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Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn

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SAP is an adaptor protein that is expressed in NK and T cells. It is mutated in humans who have X-linked lymphoproliferative (XLP) disease. By interacting with SLAM family receptors, SAP enables tyrosine phosphorylation signaling of these receptors by its ability to recruit the Src-related kinase, Fyn. Here, we analyzed the role of SAP in NK cell functions using the SAP-deficient mouse model. Our results showed that SAP was required for the ability of NK cells to eliminate tumor cells in vitro and in vivo. This effect strongly correlated with expression of CD48 on tumor cells, the ligand of 2B4, a SLAM-related receptor expressed in NK cells. In keeping with earlier reports that studied human NK cells, we showed that SAP was necessary for the ability of 2B4 to trigger cytotoxicity and IFN-γ secretion. In the absence of SAP, 2B4 function was shifted toward inhibition of NK cell–mediated cytotoxicity. By analyzing mice lacking Fyn, we showed that similarly to SAP, Fyn was strictly required for 2B4 function. Taken together, these results provide evidence that the 2B4-SAP-Fyn cascade defines a potent activating pathway of natural cytotoxicity. They also could help to explain the high propensity of patients who have XLP disease to develop lymphoproliferative disorders.

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Abbreviations used: CFSE, carboxyl fluorescein succinimidyl ester; HPS, hemophagocytic syndrome; ITAM, immunoreceptor tyrosine-based activation motif; RADCC, redirected antibody-dependent cell cytotoxicity; XLP, X-linked lymphoproliferative.

NK cells are potent effector cells of innate immunity by their ability to eliminate tumor cells and virus-infected cells. The effector functions of NK cells are controlled by activating and inhibitory receptors (1–3) that are engaged during the recognition of potential target cells. The pathways that activate natural cytotoxicity have not been elucidated completely. The C-lectin like receptor, NKG2D, plays an essential role in NK cell–mediated rejection of tumor cells, even if those cells have normal MHC class I expression. NKG2D recognizes "stress-induced" MHC class I–related molecules that are strongly up-regulated during infection or cell transformation. However, a number of tumor cells do not express NKG2D ligands, which suggest that NK cell–mediated antitumoral immunity may be activated by other ligand–receptor interactions (4, 5).

Activating NK cell receptors, such as natural cytotoxicity receptors, activating forms of killer Ig-like receptors, CD94-NKG2C, NKG2E, Ly49D, Ly49H, and Ly49P in mice, also are postulated to play an important role in the activation of natural cytotoxicity (1–3). However, recent analyses of DAP-12–deficient mice, CD3-ζ/FcR-γ-deficient mice, and Syk/Zap-70–deficient mice provided compelling evidence that immunoreceptor tyrosine-based activation motif (ITAM)-dependent signaling pathways are not essential for natural cytotoxicity against a number of tumor cells, suggesting the existence of alternative pathways (6–8).

2B4 is another activating receptor that is expressed on human and mouse NK cells. 2B4 belongs to the CD2/SLAM family of immune cell receptors (3, 9). In mice and humans, ligation of 2B4 by anti-2B4 antibodies triggers NK cell–mediated cytotoxicity and IFN-γ production (10, 11). The ligand for 2B4 is CD48, which also belongs to the CD2/SLAM family of receptors (9, 12). CD48 is expressed on B cells and T cells and its expression increases upon cell activation (12, 13). In humans, transfection of CD48 in target cells increases their lysis by NK cells (14, 15). However, direct evidence that 2B4 activates natural cytotoxicity in vivo is missing.

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2B4-mediated NK cell activation does not involve DAP-10 or ITAM-containing subunits. When phosphorylated on tyrosine, 2B4 associates with the small adaptor molecule SAP (16, 17). SAP is composed almost exclusively of a unique Src-homology 2 domain that associates with TIYxxV/I motifs found in the cytoplasmic domains of the SLAM family receptors (9, 18). In T lymphocytes, SAP is required for the function of the SLAM receptor (19). In this context, SAP behaves as an adaptor protein by promoting the selective recruitment of the Src-related kinase Fyn to SLAM. Recently, it was shown that SAP also could promote the recruitment of Fyn to 2B4 in NK cells (20). However, it is not known whether Fyn is required for the function of 2B4.

Evidence that SAP plays a key role in in vivo immune responses was provided by the finding that mutations in the *SH2D1A* gene encoding SAP caused X-linked lymphoproliferative (XLP) syndrome (9). XLP is an inherited fatal immune dysfunction of lymphocyte homeostasis that is characterized principally by an inappropriate immune response to EBV infection that leads to a massive and uncontrolled lymphoproliferation of $CD8^+$ T cells and macrophages (21, 22). If children survive this initial episode or are asymptomatic, they generally go on to develop aggressive lymphoproliferative disorders, such as lymphomas. However, the physiopathology of XLP is unclear. Abnormalities in NK cell–and T cell–mediated cytotoxicity responses have been reported (21, 23, 24). Given the crucial role of cytotoxic functions in lymphocyte homeostasis and antitumoral responses, defects in this machinery could contribute to the lymphoproliferation disorders that are observed in XLP (25). Recent studies of NK cells from humans who had XLP showed that 2B4-mediated cytotoxicity is impaired in the absence of SAP; this highlighted the crucial role of SAP in the signal transduction and the function of 2B4 (14, 16, 17). Whether similar defects exist in SAP-deficient mice has not been investigated.

In light of these observations, we took advantage of SAP-deficient mice and Fyn-deficient mice to examine the exact function of SAP in natural cytotoxicity, and to define the signaling pathways in NK cells in which SAP is involved. We showed that a 2B4/SAP/Fyn pathway is required for antitumoral natural cytotoxicity.

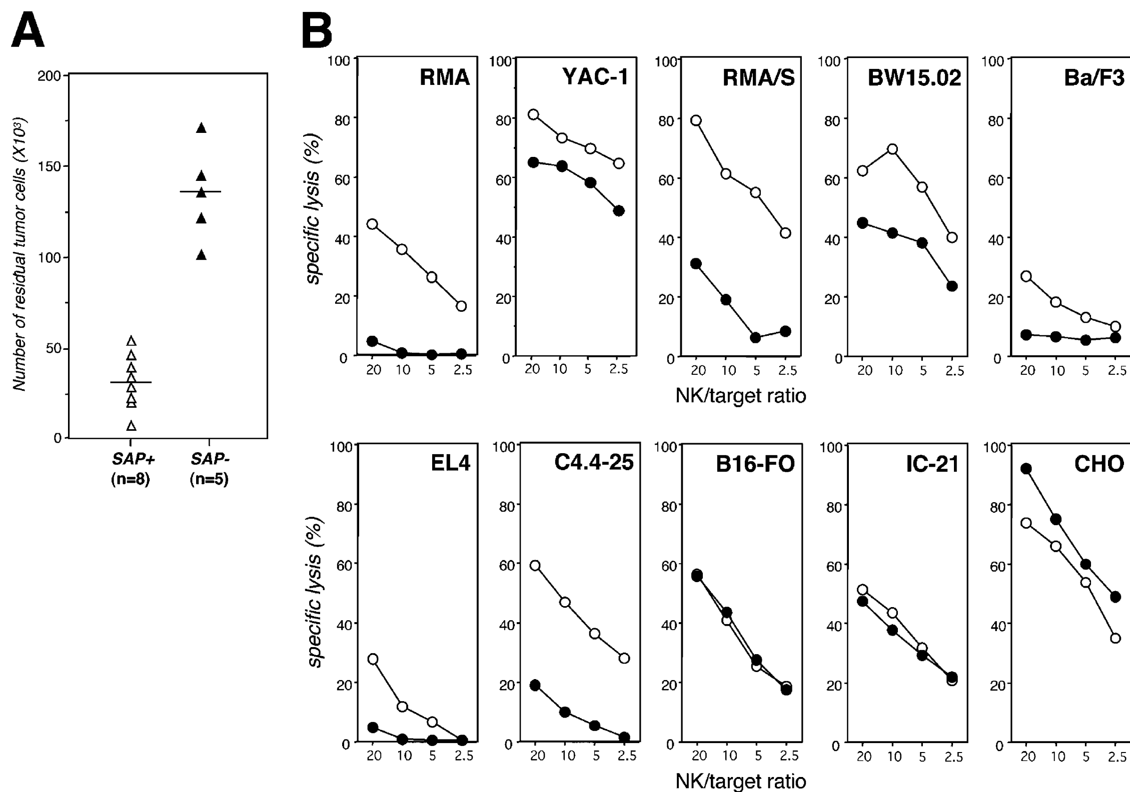

Figure 1. In vivo and in vitro natural killing responses are impaired in SAP-deficient mice. (A) The elimination of tumor cells in vivo is impaired in *SAP*⁻ mice. *SAP*⁺ and *SAP*⁻ mice were injected intraperitoneally with PKH-26–labeled RMA cells (10^6). After 24 h, the numbers of residual RMA cells recovered from the peritoneal cavity of *SAP*⁺ and *SAP*⁻ mice were determined by cytometry (see Materials and methods). The data presented are pooled from three independent experiments. Bars indicate mean values for each group of mice. (B) Natural cytotoxicity in vitro against a panel of target cells is restricted in *SAP*-deficient NK cells. The cytolytic activity of IL-2–expanded splenic NK cells from *SAP*⁺ (○) and *SAP*⁻ (●) mice was tested against the indicated target cells, at the mentioned NK/target cells ratios. For each target, data are presented as means from one experiment representative of three independent experiments.

