, hematopoietic cells expressing CD48 and when NK cells encounter EBV-infected cells. The conditions under which 2B4 initiates cytolysis are therefore unclear. Two models were proposed to answer this question. Kumar and colleagues proposed a signal strength hypothesis, whereby the number of CD48 molecules present on the target cell surface determines whether 2B4 will trigger cytolysis (Chlewicki et al., 2008). Another model, proposed by Sivori, Moretta, and colleagues, stipulates a coreceptor is required for 2B4 activating function (Sivori et al., 2000). While both models may be correct, we chose to address Sivori and Moretta's model.

Since 2B4 has functional signaling motifs in the intracellular domain, and 2B4 lacks a transmembrane charged residue for noncovalent binding to ITAM-containing adaptor proteins, the prevailing assumption is that 2B4-initiated signaling occurs independently from ITAM-containing adaptors. However, Sivori, Moretta, and colleagues (Sivori et al., 2000; Sivori et al., 1999) have identified a correlation between NKp46 expression and the outcome of 2B4 ligation in NK cells. NKp46^{high} cells respond more effectively to 2B4 stimulation for target lysis (Sivori et al., 2000). NKp46 is an immunoglobulin (Ig) family receptor expressed on all NK cells (Sivori et al., 1997), which binds vimentin (Garg et al., 2006) and viral hemagglutinin (Arnon et al., 2004; Jarahian et al., 2009). The NKp46 receptor complex

³Abbreviations used in this paper: SAP, SLAM associated Protein, SH2 domain containing protein 1A; XLP, X-linked lymphoproliferative disorder; EBV Epstein-Barr Virus; SLAM, Signaling Lymphocyte Activation Molecule; ITSM Immunoreceptor Tyrosine Switch Motif; γ , Fc ϵ RI γ , ζ , CD3 ζ ; D12, DAP12, DNA associated protein of 12 kDa; NRS, Normal Rabbit Serum; GPI – glycosylphosphatidylinositol.

includes ITAM-containing proteins FcεRIγ and CD3ζ (Augugliaro et al., 2003; Biassoni et al., 1999; Westgaard et al., 2004). We postulated that the ITAM-containing proteins within the Nkp46 receptor complex heterologously regulate 2B4 signal transduction for cytotoxicity.

Herein, we demonstrate that suppression of FcεRIγ substantially reduced 2B4-initiated cytotoxicity in all NK clones tested, whereas suppression of Nkp46 reduced 2B4-initiated cytotoxicity in a fraction of NK clones. To determine if ITAM adaptor proteins regulate 2B4 signal transduction events independent from co-receptor ligation, we suppressed CD3ζ in T cells where the ITAM-associated TCR is definitely not ligated. Significantly, the CD3ζ ITAM adaptor was the key molecule important for 2B4-initiated events in T cells. As expected, 2B4 signaling is facilitated by functional Syk and ZAP-70 kinases. We furthermore demonstrate that CD3ζ constitutively binds SAP and inducibly binds 2B4. Therefore our results identify that 2B4 functionally couples with activating immunoreceptor complexes in both NK and T cells to initiate signaling and cellular cytotoxicity.

2. Materials and Methods

2.1 Reagents and antibodies

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. Indo-1 A/M and piceatannol were obtained from Calbiochem (Gibbstown, NJ). Mouse monoclonal anti-2B4 (C1.7) was kindly provided by Dr. G. Trinchieri (NCI, Frederick, MD). Mouse monoclonal IgG1 was obtained from R&D Systems. Anti-PLCγ1, anti-PLCγ2, anti-DAP12, anti-FcR (3G8), and OKT3 antisera have been described (Ting et al., 1992; Upshaw et al., 2005). Mouse monoclonal anti-CD4 was obtained from Becton Dickinson, (San Jose, CA). Mouse monoclonal anti-phospho Erk and rabbit polyclonal anti-Erk were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Murine anti phosphotyrosine 4G10 and rabbit polyclonal anti-FcεRIγ were obtained from Upstate Biotechnology, (Lake Placid, NY). Rabbit polyclonal anti-2B4 and anti-SAP were obtained by immunization of rabbits with keyhole limpet hemocyanin-conjugated 2B4 peptide (amino acids 342-365) and SAP peptide (amino acids 99-122) respectively. Mouse monoclonal anti CD3ζ was obtained from BD Pharmingen.

2.2 Cells

Primary, nontransformed, human NK cells were cloned by limiting dilution and cultured as described (Windebank et al., 1988). These will be referred to as “NK cells” for the remainder of this manuscript. The mouse mastocytoma cell line P815 and the NK cell line NK92 were obtained from American Type Culture Collection (Manassas, VA). The HLA class I deficient 721.221 cell line was kindly provided by Dr. P. Parham (Stanford University, Stanford, CA). The Jurkat T cell line and mutagenized variants (Jrt3, PF2.4, P116, P116.C40) were kindly provided by Drs. A. Weiss (UCSF) and R.T. Abraham (Merck Pharmaceuticals).

2.3 siRNA-mediated protein suppression

The following target sequences were used to generate small interfering RNA (siRNA) duplexes purchased from Dharmacon: CD3ζ, siGENOME SMART pool MU-004257-01-005, siGENOME individual D-004257-01-0020, siGENOME individual D-004257-02-0020, targeted against NM_000734, FcεRIγ, siGENOME SMART pool MU-011856-01-0010, targeted against NM_004106, DAP12, siGENOME SMART pool MU-012607-02-0005, targeted against NM_198125, Nkp46 M-020866-01, targeted against NM_004829, negative control (siNeg) TTCTCCGAACGTGTCACGT. 200 pmol siRNA specific for each target protein was introduced by electroporation to 5×10^6 proliferating

NK cells. 500 μ l of NK cells with siRNA were loaded into a 4 mm gap cuvette and electroporated with one pulse at 295 mV for 10 msec. For experiments in which two or three proteins were targeted for siRNA suppression, the control sample was treated with 400 or 600 pmol of siNeg respectively. NK cells were then cultured in RPMI supplemented with 5% BCS, 5% FCS, glutamine, and 20 U/ml IL-2 for 48 hours. Where indicated, NK cells were infected with control WR (Western Reserve) or recombinant WR vaccinia virus expressing a designated protein. NK cells were assayed for receptor expression, intracellular protein expression, calcium mobilization, and cytotoxicity as indicated.

2.4 Chimeric receptors and recombinant vaccinia virus

Recombinant vaccinia virus encoding FLAG-tagged F.CD4.2B4, F.CD4. ζ , and F.CD4.DAP12 chimeras were generated as described (Billadeau et al., 2003) using extracellular and transmembrane regions of human CD4 and the intracellular regions of 2B4, CD3 ζ , or DAP12. Recombinant vaccinia viruses encoding EE tagged wild type SAP, SAP SH2 point mutant (SAP.R32Q), wild type Syk, and kinase inactive (KI-Syk) were generated as described (Billadeau et al., 2003; Upshaw et al., 2005). All recombinant vaccinia viruses were made using the Western Reserve (WR) strain of *vaccinia* and the pSP11 recombination substrate, except WT-Syk and KI-Syk recombinant viruses, which were made using the pSC65 vaccinia recombination vector. NK cells were infected at a multiplicity of infection of 20:1 for 5 hours in serum free media, whereas Jurkat T cells and mutant sublines were infected at 10:1 for 2 hours.

