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CUTTING EDGE

Cutting Edge: Functional Requirement for SAP in 2B4-Mediated Activation of Human Natural Killer Cells as Revealed by the X-Linked Lymphoproliferative Syndrome[1]

Stuart G. Tangye,[2]*† Joseph H. Phillips,† Lewis L. Lanier,‡ and Kim E. Nichols§

X-linked lymphoproliferative syndrome (XLP) is an immunodeficiency characterized by life-threatening infectious mononucleosis and EBV-induced B cell lymphoma. The gene mutated in XLP encodes SLAM (signaling lymphocytic activation molecule-associated protein)-associated protein (SAP), a small SH2 domain-containing protein. SAP associates with 2B4 and SLAM, activating receptors expressed by NK and T cells, and prevents recruitment of SH2 domain-containing protein tyrosine phosphatase-2 SHP-2) to the cytoplasmic domains of these receptors. The phenotype of XLP may therefore result from perturbed signaling through SAP-associating receptors. We have addressed the functional consequence of SAP deficiency on 2B4-mediated NK cell activation. Ligating 2B4 on normal human NK cells with anti-2B4 mAb or interaction with transfectants bearing the 2B4 ligand CD48 induced NK cell cytotoxicity. In contrast, ligation of 2B4 on NK cells from a SAP-deficient XLP patient failed to initiate cytotoxicity. Despite this, CD2 or CD16-induced cytotoxicity of SAP-deficient NK cells was similar to that of normal NK cells. Thus, selective impairment of 2B4-mediated NK cell activation may contribute to the immunopathology of XLP. ***The Journal of Immunology,*** **2000, 165: 2932–2936.**

It has become increasingly clear that immune homeostasis requires a delicate balance between positive and negative signals elicited by a multitude of receptors found on the surface of immune cells (1). Mutations in genes regulating intracellular signaling pathways are associated with immunodeficiency disorders such as X-linked agammaglobulinemia (2), autosomal and X-linked SCID (3, 4), and B cell lymphopenia (5). Recently, the gene mutated in the inherited immunodeficiency X-linked lymphoproliferative syndrome (XLP)[3] (reviewed in Ref. 6) was identified as SLAM (signaling lymphocytic activation molecule-associated protein)-associated protein (SAP; also known as DSHP and SH2D1A) and encodes a small SH2-domain containing protein (7–9). SAP interacts with a unique tyrosine-based motif (TxYxxV/I) present in the cytoplasmic domains of 2B4 and SLAM (7, 10–12), cell surface molecules that, when ligated, result in activation of NK cells and T cells, respectively (13–15). The association between SAP and 2B4 or SLAM prevented the recruitment of the protein tyrosine phosphatase SH2 domain-containing protein tyrosine phosphatase-2 (SHP-2) to the cytoplasmic domain of tyrosyl-phosphorylated 2B4 and SLAM (7, 10, 12). Thus, SAP may modulate signals initiated via SLAM and 2B4 by regulating the interactions between these receptors and SHP-2. Because SHP-2 exerts both positive and negative effects on different signaling pathways (16), the relative contributions of SHP-2 and SAP to cellular activation induced via 2B4 and SLAM are unclear. Specifically, it is not known whether SAP-mediated displacement of SHP-2 from SLAM or 2B4 would result in induction or termination of cellular activation. The availability of SAP-deficient lymphocytes from an XLP patient has now made it possible to determine the functional requirement for SAP in 2B4-mediated activation of human NK cells.

Materials and Methods

Antibodies

The following mAb were used in this study: anti-2B4 mAb (10, 17) (c1.7, Coulter-Immunotech, Hialeah, FL); anti-CD2 (DX10), CD16 (Leu-11a), and CD56 (Leu-19) mAb (Becton Dickinson, San Jose, CA); anti-CD48 mAb (18) (clone 6.28; provided by Dr. D. Thorley-Lawson, Tufts University School of Medicine, Boston, MA). The anti-human SAP polyclonal Ab was generated as previously described (12).