RESULTS

Natural cytotoxicity in SAP-deficient NK cells

Because previous studies reported that patients who had XLP disease could present abnormalities in NK cell–mediated cytotoxicity, we investigated the natural cytotoxicity response in SAP-deficient mice. We first examined NK cell populations in the spleen, bone marrow, liver, and thymus of SAP-deficient mice (hereafter referred to as *SAP*$^-$ mice) and wild-type littermates (hereafter referred to as *SAP*$^+$ mice; Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050449/DC1). The percentages of CD3$^-$NK1.1$^+$ NK cells in the various organs of *SAP*$^-$ mice were comparable to those observed in their *SAP*$^+$ littermates, and the expression profiles of 2B4, Ly49G2, Ly49D, Ly49A, Ly49C/I, and CD16 (FcγRIII) on splenic NK cells were similar in *SAP*$^-$ and *SAP*$^+$ mice. Therefore, SAP seems to be dispensable for the development of NK cells. The elimination of tumor cells is one of the major functions attributed to NK cells. Antitumoral NK activity can be evaluated in vivo by injecting tumor cells into the peritoneal cavity of mice. When injected into the peritoneal cavity, RMA lymphoma cells are eliminated by NK cells (6). Therefore, we evaluated the capacity of *SAP*$^-$ NK cells to eliminate RMA tumor cells in vivo. RMA cells that were labeled with PHK 26 could be detected readily among peritoneal exudate cells by cytometry based on their PKH 26 fluorescence (unpublished data). The percentage of RMA cells was determined and the number of RMA cells recovered from the peritoneal cavity was calculated (Fig. 1 A). In the control *SAP*$^+$ mice (n = 8), 30 ± 15 × 10^3 RMA cells were recovered, whereas 134 ± 26 × 10^3 RMA cells were recovered from *SAP*$^-$ mice (n = 5). Thus, *SAP*$^-$ mice eliminated RMA cells much less efficiently than wild-type mice ($P < 0.001$). This strongly suggests that antitumoral NK activity is impaired in *SAP*$^-$ mice.

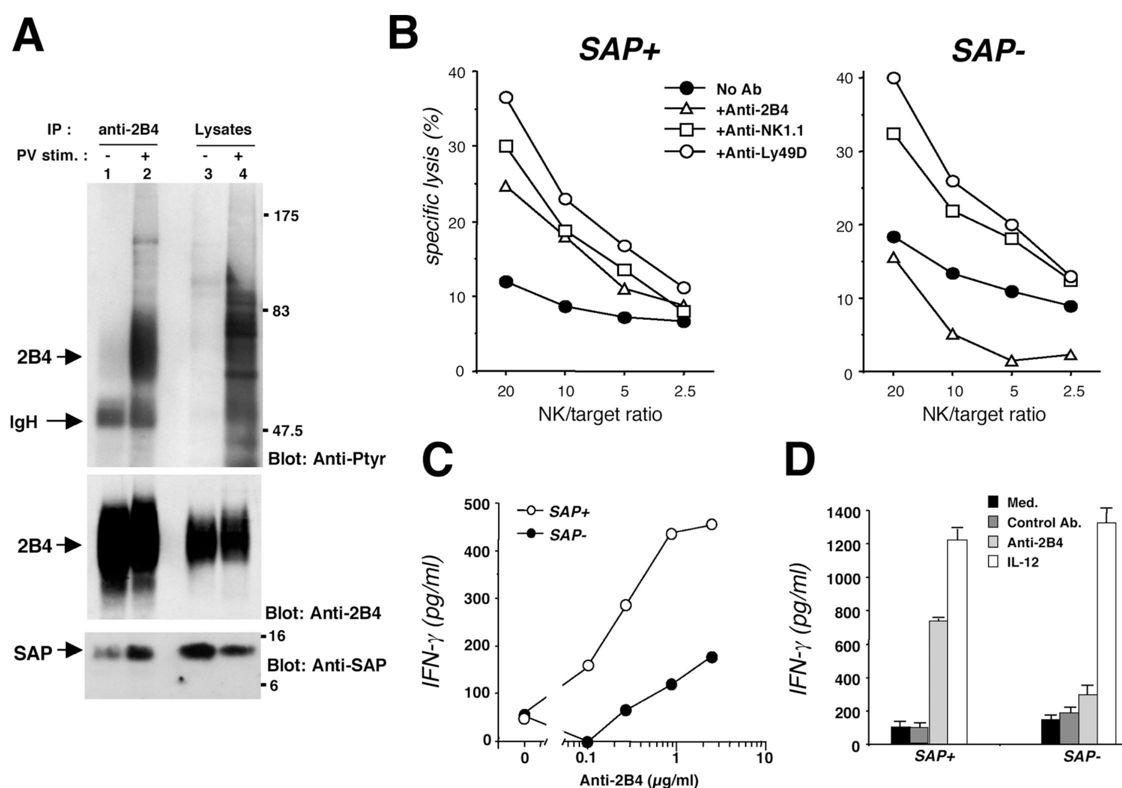

Figure 2. SAP is required for 2B4-mediated cell cytotoxicity and IFN-γ production by NK cells. (A) Association of SAP with 2B4 in mouse NK cells. IL-2–expanded splenic NK cells were treated (+) or not treated (−) with pervanadate (PV) for 10 min. 2B4 was immunoprecipitated (I.P.) and subjected to immunoblotting with anti-Ptyr antibody (top), anti-2B4 antibodies (middle), or anti-SAP antibodies (bottom). Total cell lysates were assessed in parallel. (B) The 2B4-mediated cytolytic activity by NK cells is impaired in the absence of SAP. The cytolytic activity of IL-2–expanded NK cells from *SAP*$^+$ (left) and *SAP*$^-$ (right) mice was measured in a redirected antibody-dependent cell-cytotoxicity assay. NK cells were incubated with the FcR$^+$ P815 target cells in the presence of anti-2B4, anti-Ly49D, anti-NK1.1 antibodies or medium (no Ab) at the indicated NK/target cell ratios. Data are presented as means from one representative experiment of three independent experiments. (C and D) 2B4-mediated IFN-γ production by NK cells is impaired in the absence of SAP. IFN-γ in the cell-supernatants was detected by ELISA. (C) NK cells from *SAP*$^+$ and *SAP*$^-$ mice were incubated with increased concentrations of anti-2B4 antibodies in the presence of FcR$^+$ P815 cells to trigger antibody-redirected stimulation of NK cells. Data are means from one representative experiment of two independent experiments. (D) IL-2–expanded NK cells from *SAP*$^+$ and *SAP*$^-$ mice were stimulated with medium, soluble anti-2B4 antibodies; irrelevant antibodies of the same isotype; or IL-12. Data are means ± SD from one representative experiment of three independent experiments.

To characterize this defect further, we evaluated the cytolytic activity of IL-2–expanded splenic NK cells from *SAP*$^{-}$ and *SAP*$^{+}$ mice toward various tumor cell lines in vitro. Consistent with the in vivo data (Fig. 1 A), *SAP*$^{-}$ NK cells showed an impaired cytolytic activity against RMA cells when compared with *SAP*$^{+}$ NK cells (Fig. 1 B). Similarly, the capacity of *SAP*$^{-}$ NK cells to lyse YAC-1, RMA/S, BW15.02, Ba/F3, EL4, and C4.4-25 tumor cell targets also was reduced (Fig. 1 B). In particular, the cytolytic activity involved in the killing of RMA, Ba/F3, and EL-4 was abolished almost completely in the absence of SAP. By contrast, the ability of *SAP*$^{-}$ NK cells to lyse B16-FO, IC-21, CHO, and P815 cells was comparable to that of *SAP*$^{+}$ NK cells (Fig. 1 B; see Fig. 2 B). C4.4.25 and RMA/S cells are MHC class I negative and low derivatives from EL4 and RMA cells, respectively (8). The lack of MHC class I expression renders C4.4.25 and RMA/S cells more susceptible to NK cell lysis. However, the killing of all of these cells was dependent on SAP, regardless of their expression of MHC class I. Together, these results indicate that the natural cytotoxicity of *SAP*$^{-}$ NK cells against tumor cells is restricted. Thus, SAP is required for some forms of NK cell–mediated cytotoxicity against tumor cells, including those that expressed MHC class I molecules.

2B4 functions in SAP-deficient NK cells

In human NK cells, SAP was shown to bind to tyrosine phosphorylated 2B4 and to be required for 2B4-mediated NK cell cytotoxicity (14, 16, 17). We first ascertained that SAP also bound with 2B4 in mouse NK cells. To this end, IL-2–expanded splenic NK cells were stimulated with the tyrosine phosphatase inhibitor, pervanadate, which has been used to demonstrate the association of SAP with 2B4 in human NK cells (16, 17, 26). In pervanadate-treated NK cells, 2B4 exhibited a strong increase in its phosphotyrosine content as compared with that in nonstimulated NK cells (Fig. 2 A, top, lanes 1 and 2). However, a weak tyrosine phosphorylation of 2B4 was detected in unstimulated NK cells (lane 1). A parallel anti-SAP immunoblot revealed that 2B4 was associated with SAP in nonstimulated cells (Fig. 2, bottom, lane 1). Upon pervanadate treatment, the extent of the 2B4-SAP association was enhanced strongly (Fig. 2, bottom, lane 2). Reblotting with anti-2B4 antibodies showed that similar amounts of 2B4 polypeptides were recovered by immunoprecipitation in nonstimulated and in pervanadate-stimulated cells (Fig. 2, middle, lanes 1 and 2). Thus, our results strongly suggest that in mouse NK cells, 2B4 is associated with SAP in a tyrosine phosphorylation–dependent manner.

