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Regulation of NKT cell development by SAP, the protein defective in XLP

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Abstract

The adaptor molecule SAP is expressed in T lymphocytes and natural killer (NK) cells, where it regulates cytokine production and cytotoxicity[1–3]. Here, we show that SAP, encoded by the *SH2D1A* gene locus, also has a crucial role during the development of NKT cells, a lymphocyte subset with immunoregulatory functions in response to infection, cancer and autoimmune disease[4]. Following stimulation with the NKT cell–specific agonist α-galactosyl ceramide (αGC), *Sh2d1a*$^{-/-}$ splenocytes did not produce cytokines or activate other lymphoid lineages in an NKT cell–dependent manner. While evaluating the abnormalities in αGC-induced immune responses, we observed that *Sh2d1a*$^{-/-}$ animals lacked NKT cells in the thymus and peripheral organs. The defect in NKT cell ontogeny was hematopoietic cell autonomous and could be rescued by reconstitution of SAP expression within *Sh2d1a*$^{-/-}$ bone marrow cells. Seventeen

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

individuals with X-linked lymphoproliferative disease (XLP), who harbored germline mutations in *SH2D1A*, also lacked NKT cells. Furthermore, a female XLP carrier showed completely skewed X chromosome inactivation within NKT cells, but not T or B cells. Thus, SAP is a crucial regulator of NKT cell ontogeny in humans and in mice. The absence of NKT cells may contribute to the phenotypes of SAP deficiency, including abnormal antiviral and antitumor immunity and hypogammaglobulinemia.

XLP is a rare human immunodeficiency caused by germline mutations in *SH2D1A*[5,6], the gene that encodes the signaling lymphocytic activation molecule (SLAM)-associated protein SAP[7], an adaptor molecule expressed in T and NK cells. The function of SAP in lymphocytes has primarily been illuminated through studies of SAP-deficient humans and mice. XLP patients[8] and *Sh2d1a*$^{-/-}$ animals[9,10] have normal T, B and NK cell numbers, suggesting that SAP is not required for the development of these lineages. In contrast, XLP patients and *Sh2d1a*$^{-/-}$ mice do not generate normal *in vivo* cellular and humoral immune responses following infection with certain viruses or other pathogens, and after immunization with T cell–dependent antigens[9–14]. In XLP, this is manifested as a heightened susceptibility to Epstein-Barr virus (EBV) infection, an increased development of lymphomas and hypogammaglobulinemia[15,16]. *In vitro*, SAP-deficient lymphocytes show marked functional defects, including reduced T and NK cell cytotoxicity and abnormal T cell cytokine secretion[1–3]. Thus, SAP regulates a broad spectrum of T and NK cell functions.

SAP binds to the cytoplasmic domain of the SLAM family receptors, to which it recruits the Src family tyrosine kinase Fyn[17]. This process contributes to SLAM phosphorylation and the generation of down-stream phosphotyrosine signals. Because SAP signaling depends in part on Fyn, we sought to determine whether *Sh2d1a*$^{-/-}$ and *Fyn*$^{-/-}$ animals shared phenotypic similarities. Notably, previous studies have shown that Fyn is selectively required for the development of NKT cells[18,19], a specialized lymphocyte population that regulates antiviral, antitumor and autoimmune responses[4]. As these processes are perturbed in XLP patients, we speculated that NKT cell defects might contribute to the pathogenesis of XLP.

NKT cells coexpress markers found on NK and T cells, including an invariant T-cell receptor (TCR) that recognizes CD1d (encoded by *Cd1d1*), which presents glycolipid antigens such as the synthetic lipid αGC[4]. To determine whether SAP has a role in NKT cells, we first evaluated its expression by reverse transcription polymerase chain reaction using sort-purified lymphocytes from wild-type mice. As previously reported, *Sh2d1a* transcripts were present in thymocytes and T cells, but not in B cells (Fig. 1a). *Sh2d1a* was also readily amplified from NKT cells (Fig. 1a), supporting the possibility that it might contribute to the development and function of this lineage.

Sh2d1a$^{-/-}$ T cells show disturbances in TCR-induced cytokine production[3,11,12]. Thus, we evaluated whether *Sh2d1a*$^{-/-}$ splenocytes produced cytokines normally in response to αGC. NKT cell–enriched splenocytes from wild-type and *Sh2d1a*$^{-/-}$ mice were stimulated *ex vivo* with αGC, and supernatants were evaluated for presence of interferon-γ (IFN-γ) and interleukin-4 (IL-4). Wild-type splenocytes produced cytokines in response to αGC (Fig.

1b). In contrast, *Sh2d1a*$^{-/-}$ cells did not produce detectable levels of either cytokine (Fig. 1b). To examine *in vivo* cytokine responses, we injected wildtype and *Sh2d1a*$^{-/-}$ mice with αGC. We harvested spleens 2 h later and investigated the expression of cytokine transcripts by RNase protection assay. Consistent with *in vitro* studies, αGC induced the expression of cytokine transcripts by wild-type but not *Sh2d1a*$^{-/-}$ splenocytes (Fig. 1c).

