Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product

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SAP is an adaptor protein expressed in T cells and natural killer cells. It plays a critical role in immunity, as it is mutated in humans with X-linked lymphoproliferative syndrome (XLP), a fatal immunodeficiency characterized by an abnormal response to Epstein-Barr virus (EBV) infection. SAP interacts with the SLAM family receptors and promotes transduction signal events by these receptors through its capacity to recruit and activate the Src kinase FynT. Because it has been previously established that FynT is selectively required for the development of NKT cells, we examined NKT cells in SAP-deficient mice and in humans with XLP. In the absence of SAP, the development of NKT cells is severely impaired both in mice and in humans. These results imply that SAP is a potent regulator of NKT cell development. They also identify for the first time a defect in NKT cells associated with a human primary immunodeficiency, revealing a potential role of NKT cells in the immune response to EBV.
NKT cells are not subjected to negative selection in the thymus but are positively selected by CD1d expressed on CD4+/CD8+ thymocytes in the presence of the self-glycolipid iGb3 (12, 13). It has been proposed that NKT cells might be important for the initiation and the regulation of immune responses by interplaying with innate and adaptive immune responses (9–11).

There is accumulating evidence supporting the notion that some of the signaling pathways driving NKT cell development are unique and differ from those involved in conventional T cells and NK cells (11). The Src kinase FynT has been shown to be required for NKT cell development but not for T cell and NK cell differentiation (14, 15), even though its precise role in NKT cell ontogeny is not known. Because SAP has the capacity to associate and to activate FynT (4), we examined whether SAP is required for NKT cell development. In this study, we report that the NKT cell development is severely impaired both in mice and humans lacking SAP. This is the first report to date describing an inherited fatal immunodeficiency condition in humans in which NKT cells are lacking.

RESULTS AND DISCUSSION

Lack of NKT cells in SAP-deficient mice

We first examined the amount of NKT cells in the different hematopoietic organs of SAP-deficient mice by flow cytometric analysis. The percentages of NKT cells stained by anti-

Figure 1. Defect in NKT cells in SAP-deficient mice. NKT cells were analyzed by flow cytometry in the liver, spleen, and thymus of wild-type (SAP+), SAP-deficient (SAP−), Fyn-deficient (Fyn−/−), and CD1d-deficient (CD1d−/−) mice. (A) Liver lymphocytes and splenocytes were stained with anti-CD19 and thymocytes with anti-HSA. After gating on CD19− or HSAlow cells, dot plots were constructed. Representative two-color dot plots show staining with anti-TCRβ versus anti-NK1.1 antibodies. The percentage of NKT cells corresponding to double positive cells in the circle gate is indicated in each plot. The data are representative of at least five mice per group. (B and C) Absolute numbers of NKT cells in the liver, spleen, and thymus mononuclear cells from SAP+ (black histogram), SAP− (white histogram), and Fyn−/− mice (gray histogram) stained for NK1.1 and TCRβ [B] or NK1.1 and Vβ8.2-TCR [C]. Cells were counted in each organ and absolute numbers of NKT cells were determined based on their proportion (gated on double positive cells). Numbers are mean ± SD of three mice per group. (D) Same experiment as A. Representative two-color dot plots show staining with anti-TCRβ versus anti-NK1.1 antibodies. The percentage of NKT cells corresponding to double positive cells in the circle gate is indicated in each plot. The data are representative of at least five mice per group.
TCRβ and anti-NK1.1 antibodies in the liver, spleen, and thymus of SAP-deficient mice were severely decreased when compared with wild-type (SAP⁺) mice (Fig. 1A). This decrease was similar to that found in Fyn-deficient mice (Fig. 1A). Similar results were obtained with anti-NK1.1 plus anti-Vβ8.2 antibodies (not depicted). However, the percentages of conventional T cells (TCRβ⁺ NK1.1⁺) and NK cells (TCRβ⁺ NK1.1⁺) in SAP-deficient and Fyn-deficient mice were comparable to those observed in wild-type mice (Fig. 1A). Consistent with the decreased proportions of NKT cells in SAP-deficient mice, absolute numbers of these cells in the liver, spleen, and thymus were found to be severely reduced in SAP-deficient and Fyn-deficient mice relative to wild-type mice (Fig. 1, B and C). Moreover, very low amounts of transcripts encoding the Vα14-Jα18 TCR. rearrangement were detected by semiquantitative RT-PCR in the spleen of SAP-deficient and Fyn-deficient animals when compared with wild-type animals (not depicted). Next, we examined CD1d-restricted NKT cells using CD1d tetramers loaded with αGalCer (Fig. 1D). Although CD1d-restricted NKT cells were easily detected in wild-type (SAP⁺) mice, a dramatic reduction of their frequency was observed in SAP-deficient, Fyn-deficient, and CD1d-deficient mice. Importantly, most of the residual TCRβ⁺ αGalCer-loaded CD1d⁺ cells found in SAP-deficient, Fyn-deficient, and CD1d-deficient mice appeared to be nonspecific staining, as a close proportion of these cells were detected with unloaded CD1d tetramers (Fig. 1D, right, and not depicted). These data indicate that SAP-deficient and Fyn-deficient mice lack CD1d-restricted NKT cells.