We then investigated whether SAP was required for 2B4-mediated cell cytotoxicity of IL-2–expanded splenic NK cells. We assessed 2B4-mediated cell cytotoxicity by redirected (or reverse) antibody-dependent cell cytotoxicity (RADCC) assays, using the FcR^{+} P815 mastocytoma cell line as target cells. Cross-linking of 2B4 with anti-2B4 antibodies enhanced the lysis of P815 cells by *SAP*$^{+}$ NK cells (Fig. 2 B, left), but it failed to enhance P815 killing by *SAP*$^{-}$ NK cells over the magnitude obtained in the absence of antibodies (right). The lysis of P815 cells was inhibited slightly, albeit significantly, by 2B4 stimulation in these conditions. In a related manner, we and others have observed a similar inhibitory effect in testing human NK cells that lack SAP (unpublished data; reference 16). However, the stimulation of activating NK receptors, such as NK1.1, Ly49D, and CD16 (unpublished data), on *SAP*$^{-}$ NK cells resulted in an increase in P815 lysis similar in intensity to that obtained with *SAP*$^{+}$ NK cells. Thus, SAP is not required for the activating pathways that are triggered by NKR-P1c, CD16, and Ly49D receptors, which are known to depend on ITAM-containing subunits (1).

Because 2B4 ligation also triggers IFN-γ production by NK cells, we next examined whether SAP was required for this function. The stimulation of *SAP*$^{+}$ NK cells with P815 target cells in the presence of increasing concentrations of 2B4 antibodies resulted in a marked accumulation of IFN-γ in the cell supernatant (Fig. 2 C). In contrast, this response was impaired severely with *SAP*$^{-}$ NK cells. Consistent with earlier reports (10, 11), the stimulation of *SAP+* NK cells with soluble anti-2B4 antibodies also increased IFN-γ secretion when compared with cells that were stimulated with control antibodies (Fig. 2 D). In contrast, IFN-γ production in response to soluble anti-2B4 antibodies was abolished almost completely in *SAP*$^{-}$ NK cells. Despite these differences, *SAP*$^{-}$ and *SAP*$^{+}$ NK cells displayed similar levels of IFN-γ production following stimulation with IL-12 (Fig. 2 D). Taken together, these observations clearly show that in mice, SAP is required for 2B4-mediated cell cytotoxicity and IFN-γ production by NK cells.

Regulation of natural cytotoxicity by CD48 expression on tumor cells

Because 2B4-mediated cytotoxicity is abolished in *SAP*$^{-}$ NK cells, we considered that the loss-of-function of 2B4 might account for the natural cytotoxicity defect in *SAP*$^{-}$ NK cells. In this case, only target cells killed less efficiently by *SAP*$^{-}$ NK cells would be expected to express CD48, the 2B4 ligand (12). To address this issue, the various target cell lines were examined by flow cytometry for CD48 expression. Expression of CD48 was detected readily on the membrane of YAC-1, EL4, C4.4-25, RMA, RMA-S, Ba/F3, and BW15.02 cells (Fig. 3), all of which were found to be lysed by a SAP-dependent NK cell cytotoxicity (Fig. 1). Conversely, no CD48 expression was detected on P815, B16-FO, IC-21, or CHO target cells (Fig. 3), the killing of which by NK cells does not require SAP (Fig. 1 B). Hence, CD48 expression on tumor cell lines seems to be related to the impaired capacity of *SAP*$^{-}$ NK cells to kill those cells. Therefore, these data strongly suggest that the impaired natural cytotoxicity response in *SAP*$^{-}$ mice is caused by a defect of 2B4-mediated cytotoxicity.

Because the results reported above were purely correlative, we wished to address the role of CD48 expression directly on target cells in SAP-dependent killing. To this end, C4.4-25 cells were cloned by limiting dilution, and we obtained two monoclonal cell lines that did not express CD48 (hereafter re-

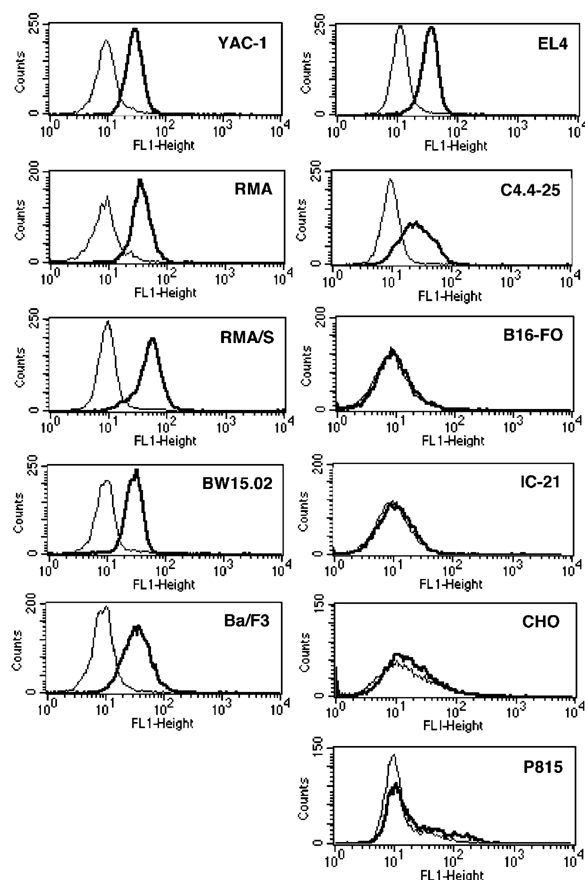

Figure 3. Cell surface expression of CD48, the 2B4 ligand, on target cell lines. Cell surface expression of CD48 was assessed by flow cytometry with anti-CD48 and FITC-conjugated anti–rat Ig (thick histogram). Controls were stained with FITC-conjugated anti–rat Ig alone (thin histogram).

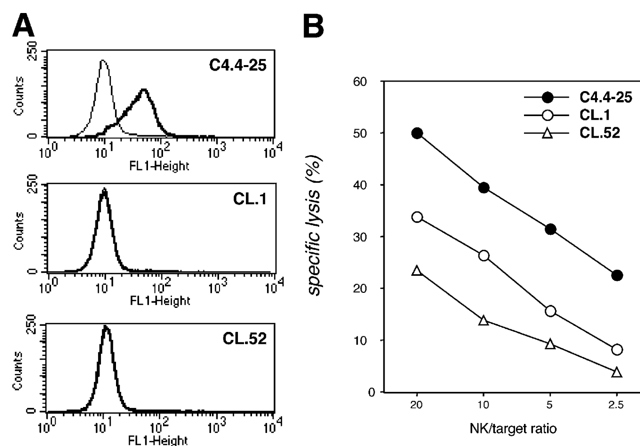

Figure 4. The lack of CD48 expression on tumor cells decreases the susceptibility of these cells to be killed by NK cells in vitro. (A) CD48 is not expressed on CL.1 and CL.52 cells which were derived from C4.4-25 cells (see Materials and methods). CD48 expression was assessed as in Fig. 3. (B) Lysis of CL.1 and CL.52 by NK cells. The cytolytic activity of IL-2–expanded splenic NK cells was tested against CL.1, CL.52, or the parental C4.4-25 target cells, at the indicated NK/target cell ratios. Data are presented as means from one experiment representative of three independent experiments.

ferred to as CL.1 and CL.52) as shown by flow cytometry analysis (Fig. 4 A). When used as target cells with SAP^+ NK cells, CL.1 and CL.52 were lysed less efficiently than the parental C4.4-25 cells (Fig. 4 B). Thus, the loss of CD48 expression on C4.4-25 target cells decreased their susceptibility to 2B4-mediated NK cell cytotoxicity. However, we could not exclude formally that the possibility that this decrease was caused by CD48-independent events that were selected for during the cloning process. To rule out this possibility, a mouse cDNA that encoded the C57BL/6 allelic form of CD48 was introduced into CL.1 and CL.52 cells by electroporation. Polyclonal populations that stably expressed CD48 were produced (hereafter referred to as CL.1-CD48 and CL.52-CD48) as depicted in Fig. 5 A. The lysis of these cell lines by SAP^+ NK cells was compared with that of the parental CD48-deficient CL.1 and CD48-deficient CL.52 cells. The killing of CL.1-CD48 and CL.52-CD48 cells by SAP^+ NK cells was greater than that of the CD48-deficient CL.1 and CL.52 cells (Fig. 5 B).

We next investigated whether SAP was required for the NK cell cytotoxicity induced by CD48 expression on CL.1-CD48 and CL.52-CD48 cells. The lysis of CL.1-CD48 and CL.52-CD48 cells and their CD48-deficient parental cells by SAP^- NK cells was examined. SAP^- NK cells lysed CL.1-CD48 and CL.52-CD48 much less efficiently than did SAP^+ NK cells. In comparison, CL.1 and CL.52 cells were lysed to a similar extent by SAP^+ NK cells and SAP^- NK cells. When compared with CL.1 and CL.52, the killing of CL.1-CD48 and CL.52-CD48 by SAP^- NK cells was diminished markedly, whereas it was increased with SAP^+ NK cells. This inhibition was consistent with the data presented in Fig. 2 B, which showed that the stimulation of 2B4 with anti-2B4 antibodies inhibited NK cell cytotoxicity of SAP^- NK cells.

Based on these results, we conclude that expression of CD48 on tumor cells increases their susceptibility to lysis by NK cells via a 2B4/SAP-dependent activating pathway. In addition, these data indicate that in the absence of SAP, the engagement of 2B4 by its ligand, CD48, on target cells results in the inhibition of NK cell cytotoxicity, as suggested previously by the analysis of human SAP^- NK cells (16).

In vivo elimination of CD48-expressing tumor cells

We then evaluated the role of CD48 expression on tumor cells in vivo during the NK cell antitumoral response. For this purpose, in vivo elimination of CL.1 and CL.1-CD48 tumor cells was examined in the peritoneal cavity of various strains of mice. Like the parental C4.4-25 cells, CL.1 and CL.1-CD48 cells do not express MHC class I molecules, and accordingly, are eliminated rapidly in the peritoneal cavity by NK cells (not depicted and Fig. 6). Equal numbers of carboxyl fluorescein succinimidyl ester (CFSE)-labeled CL.1 and CL.1-CD48 cells were mixed and injected intraperitoneally into normal SAP^+ mice.