2.5 Receptor expression, cell stimulation, immunoprecipitation, and immunoblot analysis

NK cells (clones) were infected with vaccinia virus encoding a chimeric receptor (e.g. F.CD4.2B4) for 5 hours. Chimeric receptor expression was monitored by flow cytometric surface staining. Cell stimulation, protein immunoprecipitation, and immunoblotting were performed as described (Upshaw et al., 2005). Immunoblots separated by a vertical line represent one film with intervening lanes spliced out.

2.6 Calcium mobilization assays

Intracellular calcium mobilization was measured using the ratiometric calcium indicator dye Indo-1A/M as described (Billadeau et al., 2000; Upshaw et al., 2005). Cells were stimulated with anti-2B4 or anti-CD4 antibodies and goat-anti-mouse F(AB)₂ fragment at the time indicated by the arrow in each figure.

2.7 Cytotoxicity assays

All cytotoxicity experiments were performed using NK cells cultured with ⁵¹Cr-loaded P815 target cells as described (Windebank et al., 1988) with the following modification. P815 cells were coated with monoclonal antisera specific for 2B4 (C1.7) or isotype control mouse monoclonal antibody. Endogenous 2B4-mediated cytotoxicity experiments were performed using NK cells screened for the capacity to initiate redirected antibody dependent cytotoxicity via anti-2B4 antibody at least 3 fold more than isotype control antibody. All cytotoxicity data was collected using several effector to target cell ratios in triplicate. Cytotoxicity data is presented at several effector to target ratios or as lytic units (LU). We calculated lytic units per 10⁶ cells on the basis of 20 percent cytotoxicity. Lytic units were normalized to the control sample to depict results from multiple experiments.

3. Results

3.1 NKp46 is required for 2B4-initiated cytotoxicity in a subset of NK cells

Sivori and colleagues demonstrated that 2B4-initiated cytolysis occurs most effectively in NK cells that express high levels of NKp46 (Sivori et al., 2000). However, this study relied on antibody mediated NKp46 internalization to remove NKp46 from the cell surface. We sought to confirm the requirement for NKp46 expression in 2B4-initiated cytolysis by specifically removing the NKp46 protein using siRNA. We treated IL-2 expanded human NK clones with an siRNA oligonucleotide duplex designed to specifically target NKp46 expression. We observed reduced NKp46 surface expression by flow cytometry (Fig. 1A, top panel) but 2B4 expression remained unaltered (Fig. 1A, bottom panel). NK cells suppressed for NKp46 were subjected to a chromium release assay against P815 target cells coated with anti-2B4. Removal of NKp46 substantially reduced the ability of some NK cell clones to initiate cytotoxicity via 2B4 (Fig. 1B) but not all NK cell clones (Fig. 1C). Clones one and two displayed dramatic reduction in 2B4-initiated killing, clones three and four demonstrated slight reduction, and clone five demonstrated no reduction. These data are presented in Figure 1C as lytic units (LU) normalized to the control sample in each experiment. This suggests multiple pathways can support 2B4-initiated signaling leading to cytolysis.

3.2 Cytotoxicity mediated by 2B4 is dependent upon ITAM-containing proteins

Because removal of a surface receptor removes the entire receptor complex including the associated ITAM adaptor molecules from the cell surface, we next investigated whether removal of the known ITAM-containing adaptors would likewise reduce 2B4-initiated cytotoxicity. The NKp46 complex consists of the NKp46 receptor, Fc ϵ RI γ , and CD3 ζ (Westgaard et al., 2004). Fc ϵ RI γ (γ) and CD3 ζ (ζ) are two of three ITAM-containing molecules expressed by NK cells. DAP12, the third ITAM-containing molecule, is present in other receptor complexes such as the NKG2C/CD94 complex. In order to test the role of these signaling adaptors in 2B4-initiated events, we examined NK cell function in the presence and absence of ITAM-containing proteins. We treated NK cells with siRNA duplexes (siGenome SMART pools) specific for Fc ϵ RI γ , CD3 ζ , and DAP12 and observed reduced expression of each ITAM-containing protein by western blot analysis (Fig. 1E, Supplemental Fig. 1A). We observed reduced NKp46 surface expression upon suppression of Fc ϵ RI γ , but not CD3 ζ or DAP12 (Fig. 1D). siRNA reduction of DAP12 was sufficient to reduce the surface levels of NKG2C, an NK activating receptor known to couple to DAP12 (data not shown). Likewise, suppression of CD3 ζ was sufficient to reduce surface levels of CD3 ζ in Jurkat T cells (data not shown). Suppression of Fc ϵ RI γ induced the most significant reduction in NKp46 surface expression (Fig. 1F).

We next examined the ability of NK cells suppressed for ITAM-containing proteins to initiate cytolysis via the 2B4 receptor. NK cells that killed through 2B4 were treated with siRNA duplexes to specifically suppress individual ITAM-containing proteins and assayed for their ability to kill P815 target cells coated with anti-2B4. To be complete, we tested the role of each ITAM-containing molecule in 2B4-initiated cytotoxicity. Indeed, consistent with a role for Fc ϵ RI γ in NKp46 surface expression, depletion of this ITAM adaptor, but not CD3 ζ or DAP12, appreciably affected 2B4-initiated cytolysis (Fig. 1G). Conversely, NKp46 expression is not required for ITAM-protein stability (Supplemental Fig. 1B,C). Taken together, our data suggest that 2B4-initiated cytolysis in NK cells relies on NKp46 surface expression in some clones, and relies on Fc ϵ RI γ in all clones tested.

ITAM adaptor proteins Fc ϵ RI γ and CD3 ζ , which are removed from the cell surface upon NKp46 internalization or siRNA mediated suppression, are also important for signal

transduction downstream of multiple NK cell receptors. FcεRIγ and CD3ζ are coupled to NKp46, NKp30, and CD16. FcεRIγ has additionally been reported to transduce activating signals from the KIR2DL4 receptor (Kikuchi-Maki et al., 2005). We therefore examined whether the FcεRIγ and CD3ζ associated receptor, CD16, is important for 2B4-initiated cytotoxicity. CD16 suppression does not alter 2B4-initiated cytotoxicity (Supplemental Figure 1D,E).

3.3 F.CD4.2B4 chimera accurately reflects endogenous 2B4 biology

In order to understand the mechanism by which 2B4 could be using ITAM molecules for signaling, we created a Flag epitope tagged F.CD4.2B4 chimeric receptor as described (Upshaw et al., 2005). This chimeric receptor consists of the human CD4 extracellular and transmembrane domains fused to the human 2B4 intracellular domain (Fig. 2A). We first sought to determine whether the F.CD4.2B4 chimera accurately reflects endogenous 2B4 biology. As described above, endogenous 2B4 associates with SAP via the SAP SH2 domain upon stimulation (Tangye et al., 1999). To examine this interaction with the chimeric receptor, we immunoprecipitated F.CD4.2B4 from unstimulated or pervanadate-stimulated NK cells and found that SAP was present only in the immunoprecipitates from stimulated cells (Fig. 2B). As expected, the SAP SH2 domain mutant (SAP.R32Q) did not co-immunoprecipitate with the chimeric receptor following pervanadate stimulation in COS cells (Fig. 2C). These data indicate that like endogenous 2B4 (Tangye et al., 1999), chimeric F.CD4.2B4 inducibly associates with SAP via the SAP SH2 domain upon pervanadate stimulation.

