Signaling lymphocytic activation molecule (SLAM)/SLAM-associated protein pathway regulates human B-cell tolerance

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Background: Signaling lymphocytic activation molecule (SLAM)–associated protein (SAP) can mediate the function of SLAM molecules, which have been proposed to be involved in the development of autoimmunity in mice.
Objective: We sought to determine whether the SLAM/SAP pathway regulates the establishment of human B-cell tolerance and what mechanisms of B-cell tolerance could be affected by SAP deficiency.
Methods: We tested the reactivity of antibodies isolated from single B cells from SAP-deficient patients with X-linked lymphoproliferative disease (XLP). The expressions of SAP and SLAM family members were assessed in human bone marrow–developing B cells. We also analyzed regulatory T (Treg) cell function in patients with XLP and healthy control subjects.
Results: We found that new emigrant/transitional B cells from patients with XLP were enriched in autoreactive clones, revealing a defective central B-cell tolerance checkpoint in the absence of functional SAP. In agreement with a B cell–intrinsic regulation of central tolerance, we identified SAP expression in a discrete subset of bone marrow immature B cells. SAP colocalized with SLAMF6 only in association with clustered B-cell receptors likely recognizing self-antigens, suggesting that SLAM/SAP regulate B-cell receptor–mediated central tolerance. In addition, patients with XLP displayed defective peripheral B-cell tolerance, which is normally controlled by Treg cells. Treg cells in patients with XLP seem functional, but SAP-deficient T cells were resistant to Treg cell–mediated suppression. Indeed, SAP-deficient T cells were hyperresponsive to T-cell receptor stimulation, which resulted in increased secretion of IL-2, IFN-γ, and TNF-α.
Conclusions: SAP expression is required for the counterselection of developing autoreactive B cells and prevents their T cell–dependent accumulation in the periphery. (J Allergy Clin Immunol 2014;133:1149-61.)

Key words: *SLAM-associated protein, signaling lymphocytic activation molecule, B-cell tolerance, regulatory T cells*

X-linked lymphoproliferative disease (XLP) 1 is a primary immunodeficiency caused by mutations/deletions in the *SH2D1A* gene, which encodes the signaling lymphocytic activation molecule (SLAM)–associated protein (SAP).[1-3] SAP is a single SH2 domain–containing molecule that plays a crucial role in the signaling of SLAM molecules. It might function as an adaptor for the Src family tyrosine kinase Fyn, as well as a competitor for phosphatases, such as Src homology domain 2–containing protein tyrosine phosphatase (SHP) 1 and SHP-2, thereby modulating the function of SLAM family members.[4] The SAP/SLAM pathway has been implicated in the development of autoimmunity. The mouse *Sle1b* locus, which has been linked to lupus susceptibility, contains genes encoding members of the SLAM family.[5] In the lupus-prone mouse strain NZM2410, the expression of the *Ly108.1* isoform leads to altered central B-cell tolerance mechanisms, including B-cell anergy, receptor editing, and deletion.[6] Although polymorphisms in SLAM family genes have been linked to lupus and rheumatoid arthritis in human subjects,[7,8] a direct role of the SAP/SLAM pathway in the control of B-cell tolerance in human subjects has not yet been demonstrated.

In healthy human subjects most developing autoreactive B cells are removed at 2 discrete steps.[9] First, a central tolerance checkpoint in the bone marrow between early immature and immature B cells removes most of the developing B cells that express highly polyreactive antibodies. Then a peripheral B-cell tolerance checkpoint further counterselects autoreactive new emigrant B cells before they enter the mature naive B-cell compartment.[9] The central B-cell tolerance checkpoint seems to be mostly regulated by B cell–intrinsic pathways. Alterations of the B-cell receptor (BCR) signaling pathway in patients lacking functional

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Abbreviations used
ANA: Anti-nuclear antibody
APC: Allophycocyanin
BAFF: B cell–activating factor of the TNF family
BCR: B-cell receptor
CFSE: Carboxyfluorescein succinimidyl ester
FOXP3: Forkhead box protein 3
IPEX: Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
NKT: Natural killer T
PE: Phycoerythrin
SAP: SLAM-associated protein
SHP: Src homology domain 2–containing protein tyrosine phosphatase
SLAM: Signaling lymphocytic activation molecule
TCR: T-cell receptor
TLR: Toll-like receptor
Treg: Regulatory T
Tresp: Responder T
XLP: X-linked lymphoproliferative disease

Bruton tyrosine kinase (BTK) or in healthy subjects carrying the *C1858T PTPN22* risk allele result in failure to counterselect developing autoreactive B cells in the bone marrow.[10-12] In addition, mutations in genes encoding molecules, such as IL-1 receptor–associated kinase 4, myeloid differentiation primary response gene–88, UNC-93B, and adenosine deaminase, which mediate and regulate the functions of Toll-like receptors (TLRs), potentially sensing self-antigens, also interfere with the establishment of central tolerance, especially toward nucleic acid–containing antigens.[11,13,14] Although showing normal central B-cell tolerance, CD40 ligand (CD40L)– and MHC class II–deficient patients display specific defects in the peripheral B-cell tolerance checkpoint characterized by high frequencies of autoreactive mature naive B cells correlating with low numbers of circulating $CD4^+CD25^+CD127^{lo}$ forkhead box protein 3 (FOXP3)–positive regulatory T (Treg) cells.[15] The essential role of Treg cells in regulating the peripheral B-cell tolerance checkpoint was demonstrated in FOXP3-deficient patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, who display nonfunctional Treg cells and harbor severe defects in the counterselection of autoreactive peripheral B cells.[16]