1 h after injection into normal SAP^+ mice, the proportions of CL.1 and CL.1-CD48 cells in the peritoneal cavity

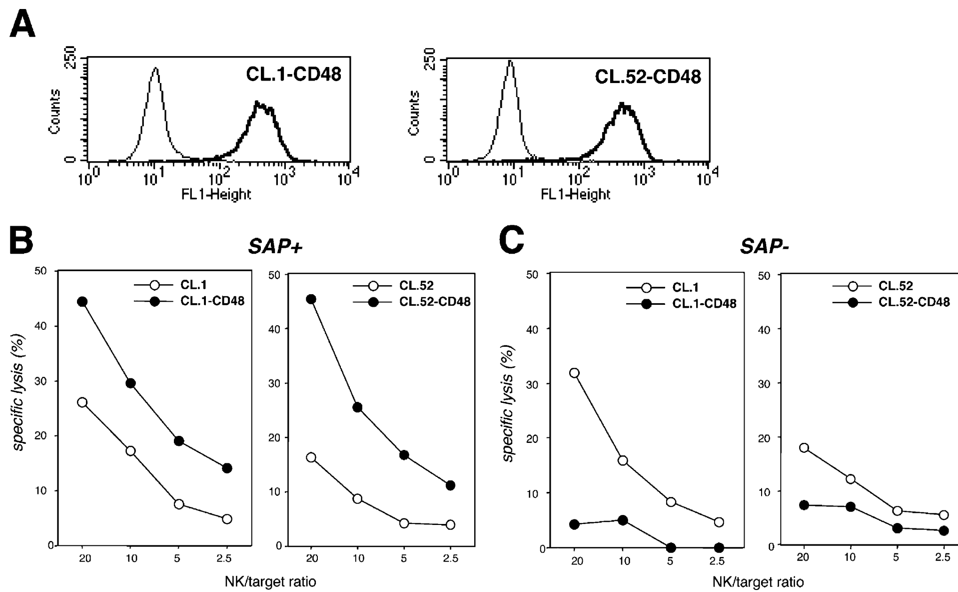

Figure 5. Influence of SAP expression on NK cell–mediated cytotoxicity toward CD48-expressing tumor cells. (A) Cell surface expression of CD48 in CL.1 and CL.52 cells stably transfected with a cDNA encoding CD48. Experiment same as in Fig. 4. (B and C) The expression of CD48 on tumor cells enhanced the susceptibility of these cells to be killed by *SAP*$^+$ NK cells, whereas it inhibited the killing of these cells by *SAP*$^-$ NK cells. The cytolytic activity of IL-2–expanded splenic NK cells from *SAP*$^+$ (B) and *SAP*$^-$ (C) mice was tested in parallel against CL.1, CL.52, CL.1-CD48, or CL.52-CD48 target cells at the indicated NK/target cell ratios. For each target, data are presented as means from one representative experiment of three independent experiments.

were similar to those in the original cell mixture, maintained in culture for the same period of time (unpublished data). By contrast, after 6 h, the proportion of CL.1-CD48 cells among the CFSE-labeled peritoneal cells had decreased significantly (Fig. 6 A). This decrease could not be explained by different rates of division of CL.1 and CL.1-CD48 cells because the proportions of these two types of cell remained unchanged in the noninjected cell mixture that was maintained in culture for 6 h (Fig. 6 A). Combined with the data from in vitro cytotoxicity assays (Fig. 5), this decrease likely reflects a more efficient in vivo killing of CL.1-CD48 cells as compared with CL.1 cells. Consistent with that, the absolute number of CL.1-CD48 cells ($49.4 \pm 30 \times 10^3$; $n = 8$) that was recovered in the peritoneal cavity was much lower than the number of CL.1 cells that was recovered ($297.2 \pm 97.5 \times 10^3$; $n = 5$; $P = 0.0038$; Fig. 6 B). Therefore, these observations suggest that CD48 expression on tumor cells increases the elimination of these cells in vivo.

To ascertain the role of NK cells in this response, the same experiments were performed in T/B cell–deficient *RAG-2*$^{-/-}$ and T/B/NK cell–deficient *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice. In *RAG-2*$^{-/-}$ mice, the proportion of CL.1-CD48 cells was shown to be decreased to a similar extent to that observed in control *SAP*$^+$ mice (Fig. 6 A). The number of CL.1-CD48 cells recovered from *RAG-2*$^{-/-}$ mice ($34.5 \pm 3.5 \times 10^3$, $n = 2$) did not differ significantly from that obtained from *SAP*$^+$ mice ($49.4 \pm 30 \times 10^3$, $n = 8$; Fig. 6 B). Thus, T and B lymphocytes are not required for the elimination of CD48-expressing tumor cells. By contrast, in *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice, which lack B, T, and NK cells, the proportion of CL.1-CD48 cells was only slightly lower than the initial proportion of CL.1-CD48 cells in the control cell mixture that was not injected. In agreement with these data, the number of CL.1-CD48 cells found in the peritoneal cavity of *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice ($221.6 \pm 104.6 \times 10^3$, $n = 5$) was larger than that in control *SAP*$^+$ mice ($49.4 \pm 30 \times 10^3$; $n = 8$; $P = 0.001$). Thus, the elimination of CL.1-CD48 cells in the peritoneal cavity primarily depends on NK cells.

We assessed the elimination of CL.1 and CL.1-CD48 cells in *SAP*$^-$ mice. CL.1-CD48 cells were eliminated much less efficiently in *SAP*$^-$ mice than in normal *SAP*$^+$ mice (Fig. 6 A). Consistent with these data, more CL.1-CD48 cells ($248.7 \pm 87.8 \times 10^3$, $n = 6$) were recovered from the peritoneal cavity in *SAP*$^-$ mice than in *SAP*$^+$ mice ($49.4 \pm 30 \times 10^3$, $n = 8$; $P = 0.0013$; Fig. 6 B). By contrast, the numbers of CL.1 cells collected from *SAP*$^-$ mice ($429.6 \pm 14 \times 10^3$, $n = 4$) and *SAP*$^+$ mice ($297.2 \pm 97.5 \times 10^3$, $n = 5$) did not differ significantly. These results are consistent with those of the cytotoxicity assays in vitro shown in Fig. 5. Lastly, the proportion of CL.1-CD48 cells in *SAP*$^-$ mice was very similar to that observed in *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice, and the number of CL.1-CD48 cells found in *SAP*$^-$ mice was not significantly different from that in *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice. To exclude that these effects were caused by a defect in the recruitment of NK cells in the peritoneal cavity of *SAP*$^-$ mice, the number of peritoneal NK cells was evaluated after injection of CL.1 and CL.1-CD48 cells. NK cells in the peritoneal fluid were detected by flow cytometry based on their expression of NK1.1

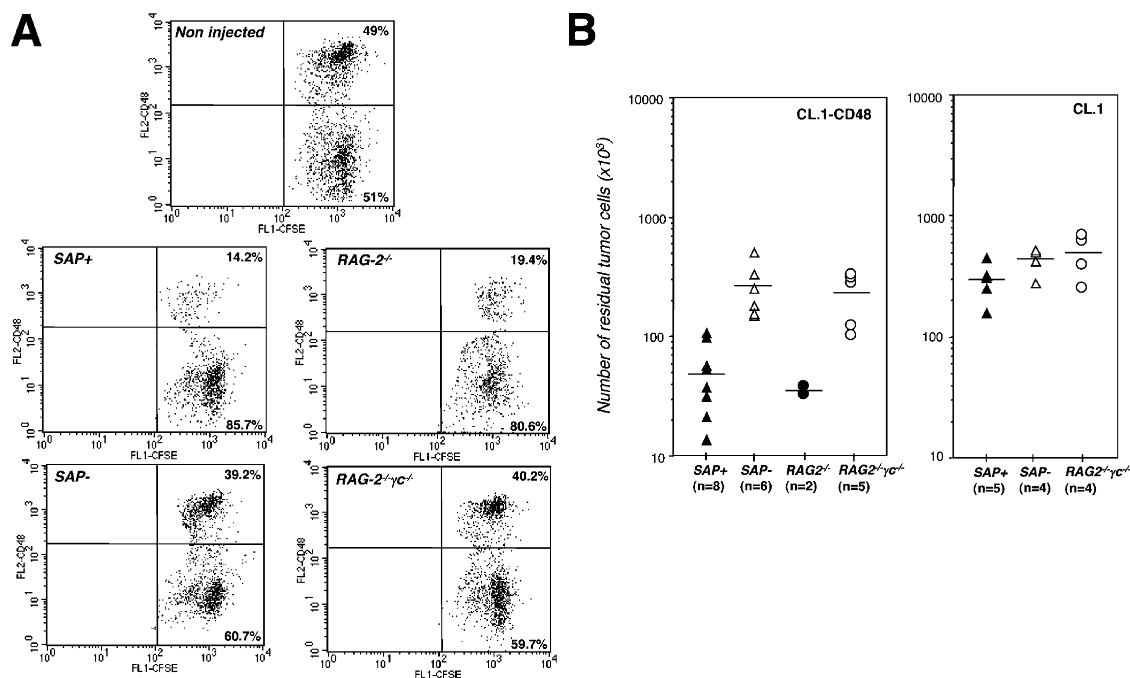

Figure 6. Increase in the in vivo elimination of CD48-expressing tumor cells by NK cells. *SAP*$^+$, *SAP*$^-$, *RAG-2*$^{-/-}$ and *RAG-2*$^{-/-}$γc$^{-/-}$ mice were injected intraperitoneally with a mixture of equal numbers of CFSE-labeled CL.1 and CFSE-labeled CL.1-CD48 tumor cells. After 6 h, peritoneal cells were recovered, counted, and stained with PE-conjugated anti-CD48 antibody. An aliquot of the cell mixture was kept in culture for 6 h (noninjected). (A) The percentages of CL.1 and CL.1-CD48 tumor cells in the CFSE-positive cell population were evaluated by flow cytometry, based on CD48 fluorescence. Representative dot-plots of gated CFSE-positive cells are shown and the injected mice are indicated. (B) Numbers of residual CL.1 cells and CL.1-CD48 cells recovered from the peritoneal cavity of *SAP*$^+$, *SAP*$^-$, *RAG-2*$^{-/-}$, and *RAG-2*$^{-/-}$γc$^{-/-}$ mice. The data presented are pooled from four independent experiments. One experiment in which only CL1-CD48 cells were injected is included. Bars indicate mean values for each group of mice.