Cells

NK cell clones and polyclonal NK cell lines were generated from normal donors and the XLP patient according to established protocols (19). All clones were cultured in Yssel's medium (20) containing 10% FBS, L-glutamine, penicillin, and streptomycin. NKL23, a subline of the transformed

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[3] Abbreviations used in this paper: XLP, X-linked lymphoproliferative syndrome; SLAM, signaling lymphocytic activation molecule; SAP, SLAM-associated protein; SHP-2, SH2 domain-containing protein tyrosine phosphatase-2.

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human NK cell line NKL (21), was cultured in IMDM supplemented with 10% FBS, L-glutamine, 100 U/ml human IL-2, penicillin, and streptomycin. PHA blasts were generated by activating PBMC with PHA (0.1 μg/ml) and IL-2 (100 U/ml) for 5 days. P815 cells stably expressing human CD48 were generated by electroporation of parental P815 cells with human CD48 cDNA subcloned into the pBJ expression vector. Positive cells were obtained initially by neomycin selection and subsequently by cell sorting.

Immunofluorescence

The expression of 2B4 on human mononuclear cells and that of CD48 on transfected P815 cells were assessed by immunofluorescence and flow cytometry using standard techniques.

Expression of SAP in human mononuclear cells

Unstimulated human mononuclear cells or PHA blasts were solubilized in lysis buffer (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, and enzyme inhibitors) (10). Whole cell lysates were electrophoresed through 4–15% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA), transferred to Immobilon polyvinylidened difluoride membranes (Millipore, Bedford, MA), and assessed for the presence of human SAP using a rabbit polyclonal antiserum and HRP-conjugated anti-rabbit Ig antiserum. The membranes were developed using enhanced chemiluminescence (Pierce, Rockford, IL) and autoradiography.

PCR analysis of SAP expression

RNA was isolated according to standard protocols and transcribed into cDNA using SuperScript II reverse transcriptase (Life Technologies, Grand Island, NY). This cDNA was then used as a template for PCR amplification of SAP or GAPDH using previously described primers and PCR conditions (10).

Cytotoxicity assays

The cytotoxicity of different NK clones and cells was assessed in triplicate using a standard 5-h ^{51}Cr release assay (22) with P815, CD48-expressing P815, or K562 cells as targets. Redirected lysis against P815 target cells was induced by adding mAb specific for CD2, CD16, or 2B4 (2 μg/ml). The E:T cell ratio for all cytotoxicity assays was 4–6:1.

Results and Discussion

Expression of human SAP in normal and XLP cells

To examine the functional consequences of SAP deficiency in human NK cells, mononuclear cells were obtained from an XLP patient (AD in Ref. 8) harboring a deletion of the X-chromosome encompassing the entire XLP locus (8). SAP expression was evaluated by preparing protein lysates from normal or XLP mononuclear cells before stimulation or after in vitro activation with PHA and IL-2. As shown in Fig. 1*a*, unstimulated cells from two healthy donors do not express appreciable amounts of SAP protein. However, following activation of normal human leukocytes, abundant amounts of SAP protein were detectable (Fig. 1*a*). This is consistent with our previous report demonstrating increased expression of SAP mRNA in PBMC following activation (8). In contrast to normal mononuclear cells, PHA-activated mononuclear cells from the XLP patient were devoid of any SAP protein (Fig. 1*a*). SAP mRNA has been detected in human T cells, T cell lines, and some EBV-transformed B cell lines (7–9); however, expression in NK cells has not been reported. PCR analysis revealed that in addition to human mononuclear cells (PBMC) and T cells (Jurkat, Th cell clone), SAP was expressed in human NK cells (NK cell lines NK92 and YT2C2, and IL-2-activated primary NK cells; Fig. 1*b*, *upper panel*). A PCR product was amplified from all cDNA samples using GAPDH-specific primers, demonstrating the integrity of each of the cDNA templates (Fig. 1*b*, *lower panel*). Thus, in addition to T cells, SAP appears to be most highly expressed in NK cells.