NKT cells are known to proliferate and produce IFN-γ and IL-4 upon engagement of their invariant TCR with CD1d-presented αGalCer (11). Stimulation of wild-type splenocytes with αGalCer resulted in a robust cell proliferation and production of IFN-γ and IL-4 (Fig. 2, A–C). By contrast, no significant cell proliferation and no production of IFN-γ and IL-4 were observed with SAP-deficient and Fyn-deficient splenocytes cultured in the presence of αGalCer (Fig. 2, A–C). As control, stimulation with anti-CD3 plus IL-2 induced a strong cell proliferation by wild-type, SAP-deficient, and Fyn-deficient splenocytes (Fig. 2 A). IFN-γ production in these conditions was found to be comparable between wild-type and SAP-deficient splenocytes, whereas it was slightly decreased with Fyn-deficient splenocytes. However, SAP-deficient and Fyn-deficient splenocytes failed to produce IL-4 upon anti-CD3 plus IL-2 stimulation. These data were consistent with recent studies showing that activated T lymphocytes from SAP- and Fyn-deficient mice have a defect in IL-4 production (6, 16). Taken together, these results indicate that the compartment of NKT cells is selectively and severely impaired in the absence of the SAP protein.

**Early block in NKT cell development in SAP-deficient mice**

Next, we examined the developmental steps of NKT cells in SAP-deficient mice. During their development in the thymus, CD1d-restricted NKT cell precursors primarily acquire the Vα14-Jα18/Vβ8 TCR that allows their subsequent selection by CD1d-presented self-glycolipid expressed by CD4⁺ CD8⁺ thymocytes (12, 13). Then, NKT cell precursors up-regulate CD44 and lastly acquire NK1.1 expression during their final maturation (17). Because CD1d-deficient mice exhibit a profound defect in NKT cell development (18), we ascertained that the defect of NKT cells in the absence of SAP was not caused by a defective CD1d expression. Expression of CD1d by thymocytes (Fig. 3A) and splenic T and B cells (Fig. 3A and not depicted) in SAP-deficient mice was found to be equivalent to that of wild-type (SAP⁺) and Fyn-deficient mice, excluding that a loss of CD1d expression accounts for the defect of NKT cells observed in SAP-deficient mice. Next, we investigated the expression of CD44 and NK1.1 by thymocytes of SAP-deficient mice that were positive for αGalCer-loaded CD1d tetramers. As shown above in Fig. 1, only a few thymocytes were positive for αGalCer-loaded CD1d tetramers in the SAP-deficient mice in comparison with wild-type (SAP⁺) mice. Nonetheless, when examined for CD44 and NK1.1 expression of the SAP protein.
expression, most of these residual CD1d tetramer\(^+\) cells in SAP-deficient mice did not up-regulate CD44 (94%), and none of them expressed NK1.1 in contrast to wild-type (SAP\(^+/+\)) cells (0 vs. 31%, respectively; Fig. 3 B). To confirm the developmental arrest of NKT cells in absence of SAP, thymocytes from SAP-deficient mice were compared with those of CD1d-deficient mice. The proportions of residual CD1d tetramer\(^+\) cells that are CD44\(^{low}\) were found to be similar in both mice (94 vs. 93%, respectively; Fig. 3 B), suggesting that the block of NKT cell development occurs at a related stage in SAP-deficient and CD1d-deficient mice.