and 2B4 and their lack of CD3 expression. CL.1 and CL.1-CD48 cells did not express 2B4 and NK1.1 (unpublished data). Injection of CL.1 and CL.1-CD48 cells in *SAP*$^+$ mice resulted in a significant increase in the number of peritoneal 2B4$^+$NK1.1$^+$CD3$^-$ NK cells ($47.6 \pm 27 \times 10^3$, $n = 9$) when compared with that observed in mice injected with PBS alone ($16.3 \pm 6.9 \times 10^3$, $n = 6$, P = 0.004). However, the number of 2B4$^+$NK1.1$^+$CD3$^-$ cells found in the peritoneal cavity of *SAP*$^-$ mice ($34.2 \pm 25.5 \times 10^3$, $n = 6$) after injection of CL.1 and CL.1-CD48 cells was not significantly different from that observed in *SAP*$^+$ mice (P = 0.47). This result indicates that the defect in the elimination of CL.1-CD48 cells in *SAP*$^-$ mice did not result from a reduced recruitment of NK cells in the peritoneal cavity. Collectively, these data clearly demonstrate that the elimination of CL.1-CD48 cells by NK cells is dependent on SAP. They also suggest that 2B4-mediated NK cell cytotoxicity is involved in the in vivo elimination of tumor cells expressing CD48.

2B4-mediated natural cytotoxicity in Fyn-deficient mice

We recently provided evidence that SAP could promote the recruitment of Fyn to 2B4 (20). Hence, we examined whether Fyn was required for 2B4-mediated natural cytotoxicity and IFN-γ production. IL-2–expanded splenic NK cells from Fyn-deficient mice were obtained. In RADCC, cross-linking of 2B4 with anti-2B4 antibodies failed to enhance P815 killing by IL-2–expanded splenic NK cells from Fyn-deficient mice when compared with wild-type NK cells. However, killing of P815 lysis by Fyn-deficient NK cells after stimulation with anti-Ly49D (Fig. 7 A) or anti-NK1.1 (not depicted) antibodies was not affected, whereas it was decreased partially upon stimulation with anti-CD16. This is consistent with the notion that CD16-mediated NK cell activation depends on Fyn and Lck Src-kinases (27). 2B4-mediated IFN-γ production also was defective in Fyn-deficient NK cells (Fig. 7 B). Killing of CD48-positive target cells, such as C4.4-25, RMA/S, and RMA cells, by Fyn-deficient NK cells was impaired strongly (Fig. 7 C). By contrast, the lysis of the CD48-negative CHO target cells was not affected. Finally, the expression of CD48 on CD48-CL.1 cells failed to enhance their killing by Fyn-deficient NK cells as compared with CL.1 cells that lack CD48 (Fig. 7 D). These data demonstrate that like SAP, Fyn is required for 2B4-mediated natural cytotoxicity.

2B4-mediated tyrosine phosphorylation in SAP-deficient and Fyn-deficient NK cells

To characterize further the defect in 2B4-mediated natural cytotoxicity in SAP-deficient and Fyn-deficient mice, the capac-

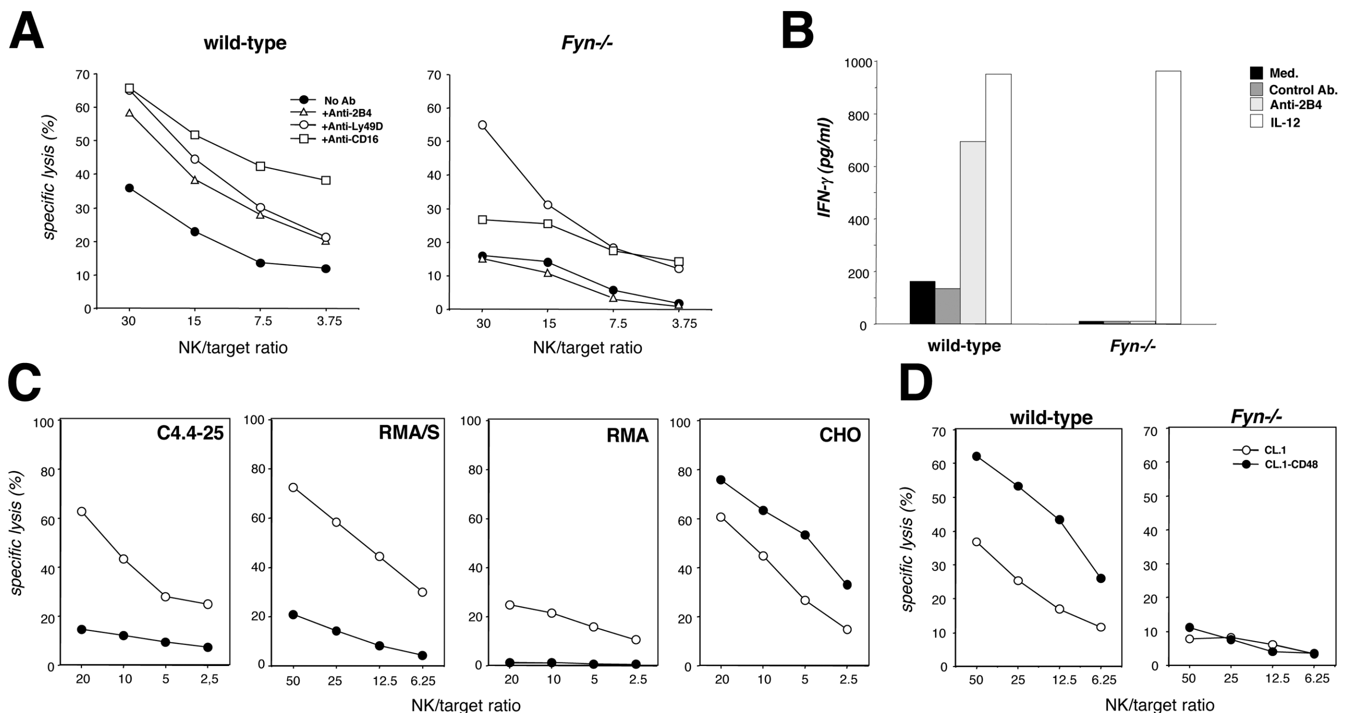

Figure 7. 2B4-mediated cell-cytotoxicity and IFN-γ production by NK cells is impaired in the absence of Fyn. (A) The cytolytic activity of IL-2–expanded NK cells from wild-type (left) and *Fyn*$^{-/-}$ mice (right) mice was measured in a RADCC assay. Experiment same as in Fig. 2. (B) 2B4-mediated IFN-γ production by IL-2–expanded NK cells. Experiment same as in Fig. 2. (C and D) Natural cytotoxicity in vitro against a panel of target cells is restricted in Fyn-deficient NK cells. (C) Cytolytic activity of IL-2–expanded splenic NK cells from wild-type (○) and *Fyn*$^{-/-}$ (●) mice against C4.4-25, RMA/S, RMA, or CHO target cells. Experiment same as in Fig. 1. (D) Cytolytic activity of IL-2–expanded splenic NK cells from wild-type (left) and *Fyn*$^{-/-}$ (right) mice was tested in parallel against CL.1 and CL.1-CD48 target cells. Experiment same as in Fig. 5.

ity of 2B4 to trigger tyrosine phosphorylation signals was examined (Fig. 8). Stimulation of wild-type NK cells with anti-2B4 antibodies increased the tyrosine phosphorylation levels of several intracellular proteins, including a p70 and a p150 kD (Fig. 8 A). In Fyn-deficient mice this signal was completely abolished, whereas it was decreased markedly in SAP-deficient mice. As described previously (20), the major substrates that were phosphorylated upon 2B4 engagement in wild-type NK cells were 2B4 itself (Fig. 8 B, top) and the inositol phosphatase, SHIP (Fig. 8 B, middle; lanes 1 and 2). In contrast, tyrosine phosphorylation of 2B4 was abolished completely in NK cells from *SAP*$^{-}$ and *Fyn*$^{-/-}$ mice (top, lanes 4–6). Similarly, phosphorylation of SHIP was reduced in *SAP*$^{-}$ and abrogated in *Fyn*$^{-/-}$ mice (middle, lanes 4–6). These data indicate that 2B4-mediated tyrosine phosphorylation signals are impaired in SAP^{-} and Fyn-deficient mice. They also argue that in the absence of SAP, 2B4-mediated natural cytotoxicity is impaired because 2B4 is uncoupled to Fyn.