The secretion of cytokines by NKT cells contributes to the *in vivo* activation of other immune cell lineages[20]. Therefore, we next investigated whether *Sh2d1a*$^{-/-}$ mice showed abnormalities in these secondary NKT cell–dependent activation events. We harvested splenocytes from wild-type and *Sh2d1a*$^{-/-}$ mice 4 h after αGC injection, and examined them for expression of the CD69 activation marker or for production of intracellular IFN-γ. CD69 expression was upregulated on the NK, T and B cells from wild-type, but not *Sh2d1a*$^{-/-}$ mice (Fig. 1d). Similarly, NK cells from wild-type, but not *Sh2d1a*$^{-/-}$ mice, showed increased production of IFN-γ (Fig. 1e).

The lack of αGC-induced responses suggested that *Sh2d1a*$^{-/-}$ mice might have qualitative or quantitative defects within the NKT cell lineage. To distinguish between these possibilities, we harvested thymocytes, splenocytes and liver lymphocytes from wild-type and *Sh2d1a*$^{-/-}$ mice and quantified NKT cells by flow cytometry. In wild-type mice, NKT cells represented 14 ± 1.8% (s.e.m.; range, 5–21%), 1.4 ± 0.17% (range, 0.8–2%) and 21 ± 15.7% (range, 4–37%) of the thymocytes, splenocytes and liver lymphocytes, respectively (Fig. 2a). In contrast, NKT cells were markedly reduced in the thymi, spleens and livers of *Sh2d1a*$^{-/-}$ animals, which contained 0.07 ± 0.05% (s.e.m.; range, 0–0.47%), 0.04 ± 0.01% (range, 0.01–0.09%) and 0.03 ± 0% NKT cells (Fig. 2a). The NKT cell percentage was similar to that observed in *Cd1d1*$^{-/-}$ animals[21–23], which lack these cells because of a failure to select NKT cell progenitors, but was unlike that observed in *Fyn*$^{-/-}$ mice, which had a small, but consistently higher percentage of NKT cells (Fig. 2a). The absolute number of NKT cells present in the thymus and spleens of *Sh2d1a*$^{-/-}$ mice was less than 2% of wildtype levels in the thymi and spleens, respectively, of *Sh2d1a*$^{-/-}$ animals (Fig. 2b). In contrast to NKT cells, *Sh2d1a*$^{-/-}$ mice showed no obvious defects in the number or maturation of conventional T or NK cells (see Supplementary Fig. 1 online).

One explanation for the defect in NKT cell development in *Sh2d1a*$^{-/-}$ mice could be a disturbance in CD1d expression or function; however, cells from wild-type and *Sh2d1a*$^{-/-}$ mice expressed similar levels of surface CD1d (Fig. 2c). Furthermore, wild-type and *Sh2d1a*$^{-/-}$ splenocytes were equally effective in presenting αGC to the Vα14^{+} NKT cell hybridoma DN3A4–1.2 and inducing hybridoma cells to produce interleukin-2 (IL-2; Fig. 2d). Thus, the reduced NKT cell number observed in *Sh2d1a*$^{-/-}$ mice does not seem to result from impaired CD1d expression or function.

NKT cell development occurs in the thymus[4], where CD1d^{+} hematopoietic cells select NKT cell progenitors. To determine whether the defect in NKT cell ontogeny resulted from abnormalities within Sap-deficient hematopoietic versus nonhematopoietic cells, we transferred a mixture of wild-type and *Sh2d1a*$^{-/-}$ bone marrow cells, which could be distinguished based on differential CD45.1 expression, into lethally irradiated wild-type mice. We harvested lymphoid organs 6–12 weeks later and compared the contribution of

wild-type and *Sh2d1a*$^{-/-}$ cells to the T-, B- and NKT-cell compartments. CD45.1^{+} wild-type and CD45.1^{-} *Sh2d1a*$^{-/-}$ cells were represented equally in the thymi and spleens of chimeric animals (Fig. 3a) and contributed similarly to T- and B-cell reconstitution (Fig. 3b). In contrast, NKT cells were derived solely from CD45.1^{+} wild-type cells (Fig. 3a), showing that *Sh2d1a*$^{-/-}$ bone marrow cells are selectively impaired in their ability to develop into NKT cells, but not T or B cells.

To verify that the defect in NKT cell ontogeny resulted from the absence of Sap expression within hematopoietic precursors, we t ransduced *Sh2d1a*$^{-/-}$ bone marrow cells *in vitro* with bicistronic retroviruses coexpressing wild-type human SAP and the green fluorescent protein (GFP) or GFP alone. Bone marrow cells were injected intravenously into lethally irradiated mice. Expression of wild-type SAP within *Sh2d1a*$^{-/-}$ bone marrow cells led to the emergence of GFP^{+} NKT cells within the lymphoid compartment of reconstituted animals (Fig. 3c). In contrast, there were no GFP^{+} NKT cells in mice that received *Sh2d1a*$^{-/-}$ bone marrow cells infected with control vector encoding GFP alone (Fig. 3c). All mice showed 58–70% GFP^{+} cells within examined organs (not shown), indicating that differences in NKT cell number were not the result of variable efficiency of retroviral infection between reconstituted animals.