Together, these results suggest that the absence of SAP leads to a severe defect in the early steps of NKT cell development before they up-regulate CD44. SAP could be involved in the intrinsic maturation of NKT cell precursors, the development of the thymic microenvironment, or both. However, we could not strictly exclude that the defect may occur in more mature cells by activation-induced cell death upon physiological antigen encounter. It is proposed that the main function of SAP is to recruit the Src kinase FynT to SLAM family receptors, allowing their coupling to intracellular pathways (19). Thus, one or several members of the SLAM family receptors might be required for normal NKT cell development. Further studies will be needed to test these possibilities.

**Absence of NKT cells in humans with an XLP**

Because the lack of SAP is responsible for XLP in humans (1, 2), we investigated whether NKT cells could be normally detected in the blood of patients with XLP. XLP patients carrying different mutations in the SAP gene were analyzed and compared with healthy age-matched individuals as well as the mother of one patient. None of the XLP patients except one (patient 4; see Materials and methods) tested in this study expressed the SAP protein in his PBLs as shown by Western blotting of cell lysates performed with anti-SAP antibodies (Fig. 4 B and not depicted). The presence of NKT cells within the PBLs of an XLP patient (patient 5), his mother, and a healthy age-matched donor was assessed by flow cytometry by staining with anti-V\(^{\beta}24\) TCR and anti-V\(^{\alpha}24\) TCR antibodies (Fig. 4 A, left) or with anti-V\(^{\beta}11\) TCR antibodies and \(\alpha\)GalCer-loaded CD1d tetramers (Fig. 4 A, middle). In the PBLs from the healthy individual and the mother of the XLP patient, NKT cells were significantly detected with both staining reagents. As a control of specificity, unloaded CD1d tetramers did not identify NKT cells in the healthy individual nor in the mother of the XLP patient (Fig. 4 A, right). In striking contrast, no NKT cells were found in the PBLs of the XLP patient. To confirm this result, PBLs from three additional patients with XLP and six healthy age-matched donors were analyzed. The proportion of NKT cells that were positive for both \(\alpha\)GalCer-loaded CD1d tetramers and anti-V\(^{\alpha}24\) TCR antibodies ranged from 0.08 to 0.18% (0.11 ± 0.04%) in control donors, whereas no detectable NKT cells were observed in XLP patients (0.01 ± 0.01%; P = 0.001; Fig. 4 C). Similar results were found by using anti-V\(^{\alpha}24\) TCR and anti-V\(^{\beta}11\) TCR antibodies.
staining (control donors: 0.18 ± 0.08%, n = 8; XLP patients: 0.00%, n = 6; P = 0.0002; Fig. 5 A). The six XLP patients tested had clinical manifestations that were diverse but typical of XLP (i.e., fulminant mononucleosis, hemophagocytic syndrome, hypogammaglobulinaemia, and lymphoma). Whatever these differences, all XLP patients were found to have this common lack of NKT cells.

To point out that the absence of NKT cells is restricted to XLP, we further examined NKT cells from patients affected with other primary immunodeficiencies such as the closely related inherited hemophagocytic lymphohistiocytic syndromes, the Chédiak–Higashi syndrome (CHS), and the familial hemophagocytic lymphohistiocytosis syndrome (FHL; reference 20). In these patients, Vα24+/Vβ11+ TCR NKT cells were significantly detectable (0.09 ± 0.06%, n = 3) compared with XLP patients (0.00%, n = 6; P = 0.009) and were found to be similar or slightly reduced relative to healthy donors (0.18 ± 0.08%, n = 8; P = 0.12; Fig. 5 A). Thus, the absence of NKT cells in XLP patients appears to be specific of this immunodeficiency condition. In addition, the absence of SAP seems to selectively impair NKT cell development because the proportions of NK cells in the PB-
MCs of XLP patients (2.9 ± 2.8%, n = 6) were comparable to those observed in patients with other immune defects (4.0 ± 2.0%, n = 3; P = 0.55) or in healthy individuals (4.6 ± 3.6%, n = 7; P = 0.34; Fig. 5 B). This is consistent with the normal development of NK cells found in SAP-deficient mice (Fig. 1).

Altogether, these data indicate that in mice and in humans, SAP is required for normal NKT cell development. NKT cells have been proposed to play critical roles in a variety of immune responses, including host defense against pathogens, regulation of autoimmunity, and tumor surveillance (10, 11, 21, 22). In this report, we showed that patients suffering from XLP are devoid of NKT cells, and because XLP is a severe immunodeficiency characterized by an extreme sensitivity to EBV infection, it is tempting to speculate that NKT cells may play an essential function in the control of EBV infection. Future studies should be aimed at addressing this important issue.