DISCUSSION

In this study we showed that SAP-deficient and Fyn-deficient mice exhibit an impaired natural cytotoxicity response to tumor cells that express CD48. Because CD48 is the ligand of 2B4, and none of the CD48-negative target cell lines that we examined was killed via a SAP-dependent pathway, we conclude that the defect in natural cytotoxicity of *SAP*$^{-}$ NK cells resulted from a loss-of-function of 2B4. However, SAP also binds to other SLAM family receptors, and recent studies showed that SAP is required for the biologic functions of at least three of these receptors, including 2B4, SLAM, and NTB-A. Therefore, one could argue that the defect of *SAP*$^{-}$ NK cells is not restricted to 2B4. In addition to 2B4, murine NK cells were shown to express the SLAM family receptors, CD84 and CRACC, but not NTB-A, Ly9, and SLAM (unpublished data). Recent reports showed that CRACC triggers cell cytotoxicity by human NK cells, but that this activity does not require SAP (26). This finding was explained by the inability of CRACC to associate with SAP in humans and in mice (unpublished data). The function of CD84 in NK cells is unknown. However, its seems to be similar to that of SLAM in T cells, which consists of the regulation of TCR-mediated IFN-γ production (9). Accordingly, in mouse NK cells, 2B4 seems to be the only SLAM family receptor expressed that is involved in SAP-dependent cell cytotoxicity.

The impairment of the cytolytic activity of *SAP*$^{-}$ NK cells is restricted to CD48-expressing tumor cells, although this defect was variable, depending on the tumor cell line. This variability could not be explained by differences in CD48 expression between tumor cell lines, because all ex-

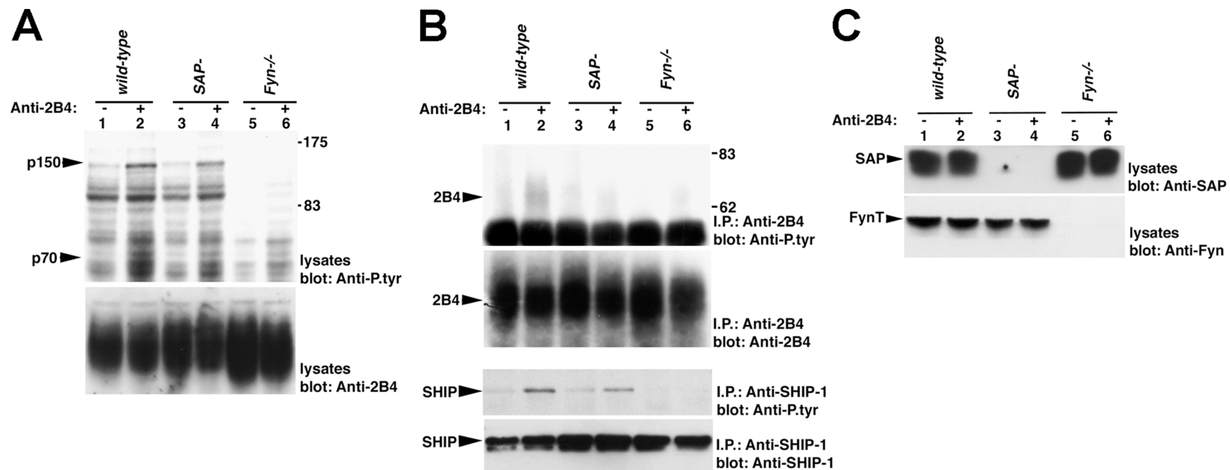

Figure 8. Fyn and SAP are required for 2B4-mediated tyrosine phosphorylation signals. (A) Overall tyrosine phosphorylation. IL-2–expanded splenic NK cells from wild-type, *SAP*$^{-}$, and *Fyn*$^{-/-}$ mice were stimulated (+) or not (−) for 5 min with biotinylated anti-2B4 antibodies and avidin. Protein tyrosine phosphorylation was examined by immunoblotting of total cell lysates with anti-P.tyr antibody (top). The abundance of 2B4 in the lysates was verified by reprobing the membrane with anti-2B4 antibodies (bottom). (B) Tyrosine phosphorylation of SHIP and 2B4. Experiment same as (A). Tyrosine phosphorylation of 2B4 (top) and SHIP (middle) was analyzed by probing anti-2B4 and anti-SHIP antibody immunoprecipitates (I.P.) with anti-P.tyr antibody. The abundance of 2B4 and SHIP was verified by reprobing the membranes with anti-2B4 and anti-SHIP antibodies, respectively. (C) Expression of SAP and Fyn in NK cells from wild-type, *SAP*$^{-}$, and *Fyn*$^{-/-}$ mice. Experiment same as (A) and (B). The abundance of SAP and Fyn was verified in total cell lysates by immunoblotting with anti-SAP (top) and Fyn (bottom) antibodies.

pressed similar magnitudes of CD48. These tumor cells also expressed ligands other than CD48 that might trigger NK cell cytotoxicity. The impact of 2B4 stimulation on NK cell activation probably depends on the nature of the other NK receptors engaged. YAC-1 cells express high level of ligands for NKG2D, and YAC-1 cell lysis is mostly dependent on NKG2D receptor activation (4, 6). Likewise, the lysis of BW 15.02 cells depends on activation of the stimulatory NK cell receptor, NKp46 (3, 7). Presumably, in these cases, NK cells are activated via the coengagement of 2B4 and NKG2D or NKp46 receptors as it was reported previously for human NK cells (28). In this context, NKG2D and NKp46 receptors may play a predominant role and account for the slight dependence of YAC-1 and BW 15.02 cells lysis on 2B4-mediated NK cell cytotoxicity. By contrast, the lysis of RMA, RMA/S, EL4, C4.4-25, and BaF/3 cells was heavily dependent on 2B4 activation. Until now, the activating pathways in NK cells involved in triggering the lysis of these target cells were unknown. These tumor cells express no known NKG2D ligands (4, 5), and a recent report showed that the lysis of these tumor cells does not require ITAM-bearing NK receptors because these cells normally are killed by *Syk*$^{-/-}$ *ZAP-70*$^{-/-}$ NK cells (8). These observations imply that in the absence of activating ligands for NKG2D and ITAM-bearing NK receptors, CD48 expression on tumors cells is a major trigger of NK cell activation via a 2B4/SAP/Fyn pathway. Along the same line, in patients who have XLP, defects in 2B4-mediated antitumoral NK activity may account for the appearance of lymphomas in 30% of patients (9, 21).

The mechanism by which 2B4 initiates signaling events is distinct from that used by the NKG2D-DAP-10 receptor complex and NK cell receptors associated with ITAM-bearing subunits. The cell cytotoxicity triggered by NKG2D-DAP-10 depends directly on the binding of PI3 kinase to DAP-10 (1, 3). For ITAM-associated receptors, Syk/ZAP protein tyrosine kinases are recruited to phosphorylated ITAMs and are responsible for the propagation of downstream signals. In T lymphocytes, we previously showed that SAP is required for the function of SLAM by its capacity to promote the selective recruitment and activation of the Src-PTK Fyn (29). We and others have proposed that SAP might have a similar role in the signal transduction via the other SLAM family receptors. We recently provided evidence that SAP is indeed involved in the recruitment of Fyn to 2B4 allowing by this way 2B4-mediated tyrosine phosphorylation signals (20). In this previous study, the association between Fyn and 2B4 was observed in a human NK cell line. In the present study, we failed to detect 2B4-Fyn interaction in mouse NK cells. It is possible that this association is less stable in mouse than in humans. However, Fyn seems to be essential for 2B4 function in mouse as NK cells from *Fyn*$^{-/-}$ mice exhibit defective 2B4-mediated cell cytotoxicity. Intriguingly, in *Fyn*$^{-/-}$ NK cells, 2B4-mediated tyrosine phosphorylation signals was found to be almost completely abolished while it was only decreased in *SAP*$^{-}$ NK cells suggesting SAP-independent role(s) of Fyn. Fyn also is required for the function of other activating NK cell receptors such as CD16 (FcγRIII) that may be engaged by anti-2B4 antibodies via their Fc portion (27). One other possibility is that Fyn is required for activating and inhibitory functions of 2B4. Our results strongly suggest that 2B4-mediated inhibition of cytotoxicity in *Fyn*$^{-/-}$ NK cells is defective (Fig. 7). Finally, it also is possible that Fyn is in-

volved in 2B4-mediated tyrosine phosphorylation of substrates, such as LAT and PLC-γ1, which seem not to depend on SAP (20). In connection with that, we observed a weak killing against CL.1 cells by the $Fyn^{-/-}$ NK cells when compared with that obtained with SAP^{-} NK cells and wild-type NK cells (Figs. 5 and 7). Similarly, a decreased killing against P815 (Fig. 7), YAC-1, and B16-FO (not depicted) cells also was noticed in the absence of Fyn. These effects could be also explained by the fact that Fyn is involved in other activating pathways in NK cells that do not depend on SAP, such as those triggered by CD16 and DNAM-1, as mentioned above (27, 30). These pathways may be required for efficient killing of some target cells. Also, these observations are consistent with recent studies that showed that the absence of Fyn results in T cell activation defects that are more severe than those observed in SAP-deficient T cells (31).

Our results clearly demonstrate that 2B4 behaves as an inhibitory and an activating receptor, depending of the presence of SAP. The inability of *SAP*$^{-}$ NK cells to lyse CD48-expressing target cells combines two effects: the loss of 2B4-mediated stimulatory signals and the ability of 2B4 to mediate inhibitory signals in the absence of SAP that turn off NK cell cytotoxicity. The first evidence for an inhibitory role of 2B4 in the absence of SAP was provided by studies of patients with XLP, which showed that 2B4 has the capacity to inhibit activating signals triggered by natural cytotoxicity receptors (16). Anti-2B4 antibodies also enhanced cell cytotoxicity of NK cells from patients who had XLP toward EBV-infected cells, presumably by blocking 2B4-CD48 interactions. Importantly, this inhibition could compromise further the ability of NK cells of patients who have XLP to eliminate EBV-infected cells or tumor cells. The inhibitory function of 2B4 was supported further by the recent characterization of mice lacking 2B4. 2B4-deficient NK cells exhibited increased NK cell–mediated cytotoxicity and IFN-γ production (32, 33). However, in these studies, 2B4 was shown to be primarily an inhibitory receptor, which could be viewed as contradictory to our data and the data obtained in humans. The dominant inhibitory effect of 2B4 in these recent reports might be explained by a low expression of SAP in NK cells that were used in these studies. Consistent with this, the authors showed that bone marrow–derived NK cells that lacked *SAP* RNA expression exhibited a strong 2B4-mediated inhibition, whereas LAK cells that had detectable *SAP* RNA showed a 2B4-mediated inhibition to a lesser extent. Based on these observations and our data, it is very likely that there is a threshold in SAP expression below which the activating function of 2B4 is shifted toward inhibition. SAP is expressed in limiting quantities in NK cells, and small variations in the amount of SAP may change the function of 2B4. This also is supported by the recent analysis of NK cells from transgenic mice for SAP under the CD2 promoter. NK cells from these mice overexpress SAP and have enhanced 2B4-mediated natural cytotoxicity (Veillette et al., unpublished observations).