Downstream of SAP, endogenous 2B4 activates PLCγ leading to calcium mobilization critical for cytotoxic granule release. To determine whether the F.CD4.2B4 chimera provides an accurate model of endogenous 2B4 signal transduction, we compared the activation of PLCγ2 following stimulation of either endogenous 2B4 or the F.CD4.2B4 chimera. Stimulation of either receptor led to PLCγ2 tyrosine phosphorylation (Fig. 2D,E) in NK cells and the mobilization of intracellular calcium (Fig. 2F,G) in both NK and Jurkat T cells. 2B4 is expressed endogenously in some T cell subsets (Dupre et al., 2005; Sharifi et al., 2004) so we examined whether Jurkat T cells could be a good model to study 2B4 signaling using our chimeric receptor. Importantly, endogenous CD4, which is expressed at low levels in our Jurkat T cells (Supplemental Fig. 2D), does not contribute to signal transduction as control (WR) infected cells do not mobilize intracellular calcium in response to anti-CD4 crosslinking (Fig. 2G). Together, these data indicate that the F.CD4.2B4 chimeric receptor provides an accurate model of endogenous 2B4 signaling via SAP, PLCγ, and calcium mobilization.

3.4 2B4 signaling to PLCγ and calcium is dependent on surface expression of the TCR complex

NK cells express multiple activating receptors that interact with distinct ITAM bearing signaling adaptor proteins. T cells, on the other hand, have only one ITAM-associated immunoreceptor, the TCR. In order to gain a better understanding as to the broader use of ITAMs in 2B4 signaling, we examined 2B4 in context of the TCR. In fact, in stark contrast to CD4 crosslinking in (WR) control virus infected cells, crosslinking of the F.CD4.2B4 chimera induced activation of PLCγ1, although not to the extent seen with ligation of the TCR by OKT3 (Fig 3A). Consistent with the activation of PLCγ1, ligation of the F.CD4.2B4 chimera also induced calcium mobilization (Fig 3B). To investigate whether the presence of the TCR is required for 2B4 signal transduction, we utilized the TCRβ deficient Jurkat subline, Jrt3. These cells have significantly reduced cell surface expression of the entire TCR complex (Goldsmith and Weiss, 1987) although they express normal levels of CD3ζ (Supplemental Fig 2C). In contrast to the parental Jurkat line, Jrt3 cells were unable

to activate PLC γ 1 or stimulate robust calcium mobilization following F.CD4.2B4 crosslinking (Fig. 3A,B). Importantly, crosslinking of the 2B4 chimera resulted in PLC γ 1 activation and calcium mobilization in the TCR β reconstituted (PF2.4) cells (Fig. 3A,B). Since it was unclear whether it was merely TCR expression or a component of the CD3 complex that was involved in mediating 2B4 signaling, we suppressed CD3 ζ and examined the ability of the 2B4 chimera to induce calcium mobilization. Significantly, CD3 ζ suppression (Supplemental Fig. 2A,B) reduced calcium mobilization in response to F.CD4.2B4 stimulation (Fig. 3C, left). F.CD4.2B4 was expressed equivalently in all groups (Fig. 3C, right). Together, these data suggest that the TCR-ITAM complex is required for 2B4 signal transduction in Jurkat T cells, analogous to the requirement for ITAM complexes for 2B4-initiated cytotoxicity in NK cells.

3.6 Syk family kinases facilitate 2B4-induced calcium mobilization and cytotoxicity

Our data suggest that 2B4 utilizes ITAM-bearing immunoreceptor complexes containing CD3 ζ and Fc ϵ RI γ for signaling and cytotoxicity. We therefore explored whether proteins known to signal specifically downstream of ITAM-containing molecules are also involved in 2B4-initiated cytotoxicity. Syk is involved in natural cytotoxicity (Brumbaugh et al., 1997) but not NKG2D specific cytotoxicity (Billadeau et al., 2003). We postulated a specific role for Syk family kinases in human 2B4 function due to the dependence on ITAM molecules. We therefore inhibited both Syk and ZAP-70 using piceatannol and measured endogenous 2B4-mediated cytotoxicity. As can be seen in Figure 4A, piceatannol blocked 2B4-mediated cytotoxicity in a dose-dependent manner. However, piceatannol may have off-target effects. We therefore examined F.CD4.2B4 signaling in the P116 Jurkat mutant subline, which lacks the relevant Syk family kinase Zap70. Compared to wild type Jurkat cells, which mobilize calcium in response to crosslinking of the F.CD4.2B4 chimera, P116 cells were unable to mobilize calcium in response to F.CD4.2B4 crosslinking (Fig. 4B). This defect was restored in ZAP-70 reconstituted (P116.c40) cells (Fig. 4B) indicating a requirement for this kinase in mediating 2B4-initiated signaling to calcium mobilization.

In order to address the role of Syk in 2B4 events leading to cytotoxicity in NK cells, we expressed wild type Syk (WT-Syk) or a kinase inactive mutant of Syk (KI-Syk) and analyzed F.CD4.2B4-initiated cytotoxicity. Significantly, over expression of WT-Syk substantially increased the ability of NK cells to initiate cytotoxicity via F.CD4.2B4, whereas, KI-Syk was unable to enhance F.CD4.2B4-initiated cytotoxicity in NK cells (Fig. 4C). Together these data demonstrate that Syk family kinases are able to mediate 2B4 signal transduction and more broadly suggest the involvement of Syk family kinases in 2B4-initiated signaling utilizing ITAM-containing immunoreceptors.

3.6 2B4 and SAP associate with ITAM-containing receptors

We hypothesized a physical association in addition to the functional dependence between 2B4 and the ITAM complexes. Since SAP is needed for proximal signal transduction, cytokine production, and adhesion to B cells upon TCR stimulation (Cannons et al., 2004; Nakamura et al., 2001), we explored whether the 2B4-SAP complex is physically linked with ITAM-bearing immunoreceptors during signaling. To examine this, NK cells were left unstimulated or stimulated with the CD48 bearing target cell 721.221. Cell lysates were immunoprecipitated for CD3 ζ and probed for SAP. As shown in Figure 5A, there was detectable association of SAP with CD3 ζ which did not increase upon stimulation. This association is specific, since the F.CD4. ζ chimeric receptor associates with SAP whereas the F.CD4.DAP12 chimeric receptor does not (Fig. 5B). Additionally, NK cells expressing F.CD4. ζ demonstrated an interaction of F.CD4. ζ -SAP that was not further stimulated with pervanadate (Fig. 5C), suggesting that the interaction of SAP with the ITAM is constitutive. We next explored whether CD3 ζ physically interacts with 2B4. To test for this association,

NK cells were stimulated with the 721.221 target cell or were left unstimulated. Cell lysates were immunoprecipitated for CD3 ζ or normal rabbit serum (NRS) and probed for 2B4. Significantly, the 2B4 receptor co-immunoprecipitated with CD3 ζ without stimulation and this association increased upon stimulation with 721.221 cells (Fig. 5D). This interaction is specific because immunoprecipitation of DAP12 demonstrates no association between DAP12 and 2B4 (C.C.C. unpublished observations). Similar to endogenous 2B4, the chimera F.CD4.2B4 co-immunoprecipitates with CD3 ζ upon pervanadate stimulation (Fig. 5E). Taken together, these data suggest that CD3 ζ associates constitutively with SAP and inducibly with 2B4.