To determine whether human NKT cell homeostasis is also SAP dependent, we examined the number of peripheral blood NKT cells in 17 XLP patients of differing genotypes (Fig. 4a). In healthy donors, 0.18 ± 0.14% (s.e.m.; range, 0.02–0.16%) of the CD3^{+} lymphocytes were NKT cells (Fig. 4b). The percentage of NKT cells was markedly reduced in the XLP patients, and ranged between 0 and 0.01% of the CD3^{+} cells. When analyzed for absolute number, normal donors had ~580 NKT cells/ml of blood (range, 120–1596; Fig. 4c), whereas XLP patients had ~19 NKT cells/ml (range, 0–58), which represents a 97% reduction in these cells. Analysis of an XLP cord blood sample also showed diminished NKT cells (data not shown), suggesting that the NKT cell defect is a primary event from birth and not the result of other XLP manifestations. Thus, germline *SH2D1A* mutations lead to a deficiency in NKT cells, suggesting that the loss of functional SAP protein severely compromises NKT cell development in humans.

Because *SH2D1A* mutations seemed to disrupt human NKT cell ontogeny, we hypothesized that NKT cells from female XLP carriers might exclusively express the X chromosome harboring the wild-type *SH2D1A* allele. Therefore, we used the human androgen receptor assay[24] to analyze X chromosome inactivation within sort-purified peripheral blood lymphocyte subsets from a normal female control and an XLP carrier. PCR amplification of non-*Hpa*II-digested genomic DNA from T, B and NKT cells from the control and the XLP carrier resulted in two distinct products, which represented the maternal and paternal androgen receptor alleles (Fig. 4d). In the control, both products were also detected following amplification *of Hpa*II-digested DNA, indicating random X chromosome inactivation within all lineages (Fig. 4d). Whereas X chromosome inactivation was random in XLP carrier T and B cells (Fig. 4d), it was highly skewed within NKT cells, in which only a single androgen receptor allele was amplified. These genetic data support the hypothesis that SAP is dispensable for human T- and B-cell development but is required for NKT cell ontogeny.

This study shows a new role for SAP during the development of NKT cells. The similar NKT cell phenotypes in *Sh2d1a*$^{-/-}$ and *Fyn*$^{-/-}$ mice suggest that the SAP-Fyn signaling axis might regulate important aspects of NKT cell ontogeny. SAP might target active Fyn to the SLAM-related receptors, which could promote the survival or expansion of NKT cell progenitors. At present, the identity of the SLAM receptor(s) regulating NKT cell ontogeny is not known. A recent report suggests that the crucial receptor may not be SLAM (encoded by *Slamf1*) itself, as *Slamf1*$^{-/-}$ mice rapidly produce IL-4 following injection of antibody specific for CD3 (ref. 25). This rapid induction of IL-4 synthesis is a hallmark of functional NKT cells.

Similar to SAP-deficient mice, no NKT cells were detectable in human XLP patients. This was a consistent finding regardless of *SH2D1A* genotype, age, prior exposure to EBV, history of lymphoma or presence of hypogammaglobulinemia. These data differ from previous studies, which did not show consistent defects in the number of other lymphocytes in XLP patients[9,10], and suggest that a reduction in NKT cells is a universal feature among affected individuals. Together with clinical data, the quantification of NKT cells may facilitate the diagnosis of XLP, a disorder for which a rapid diagnostic test is not yet available.

Our observations suggest that the lack of NKT cells may contribute to the pathogenesis of XLP. For example, *Cd1d1*$^{-/-}$ mice, which lack NKT cells, produce elevated levels of cytokines and have an increased number of CD8^{+} T cells after they are infected with the lymphocytic choriomeningitis virus (LCMV)[26]. This phenotype resembles that of *Sh2d1a*$^{-/-}$ mice[9,10] and suggests that the absence of NKT cells might perturb the immune response against LCMV. It remains possible that the absence of NKT cells also contributes to an exacerbated T-cell expansion in EBV-infected XLP patients. NKT cells can protect against tumor formation or metastasis[27]. Because individuals with XLP lack NKT cells, they may be unusually sensitive to environmental or other influences that trigger lymphoma development. Human NKT cell clones induce *in vitro* B-cell proliferation and enhance immunoglobulin secretion[28]. Thus, the lack of NKT cells might contribute to hypogammaglobulinemia in affected patients. Because *Cd1d1*$^{-/-}$ mice show no defects in humoral responses, the reduced immunoglobulin production in SAP-deficient humans and mice might result from combined defects in T and possibly NKT cells.

In conclusion, SAP is a crucial regulator of NKT cell development in humans and in mice. Future studies that examine the interactions between SAP, Fyn and the SLAM family receptors during NKT cell development and mature NKT cell activation will clarify the signaling mechanisms governing NKT cell differentiation and function. This information will enhance our understanding of NKT cells and may facilitate the development of new treatments for XLP and other human diseases associated with qualitative or quantitative NKT cell defects.

METHODS

Mouse strains.