To determine the role of the SAP/SLAM pathway in the establishment of human B-cell tolerance checkpoints, we analyzed the repertoire and reactivity of antibodies expressed by single new emigrant/transitional and mature naive B cells from SAP-deficient patients with XLP. We found that SAP is expressed by a discrete population of developing immature B cells and is required for central B-cell tolerance. We also found that SAP expression in T cells likely prevents the accumulation of autoreactive mature naive B cells, further suggesting the importance of B-cell/T-cell interactions for the establishment of peripheral B-cell tolerance.

METHODS

Patients

Information from patients with XLP is included in Table E1 in this article's Online Repository at www.jacionline.org. Healthy donors were previously reported.[9,10,12-17] None of the patients with XLP experienced accelerated phases if they encountered EBV, and none of the 4 patients with XLP analyzed for antibody reactivity displayed the *C1858T PTPN22* risk allele, which by itself interferes with the counterselection of developing autoreactive B cells.[10-12] All samples were collected in accordance with institutional review board–reviewed protocols.

Cell staining and sorting, cDNA, RT-PCR, antibody production, ELISAs, and indirect fluorescence assays

Single $CD21^{lo}CD10^{++}IgM^{hi}CD27^-$ new emigrant/transitional and $CD21^+CD10^-IgM^+CD27^-$ peripheral mature naive B cells from patients and control donors were sorted on a FACSVantage (Becton Dickinson, Mountain View, Calif) into 96-well PCR plates, and antibody reactivities were tested as previously described.[9] The following antibodies were used for flow cytometric staining: CD19–allophycocyanin (APC)-Cy7, CD19-Pacific Blue, CD27-PerCP-Cy5.5, CD10–phycoerythrin (PE), CD10-PE-Cy7, IgM–fluorescein isothiocyanate, CD21-APC, CD4-APC-Cy7, CD25-PE-Cy7, CD127-PerCP-Cy5.5, CD45RO-Pacific Blue, CD48–fluorescein isothiocyanate, CD150-PE, CD352-PE, CD319-PE, CD244-APC (all from BioLegend, San Diego, Calif), CD3-eFluor605NC (eBioscience, San Diego, Calif), CD21-BDHorizonV450, and CD84-PE (Becton Dickinson). The anti-SAP mAb (clone1C9; Abnova, Taipei, Taiwan) was biotinylated with the Fluoreporter Mini-biotin-XX protein labeling kit (Molecular Probes, Eugene, Ore). Intracellular staining for FOXP3–Alexa Fluor 488 (clone PCH101, eBioscience) and Helios-AlexaFluor647 (BioLegend) was performed with the FOXP3/Transcription Factor Staining Buffer Set (eBioscience), according to the manufacturer's instructions. For intracellular cytokine detection, $CD4^+$ responder T (Tresp) cells activated for 4 days *in vitro* were then stimulated with 30 nmol/L phorbol 12-myristate 13-acetate and 200 nmol/L ionomycin for 4 hours in the presence of GolgiStop (BD Biosciences), and intracellular staining of cytokines (IFN-γ and TNF-α) was performed with FOXP3 staining buffers (eBioscience) and the following antibodies: IFN-γ (clone 4S.B3, eBioscience) and TNF-α (clone Mab11, eBioscience).

Real-time RT-PCR analysis

$CD19^+$ cells from fetal liver, fetal bone marrow, or peripheral blood of healthy donors were enriched with CD19 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Peripheral mature naive B cells were further sorted to exclude the presence of $CD3^+$ T cells, which represent the majority of circulating lymphocytes. Total RNA was then extracted with the Absolutely RNA Microprep Kit (Agilent Technologies), and 150-ng RNA samples were reverse transcribed by using random hexamers (Applied Biosystems, Santa Clara, Calif) and the SuperScript III Reverse Transcriptase kit (Invitrogen, San Diego, Calif). For mRNA gene expression assays, probes were purchased from Applied Biosystems (Foster City, Calif; *SH2D1A [SAP]*: Hs00158978_m1, *CD3E*: Hs01062241_m1, and *HPRT1*: Hs02800695_m1), and reactions were run on a 7500 Real-Time PCR system (Applied Biosystems) in duplicate. Values are represented as the difference in cycle threshold values normalized to *HPRT1* for each sample before comparisons between fetal samples and peripheral B and T cells.