4. Discussion

While it is appreciated that 2B4 can act as a co-stimulatory molecule enhancing activating receptor signals (Bryceson et al., 2006a; Bryceson et al., 2006b; Chen et al., 2007), how these activating receptors might impact SLAM family activation are unclear. We utilized gene targeted cell lines and protein specific suppression to delineate molecules involved in 2B4 signal transduction and cytotoxicity. Here we demonstrate that 2B4 functionally links to ITAM-dependent NKp46 or TCR receptor complexes in NK cells or T cells, respectively. Our study suggests that signaling by the SLAM family member 2B4 utilizes ITAM adaptor molecules and Syk family kinases.

Based on our studies of human NK cells, one would predict that murine NK cells also rely on ITAM-containing adaptors to initiate cytolysis via 2B4. Indeed, NK cells from Fc ϵ RI γ , CD3 ζ , and DAP12 triple knockout mice are unable to initiate cytolysis against the CD48 bearing RMA and RMA-S target cells (Chiesa et al., 2006) suggesting the ITAM adaptors are utilized for 2B4-initiated cytolysis in mouse NK cells. SAP is also implicated in killing the RMA and RMA-S target cells (Bloch-Queyrat et al., 2005) further suggesting a collaboration between SAP and the ITAM adaptors for 2B4-initiated cytolysis. Chiesa and colleagues went on to identify which ITAM adaptors are utilized to kill the CD48-bearing RMA and RMA-S cells. Fc ϵ RI γ and CD3 ζ , are implicated in this murine model of 2B4 initiated cytolysis (Chiesa et al., 2006), mirroring our results using human NK and T cells.

We observed 2B4 dependence on NKp46 substantially in two of five NK clones tested (Fig 1C) and dependence on Fc ϵ RI γ in all of the clones tested (11 of 11). This implies the NKp46-Fc ϵ RI γ -CD3 ζ complex is important, but also suggests additional roles for Fc ϵ RI γ . In fact, suppression of the common signaling adaptor, Fc ϵ RI γ , has a more substantial defect on 2B4-initiated cytolysis compared to suppression of individual NKp46 or CD16 receptors in NK cells. Fc ϵ RI γ and CD3 ζ couple with several cell surface receptors, including NKp46, NKp30, CD16 and KIR2DL4. In T cells the situation is simplified since the TCR is the only ITAM containing receptor complex and removal of the TCR-CD3 complex from the cell surface by reducing TCR β is sufficient to observe 2B4 signaling defects. While we consistently observed reduced calcium mobilization in Jurkat cells suppressed for CD3 ζ , the defect was never as pronounced as in the Jrt3 cell line. Thus, it remains possible that either the level of CD3 ζ suppression was not optimal or that the remaining ITAM molecules in the TCR-CD3 complex can also functionally couple to 2B4 in the absence of CD3 ζ .

It has recently been shown that TCR crosslinking can result in the tyrosine phosphorylation of SLAM family members. In fact, TCR stimulation induces SLAM (CD150) phosphorylation and TCR-SLAM colocalization (Howie et al., 2002), as well as SAP-NTB-A association and TCR-NTB-A colocalization (Snow et al., 2009). Interestingly, XLP patient T cells are unable to colocalize the TCR with NTB-A (Snow et al., 2009) suggesting that TCR-stimulated TCR-NTB-A colocalization is SAP mediated. SAP deficient T cells were also unable to polarize 2B4, perforin, and granzymes to CD48 bearing targets (Dupre

et al., 2005). Our data identify that 2B4 and SAP biochemically associate with activating immunoreceptor component CD3 ζ , which bolsters microscopy colocalization studies asserting an association between other SLAM family members and the TCR. Therefore SAP binding to 2B4 and ITAMs may regulate 2B4 recruitment to the cytotoxic synapse.

Outside the SLAM family, CD59 is a GPI-anchored receptor that couples with NKp46 but not CD16 (Marcenaro et al., 2003). Complement protein 8 (C8)-induced CD59 clusters recruit single molecules of PLC γ 2 for IP₃ generation and calcium release (Suzuki et al., 2007a; Suzuki et al., 2007b). Suzuki and colleagues hypothesized this small PLC γ 2 signal may be stabilized under certain conditions to yield a more robust calcium response (Suzuki et al., 2007a). We demonstrate that 2B4 couples with NKp46 but not CD16 (Supplemental Figure 1D,E), providing a potential platform for CD59 signal amplification.

SLAM family members including 2B4 are known co-stimulatory molecules in both NK cells and T cells when both types of receptor are ligated. Here, we characterize 2B4-specific cytotoxicity by co-incubating NK cells with P815 target cells coated with anti-2B4 antibody. Co-incubation with P815 cells may provide the natural ligand for other receptors and it has been postulated that P815 cells contain a ligand for NKp46 (Sivori et al., 1999). In fact, depletion of NKp46 decreases basal killing of P815 cells by NK cells (A.T.B. and D.D.B., unpublished observation). Thus in this system, 2B4 is likely acting as a co-stimulatory receptor with NKp46, however, our results in the Jurkat mutant sublines where we initiated signaling exclusively through the chimeric F.CD4.2B4 receptor indicate that 2B4 needs TCR, CD3 ζ and ZAP-70 molecules to couple to PLC γ 1 activation and calcium mobilization. Importantly, these findings are specific for 2B4 signaling, evidenced by the lack of signals generated in control WR vaccinia virus infected cells. Therefore 2B4 signal transduction in the absence of target cells represents 2B4 utilization of the ITAM pathway independent from “costimulatory” signaling.

Pessino and colleagues (Pessino et al., 1998) show that NKp46 can be driven to express at the surface of COS cells independently of ITAM adaptor expression, but did not address whether either ITAM adaptor is responsible for trafficking endogenous NKp46. Fc ϵ RI γ may be crucial for transport or maintenance of NKp46 at the cell surface, whereas CD3 ζ exists in the NKp46 receptor complex but is not critical for NKp46 expression. This possibility is the natural prediction of two studies by Verneris and colleagues who compared NKp46 expression in umbilical cord blood cell populations, noting low expression in Fc ϵ RI γ ^{low} cells (Grzywacz et al., 2006; Tang et al., 2008). We observed almost complete NKp46 surface reduction upon Fc ϵ RI γ suppression but only minimally upon CD3 ζ suppression (Fig. 1F), although CD3 ζ suppression was sufficient to reduce surface CD3 ϵ expression 2 fold (A.T.B. and D.D.B. unpublished observation). These studies suggest the Fc ϵ RI γ and CD3 ζ adaptor proteins likely have unique roles in receptor expression and signal transduction.

Ultimately the role for the ITAM adaptors downstream of 2B4 ligation is an open question. Izawa and colleagues postulated ITAM adaptors confer an activating capability upon SLAM family member LMIR3 ligation (Izawa et al., 2009). What precise capability that would mean for 2B4 is not known. Syk and Zap-70 deficient mouse NK cells have unaltered ability to initiate cytolysis via 2B4 (Colucci et al., 2002; Colucci et al., 1999). This discrepancy may be due to the presence of one 2B4 isoform (long) in humans and two isoforms (long and short) in mice (Stepp et al., 1999). Syk may play a dominant role in human NK cells, while Syk may work together with other kinases for mouse natural cytotoxicity. Colucci and colleagues identified an additive role for Syk and Src kinases in mouse natural cytotoxicity (Colucci et al., 2002). We demonstrate a role for Syk/Zap-70 in human 2B4 signaling and cytolysis. Furthermore, Syk mediates SLAM (CD150)-stimulated AKT phosphorylation

(Mikhalap et al., 2004). Based on these results, we postulate that molecules associated with the ITAM complex (such as Syk/ZAP-70) are brought into proximity with molecules associated with the 2B4 complex (such as LAT (Bottino et al., 2000; Klem et al., 2002)) upon cellular stimulation. In fact, CD2 and CD48 recruit LAT to the TCR (Muhammad et al., 2009).