Sh2d1a$^{-/-}$ (ref. 12) and *Fyn*$^{-/-}$ (ref. 29) mice were generated as described and have been backcrossed to C57BL/6 mice for over five generations. C57BL/6 mice congenic for CD45.1, *Rag1*$^{-/-}$, and 129/SvJ mice were purchased from Jackson Laboratories. Mice were housed at the Wistar Institute or the University of Pennsylvania under pathogen-free conditions. Experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the Wistar Institute and the US National Institutes of Health.

Human patients.

This study investigated 17 XLP patients, 4 XLP carriers and 10 healthy individuals. Thirteen XLP patients harbored germline *SH2D1A* point mutations, and three patients had deletions of the *SH2D1A* gene. One patient met the criteria for XLP and lacked SAP protein expression in peripheral blood lymphocytes; however, no *SH2D1A* mutation could be identified. Six patients had a history of EBV exposure, eight were hypogammaglobulinemic and three had been treated for lymphoma. Clinical details were not available for four patients; however, no patients were receiving chemotherapy or immunosuppression at the time of these investigations. Participants gave informed consent, per guidelines of the Institutional Review Boards of the Children's Hospital of Philadelphia and the Centenary Institute. Lymphocyte DNA was isolated and analyzed for *SH2D1A* mutations as described[6].

Quantification of NK, T and NKT cells.

To quantify mouse cells, we generated single-cell suspensions from the thymi, spleens, bone marrow and peripheral blood of wild-type, *Sh2d1a*$^{-/-}$, *Fyn*$^{-/-}$ and *Cd1d1*$^{-/-}$ mice. Liver lymphocytes were isolated as previously reported[18]. Cells were stained using antibodies with or without phycoerythrin-conjugated αGC-loaded CD1d tetramers. Antibodies purchased from BD PharMingen included: CD24/HSA-FITC (clone M1/69), CD24/HSA-biotin (30-F1), TCRβ-CyC (H57-597), NK1.1-PE/FITC (PK 136), CD1d-FITC (1B1), CD4-perCP (RM4.5), CD8α-APC (53-6.7), CD45.1-FITC (A20), Ly5.1-FITC (30-F11), CD11b-FITC/APC (M1/70), CD43-FITC (S7), CD49b-FITC/PE (DX5), CD45R/B220-FITC (RA3-6B2), CD117/c-kit-FITC (2B8), CD51-PE (RMV-7), CD69-FITC (H1.2F3), CD122-FITC (TM-β1) and APC-streptavidin.

To evaluate human cells, we isolated peripheral blood mononuclear cells by Ficoll-Paque (Pharmacia LKB) centrifugation from normal donors and XLP patients. We stained cells using Vα24-FITC (clone C15) and Vβ11-PE (clone C21; Beckman Coulter) antibodies, plus CD3-APC (clone UCHT1; BD PharMingen). NKT cells were also stained with CD3-APC and 6B11-PE (BD Pharmingen), which recognizes a unique epitope within the Vα24-JαQ junction of the invariant TCR used by human NKT cells.

We performed flow cytometry using a FACSCalibur flow cytometer (Beckton Dickinson) and analyzed the data using CellQuest software (Beckton Dickinson). Mouse NKT cells

were defined as HSA^{low} Tet^+ $TCR\beta^+$ cells. Human NKT cells were $CD3^+$ $V\alpha24^+$ $V\beta11^+$ or $CD3^+$ $6\beta11^+$ cells.

RT-PCR analysis of *Sh2d1a* expression.

T, B and NKT cell subsets were sort-purified from the thymi and spleens of wild-type C57BL/6 mice. RNA was isolated from purified cells and from unpurified wild-type and *Sh2d1a*$^{-/-}$ thymocytes using RNA STAT-60 (Tel-Test, Inc.), according to manufacturer's protocols. RNA was reverse transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences). To detect *Sh2d1a* transcripts, 2 μl of cDNA was used as a template in a PCR reaction employing primers complementary to the 5′ and 3′ regions of the mouse *Sh2d1a* coding sequence (PCR primers and conditions available upon request).

αGC-induced cytokine production and secondary immune cell activation.

For analysis of *in vitro* αGC-induced cytokine production, we depleted splenocytes of $CD8^+$ and $B220^+$ cells using antibody-coated magnetic beads. We plated cells in 96-well dishes at 100,000 cells/well and cultured them at 37 °C, 5% CO_2 in RPMI 1640 medium (Cellgro), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), antibiotics and glutamine (Gibco Invitrogen). To stimulate NKT cells, we added 100 ng/ml of αGC to appropriate wells. We harvested culture supernatants and measured cytokine secretion by ELISA (R&D Systems).

To evaluate *in vivo* cytokine production, we injected mice intravenously with αGC (3 μg) or vehicle. We removed spleens 2 h later. Total RNA was isolated using RNA STAT-60 (Tel-Test). To measure the levels of cytokine transcripts, RNA was analyzed using the RiboQuant RNase protection assay system (probe set m-CK-1; BD PharMingen). To evaluate the secondary activation of other immune cell types, we injected mice with αGC and 4 h later, we harvested splenocytes and analyzed them for surface expression of CD69. Splenocytes were also permeabilized (Cytofix/Cytoperm kit, BD Pharmingen) and stained for intracellular IFN-γ.