Immunofluorescence

PBMCs depleted from $CD20^+$ B cells, purified naive B cells (EasySep Naive B cell isolation kit), or pre-enriched $CD34^-CD19^+$ bone marrow B cells were washed in PBS 1× and deposited on poly-L-lysine–coated glass slides (Sigma-Aldrich, St Louis, Mo) in a cytospin centrifuge (Thermo, Waltham, Mass). Acetone-fixed cells were stained with mouse anti-IgM (Santa Cruz Biotechnology, Santa Cruz, Calif), anti-Igκ (BD Biosciences), anti-Igλ (BD Biosciences), VpreB (BioLegend), or anti-SLAMF6/CD352 (BioLegend); rat anti-SAP–PE (clone 1D12, Santa Cruz Biotechnology) or rat isotype control–PE (eBioscience); or 4′-6-diamidino-2-phenylindole dihydrochloride or rabbit anti-Igκ and anti-Igλ (DAKO, Glostrup, Denmark). Primary antibodies were revealed with donkey anti-mouse Alexa Fluor 488

(Invitrogen, Molecular Probes); goat anti-rat–PE (Santa Cruz Biotechnology), followed by donkey anti-goat PE (Jackson ImmunoResearch, West Grove, Pa); and donkey anti-rabbit Alexa Fluor 350 (Invitrogen, Molecular Probes). The percentage of cells presenting a clustered pattern of either IgM or Igκ/Igλ was estimated by counting the number of cells with clustered versus diffuse/nonclustered IgM or Igκ/Igλ cells in the SAP^+ and SAP^- cells. Slides were visualized under a Zeiss AxioObserver.Z1 microscope (Carl Zeiss, Oberkochen, Germany).

In vitro Treg cell suppression assay

$CD4^+$ T cells were enriched with the EasySep Human $CD4^+$T cells kit (STEMCELL Technologies, Vancouver, British Columbia, Canada) and either stained for fluorescence-activated cell sorting of $CD4^+CD25^{hi}CD127^{lo/-}$ Treg cells or depleted from $CD25^+$ cells with anti-human CD25 microbeads (Miltenyi Biotec) to obtain the Tresp cell fraction. $CD4^+CD25^-$ Tresp cells were labeled with CellTrace carboxyfluorescein succinimidyl ester (CFSE; InvivoGen, San Diego, Calif). Cocultures of Treg and Tresp cells at a 1:1 ratio with or without the addition of cytokines at 50 ng/mL concentration (PeproTech, Rocky Hills, NJ) were stimulated with the Treg Suppression Inspector Human kit (Miltenyi Biotec). Proliferation of the viable Tresp cells was analyzed by means of CFSE dilution at day 4.5.

Cytokine detection

Serum B cell–activating factor of the TNF family (BAFF) concentrations were determined by using ELISA, according to the manufacturer's instruction (R&D Systems, Minneapolis, Minn). Cytokines (IL-2, IL-4, IL-6, IL-7, IL-10, IL-13, IFN-γ, TNF-α, and GM-CSF) in plasma or culture supernatants were measured with the High Sensitivity Human Cytokine Magnetic Bead kit (Millipore, Temecula, Calif) by using a Luminex200. IL-21 was measured with the Human IL-21 ELISA Ready-Set-Go kit (eBioscience).

κ-Deleting recombination excision circle assay

The ratio of κ-deleting recombination excision circle joints (signal joint) to the Jκ-Cκ recombination genomic joints (coding joint) was determined, as previously described.[18,19] The number of cell divisions was calculated by subtracting the cycle threshold of the PCR detecting the coding joint from that of the PCR detecting the signal joint.

Statistical analysis

Differences between patients and healthy donors were analyzed for statistical significance with unpaired Student *t* tests by using GraphPad Prism (GraphPad Software, La Jolla, Calif).

RESULTS

Defective central B-cell tolerance checkpoint in patients with XLP

Most developing B cells that express polyreactive and anti-nuclear antibodies (ANAs) are removed at a central B-cell tolerance checkpoint in the bone marrow. To assess whether central tolerance is functional in SAP-deficient patients with XLP, we cloned antibodies expressed by single $CD19^+CD10^{++}CD21^{lo}IgM^{hi}CD27^-$ new emigrant/transitional B cells from 8 patients with XLP (see Tables E1-E9 in this article's Online Repository at www.jacionline.org) and analyzed their immunoglobulin gene repertoire. Although the immunoglobulin heavy chain gene segment repertoires showed no differences between healthy donors and patients with XLP (see Fig E1 in this article's Online Repository at www.jacionline.org), we found that both Igκ and Igλ variable gene uses were biased in new emigrant/transitional B cells from patients with XLP (see Fig E2 in this article's Online Repository at www.jacionline.org). D reading frames favoring hydrophobic amino acids have been suggested to favor self-reactivity.[10,20] Remarkably, the D6-6 gene is the only D gene that was virtually never found using its hydrophobic reading frame in new emigrant B cells from 11 healthy donors (see Fig E3 in this article's Online Repository at www.jacionline.org). In contrast, D6-6 was used as frequently in its hydrophobic and hydrophilic reading frame in B-cell counterparts from patients with XLP, a feature that we previously observed in other patients with defective central B-cell tolerance.[10,21] In addition, antibodies with long immunoglobulin heavy chain and Igλ but not Igκ CDR3s were frequently expressed in new emigrant/transitional B cells from patients with XLP, another feature that favors self-reactivity normally counterselected in the bone marrow, suggesting an altered central B-cell tolerance checkpoint in these patients (Fig 1, *A-C*).[9] In agreement with these observations, we found that the frequency of polyreactive clones was significantly higher in all SAP-deficient patients with XLP (average of 26.3%) compared with that seen in healthy control subjects (average of 7.3%, $P < .0001$; Fig 1, *D* and *E*). However, the frequency of ANA clones was not increased, which suggests that ANA-expressing B cells are properly removed in the bone marrow of patients with XLP (Fig 1, *F*). In conclusion, we found that the central B-cell tolerance checkpoint is impaired in the absence of functional SAP.