We envision several outcomes from the hypothetical coupling of the 2B4/LAT (Bottino et al., 2000; Klem et al., 2002) and SAP/ITAM/Syk complexes. Cannons and colleagues (Cannons et al.) have shown a selective adhesion defect in SAP deficient T cells upon TCR stimulation. Of note, ITAM containing molecules are known to regulate adhesion in a number of systems (Abtahian et al., 2006; Ivashkiv, 2009; Jakus et al., 2007; Mocsai et al., 2006; Mocsai et al., 2004). Therefore we predict a defect in 2B4-mediated adhesion in the absence of SAP and ITAM adaptors, consistent with the calcium mobilization and cytotoxicity defects. Indeed, calcium mobilization in NK cells is required for both integrin-mediated adhesion (Onley et al., 2000) to target cells and granule release. Alternatively, ITAM adaptors may regulate the adhesion properties of 2B4 itself. Indeed, 2B4 is a member of the CD2 adhesion receptor family.

The outcome of the 2B4-ITAM relationship would be clarified if we observed ITAM phosphorylation upon 2B4 stimulation. If 2B4 induces ITAM phosphorylation, one would expect 2B4 to activate proximal signaling and cytotoxicity as efficiently as traditional ITAM-dependent receptors such as CD16. This expectation is not the case. If 2B4 signals via the ITAM adaptors using a mechanism distinct from traditional ITAM-dependent receptor CD16, then one would predict unique 2B4 signaling and cytotoxic function compared to CD16 stimulation. This is the case, as 2B4 crosslinkage induces much smaller amplitude proximal signals and cytotoxicity compared to CD16. Therefore 2B4 utilizes ITAM adaptor proteins distinct from traditional ITAM-dependent CD16. Our findings are analogous to those identified downstream of the CXCR4 chemokine receptor, which binds SDF-1 α and utilizes the TCR to transduce calcium mobilization and prolonged Erk activation independent from detectably enhanced ITAM phosphorylation (Kumar et al., 2006).

We envision that 2B4 utilizes the constitutively phosphorylated ITAM adaptor molecules (van Oers et al., 1993) for low amplitude signaling. Low amplitude ITAM signals utilize Syk (Chen et al., 2008) and ZAP-70 (Kremer et al., 2003) as we have observed with 2B4. Perhaps these submaximally phosphorylated ITAM adaptors confer the ability to calibrate 2B4 receptor stimulation (Chlewicki et al., 2008) and distinguish between CD48^{low} hematopoietic cells from CD48^{high} EBV-infected cells. That is, perhaps submaximally phosphorylated ITAM adaptors do not transduce signals until they are clustered with sufficient 2B4 aggregation by CD48^{high} EBV transformed cells.

Dissecting these potential roles for ITAM adaptors in SLAM family signaling will provide further understanding of the 2B4 activating versus inhibitory balance that has stymied researchers for years. If SAP indeed links 2B4 with ITAM adaptors for positive signaling, another intriguing possibility is that EAT-2 prohibit SAP-ITAM association and thus keep 2B4 in an inhibitory state. Taken together, our findings illustrate the interconnected nature of receptor networks and provide some guidance to therapeutic development for NK and T cell responses in XLPD patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Abtahian F, Bezman N, Clemens R, Sebzda E, Cheng L, Shattil SJ, Kahn ML, Koretzky GA. Evidence for the requirement of ITAM domains but not SLP-76/Gads interaction for integrin signaling in hematopoietic cells. *Mol Cell Biol.* 2006; 26:6936–49. [PubMed: 16943434]
- Arnon TI, Achdout H, Lieberman N, Gazit R, Gonen-Gross T, Katz G, Bar-Ilan A, Bloushtain N, Lev M, Joseph A, Kedar E, Porgador A, Mandelboim O. The mechanisms controlling the recognition of tumor- and virus-infected cells by NKp46. *Blood.* 2004; 103:664–72. [PubMed: 14504081]
- Augugliaro R, Parolini S, Castriconi R, Marcenaro E, Cantoni C, Nanni M, Moretta L, Moretta A, Bottino C. Selective cross-talk among natural cytotoxicity receptors in human natural killer cells. *Eur J Immunol.* 2003; 33:1235–41. [PubMed: 12731048]
- Biassoni R, Pessino A, Bottino C, Pende D, Moretta L, Moretta A. The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur J Immunol.* 1999; 29:1014–20. [PubMed: 10092106]
- Billadeau DD, Mackie SM, Schoon RA, Leibson PJ. Specific subdomains of Vav differentially affect T cell and NK cell activation. *J Immunol.* 2000; 164:3971–81. [PubMed: 10754287]
- Billadeau DD, Upshaw JL, Schoon RA, Dick CJ, Leibson PJ. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol.* 2003; 4:557–64. [PubMed: 12740575]
- Bloch-Queyrat C, Fondaneche MC, Chen R, Yin L, Relouzat F, Veillette A, Fischer A, Latour S. Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn. *J Exp Med.* 2005; 202:181–92. [PubMed: 15998796]
- Bottino C, Augugliaro R, Castriconi R, Nanni M, Biassoni R, Moretta L, Moretta A. Analysis of the molecular mechanism involved in 2B4-mediated NK cell activation: evidence that human 2B4 is physically and functionally associated with the linker for activation of T cells. *Eur J Immunol.* 2000; 30:3718–22. [PubMed: 11169415]
- Brumbaugh KM, Binstadt BA, Billadeau DD, Schoon RA, Dick CJ, Ten RM, Leibson PJ. Functional role for Syk tyrosine kinase in natural killer cell-mediated natural cytotoxicity. *J Exp Med.* 1997; 186:1965–74. [PubMed: 9396765]
- Bryceson YT, March ME, Ljunggren HG, Long EO. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev.* 2006a; 214:73–91. [PubMed: 17100877]
- Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood.* 2006b; 107:159–66. [PubMed: 16150947]
- Cannons JL, Qi H, Lu KT, Dutta M, Gomez-Rodriguez J, Cheng J, Wakeland EK, Germain RN, Schwartzberg PL. Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity.* 32:253–65. [PubMed: 20153220]
- Cannons JL, Yu LJ, Hill B, Mijares LA, Dombroski D, Nichols KE, Antonellis A, Koretzky GA, Gardner K, Schwartzberg PL. SAP regulates T(H)2 differentiation and PKC-theta-mediated activation of NF-kappaB1. *Immunity.* 2004; 21:693–706. [PubMed: 15539155]
- Chen L, Monti S, Juszczynski P, Daley J, Chen W, Witzig TE, Habermann TM, Kutok JL, Shipp MA. SYK-dependent tonic B-cell receptor signaling is a rational treatment target in diffuse large B-cell lymphoma. *Blood.* 2008; 111:2230–7. [PubMed: 18006696]
- Chen R, Relouzat F, Roncagalli R, Aoukaty A, Tan R, Latour S, Veillette A. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol Cell Biol.* 2004; 24:5144–56. [PubMed: 15169881]
- Chen X, Trivedi PP, Ge B, Krzewski K, Strominger JL. Many NK cell receptors activate ERK2 and JNK1 to trigger microtubule organizing center and granule polarization and cytotoxicity. *Proc Natl Acad Sci U S A.* 2007; 104:6329–34. [PubMed: 17395718]

