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.

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 antitumor 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.
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 lymphoproliferative 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.
To characterize this defect further, we evaluated the cytolytic activity of IL-2–expanded splenic NK cells from SAP<sup>−/−</sup> and SAP<sup>+/+</sup> mice toward various tumor cell lines in vitro. Consistent with the in vivo data (Fig. 1 A), SAP<sup>−</sup> NK cells showed an impaired cytolytic activity against RMA cells when compared with SAP<sup>+</sup> NK cells (Fig. 1 B). Similarly, the capacity of SAP<sup>−</sup> 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<sup>+</sup> NK cells to lyse B16-FO, IC-21, CHO, and P815 cells was comparable to that of SAP<sup>+</sup> 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<sup>−</sup> 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 photostability 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<sup>+</sup> P815 mastocytoma cell line as target cells. Cross-linking of 2B4 with anti-2B4 antibodies enhanced the lysis of P815 cells by SAP<sup>+</sup> NK cells (Fig. 2 B, left), but it failed to enhance P815 killing by SAP<sup>−</sup> 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<sup>−</sup> NK cells resulted in an increase in P815 lysis similar in intensity to that obtained with SAP<sup>+</sup> NK cells. Thus, SAP is not required for the activating pathways that are triggered by NKRs–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<sup>−</sup> 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<sup>+</sup> NK cells. Consistent with earlier reports (10, 11), the stimulation of SAP<sup>+</sup> 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<sup>−</sup> NK cells. Despite these differences, SAP<sup>−</sup> and SAP<sup>+</sup> 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<sup>−</sup> NK cells, we considered that the loss–of–function of 2B4 might account for the natural cytotoxicity defect in SAP<sup>−</sup> NK cells. In this case, only target cells killed less efficiently by SAP<sup>−</sup> NK cells would be expected to express CD48 (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<sup>−</sup> NK cells to kill those cells. Therefore, these data strongly suggest that the impaired natural cytotoxicity response in SAP<sup>−</sup> 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-
ferred to as CL.1 and CL.52) as shown by flow cytometry analysis (Fig. 4 A). When used as target cells with SAP/H11001 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/H11001 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...
Thus, T and B lymphocytes are not required for the elimination of CL.1-CD48 cells recovered from mice. Experiment same as in Fig. 4. (B and C) 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 number of CL.1-CD48 cells (49.4 ± 30 × 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 ± 97.5 × 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−/−γc−/− 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 ± 3.5 × 10^3; n = 2) did not differ significantly from that obtained from SAP+ mice (49.4 ± 30 × 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−/−γc−/− 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−/−γc−/− mice (221.6 ± 104.6 × 10^3; n = 5) was larger than that in control SAP+ mice (49.4 ± 30 × 10^3; n = 8; P = 0.0013). 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 ± 87.8 × 10^3; n = 6) were recovered from the peritoneal cavity in SAP+ mice than in SAP− mice (49.4 ± 30 × 10^3; n = 8; P = 0.0013; Fig. 6 B). By contrast, the numbers of CL.1 cells collected from SAP+ mice (429.6 ± 14 × 10^3; n = 4) and SAP− mice (297.2 ± 97.5 × 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−/−γc−/− mice, and the number of CL.1-CD48 cells found in SAP+ mice was not significantly different from that in RAG−2−/−γc−/− 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
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/H11001 mice resulted in a significant increase in the number of peritoneal 2B4/NK1.1/CD3/NK cells (47.6 ± 27 ± 10^3, n = 9) when compared with that observed in mice injected with PBS alone (16.3 ± 6.9 ± 10^3, n = 6, P = 0.004). However, the number of 2B4/NK1.1/CD3/NK cells found in the peritoneal cavity of SAP/mice (34.2 ± 25.5 ± 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-
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 \( \text{SAP}^{-/-} \) and \( \text{Fyn}^{-/-} \) mice (top, lanes 4–6). Similarly, phosphorylation of SHIP was reduced in \( \text{SAP}^{-/-} \) and abrogated in \( \text{Fyn}^{-/-} \) mice (middle, lanes 4–6). These data indicate that 2B4-mediated tyrosine phosphorylation signals are impaired in SAP-deficient 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-deficient 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-deficient 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-\( \gamma \) 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-deficient 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-
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 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-

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.

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involved 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) 1−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+/− litters) were used for experiments. C57BL/6 RAG-2−/− mice were obtained from Charles River Laboratories. C57BL/6 RAG-2−/−yc−/− 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 Experiments.

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-CD4, anti-CD16, anti-CD3, anti-CD45, anti-DX5, and anti-NK1.1 from BD Biosciences. APC-Streptavidin and FITC-conjugated secondary antibodies (FITC-conjugated goat anti–rat Ig or FITC-conjugated goat anti–mouse Ig obtained from Jackson Immunoresearch Laboratories) were used to reveal biotin-conjugated and nonconjugated antibodies, respectively. Low-affinity Fcy receptors were blocked with 10 µg/ml of 2.4G2 to prevent nonspecific binding.

Cells. YAC-1 (H-2b), RMA (H-2b), RMA/S (H-2b), EL4 (H-2b), C4.4-25 (H-2b), Ba/F3 (H-2b), IC-21 (H-2a), BW51.20 (H-2a), B16-FO (H-2b), P815 (H-2b), 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-CD19, anti-CD4, and anti-CD16 antibodies through 30-µm cell columns that were coated with anti-PE antibodies (Miltenyi Biotec). The splenic NK cells were obtained by the selective depletion of CD4+CD8+ T cells in RPMI 1640–GLUTAMAX supplemented with 10% FCS and antibiotics, 10−5 M 2-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, Ly49I/C, CD16, 2B4, CD12, CD45, DX5, NK1.1, NK2G2, and CD3) were noticeable between IL-2-expanded splenic NK cells from SAP−/−, SAP+/−, and Fyn+/− mice (unpublished data).

eDNA constructs and transfections. The mouse CD48 cDNA was cloned from C57BL/6 thymocytes by RT-PCR using the following primers: sense oligonucleotide: 5′-AGAAAGATGTGCTTCATAAAACAGGG-3′ and antisense oligonucleotide: 5′-TTGTCAGGTTAACAGGATCCTGTG-3′. The mouse CD48 cDNA was inserted into the vector pSRα-Puro, which contains the puromycin resistance gene. Stable transfecants 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 51Cr release assay. In brief, target cells (5 × 104) labeled with 51Cr 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 × 104) were stimulated at 37°C with 5 µg/ml of anti-2B4 or control anti-H-2Dd antibodies (mouse IgG2b), with IL-12 (5 ng/ml; Valbiotech), or in a RADCC assay (see previous paragraph). In brief, NK cells (6 × 104) were incubated with the P815 cells (6 × 104) 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 (104) 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 FACSscan flow cytometer (Becton Dickinson).

The elimination of CL.1 and CL.1-CD48 tumor cells was evaluated by labeling cells (107/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 × 106 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.

We thank P. Rey and B. Pasquier for discussions and for critical reading of the text. We also thank F. Colucci and J. Di Santo for discussions, useful advice, and for providing cell lines and mice; J.-J. Medard and A. Rezard for excellent technical assistance; and G. Romeo and S. Tavligian for their support.

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale and the Association pour la Recherche contre le Cancer, GIS-Institut des Maladies Rares, and the Association for International Cancer Research. S. Latour is a scientist from the Centre National pour la Recherche Scientifique. A. Veillette is a senior investigator at the Canadian Institutes of Health Research. The authors have no conflicting financial interests.

Submitted: 28 February 2005
Accepted: 25 May 2005

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