2B4 is expressed on human mononuclear cells from XLP patients

We recently reported that the c1.7 mAb was specific for human 2B4 (10). Using this mAb, we found that 2B4 was expressed on all

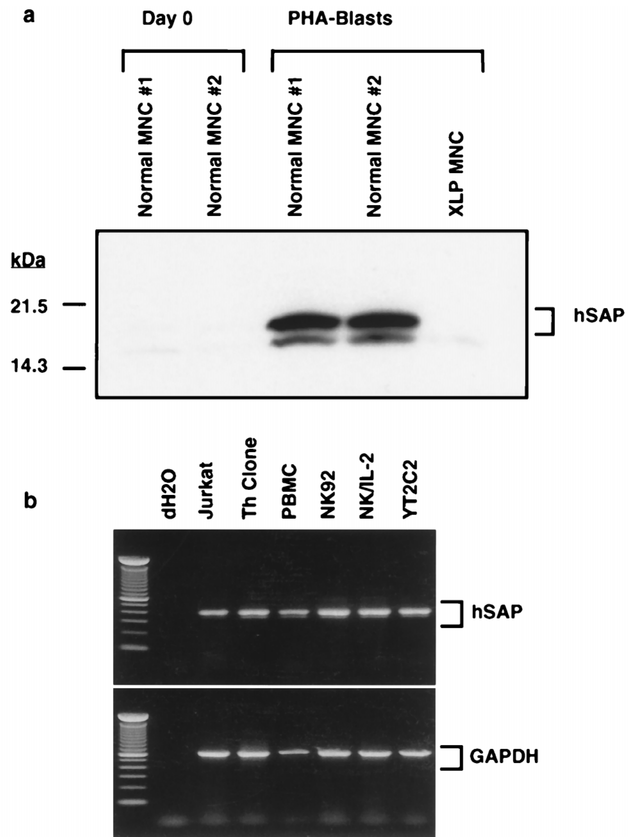

FIGURE 1. Expression of SAP in human cells. *a*, Mononuclear cells from two normal donors and an XLP patient were lysed in 1% Nonidet P-40 buffer containing 10 mM Tris-HCl (pH 7.8) and 150 mM NaCl on day 0 or following 5 days of activation with PHA and IL-2. Cell lysates were analyzed for the presence of SAP by SDS-PAGE and Western blotting, using rabbit anti-human SAP polyclonal antiserum. *b*, cDNA was prepared from different human cell types and used as a template in PCR for the amplification of SAP or GAPDH. The SAP PCR product migrates with an apparent m.w. of ~400 bp. dH_2O, Distilled H_2O.

normal human NK cells, monocytes, and some $CD8^+$ T cells, but not normal B or $CD4^+$ T cells (Fig. 2*a*) (15, 17). The expression of 2B4 on leukocytes from the XLP patient was identical to that in normal healthy donors (Fig. 2*b*). Thus, native SAP is not required for surface expression of 2B4.

Anti-2B4 mAb-induced redirected cytotoxicity of human NK cells is impaired in XLP

The original description of c1.7 was based on its ability to increase redirected cytotoxicity of human NK cell clones when presented on the FcR-bearing target cell P815 (17). The expression of SAP in NK cells and its association with 2B4 suggest that it may function in the 2B4 signaling pathway. This was assessed using normal and SAP-deficient XLP NK cells. Ligating 2B4 with c1.7 increased the cytotoxicity of normal NK cell clones and a polyclonal NK cell line generated from normal donors against P815 target cells (Fig. 3). This level of cytotoxicity was comparable to that induced with mAbs against CD16 (Fig. 3, *a* and *b*) and CD2 (Fig. 3*b*), well-characterized activating structures expressed by human NK cells (23). In contrast, the cytotoxicity of NK cell clones and polyclonal NK cell lines generated from an XLP patient was unaffected by anti-2B4 mAb (Fig. 3). Strikingly, SAP-deficient NK cells were capable of exhibiting redirected lysis when activated via receptors that do not appear to associate with SAP, namely CD2