- Chiesa S, Mingueneau M, Fuseri N, Malissen B, Raulet DH, Malissen M, Vivier E, Tomasello E. Multiplicity and plasticity of natural killer cell signaling pathways. *Blood*. 2006; 107:2364–72. [PubMed: 16291591]
- Chlewicki LK, Velikovskiy CA, Balakrishnan V, Mariuzza RA, Kumar V. Molecular basis of the dual functions of 2B4 (CD244). *J Immunol*. 2008; 180:8159–67. [PubMed: 18523281]
- Colucci F, Schweighoffer E, Tomasello E, Turner M, Ortaldo JR, Vivier E, Tybulewicz VL, Di Santo JP. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat Immunol*. 2002; 3:288–94. [PubMed: 11836527]
- Colucci F, Turner M, Schweighoffer E, Guy-Grand D, Di Bartolo V, Salcedo M, Tybulewicz VL, Di Santo JP. Redundant role of the Syk protein tyrosine kinase in mouse NK cell differentiation. *J Immunol*. 1999; 163:1769–74. [PubMed: 10438908]
- Dupre L, Andolfi G, Tangye SG, Clementi R, Locatelli F, Arico M, Aiuti A, Roncarolo MG. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. *Blood*. 2005; 105:4383–9. [PubMed: 15677558]
- Eissmann P, Beauchamp L, Wooters J, Tilton JC, Long EO, Watzl C. Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244). *Blood*. 2005; 105:4722–9. [PubMed: 15713798]
- Garg A, Barnes PF, Porgador A, Roy S, Wu S, Nanda JS, Griffith DE, Girard WM, Rawal N, Shetty S, Vankayalapati R. Vimentin expressed on Mycobacterium tuberculosis-infected human monocytes is involved in binding to the Nkp46 receptor. *J Immunol*. 2006; 177:6192–8. [PubMed: 17056548]
- Goldsmith MA, Weiss A. Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. *Proc Natl Acad Sci U S A*. 1987; 84:6879–83. [PubMed: 3309950]
- Grzywacz B, Kataria N, Sikora M, Oostendorp RA, Dzierzak EA, Blazar BR, Miller JS, Verneris MR. Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells. *Blood*. 2006; 108:3824–33. [PubMed: 16902150]
- Gu C, Tangye SG, Sun X, Luo Y, Lin Z, Wu J. The X-linked lymphoproliferative disease gene product SAP associates with PAK-interacting exchange factor and participates in T cell activation. *Proc Natl Acad Sci U S A*. 2006; 103:14447–52. [PubMed: 16983070]
- Howie D, Simarro M, Sayos J, Guirado M, Sancho J, Terhorst C. Molecular dissection of the signaling and costimulatory functions of CD150 (SLAMF7): CD150/SAP binding and CD150-mediated costimulation. *Blood*. 2002; 99:957–65. [PubMed: 11806999]
- Ivashkiv LB. Cross-regulation of signaling by ITAM-associated receptors. *Nat Immunol*. 2009; 10:340–7. [PubMed: 19295630]
- Izawa K, Kitaura J, Yamanishi Y, Matsuoka T, Kaitani A, Sugiuchi M, Takahashi M, Maehara A, Enomoto Y, Oki T, Takai T, Kitamura T. An activating and inhibitory signal from an inhibitory receptor LMIR3/CLM-1: LMIR3 augments lipopolysaccharide response through association with FcRgamma in mast cells. *J Immunol*. 2009; 183:925–36. [PubMed: 19561101]
- Jakus Z, Fodor S, Abram CL, Lowell CA, Mocsai A. Immunoreceptor-like signaling by beta 2 and beta 3 integrins. *Trends Cell Biol*. 2007; 17:493–501. [PubMed: 17913496]
- Jarahian M, Watzl C, Fournier P, Arnold A, Djandji D, Zahedi S, Cerwenka A, Paschen A, Schirmacher V, Momburg F. Activation of natural killer cells by newcastle disease virus hemagglutinin-neuraminidase. *J Virol*. 2009; 83:8108–21. [PubMed: 19515783]
- Kikuchi-Maki A, Catina TL, Campbell KS. Cutting edge: KIR2DL4 transduces signals into human NK cells through association with the Fc receptor gamma protein. *J Immunol*. 2005; 174:3859–63. [PubMed: 15778339]
- Kim HS, Das A, Gross CC, Bryceson YT, Long EO. Synergistic signals for natural cytotoxicity are required to overcome inhibition by c-Cbl ubiquitin ligase. *Immunity*. 2005; 22:175–86. [PubMed: 16189481]
- Klem J, Verrett PC, Kumar V, Schatzle JD. 2B4 is constitutively associated with linker for the activation of T cells in glycolipid-enriched microdomains: properties required for 2B4 lytic function. *J Immunol*. 2002; 169:55–62. [PubMed: 12077228]
- Kremer KN, Humphreys TD, Kumar A, Qian NX, Hedin KE. Distinct role of ZAP-70 and Src homology 2 domain-containing leukocyte protein of 76 kDa in the prolonged activation of