To measure the functional status of surface CD1d, we incubated 2×10^6 splenocytes for 4 h at 37 °C in RPMI medium containing 10% FBS, antibiotics, glutamine and 100 ng/ml αGC or vehicle. Washed splenocytes (1×10^5) were incubated with DN3A4–1.2 NKT hybridoma cells (5×10^4) in a volume of 200 μl of RPMI medium containing 10% FBS, antibiotics and glutamine. After 18 h, we harvested supernatants and measured IL-2 secretion by ELISA (R&D Systems).

Generation of bone marrow chimeras.

Bone marrow cells were obtained from $CD45.1^+$ wild-type and $CD45.1^-$ *Sh2d1a*$^{-/-}$ mice. Cells were mixed at a ratio of 1:1, and 1×10^6 cells were injected intravenously into lethally irradiated C57BL/6 ($n = 4$), 129/SvJ ($n = 4$) or *Rag1*$^{-/-}$ ($n = 4$) recipients. Mice were maintained on sterile water containing the antibiotic Bactrim for 2–3 weeks, and then were switched to sterile water. After 6–8 weeks, we determined donor chimerism and NKT cell reconstitution by flow cytometry.

Retroviral infection of *Sh2d1a*$^{-/-}$ bone marrow cells and generation of bone marrow reconstituted animals.

Retroviruses were generated as described[3]. For bone marrow transfer experiments, we injected *Sh2d1a*$^{-/-}$ mice intraperitoneally with 5 mg of 5-flourouracil (American Pharmaceutical Partners, Inc.). We harvested bone marrow cells 4–5 d later, and infected them *in vitro* with retroviruses[30]. We injected 1×10^6 cells intravenously into lethally irradiated recipients, which were maintained as described[30]. After 6–12 weeks, we analyzed NKT cells.

X chromosome inactivation studies.

X chromosome inactivation was d etermined using the human androgen receptor assay[24]. We isolated genomic DNA from sort-purified T, B and NKT cells from a normal female control and a female XLP carrier who was heterozygous for a G383C substitution in *SH2D1A*. We digested 200 ng of DNA with *Rsa*I for 12 h at 37 °C. We then digested half of the DNA with the methylation-sensitive enzyme *Hpa*II for 5 h at 37 °C. *Hpa*II-digested and nondigested DNA samples served as templates to PCR amplify across a $(CAG)_n$ repeat within exon 1 of the human androgen receptor gene using f luorescent primers (sequences available upon request). PCR products were evaluated using an ABI 3100 DNA Fragment Analyzer (Applied Biosystems), and data were a nalyzed using Genescan and Genotyper software (Applied Biosystems).

If randomly inactivated, a proportion of the methylated maternal and paternal X chromosomes will remain intact after *Hpa*II digestion and can serve as templates for PCR amplification. The maternal and paternal alleles are then distinguished based on variations in the sizes of the amplified products, which reflect differences in the lengths of the maternal and paternal $(CAG)_n$ repeats. If nonrandomly inactivated, the allele on the active X chromosome will be completely digested by *Hpa*II. Only the inactive allele will be amplified, resulting in a single PCR product.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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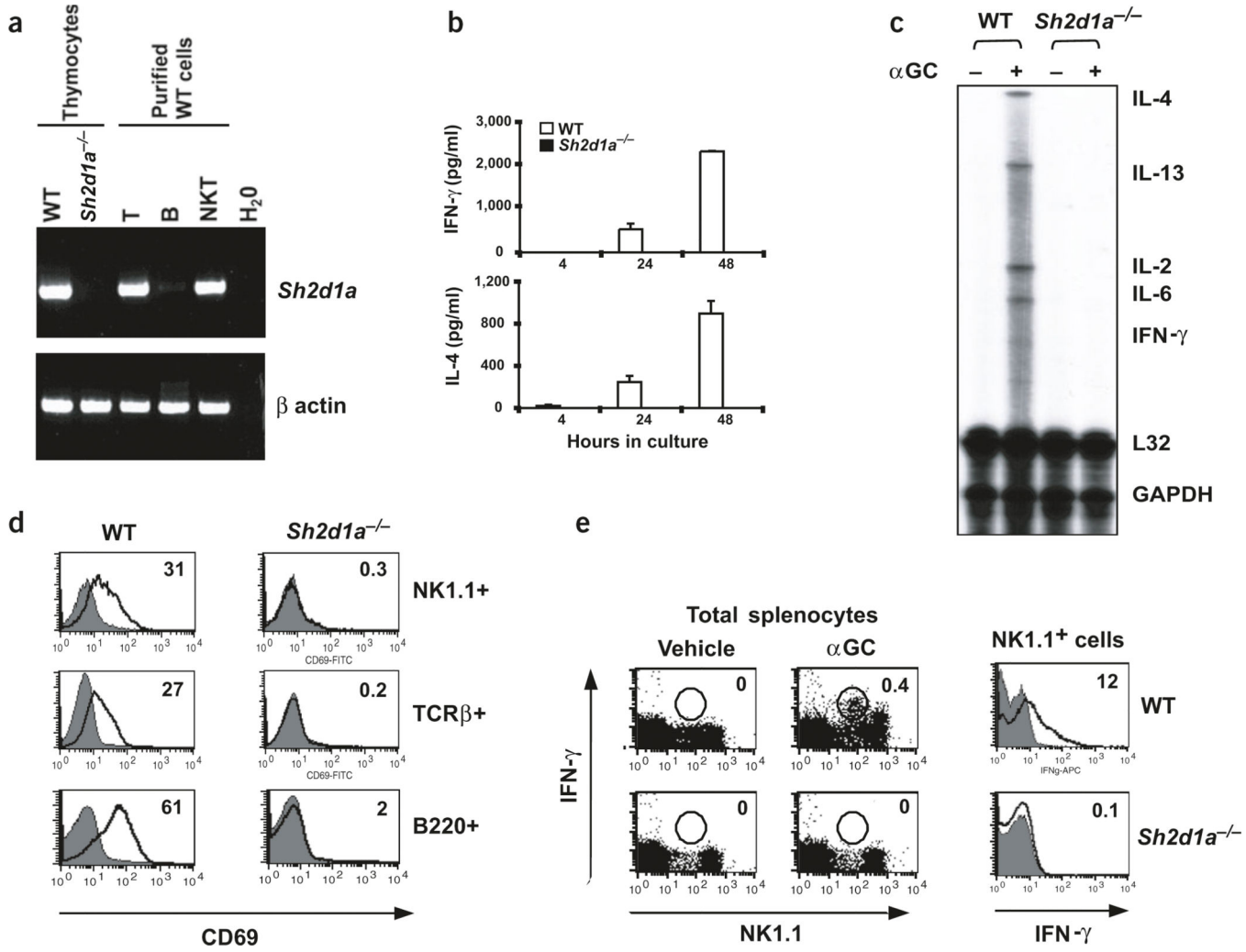