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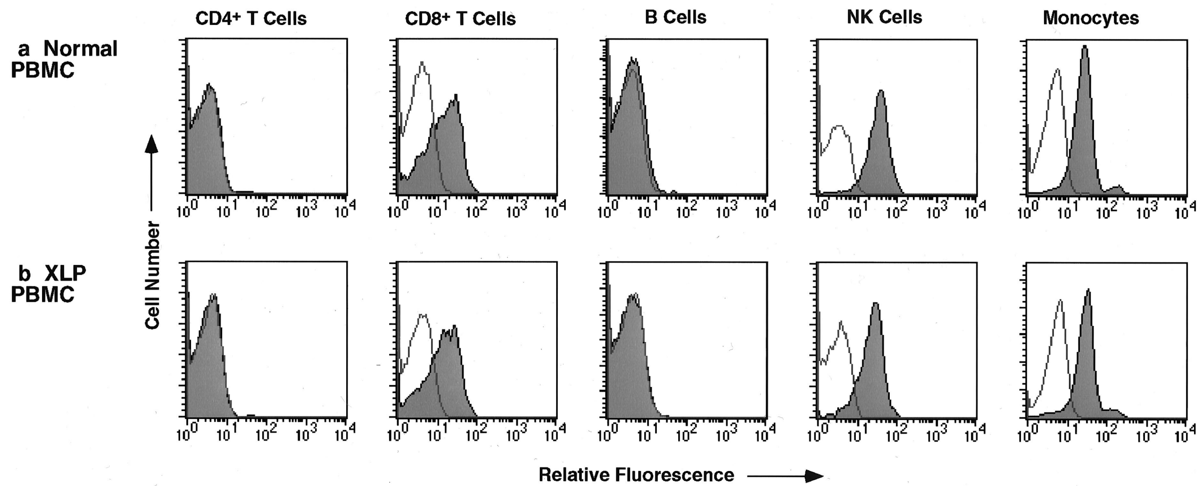

FIGURE 2. SAP is not required for the expression of 2B4. Mononuclear cells from a normal healthy donor (*a*) or a SAP-deficient XLP patient (*b*) were incubated with either PE-conjugated isotype control mAb (□) or PE-conjugated anti-2B4 mAb (c1.7; ■) and FITC-conjugated mAb specific for CD4, CD8, CD14, CD19, or CD56. The expression of 2B4 on the different leukocyte subsets of was determined by gating on each population of FITC-positive cells.

and CD16. Cytotoxicity induced in XLP NK cells under these conditions was comparable to that in the normal NK cells used in this study (Fig. 3). Thus, SAP appears to be essential for the initiation of activation signals delivered via 2B4. Although it is unclear how SAP initiates 2B4-mediated signaling, we proposed that