SAP is expressed in a discrete population of immature B cells

Central B-cell tolerance is believed to be regulated by intrinsic B-cell factors, including BCRs and TLRs, which sense and bind to self-antigens at the immature B-cell stage in bone marrow.[11] SAP expression in B cells is controversial and might be restricted to a subset of activated germinal center B cells.[22-24] However, SAP expression in bone marrow B cells has not been characterized. Purified $CD19^+$ precursor B cells from fetal or adult bone marrow showed SAP expression in approximately 10% of IgM^+ cells, whereas SAP expression was not detected in peripheral blood B cells and only found in $CD3^+$ T cells (Fig 2, *A* and *B*, and see Fig E4, *A*, in this article's Online Repository at www.jacionline.org).[23,24] SAP expression was restricted to $Ig\kappa^+/Ig\lambda^+$ immature B cells and not found in $VpreB^+$ pre-B cells (Fig 2, *B*, and see Fig E4, *B* and *C*). In addition, SAP^+ immature B cells from fetal and adult bone marrow preferentially showed a pattern of clustered IgM or Igκ/Igλ, suggesting that these BCRs were cross-linked by self-antigens (Fig 2, *C*, and see Fig E4, *B* and *C*). In contrast, SAP^- immature B cells displayed nonclustered BCRs, suggesting that these B cells do not bind self-antigens (Fig 2, *C*, and see Fig E4, *B* and *C*). To further identify SAP expression in human B-cell precursors, we assessed the presence of SAP transcripts in fetal liver and fetal bone marrow $CD19^+$ B-cell precursors and mature naive B cells that were further sorted to exclude any T-cell contamination (Fig 2, *D*). We found that SAP transcripts could be detected in $CD19^+$ B-cell precursors from fetal liver and bone marrow but not in peripheral mature naive B cells (Fig 2, *E*). The absence of CD3E transcripts in all these $CD19^+$ fractions further attested to the absence of T-cell contamination (Fig 2, *F*). SAP expression was 100-fold less in $CD19^+$ fetal liver and bone marrow cells than in T cells but is consistent with SAP being only expressed in a discrete population (10%) of