Figure 1.

Absence of αGC induced immune cell activation in *Sh2d1a*$^{-/-}$ mice. (**a**) *Sh2d1a* mRNA expression in wild-type (WT) and *Sh2d1a*$^{-/-}$ thymocytes, and purified T, B and NKT cells. (**b**) *In vitro* αGC-induced cytokine secretion by wild-type and *Sh2d1a*$^{-/-}$ splenocytes. (**c**) *In vivo* αGC-induced cytokine response in wild-type and *Sh2d1a*$^{-/-}$ mice, as measured by RNase protection. (**d**,**e**) Activation of immune cells in vehicle- (filled histograms) or αGC-injected (open histograms) wild-type and *Sh2d1a*$^{-/-}$ mice. (**d**) Expression of CD69 on splenocytes. The percentage of CD69^{+} cells following αGC injection is shown. (**e**) Intracellular IFN-γ production by TCRβ^{-} NK1.1^{+} NK cells. IFN-γ-positive cells are shown as a percentage of total splenocytes (dot plots) or NK cells (histograms).

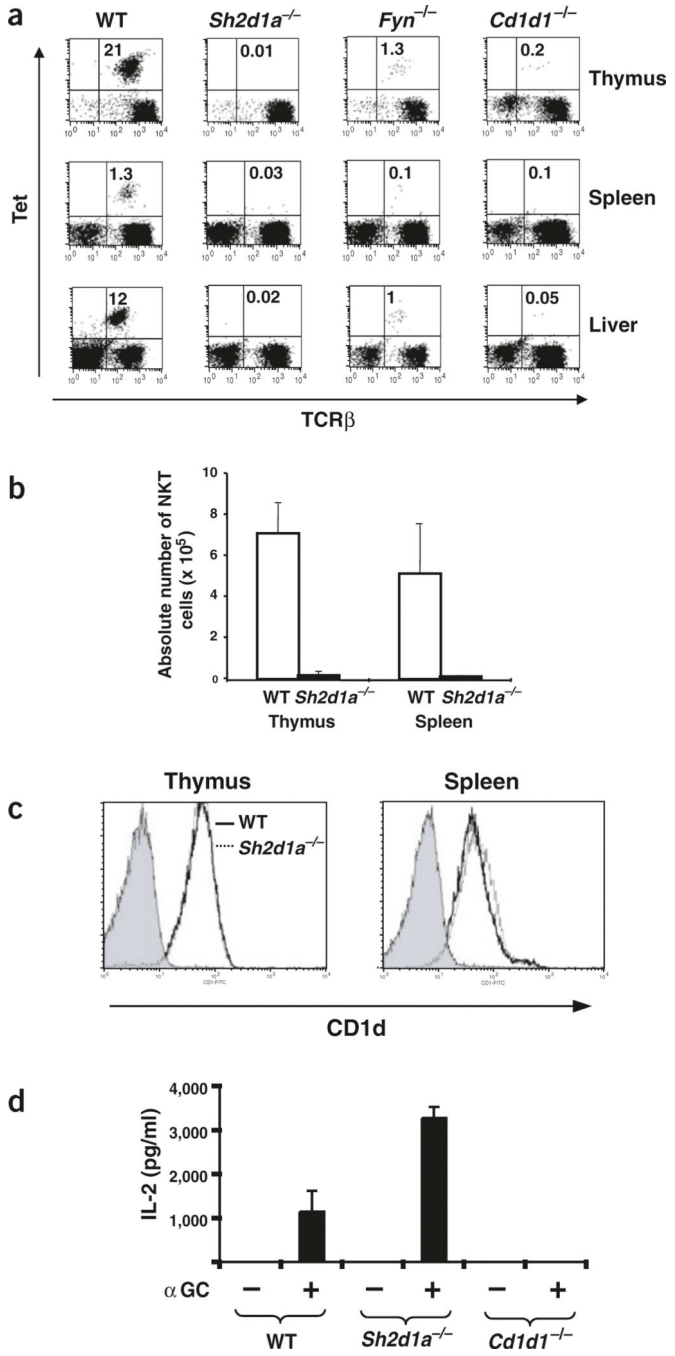

Figure 2.

Sh2d1a$^{-/-}$ mice lack NKT cells. (**a**) Flow cytometric plots showing the percentages of HSA^{low} Tet^{+} TCRβ^{+} NKT cells in wild-type, *Sh2d1a*$^{-/-}$, *Fyn*$^{-/-}$ and *Cd1d1*$^{-/-}$ mice. (**b**) Absolute numbers of NKT cells in the thymi and spleens of wild-type ($n = 10$) and *Sh2d1a*$^{-/-}$ mice ($n = 10$). Differences in NKT cell number were statistically significant in both organs; Mann-Whitney test, $P < 0.001$. (**c**) CD1d expression on thymocytes and splenocytes from wild-type (open histogram), *Sh2d1a*$^{-/-}$ (dotted open histogram) and *Cd1d1*$^{-/-}$ (filled histogram) mice. (**d**) IL-2 secretion by DN3A4–1.2 Vα14^{+} NKT cell

hybridoma cells after culture with vehicle or αGC-pulsed wild-type, *Sh2d1a*$^{-/-}$ or *Cd1d1*$^{-/-}$ splenocytes.

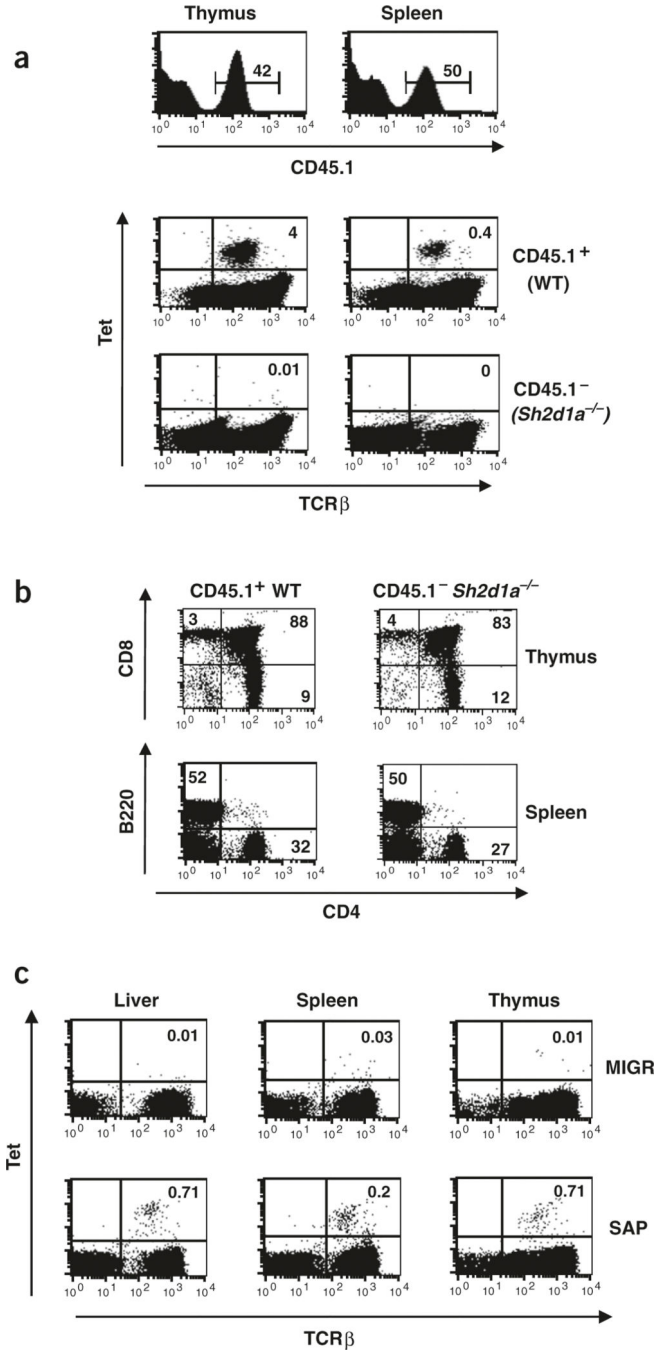

Figure 3.