The molecular basis of the 2B4 inhibitory function is not well-understood. Reports have shown that 2B4 has the capacity to associate with the protein tyrosine phosphatases, SHP-1 and SHP-2, and that this interaction is blocked by SAP (16, 34). However, these findings were not confirmed in other studies (17, 26). Nonetheless, Src-homology 2–containing inhibitory molecules may interact with 2B4 in the absence of SAP. It should be pointed out that EAT-2, a SAP-related protein, is expressed in NK cells (26, 32). Similar to SAP, EAT-2 has the capacity to bind to SLAM family receptors, including 2B4 (18). Therefore, one possibility is that when bound to 2B4, EAT-2 provided a signal that led to the inhibition of NK cell activation. Additional studies are needed to determine the precise biochemical mechanism by which 2B4 inhibits NK activation.

Despite identification of the gene that is responsible for XLP disease, the immune defects that cause XLP have not been characterized unambiguously. In particular, susceptibility to EBV infection and hemophagocytic syndrome (HPS; or virus-associated hemophagocytic syndrome) that occur in most cases, are poorly understood. It was hypothesized that this phenotype might stem from a defect in T helper (Th) 2–type responses (21, 22). As a consequence, Th1-type responses would be prolonged inappropriately, and hence, could account for the excessive accumulation of activated $CD8^{+}$ T cells and macrophages that occur in HPS. Studies of *SAP*$^{-}$ mice confirmed that SAP is necessary for normal Th2 responses (31, 35). However, one limitation of the mouse model is that mice are not susceptible to EBV infection and do not develop HPS. The HPS observed in XLP disease is very similar to that observed in the inherited immunodeficiencies that lead to defective cytotoxic functions of $CD8^{+}$ T cells and NK cells (25). The molecular elucidation of these diseases has highlighted the crucial role of T and NK cell cytotoxic functions in immune homeostasis, particularly after antiviral responses. Thus, because the loss of cytotoxic functions leads to a defect in homeostatic balance that results in HPS, one attractive possibility is that the blocking of NK cytotoxicity by 2B4 could be one of the major factors of HPS manifestations in XLP.

CD48 is expressed strongly on B cells when infected with EBV. Notably, this molecule was described first 20 yr ago as a specific marker of B cell transformation by EBV (13). It also was reported that the increase in CD48 expression observed during EBV infection was dependent on a regulatory element in the CD48 promoter that is activated in the presence of EBV (36). During EBV infection, the increased CD48 expression on EBV-infected B cells is likely to lead to the sustained engagement of 2B4 on NK cells, and, possibly, on $CD8^{+}$ T cells expressing 2B4 (23, 24). In a normal setting, such stimulation of 2B4 would facilitate the elimination of infected B cells by activating the cytolytic activities of NK cells and maybe also $CD8^{+}$ T cells. Conversely, in patients who have XLP, this would lead to an inhibition of NK cell cytotoxicity. Further experiments are required to test this model.

MATERIALS AND METHODS

Mice. SAP-deficient (*SAP*$^{-}$) and Fyn-deficient (*Fyn*$^{-/-}$) mice have been described elsewhere (37, 38). SAP-deficient mice were backcrossed to C57BL/6 mice for 10 generations. Male mice (*SAP*$^{-}$ and their *SAP*$^{+}$ littermates) were used for experiments. C57BL/6 *RAG-2*$^{-/-}$ mice were obtained from Charles River Laboratories. C57BL/6 *RAG-2*$^{-/-}$$\gamma$c$^{-/-}$ mice were provided by F. Colucci (Institut Pasteur, Paris, France). All mice were 8–12 wk of age. Mouse studies were approved by the French Ministry of Agriculture for Animal Experimentations.

Flow cytometry analysis. The following mAbs, unconjugated or conjugated to FITC, PE, or biotin were used in this study: anti-NK1.1, anti-CD2, anti-2B4, anti-DX5, anti-CD16/CD32, anti-Ly49C/I, anti-LY49G2, anti-Ly49A, anti-Ly49D, anti-CD45R, anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-TCRβ, anti-H-2K^{b}, anti-H-2D^{b}, and anti-CD48 (all obtained from BD Biosciences). APC-Streptavidin and FITC-conjugated secondary antibodies (FITC-conjugated goat anti–rat Ig or FITC-conjugated rat anti–mouse Ig obtained from Jackson ImmunoResearch Laboratories) were used to reveal biotin-conjugated and nonconjugated antibodies, respectively. Low-affinity Fcγ receptors were blocked with 10 μg/ml of 2.4G2 to prevent nonspecific binding.

Cells. YAC-1 (H-2^{a}), RMA (H-2^{b}), RMA/S (H-2^{b}), EL4 (H-2^{b}), C4.4-25 (H-2^{b}), Ba/F3 (H-2^{d}), IC-21 (H-2^{b}), BW51.05 (H-2^{b}), B16-FO (H-2^{b}), P815 (H-2^{d}), and CHO cell lines were grown in RPMI-1640–GLUTAMAX supplemented with 10% FCS and antibiotics (all reagents were obtained from GIBCO BRL). The CD48^{-} cell lines, CL.1 and CL.52, were obtained by the limiting dilution of C4.4-2 cells.

IL-2–expanded NK cells were obtained from splenocytes. T and B cells were removed by passing splenocytes stained with PE-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-CD19 antibodies through magnetic columns that were coated with anti-PE antibodies (Miltenyi Biotec). The splenic NK cells were expanded in RPMI 1640–GLUTAMAX supplemented with 10% FCS, antibiotics, 10^{-5} M β-mercaptoethanol (Sigma-Aldrich), and 1,000 IU/ml of recombinant IL-2 for 6–12 d. Greater than 98% of the cells obtained with this protocol were CD3^{-}, NK1.1^{+}, 2B4^{+}, and CD16^{+}. No differences in the expression patterns of 12 markers tested (including Ly49G2, Ly49D, Ly49A, Ly49C/I, CD16, 2B4, CD2, CD45, DX5, NK1.1, NKG2D, and CD3) were noticeable between IL-2–expanded splenic NK cells from *SAP*$^{+}$, *SAP*$^{-}$, and *Fyn*$^{-/-}$ mice (unpublished data).

cDNA constructs and transfections. The mouse CD48 cDNA was cloned from C57BL/6 thymocytes by RT-PCR using the following primers: sense oligonucleotide: 5′-AGGAAGATGTGCTTCATAAAACAGGG-3′; antisense oligonucleotide: 5′-TTGTCAGGTTAACAGGATCCTGTG-3′. The mouse CD48 cDNA was inserted into the vector pSRα-Puro, which contains the puromycin resistance gene. Stable transfectants expressing CD48 (CL.1-CD48 and CL.52-CD48) were obtained by selection in medium containing puromycin (5 μg/ml) as described previously (19).

Immunoprecipitation and immunoblots. Immunoprecipitations and immunoblots were performed as described previously (19, 20).

NK cell cytotoxicity in vitro assays. The cytolytic activity of NK cells was evaluated with a standard 4-h ^{51}Cr release assay. In brief, target cells (5×10^{3}) labeled with ^{51}Cr were incubated with NK cells for 4 h at 37°C at the indicated effector/target ratio. For RADCC assays, NK cells were incubated with 5 μg/ml of anti-2B4, anti-NK1.1, or anti-CD16 for 30 min at 4°C, washed, and then mixed with the P815 FcR^{+}-labeled target cells. The percentage of specific lysis was calculated according to the standard formula: (experimental − spontaneous release)/(total − spontaneous release). All assays were done in triplicate.

Cytokine production assays. NK cells (6×10^{4}) were stimulated at 37°C with 5 μg/ml of anti-2B4 or control anti–H-2D^{b} antibodies (mouse IgG2b), with IL-12 (5 ng/ml; Valbiotech), or in a RADCC assay (see previous paragraph). In brief, NK cells (6×10^{4}) were incubated with the P815 cells (6×10^{3}) in the presence of various concentrations of anti-2B4 antibodies. After 24 h of stimulation, cell-free supernatants were collected and assayed for IFN-γ by ELISA (R&D Systems). All assays were done in triplicate.

In vivo tumor elimination. RMA cells (10^{6}) were labeled with the vital dye PKH-26 (5 μM) according to the protocol of the manufacturer (Sigma-Aldrich). Cells were washed, resuspended in PBS, and injected into the peritoneal cavity of mice. 24 h after the injection; mice were killed, and the peritoneal cells were recovered, washed, and counted. The proportion of cells that were PKH-26 positive was determined with a FACScan flow cytometer (Becton Dickinson).