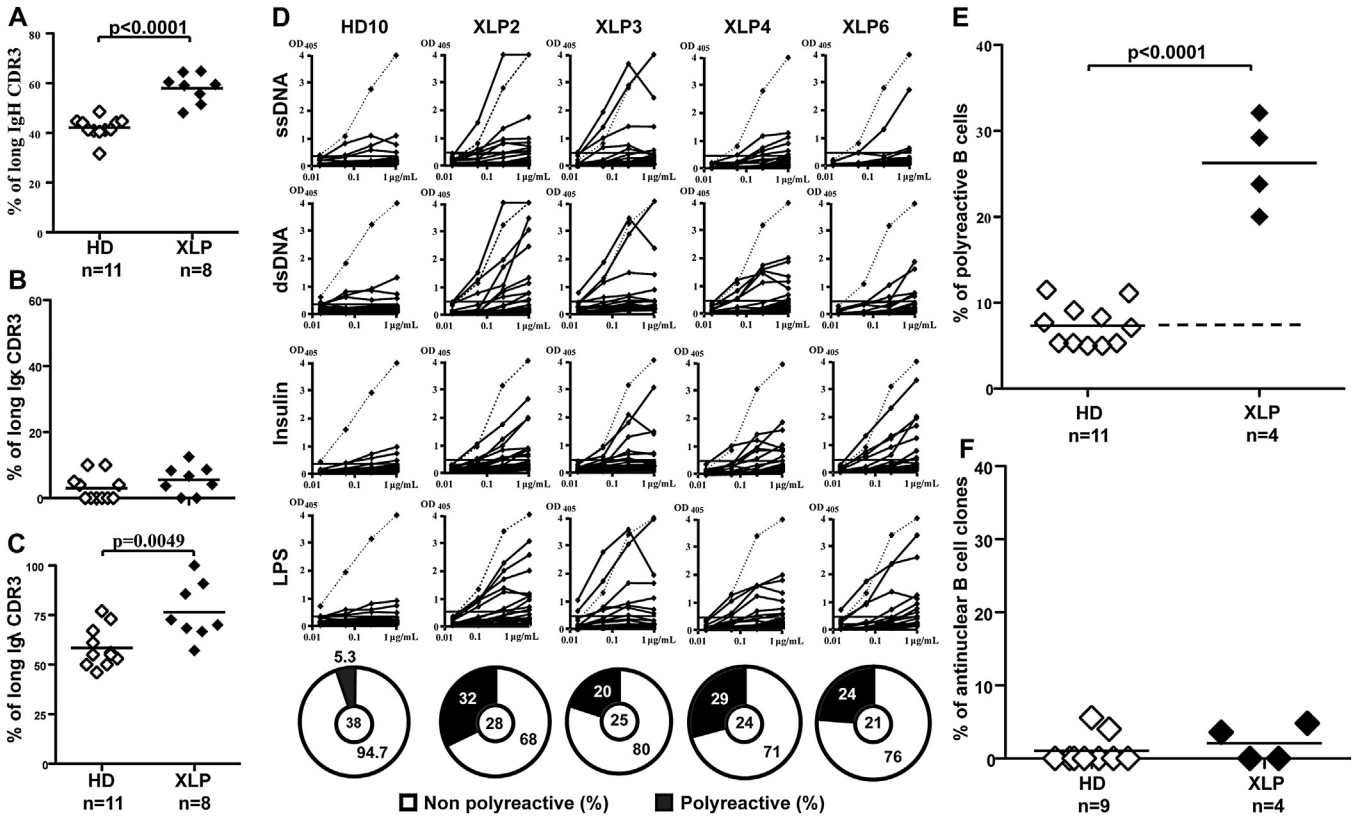

FIG 1. The central B-cell tolerance checkpoint is not functional in patients with XLP. The frequencies of long IgH (≥15 amino acids; **A**) or long Igκ **(B)** or Igλ (**C;** ≥11 amino acids) in new emigrant/transitional B cells are represented for 11 healthy donors and 8 patients with XLP. Each *diamond* represents a subject. The average is shown with a *bar*. **D-F,** Antibodies from new emigrant/transitional B cells from patients with XLP were tested by means of ELISA for reactivity against single-stranded DNA *(ssDNA)*, double-stranded DNA *(dsDNA)*, insulin, and LPS (Fig 1, *D*), and the frequencies of polyreactive and anti-nuclear clones are displayed in Fig 1, *E* and *F*, respectively. *Dotted line*, ED38 positive control; *horizontal line*, cutoff OD_{405} for positive reactivity. For each subject, the frequency of polyreactive *(solid area)* and nonpolyreactive *(open area)* clones is summarized in pie charts, with the total number of clones tested indicated in the *center*.

$CD19^+IgM^+Ig\kappa^+/Ig\lambda^+$ immature B cells, which itself only represents approximately 20% to 30% of $CD19^+$ cells (Fig 2, *E*, and see Fig E4, *D*). Hence SAP is expressed by a discrete subset of developing immature B cells, which are likely autoreactive and undergoing central selection.

SLAMF6 colocalizes with SAP when segregating with autoreactive BCRs

We assessed the expression during B-cell differentiation of SLAM family members that might require SAP to mediate their function.[4] SLAMF1/CD150, SLAMF2/CD48, and SLAMF6/CD352 (human NTB-A/mouse Ly108) expression increased with B-cell maturation (low in pre-B cells, intermediate in immature B cells in the bone marrow, and high in mature naive B cells in peripheral blood), whereas SLAMF5/CD84 expression seems to be increased at the immature B-cell stage (Fig 3, *A*). SLAMF4/CD244 and SLAMF7/CD319 (CRACC) are not expressed or expressed at very low levels, respectively, suggesting that these SLAM members are unlikely to play a role in central B-cell selection (Fig 3, *A*). Because *Slamf6* (mouse Ly108) is required for proper central B-cell tolerance mechanisms in mice[6] and because SLAMF6 (human NTB-A) seems to be one of the SLAM family members expressed at the highest level on immature B cells, we analyzed its expression using immunofluorescence in $SAP^+Ig\kappa/Ig\lambda^+$ immature B cells. We found in all analyzed cells that SLAMF6/CD352 and SAP only associated with each other when cosegregating with clustered BCRs (Fig 3, *B*, middle and bottom). In contrast, SLAMF6/CD352 and SAP never colocalized in immature B cells with diffuse Igκ/Igλ expression (Fig 3, *B*, top). We conclude that SAP might be recruited by SLAMF6/CD352 to BCR clusters in autoreactive immature B cells and might regulate BCR signaling and the induction of central B-cell tolerance mechanisms in human bone marrow.

Defective peripheral B-cell tolerance checkpoint in patients with XLP

To analyze the effect of SAP deficiency on the peripheral B-cell tolerance checkpoint, using ELISA, we tested the reactivity of recombinant antibodies from $CD19^+CD10^-CD21^+IgM^+CD27^-$ mature naive B cells from patients with XLP (see Tables E10-E13 in this article's Online Repository at www.jacionline.org) against HEp-2 cell lysates (Fig 4, *A*). We found that the frequency of mature naive B cells that expressed HEp-2–reactive antibodies was significantly increased in all 4 patients with XLP tested (38% to 64%) compared with