The defect in NKT cell ontogeny is hematopoietic cell-autonomous and rescued by expression of wild-type SAP. (**a**,**b**) Bone marrow chimeric mice ($n = 12$) were generated using CD45.1$^+$ wild-type and CD45.1$^-$ *Sh2d1a*$^{-/-}$ cells. (**a**) Flow cytometric plots showing percent donor chimerism (top) and percent HSA^{low} Tet$^+$ TCRβ$^+$ NKT cells in CD45.1$^+$ (wild-type) and CD45.1$^-$ (*Sh2d1a*$^{-/-}$) lymphocyte populations (bottom). (**b**) Percentages of B cells and T cell subsets in bone marrow chimeras. (**c**) Sap-deficient bone marrow cells were infected *in vitro* with retroviruses encoding GFP (MIGR; $n = 8$) or GFP plus

wild-type human SAP (SAP; $n = 8$) and were transferred into irradiated wild-type recipients. Percentages of GFP^+ HSA^{low} Tet^+ $TCR\beta^+$ NKT cells in the organs of reconstituted animals are indicated.

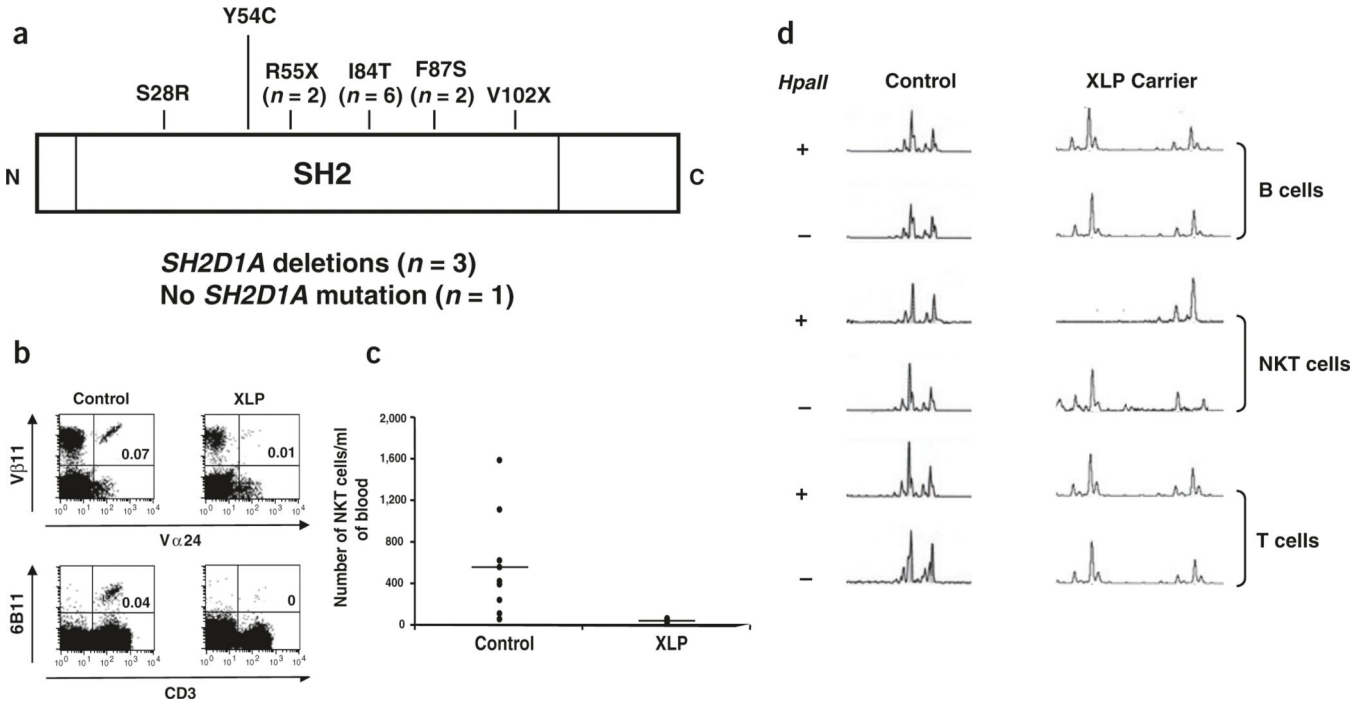

Figure 4.

XLP patients have reduced NKT cells and a female XLP carrier shows skewed X chromosome inactivation in NKT cells. (**a**) Genotypes of the 17 XLP patients examined. (**b**) Representative flow cytometric plots showing percentages of $CD3^+$ $V\alpha24^+$ $V\beta11^+$ or $CD3^+$ $6B11^+$ NKT cells in one control and one XLP patient. (**c**) The absolute number of NKT cells in 10 controls and 17 XLP patients; Mann-Whitney test, $P = 0.002$. (**d**) X chromosome inactivation in T, B and NKT cells from a female control and an XLP carrier, as determined using the human androgen receptor assay. After PCR amplification of non-*Hpa*II-digested DNA (–), products of two lengths were obtained from control and XLP carrier T, B and NKT cells, indicating polymorphic maternal and paternal androgen receptor alleles. After amplification of *Hpa*II-digested DNA (+), two alleles were obtained from control lymphocytes and XLP carrier T and B cells, indicating random X inactivation within these lineages. Only one allele was amplified from *Hpa*II-digested NKT cell DNA from the XLP carrier, indicating skewed X chromosome inactivation.