- extracellular signal-regulated protein kinase by the stromal cell-derived factor-1 α /CXCL12 chemokine. *J Immunol.* 2003; 171:360–7. [PubMed: 12817019]
- Kumar A, Humphreys TD, Kremer KN, Bramati PS, Bradfield L, Edgar CE, Hedin KE. CXCR4 physically associates with the T cell receptor to signal in T cells. *Immunity.* 2006; 25:213–24. [PubMed: 16919488]
- Li C, Schibli D, Li SS. The XLP syndrome protein SAP interacts with SH3 proteins to regulate T cell signaling and proliferation. *Cell Signal.* 2009; 21:111–9. [PubMed: 18951976]
- Marcenaro E, Augugliaro R, Falco M, Castriconi R, Parolini S, Sivori S, Romeo E, Millo R, Moretta L, Bottino C, Moretta A. CD59 is physically and functionally associated with natural cytotoxicity receptors and activates human NK cell-mediated cytotoxicity. *Eur J Immunol.* 2003; 33:3367–76. [PubMed: 14635045]
- Mikhalap SV, Shlapatska LM, Yurchenko OV, Yurchenko MY, Berdova GG, Nichols KE, Clark EA, Sidorenko SP. The adaptor protein SH2D1A regulates signaling through CD150 (SLAMF6) in B cells. *Blood.* 2004; 104:4063–70. [PubMed: 15315965]
- Mocsai A, Abram CL, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol.* 2006; 7:1326–33. [PubMed: 17086186]
- Mocsai A, Humphrey MB, Van Ziffle JA, Hu Y, Burghardt A, Spusta SC, Majumdar S, Lanier LL, Lowell CA, Nakamura MC. The immunomodulatory adapter proteins DAP12 and Fc receptor gamma-chain (FcRgamma) regulate development of functional osteoclasts through the Syk tyrosine kinase. *Proc Natl Acad Sci U S A.* 2004; 101:6158–63. [PubMed: 15073337]
- Morra M, Simarro-Grande M, Martin M, Chen AS, Lanyi A, Silander O, Calpe S, Davis J, Pawson T, Eck MJ, Sumegi J, Engel P, Li SC, Terhorst C. Characterization of SH2D1A missense mutations identified in X-linked lymphoproliferative disease patients. *J Biol Chem.* 2001; 276:36809–16. [PubMed: 11477068]
- Muhammad A, Schiller HB, Forster F, Eckerstorfer P, Geyeregger R, Leksa V, Zlabinger GJ, Sibilia M, Sonnleitner A, Paster W, Stockinger H. Sequential cooperation of CD2 and CD48 in the buildup of the early TCR signalosome. *J Immunol.* 2009; 182:7672–80. [PubMed: 19494291]
- Nakajima H, Cella M, Bouchon A, Grierson HL, Lewis J, Duckett CS, Cohen JI, Colonna M. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur J Immunol.* 2000; 30:3309–18. [PubMed: 11093147]
- Nakamura H, Zarycki J, Sullivan JL, Jung JU. Abnormal T cell receptor signal transduction of CD4 Th cells in X-linked lymphoproliferative syndrome. *J Immunol.* 2001; 167:2657–65. [PubMed: 11509608]
- Onley DJ, Knight CG, Tuckwell DS, Barnes MJ, Farndale RW. Micromolar Ca^{2+} concentrations are essential for Mg^{2+} -dependent binding of collagen by the integrin $\alpha 2\beta 1$ in human platelets. *J Biol Chem.* 2000; 275:24560–4. [PubMed: 10827198]
- Parolini S, Bottino C, Falco M, Augugliaro R, Giliani S, Franceschini R, Ochs HD, Wolf H, Bonnefoy JY, Biassoni R, Moretta L, Notarangelo LD, Moretta A. X-linked lymphoproliferative disease. 2B4 molecules displaying inhibitory rather than activating function are responsible for the inability of natural killer cells to kill Epstein-Barr virus-infected cells. *J Exp Med.* 2000; 192:337–46. [PubMed: 10934222]
- Pessino A, Sivori S, Bottino C, Malaspina A, Morelli L, Moretta L, Biassoni R, Moretta A. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med.* 1998; 188:953–60. [PubMed: 9730896]
- Roda-Navarro P, Mittelbrunn M, Ortega M, Howie D, Terhorst C, Sanchez-Madrid F, Fernandez-Ruiz E. Dynamic redistribution of the activating 2B4/SAP complex at the cytotoxic NK cell immune synapse. *J Immunol.* 2004; 173:3640–6. [PubMed: 15356108]
- Roncagalli R, Taylor JE, Zhang S, Shi X, Chen R, Cruz-Munoz ME, Yin L, Latour S, Veillette A. Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor. *Nat Immunol.* 2005; 6:1002–10. [PubMed: 16127454]
- Sayos J, Nguyen KB, Wu C, Stepp SE, Howie D, Schatzle JD, Kumar V, Biron CA, Terhorst C. Potential pathways for regulation of NK and T cell responses: differential X-linked

- lymphoproliferative syndrome gene product SAP interactions with SLAM and 2B4. *Int Immunol.* 2000; 12:1749–57. [PubMed: 11099315]
- Sharifi R, Sinclair JC, Gilmour KC, Arkwright PD, Kinnon C, Thrasher AJ, Gaspar HB. SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease. *Blood.* 2004; 103:3821–7. [PubMed: 14726378]
- Sivori S, Parolini S, Falco M, Marcenaro E, Biassoni R, Bottino C, Moretta L, Moretta A. 2B4 functions as a co-receptor in human NK cell activation. *Eur J Immunol.* 2000; 30:787–93. [PubMed: 10741393]
- Sivori S, Pende D, Bottino C, Marcenaro E, Pessino A, Biassoni R, Moretta L, Moretta A. NKp46 is the major triggering receptor involved in the natural cytotoxicity of fresh or cultured human NK cells. Correlation between surface density of NKp46 and natural cytotoxicity against autologous, allogeneic or xenogeneic target cells. *Eur J Immunol.* 1999; 29:1656–66. [PubMed: 10359120]
- Sivori S, Vitale M, Morelli L, Sanseverino L, Augugliaro R, Bottino C, Moretta L, Moretta A. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med.* 1997; 186:1129–36. [PubMed: 9314561]
- Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, van Hoff J, Dhar D, Nichols KE, Filipovich AH, Su HC, Bleasing JJ, Lenardo MJ. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest.* 2009; 119:2976–89. [PubMed: 19759517]
- Stepp SE, Schatzle JD, Bennett M, Kumar V, Mathew PA. Gene structure of the murine NK cell receptor 2B4: presence of two alternatively spliced isoforms with distinct cytoplasmic domains. *Eur J Immunol.* 1999; 29:2392–9. [PubMed: 10458751]
- Suzuki KG, Fujiwara TK, Edidin M, Kusumi A. Dynamic recruitment of phospholipase C gamma at transiently immobilized GPI-anchored receptor clusters induces IP3-Ca2+ signaling: single-molecule tracking study 2. *J Cell Biol.* 2007a; 177:731–42. [PubMed: 17517965]
- Suzuki KG, Fujiwara TK, Sanematsu F, Iino R, Edidin M, Kusumi A. GPI-anchored receptor clusters transiently recruit Lyn and G alpha for temporary cluster immobilization and Lyn activation: single-molecule tracking study 1. *J Cell Biol.* 2007b; 177:717–30. [PubMed: 17517964]
- Tang Q, Grzywacz B, Wang H, Kataria N, Cao Q, Wagner JE, Blazar BR, Miller JS, Verneris MR. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only NKp30 is functional. *J Immunol.* 2008; 181:4507–15. [PubMed: 18802053]
- Tangye SG, Lazetic S, Woollatt E, Sutherland GR, Lanier LL, Phillips JH. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J Immunol.* 1999; 162:6981–5. [PubMed: 10358138]
- Tassi I, Colonna M. The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells. *J Immunol.* 2005; 175:7996–8002. [PubMed: 16339536]
- Ting AT, Karnitz LM, Schoon RA, Abraham RT, Leibson PJ. Fc gamma receptor activation induces the tyrosine phosphorylation of both phospholipase C (PLC)-gamma 1 and PLC-gamma 2 in natural killer cells. *J Exp Med.* 1992; 176:1751–5. [PubMed: 1281218]
- Upshaw JL, Schoon RA, Dick CJ, Billadeau DD, Leibson PJ. The isoforms of phospholipase C-gamma are differentially used by distinct human NK activating receptors. *J Immunol.* 2005; 175:213–8. [PubMed: 15972651]
- van Oers NS, Tao W, Watts JD, Johnson P, Aebersold R, Teh HS. Constitutive tyrosine phosphorylation of the T-cell receptor (TCR) zeta subunit: regulation of TCR-associated protein tyrosine kinase activity by TCR zeta. *Mol Cell Biol.* 1993; 13:5771–80. [PubMed: 7689151]
- Wang D, Feng J, Wen R, Marine JC, Sangster MY, Parganas E, Hoffmeyer A, Jackson CW, Cleveland JL, Murray PJ, Ihle JN. Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. *Immunity.* 2000; 13:25–35. [PubMed: 10933392]
- Westgaard IH, Berg SF, Vaage JT, Wang LL, Yokoyama WM, Disson E, Fossum S. Rat NKp46 activates natural killer cell cytotoxicity and is associated with FcepsilonRIgamma and CD3zeta. *J Leukoc Biol.* 2004; 76:1200–6. [PubMed: 15356098]

Windebank KP, Abraham RT, Powis G, Olsen RA, Barna TJ, Leibson PJ. Signal transduction during human natural killer cell activation: inositol phosphate generation and regulation by cyclic AMP. *J Immunol.* 1988; 141:3951–7. [PubMed: 2846696]

FIGURE 1.