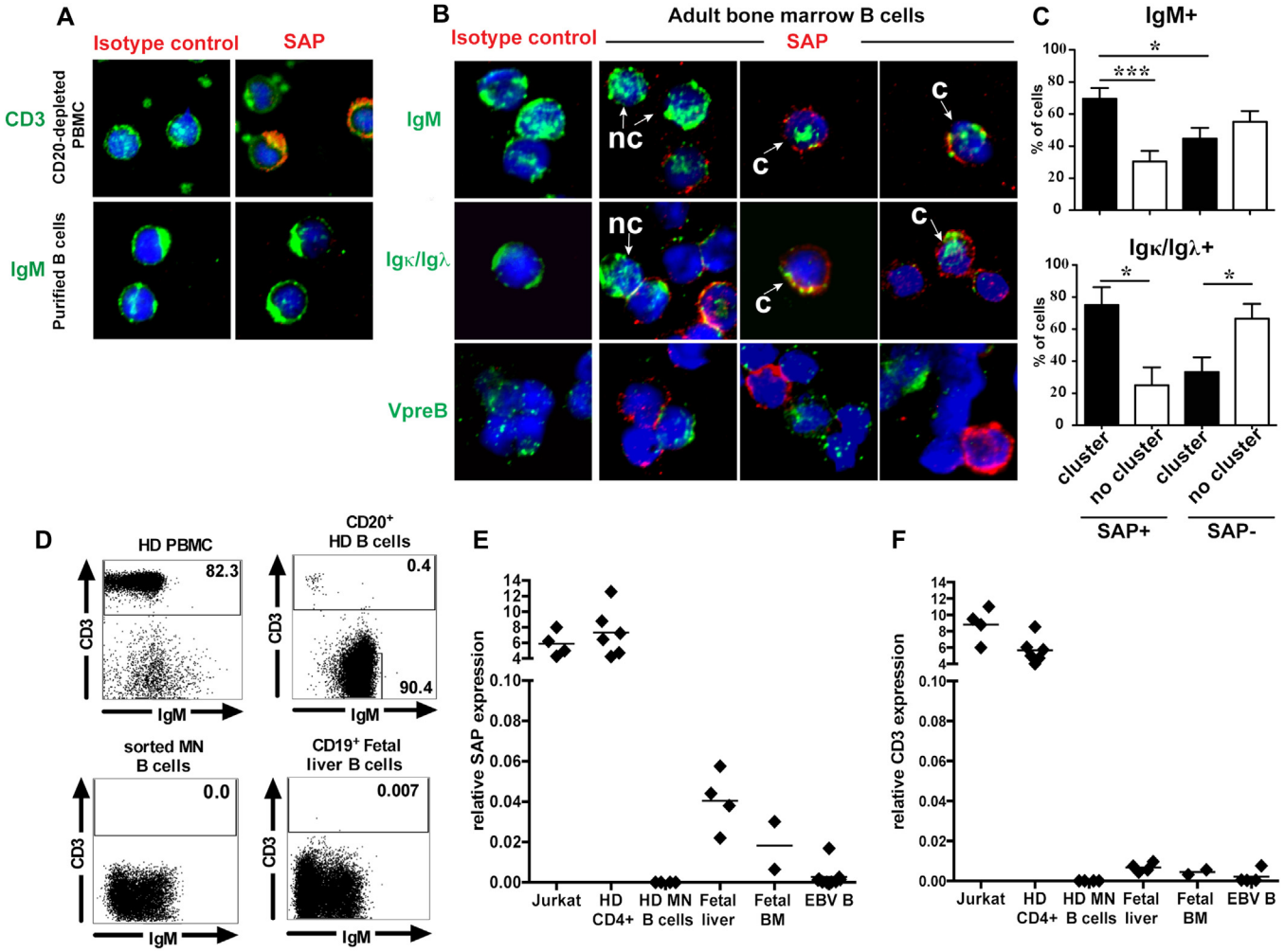

FIG 2. SAP is expressed by some immature B cells. **A,** PBMCs depleted from $CD20^+$ B cells or purified naive B cells were stained for CD3 or IgM *(green)* or SAP or isotype control *(red)*. **B,** $CD19^+$ adult bone marrow cells were stained for IgM, Igκ and Igλ, or VpreB *(green)* and SAP or isotype control *(red)*. **C,** Proportion of cells in SAP^+ versus SAP^- cells that displayed a clustered versus not clustered IgM and Igκ/Igλ staining pattern. $*P < .05$ and $***P < .001$. **D,** Dot plots show the proportion of $CD3^+$ T cells in PBMCs from healthy donors *(HD PBMC)*, CD20-enriched B cells using magnetic beads, sorted mature naive *(MN)* B cells, and $CD19^+$ fetal liver cells. **E** and **F,** *SAP* (Fig 2, *E*) and *CD3E* (Fig 2, *F*) gene expression was assessed by using quantitative RT-PCR in mature naive B cells, $CD19^+$ fetal liver and bone marrow cells, and EBV cell lines. Jurkat and healthy donor *(HD)* $CD4^+$ T cells were used as positive controls for both *SAP* and *CD3E* expression.

that seen in healthy donors (17% to 26%, $P < .0001$; Fig 4, *B*). Consistent with these data, the frequency of mature naive polyreactive B cells was higher in patients with XLP than in healthy donors ($P < .0001$; Fig 4, *C*). However, there was no increase in ANA clones in the mature naive B-cell compartment of patients with XLP (Fig 4, *D*). Thus the high frequencies of HEp-2–reactive and polyreactive mature naive B cells in patients with XLP reveal a defective peripheral B-cell tolerance checkpoint in the absence of functional SAP expression.