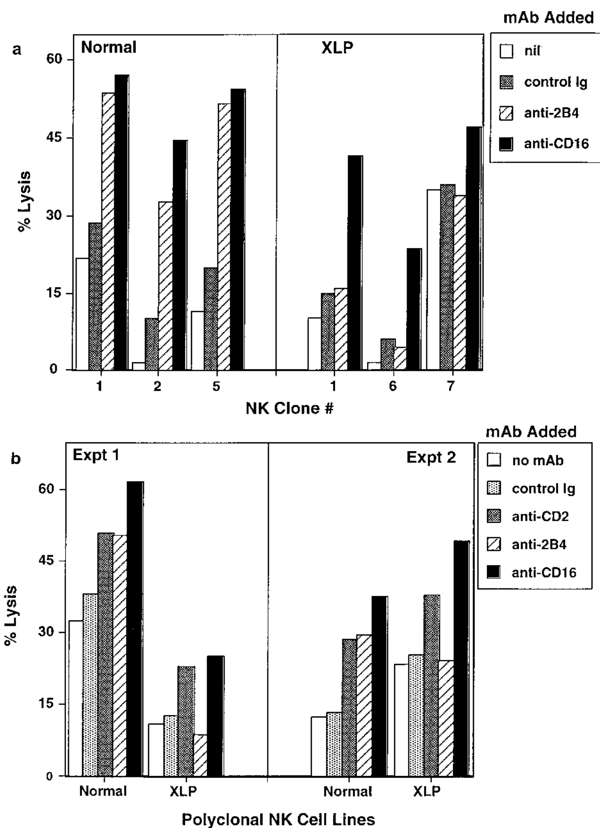

FIGURE 3. Anti-2B4 mAb-induced redirected cytotoxicity is impaired in human XLP NK cells. NK cell clones (*a*; three representative from donor and patient) or polyclonal NK cell lines derived from a normal donor or a SAP-deficient XLP patient (*b*) were assessed for cytotoxicity against P815 target cells in the absence or the presence of an isotype control mAb or mAb specific for CD2, CD16, or 2B4 (c1.7). Cytotoxicity was assessed in a standard ^{51}Cr release assay. Results are expressed as the percent lysis of target cells. Each value represents the mean of triplicate cultures; the triplicate determinations demonstrated <10% variation. For *b*, similar results were obtained in a third independent experiment.

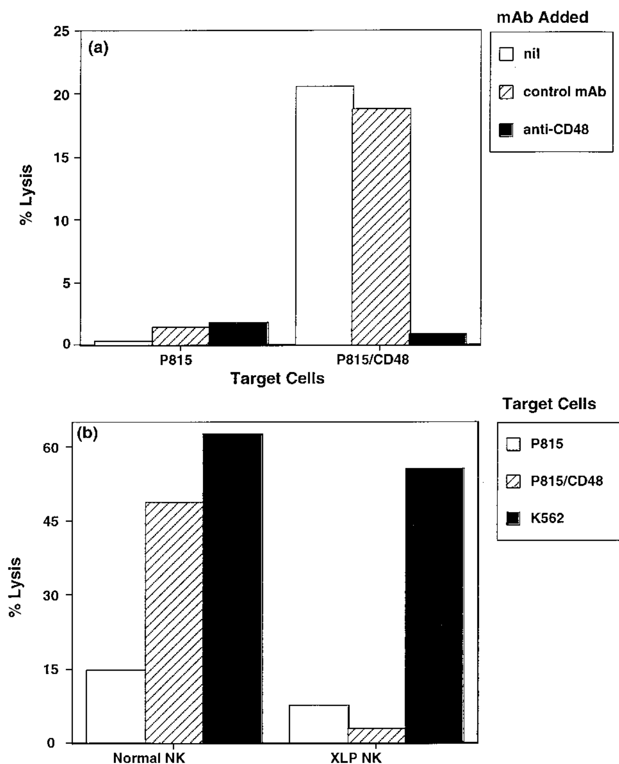

FIGURE 4. SAP is required for 2B4-mediated lysis of CD48-transfected target cells. *a*, NKL23 cells were incubated with parental or CD48-transfected P815 cells at an E:T cell ratio of 5:1 in the absence or the presence of a control mAb or a blocking anti-CD48 mAb (20 μg/ml). *b*, Polyclonal NK cell lines derived from a normal donor or an XLP patient were assessed for cytotoxicity against the following target cells: P815 (□), CD48-expressing P815 (▨), or K562 (■). Cytotoxicity was assessed in a standard ^{51}Cr release assay. Each value represents the mean of triplicate cultures; the triplicate determinations demonstrated <10% variation. Results are expressed as the percent lysis of target cells and are representative of two or more independent experiments.