NKp46 immunoreceptor complex is required for 2B4-initiated cytotoxicity in some NK cells. (A) NK cells were electroporated with siRNA specific for NKp46. NKp46 protein reduction is evident by NKp46 surface staining and flow cytometric analysis (top). 2B4 surface expression was not affected (bottom). (B,C) NK cells treated as in (A) were subjected to a standard chromium release assay using P815 targets coated with anti-2B4 (C1.7). Chromium release results from one experiment are depicted at several effector – target cell ratios (B), and from 5 experiments represented as lytic units (LU) normalized to the siNeg control (C). (D-G), NK cells were electroporated with siRNA specific for FcεRIγ, CD3ζ, or DAP12. (D) The electroporated cells were stained for NKp46 surface expression

and analyzed by flow cytometry. One representative experiment of five is shown. (E) Protein suppression was analyzed by immunoblotting. One representative of five to eleven experiments is shown. (F) The NKp46 MFI for each sample was compared to the NKp46 MFI for the negative control sample. These MFI pairs from over five experiments were compared and a p-value was calculated using the Wilcoxon-Matched-Pairs-Test. *Indicates p-value = 0.001. Results were normalized to siNeg control. (G) NK cells suppressed for ITAM proteins were subjected to a chromium release assay as in (A). The result (LU) for each experimental sample was compared to the siNeg control sample. These LU pairs from the indicated number of experiments were compared and a p-value was calculated using the Wilcoxon-Matched-Pairs-Test. **indicates p = 0.02. Results were normalized to the siNeg control.

FIGURE 2.

F.CD4.2B4 provides an accurate depiction of endogenous 2B4 signaling. (A) Schematic depicting the chimeric 2B4 (F.CD4.2B4) domain structure. (B) NK cells were infected with the chimeric 2B4 (F.CD4.2B4) virus for 5hr and then were stimulated for 5min with pervanadate (PV). Cell lysates were immunoprecipitated with anti-FLAG or control IgG antibody and the immunoblot was probed sequentially for SAP and phosphotyrosine (p-Tyr) (F.CD4.2B4). (C) COS cells were infected with vaccinia virus encoding the chimeric 2B4 (F.CD4.2B4) and either wild type SAP or SH2 mutant (SAP.R32Q) for 2hr. Cells were stimulated for 5min with PV. Cell lysates were immunoprecipitated for anti-SAP or normal rabbit serum and probed sequentially for FLAG and SAP. (D) NK cells were stimulated with anti-2B4 (C1.7) or positive control anti-FcR (3G8) / goat anti mouse antibodies for 1 or 5 min. Cell lysates were immunoprecipitated with anti-PLCγ2 antibody and immunoblots were probed sequentially for p-Tyr and PLCγ2. (E) NK cells expressing F.CD4.2B4 were stimulated as indicated and immunoblots were probed sequentially for p-Tyr and PLCγ2.

(F) Endogenous 2B4 expressing NK cells were stimulated with anti-2B4 (C1.7)/goat anti-mouse antibodies and calcium mobilization was measured using Indo-1 labeling and flow cytometry. (G) Jurkat cells expressing F.CD4.2B4 or control vaccinia virus WR (Western Reserve) were stimulated with anti-CD4/goat anti-mouse antibodies, and analyzed as in (F).

FIGURE 3.

TCR immunoreceptor complex is required for 2B4-initiated PLC γ activation and calcium mobilization. (A) Jurkat, Jrt3 (TCR β deficient), and PF2.4 (TCR β reconstituted) cells expressing F.CD4.2B4 were stimulated with anti-CD3 or anti-CD4 / goat anti-mouse antibodies for the indicated time. Cell lysates were immunoprecipitated for PLC γ 1 and immunoblots were sequentially probed for p-Tyr and PLC γ 1. Results are representative of three independent experiments. (B) The indicated cells expressing F.CD4.2B4 or WR control virus were stimulated with anti-CD4 and calcium mobilization was measured. Cells were phenotyped for receptor expression (lower right). Histograms represent F.CD4.2B4 infected cells as follows: isotype, dark shade; Jurkat, black line; Jrt3, light shade; PF2.4,

dotted line. Results represent one of four experiments. (C) Jurkat cells were electroporated with two distinct siRNA oligos specific for CD3 ζ (ζ -1 or ζ -2) or siNeg. 24 hours after electroporation, each group was infected with either F.CD4.2B4 or WR vaccinia viruses, stimulated with anti-CD4, and calcium mobilization was measured (left). Jurkat cells were stained for CD4 surface expression (right). Histograms represent: isotype, dark shade; siNeg, black line; si ζ -1, light shade; si ζ -2, dotted line. Results depict one experiment representative of two experiments.

FIGURE 4.

Syk family kinases facilitate 2B4-induced calcium mobilization and cytotoxicity. (A) NK cells were treated with the indicated concentrations of piceatannol for 30 minutes prior to a standard chromium release assay using P815 target cells coated with anti-2B4. Results depict normalized LU from four to six experiments. The LU value for the piceatannol treated cells was compared to the control treated cells and a p-value was calculated using the two-tailed student's t-test. * indicates $p = 0.05$. ** indicates $p = 0.04$. *** indicates $p = 0.03$. (B) Jurkat, P116 (Zap-70 deficient), and P116.C40 (Zap-70 reconstituted) cells expressing F.CD4.2B4 or WR control virus were stimulated with anti-CD4 and monitored for calcium mobilization. Cells were phenotyped for receptor expression. Histograms represent F.CD4.2B4 infected cells as follows: isotype, dark shade; Jurkat, black line; P116, light shade; P116.C40, dotted line. Results depict one of three representative experiments. (C) NK cells infected with recombinant vaccinia virus expressing psc65 control, wild type Syk (WT Syk), or kinase inactive Syk (KI Syk), were subjected to a standard chromium release assay against P815 target cells coated with anti-2B4. Results depict LU normalized to the control psc65 sample for four NK clones from two different donors.

FIGURE 5.

SAP constitutively associates with ITAM-containing receptors. (A) NK-92 cells were stimulated with 721 target cells for 5 min. Cell lysates were immunoprecipitated with anti- ζ or normal rabbit serum (NRS). The immunoblot was probed sequentially for SAP or ζ . (B) COS-1 cells were infected with vaccinia virus expressing the chimeric receptors F.CD4. ζ or F.CD4.DAP12. Cell lysates were immunoprecipitated with anti-SAP or NRS, and immunoblots were probed sequentially for Flag and EE (SAP). (C) NK cells expressing F.CD4. ζ were treated with PV for 5 min. Cell lysates were immunoprecipitated with anti-Flag or isotype control, and the immunoblot was probed sequentially for SAP and pTyr to detect phosphorylated F.CD4. ζ . (D) NK92 cells were stimulated with fixed 721 target cells for 5 minutes, and cell lysates were immunoprecipitated with anti- ζ or NRS. The immunoblot was probed sequentially for 2B4, pTyr, and ζ . (E) Jurkat cells expressing F.CD4.2B4 were lysed, immunoprecipitated with anti- ζ or NRS, and probed for Flag and ζ .