Patients with XLP display normal serum BAFF concentration and Treg cell frequency

Defects in the human peripheral B-cell tolerance checkpoint seem to correlate with increased serum BAFF concentrations and decreased Treg cell frequencies.[11] However, more recent analyses revealed that these defects can occur in the absence of BAFF dysregulation.[15,16] Indeed, we found that patients with XLP displayed normal serum BAFF concentrations comparable with those of healthy donors, revealing that BAFF does not account for the defective peripheral B-cell tolerance checkpoint in patients with XLP (Fig 5, *A*). Treg cells play an essential role in preventing the accumulation of autoreactive mature naive B cells in the periphery and might control their homeostatic expansion, as is demonstrated by FOXP3-deficient patients with IPEX syndrome who display a defective peripheral B-cell tolerance checkpoint.[16] SAP-deficient naive B-cell subsets showed a normal proliferative history, suggesting normal Treg cell functions in patients with XLP (Fig 5, *B*). In agreement with this observation, normal Treg cell frequencies and phenotypes were found in patients with XLP, as evidenced

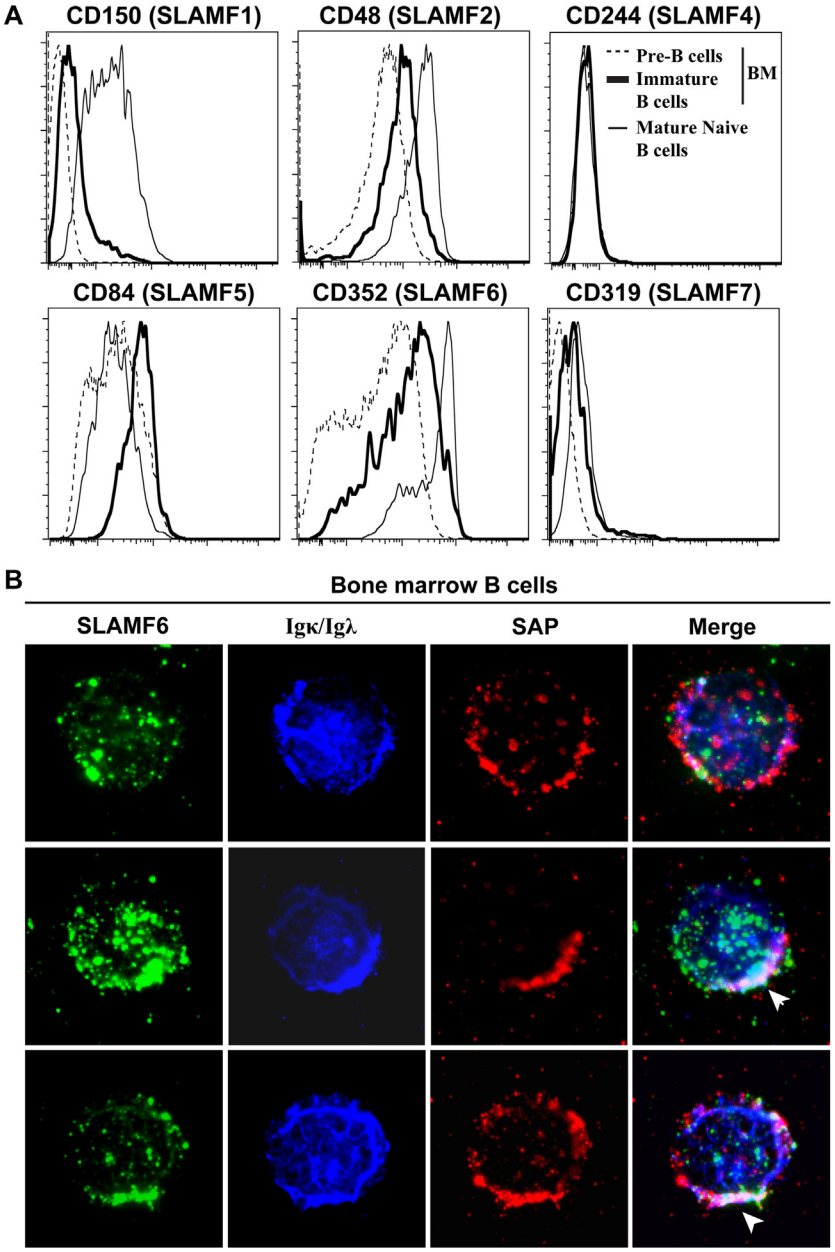

FIG 3. SAP and SLAMF6 colocalize in B cells with aggregated BCRs. **A,** Overlays display the expression of SLAM family members by $CD19^+CD27^-CD10^+IgM^-$ pre-B cells *(dotted line)* and $CD19^+CD27^-CD10^+IgM^+$ immature B cells *(bold line)* from bone marrow *(BM)* and peripheral $CD19^+CD27^-CD10^-IgM^+$ mature naive B cells *(thin line)*. **B,** $CD19^+$ enriched bone marrow B cells were stained for SLAMF6 *(green)*, Igκ/Igλ *(blue)*, and SAP *(red)*. Representative cells without *(top row)* or with (*middle* and *bottom rows*) costaining of SLAMF6, Igκ/Igλ, and SAP are shown. Costaining of clustered Igκ/Igλ, SLAMF6, and SAP appears *white* as the superposition of *green*, *red*, and *blue* and is indicated by an *arrow*.

by the normal expression of Helios, CD25/IL-2 receptor α, CD127/IL-7 receptor α, and CD45RO (Fig 5, *C-I*). In contrast, non–Treg cell $FOXP3^-Helios^-CD4^+$ populations from patients with XLP displayed a dysregulated phenotype characterized by an increased expression of CD127/IL-7 receptor α (patients with XLP: mean fluorescence intensity = 1012 vs healthy donors: mean fluorescence intensity = 744; $P = .0006$) and increased frequencies of $CD45RO^+$ memory cells (Fig 5, *F-I*). Hence SAP-deficient Treg cells display a normal phenotype, suggesting that these T cells might be functional in patients with XLP, whereas non-Treg $CD4^+$ T cells show an altered phenotype in these patients.