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SAP may mediate 2B4-induced activation by competing with SHP-2, thereby preventing an SHP-2-dependent inhibitory signal (24). However, it cannot be discounted that SAP may recruit additional downstream effector molecules that are involved in delivering a 2B4-dependent positive signal. It is interesting to note that previous reports have demonstrated that anti-2B4 mAb induced cytotoxicity of in vitro-activated, but not resting, mouse NK cells (25, 26). Our findings that SAP is induced following lymphocyte activation (Fig. 1*a*) and that SAP is required for 2B4 function (Fig. 3) may explain the requirement for cellular activation for the effect of ligating 2B4 to be apparent. Similarly, previous reports demonstrate that the activation of T cell clones with anti-SLAM mAb diminishes with time, despite unchanged expression of SLAM on the cell surface (27). It would therefore be of interest to determine the expression of SAP in these activated T cells. Based on our results, it would be predicted that the unresponsiveness of human T cells to anti-SLAM mAb would correlate with a down-regulation of SAP expression. This would be consistent with the finding that overexpression of SAP enhanced signaling via SLAM in transfected Jurkat cells (7). However, SAP interacts with SLAM and 2B4 by tyrosine phosphorylation-independent and -dependent mechanisms (7, 10–12), respectively, and it is currently not known whether signaling pathways elicited via 2B4 and SLAM will have the same functional requirement for SAP.

XLP NK cells fail to lyse CD48-expressing target cells

CD48 has recently been identified as the ligand of human and mouse 2B4 (28). We generated P815 cells expressing human CD48 to test whether ligating 2B4 with its natural ligand might induce NK cell cytotoxicity. When used in a cytotoxicity assay, the transformed human NK cell line NKL23 failed to lyse parental P815 target cells (Fig. 4*a*). However, cytotoxic activity of NKL23 was induced following expression of human CD48 by P815 target cells (Fig. 4*a*). The enhanced killing of CD48-expressing target cells was specific, because the cytotoxicity of NKL23 cells against CD48-expressing P815 cells was reduced to that observed against parental P815 cells in the presence of a blocking anti-human CD48 mAb (18), yet was unaffected by a control mAb (Fig. 4*a*). When a normal polyclonal NK cell line was tested in this assay, lysis of $CD48^+$ P815 target cells was approximately 40% greater than that of parental target cells (Fig. 4*b*). Consistent with the findings using anti-2B4 mAb, the XLP polyclonal NK cell line failed to lyse CD48-expressing P815 cells. However, lysis of the NK-sensitive target cell line K562 by the XLP NK cell line was similar to that induced by normal NK cell lines (Fig. 4*b*). Taken together, our results demonstrate that NK cells from XLP patients are indeed capable of killing; however, the absence of SAP selectively cripples the 2B4-mediated activation pathway.

The function of NK cells during the primary immune response to EBV as well as in the subsequent control of latent EBV-transformed B cells is unclear (29). However, the observations that 2B4 can interact with SAP (10) and that XLP patients often succumb to infection with EBV suggest that the 2B4 pathway may play an important role during the immune response toward EBV. Curiously, CD48 was identified as an Ag whose expression on EBV-transformed B cells is at least 10-fold greater than that on EBV-negative B cells (30). Because CD48 is the ligand for 2B4 (28), it is tempting to speculate that the up-regulation of CD48 on EBV-transformed B cells may act as a signal to specifically activate NK cells via 2B4 and induce lysis of the transformed cells. Reduced 2B4-mediated activation of XLP NK cells might explain the increased incidence of fulminant infectious mononucleosis and EBV-associated malignant B cell neoplasms observed in these patients (6, 31). The inability to specifically activate NK cells via the interaction between 2B4 and CD48 may contribute to the pathogenesis and progression of XLP.

Note added in proof. Our findings are in accordance with the recent reports by Nakajima et al. (32) and Parolini et al. (33). However, in contrast to Parolini et al., we, and Nakajima et al., did not observe an inhibitory function of the 2B4 receptor in XLP patients deficient in SAP.

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