The elimination of CL.1 and CL.1-CD48 tumor cells was evaluated by labeling cells (10^{7}/ml) with the vital dye, CFSE (20 μM, Molecular Probes), in PBS for 15 min at room temperature. Cells were washed extensively, and equal numbers of CFSE-labeled CL.1 and CL.1-CD48 cells were mixed in PBS (1.5×10^{6} of each cell type in a final volume of 300 μl) and injected into the peritoneal cavity. An aliquot of mixed cells was kept in culture. After 6 h, peritoneal cells were recovered, washed, counted, and stained with a PE-conjugated anti-CD48. As a control, the mixed cells that had been kept in culture also were stained. Based on fluorescence, the percentages of CL.1-CD48 cells (positive for CFSE and CD48) and CL.1 cells (positive for CFSE and negative for CD48) among the peritoneal cells were determined by flow cytometry. Data were acquired and analyzed with CellQuest software (Becton Dickinson).

NK cell counts in the peritoneal cavity were calculated from the percentage of NK cells among peritoneal cells as determined by flow cytometry after staining with FITC-conjugated anti-2B4 and APC-conjugated anti-NK1.1 antibodies. These cells were negative for CD3.

Statistical analysis. Student's *t* tests were performed with InStat software.

Online supplemental material. Fig. S1 shows the proportions of NK cells in the thymus, bone marrow, liver, and spleen of wild-type and SAP-deficient mice, and the expression NK cell receptors on splenic NK cells from wild-type and SAP-deficient mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050449/DC1.

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REFERENCES

1. Lanier, L.L. 2003. Natural killer cell receptor signaling. *Curr. Opin. Immunol.* 15:308–314.
2. Colucci, F., J.P. Di Santo, and P.J. Leibson. 2002. Natural killer cell activation in mice and men: different triggers for similar weapons? *Nat. Immunol.* 3:807–813.
3. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M.C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19:197–223.
4. Diefenbach, A., A.M. Jamieson, S.D. Liu, N. Shastri, and D.H. Raulet. 2000. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* 1:119–126.
5. Diefenbach, A., J.K. Hsia, M.Y. Hsiung, and D.H. Raulet. 2003. A

novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity. *Eur. J. Immunol.* 33:381–391.
6. Zompi, S., J.A. Hamerman, K. Ogasawara, E. Schweighoffer, V.L. Tybulewicz, J.P. Di Santo, L.L. Lanier, and F. Colucci. 2003. NKG2D triggers cytotoxicity in mouse NK cells lacking DAP12 or Syk family kinases. *Nat. Immunol.* 4:565–572.
7. Tomasello, E., P.O. Desmoulins, K. Chemin, S. Guia, H. Cremer, J. Ortaldo, P. Love, D. Kaiserlian, and E. Vivier. 2000. Combined natural killer cell and dendritic cell functional deficiency in KARAP/DAP12 loss-of-function mutant mice. *Immunity.* 13:355–364.
8. Colucci, F., E. Schweighoffer, E. Tomasello, M. Turner, J.R. Ortaldo, E. Vivier, V.L. Tybulewicz, and J.P. Di Santo. 2002. Natural cytotoxicity uncoupled from the Syk and ZAP-70 intracellular kinases. *Nat. Immunol.* 3:288–294.
9. Engel, P., M.J. Eck, and C. Terhorst. 2003. The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. *Nat. Rev. Immunol.* 3:813–821.
10. Valiante, N.M., and G. Trinchieri. 1993. Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes. *J. Exp. Med.* 178:1397–1406.
11. Garni-Wagner, B.A., A. Purohit, P.A. Mathew, M. Bennett, and V. Kumar. 1993. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J. Immunol.* 151:60–70.
12. Brown, M.H., K. Boles, P.A. van der Merwe, V. Kumar, P.A. Mathew, and A.N. Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* 188:2083–2090.
13. Thorley-Lawson, D.A., R.T. Schooley, A.K. Bhan, and L.M. Nadler. 1982. Epstein-Barr virus superinduces a new human B cell differentiation antigen (B-LAST 1) expressed on transformed lymphoblasts. *Cell.* 30:415–425.
14. Tangye, S.G., J.H. Phillips, L.L. Lanier, and K.E. Nichols. 2000. Functional requirement for SAP in 2B4-mediated activation of human natural killer cells as revealed by the X-linked lymphoproliferative syndrome. *J. Immunol.* 165:2932–2936.
15. Nakajima, H., M. Cella, H. Langen, A. Friedlein, and M. Colonna. 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. *Eur. J. Immunol.* 29:1676–1683.
16. Parolini, S., C. Bottino, M. Falco, R. Augugliaro, S. Giliani, R. Franceschini, H.D. Ochs, H. Wolf, J.Y. Bonnefoy, R. Biassoni, et al. 2000. 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.* 192:337–346.
17. Nakajima, H., M. Cella, A. Bouchon, H.L. Grierson, J. Lewis, C.S. Duckett, J.I. Cohen, and M. Colonna. 2000. Patients with X-linked lymphoproliferative disease have a defect in 2B4 receptor-mediated NK cell cytotoxicity. *Eur. J. Immunol.* 30:3309–3318.
18. Latour, S., and A. Veillette. 2004. The SAP family of adaptors in immune regulation. *Semin. Immunol.* 16:409–419.
19. Latour, S., G. Gish, C.D. Helgason, R.K. Humphries, T. Pawson, and A. Veillette. 2001. Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. *Nat. Immunol.* 2:681–690.
20. Chen, R., F. Relouzat, R. Roncagalli, A. Aoukaty, R. Tan, S. Latour, and A. Veillette. 2004. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol. Cell. Biol.* 24:5144–5156.
21. Seemayer, T.A., T.G. Gross, R.M. Egeler, S.J. Pirruccello, J.R. Davis, C.M. Kelly, M. Okano, A. Lanyi, and J. Sumegi. 1995. X-linked lymphoproliferative disease: twenty-five years after the discovery. *Pediatr. Res.* 38:471–478.
22. Gaspar, H.B., R. Sharifi, K.C. Gilmour, and A.J. Thrasher. 2002. X-linked lymphoproliferative disease: clinical, diagnostic and molecular perspective. *Br. J. Haematol.* 119:585–595.
23. Dupre, L., G. Andolfi, S.G. Tangye, R. Clementi, F. Locatelli, M. Arico, A. Aiuti, and M.G. Roncarolo. 2005. SAP controls the cytolytic activity of CD8+ T cells against EBV-infected cells. *Blood.* 105:4383–4389.
24. Sharifi, R., J.C. Sinclair, K.C. Gilmour, P.D. Arkwright, C. Kinnon, A.J. Thrasher, and H.B. Gaspar. 2004. SAP mediates specific cytotoxic T-cell functions in X-linked lymphoproliferative disease. *Blood.* 103:3821–3827.
25. de Saint Basile, G., and A. Fischer. 2003. Defective cytotoxic granule-mediated cell death pathway impairs T lymphocyte homeostasis. *Curr. Opin. Rheumatol.* 15:436–445.
26. Bouchon, A., M. Cella, H.L. Grierson, J.I. Cohen, and M. Colonna. 2001. Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family. *J. Immunol.* 167:5517–5521.
27. van Oers, N.S., B. Lowin-Kropf, D. Finlay, K. Connolly, and A. Weiss. 1996. alpha beta T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. *Immunity.* 5:429–436.
28. Sivori, S., S. Parolini, M. Falco, E. Marcenaro, R. Biassoni, C. Bottino, L. Moretta, and A. Moretta. 2000. 2B4 functions as a co-receptor in human NK cell activation. *Eur. J. Immunol.* 30:787–793.
29. Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P.L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. *Nat. Cell Biol.* 5:149–154.
30. Shibuya, A., D. Campbell, C. Hannum, H. Yssel, K. Franz-Bacon, T. McClanahan, T. Kitamura, J. Nicholl, G.R. Sutherland, L.L. Lanier, and J.H. Phillips. 1996. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity.* 4:573–581.
31. Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. *Immunity.* 21:707–717.
32. Lee, K.M., M.E. McNerney, S.E. Stepp, P.A. Mathew, J.D. Schatzle, M. Bennett, and V. Kumar. 2004. 2B4 acts as a non-major histocompatibility complex binding inhibitory receptor on mouse natural killer cells. *J. Exp. Med.* 199:1245–1254.
33. Mooney, J.M., J. Klem, C. Wulfing, L.A. Mijares, P.L. Schwartzberg, M. Bennett, and J.D. Schatzle. 2004. The murine NK receptor 2B4 (CD244) exhibits inhibitory function independent of signaling lymphocytic activation molecule-associated protein expression. *J. Immunol.* 173:3953–3961.
34. Tangye, S.G., S. Lazetic, E. Woollatt, G.R. Sutherland, L.L. Lanier, and J.H. Phillips. 1999. Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP. *J. Immunol.* 162:6981–6985.
35. Wu, C., K.B. Nguyen, G.C. Pien, N. Wang, C. Gullo, D. Howie, M.R. Sosa, M.J. Edwards, P. Borrow, A.R. Satoskar, et al. 2001. SAP controls T cell responses to virus and terminal differentiation of TH2 cells. *Nat. Immunol.* 2:410–414.
36. Klaman, L.D., and D.A. Thorley-Lawson. 1995. Characterization of the CD48 gene demonstrates a positive element that is specific to Epstein-Barr virus-immortalized B-cell lines and contains an essential NF-kappa B site. *J. Virol.* 69:871–881.
37. Yin, L., U. Al-Alem, J. Liang, W.M. Tong, C. Li, M. Badiali, J.J. Medard, J. Sumegi, Z.Q. Wang, and G. Romeo. 2003. Mice deficient in the X-linked lymphoproliferative disease gene sap exhibit increased susceptibility to murine gammaherpesvirus-68 and hypo-gammaglobulinemia. *J. Med. Virol.* 71:446–455.
38. Gadue, P., N. Morton, and P.L. Stein. 1999. The Src family tyrosine kinase Fyn regulates natural killer T cell development. *J. Exp. Med.* 190:1189–1196.