MATERIALS AND METHODS

Patients. XLP patients have been genotyped for SAP/SH2D1A and were found to be mutated in SAP resulting in the following amino acid changes in SAP: SAP X129R (patient 1), SAP R55X (patient 2), SAP E67G (patient 3), and SAP R55P (patient 4). Patient 5 had a deletion of the third exon of SAP and patient 6 had a single nucleotide insertion causing a frameshift that leads to a stop codon. The following are the clinical features of the XLP patients: patient 1 developed hypogammaglobulinemia; patients 2, 4, and 6 had a fulminant infectious mononucleosis with a hemophagocytic syndrome; patients 3 and 5 had lymphoma with hypogammaglobulinemia. Two patients with CHS and one patient with FHL who developed hemophagocytic syndrome were also analyzed. Ages of the individuals ranged from 1 to 27 yr old for healthy age-matched donors, 4 to 20 yr old for XLP patients, and 2 mo to 3 yr old for CHS and FHL patients. The mother of patient 5 was 42 yr old. Patient or families provided informed consent for the study in accordance with the Declaration of Helsinki. This study was approved by the INSERM Institutional Review Board.

Animals. SAP-deficient (SAP−/−) mice, Fyn-deficient (Fyn−/−) mice, and CD1d-deficient (CD1d−/−) mice have been described elsewhere (15, 23, 24). Male SAP-deficient mice and male wild-type (SAP+/+) littermates were typed by PCR. All mice used in this study were from 8 to 11 wk of age. Mouse studies were performed under the institutional animal care and use guidelines.

Antibodies and reagents. The following mAbs conjugated to FITC, PE, APC, or biotin were used: anti-NK1.1 (PK136), anti-TCRβ (H57-597), anti-Vβ8.1/8.2 TCR (MR5-2), anti-CD4 (RM4-5), anti-CD8 (53-5-8), anti-CD19 (ID3), anti-CD44 (IM7), anti-HSA (M1/69), and anti-CD1d (B1; all from BD Biosciences). PerCP- or PE-conjugated streptavidin were from BD Biosciences. The mAbs used for experiments in humans were as follows: anti-Vβ11 TCR (C21) and anti-Vß24 TCR (C15) from Beckman Coulter and anti-CD5 (SK7) and anti-CD56 (MY31) from BD Biosciences. CD1d tetramers and αGalCer were provided by A. Bendelac (University of Chicago, Chicago, IL). αGalCer-loaded CD1d tetramers were prepared as described previously (24).

Cell preparation and flow cytometry. Single cell suspensions were prepared from the liver, spleen, and thymus as described previously (14). Human PBMCs were isolated from blood samples by the standard Ficoll-Paque method (Axon-Shield PoC AS; Lymphoprep). Murine single cell suspensions or PBMCs were washed twice in PBS containing 2% FCS and 0.1% NaN3 before staining with the indicated reagents. For mouse analysis, cells were first preincubated with anti-FcγRII/III antibodies (2.4G2) to block Fcγ receptors before staining. Finally, cells were analyzed using a FACS Calibur and CELLQuest software (Becton Dickinson).

Cell proliferation and cytokine production. Spleen cell suspensions were incubated in complete medium supplemented or not with 100 ng/ml αGalCer or stimulated with 3 μg/ml of immobilized anti-CD3 (145-2C11) in the presence of 100 IU/ml of recombinant IL-2. After 36 h in culture, cells were labeled with [3H]thymidine for 12 h, harvested, and counted in a microbeta platelet counter (Wallac). Supernatants were collected after 48 h of stimulation with αGalCer and were tested for IL-4 and IFN-γ contents by ELISA according to the manufacturer’s instructions (R&D Systems). All assays were performed in duplicate.

Western blot. Immunoblots were performed as described previously (3). Polyclonal antibodies to human SAP were produced by immunizing rabbits with a bacterial fusion protein containing the entire human SAP protein.

Semi quantitative RT-PCR. The transcripts encoding the Vα14-Jα18 TCR rearrangement were detected by RT-PCR. In brief, 5 μg of total RNA was reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen), and the cDNAs were amplified by PCR using specific primers as described previously (18).

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

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