SAP-deficient T cells are resistant to *in vitro* suppression by Treg cells

Control and SAP-deficient Treg cells were then tested *in vitro* for their ability to suppress Tresp cell proliferation. $CD3^+CD4^+CD25^{hi}CD127^{-/lo}$ Treg cells were sorted by using flow cytometry and cultured with CFSE-labeled

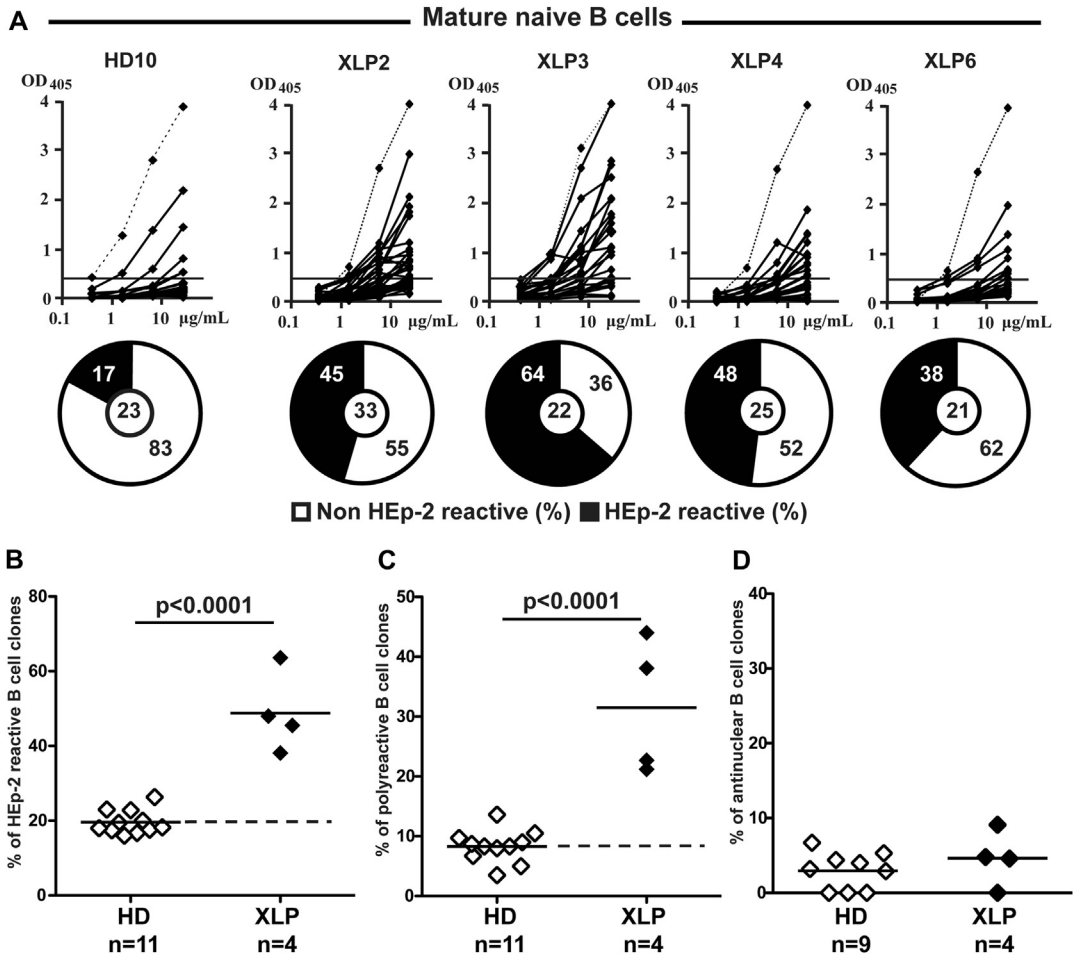

FIG 4. Defective peripheral B-cell tolerance checkpoint in patients with XLP. **A,** Antibodies from mature naive B cells from patients with XLP were tested by using ELISA for anti–HEp-2 cell reactivity. **B-D,** Frequencies of HEp-2–reactive (Fig 4, *B*), polyreactive (Fig 4, *C*), and anti-nuclear mature (Fig 4, *D*) naive B cells. *Dotted line,* ED38 positive control; *horizontal lines,* cutoff OD_{405} for positive reactivity. For each subject, the frequency of HEp-2–reactive *(solid area)* and non–HEp-2–reactive *(open area)* clones is summarized in pie charts, with the total number of clones tested indicated in the *centers. HD,* Healthy donors.

$CD3^+CD4^+CD25^-$ Tresp cells in the presence of anti-CD2–, anti-CD3–, and anti-CD28–coated beads. The proliferation of healthy donor Tresp cells was inhibited by the addition of Treg cells, either from healthy control subjects or patients with XLP (Fig 6, *A* and *C*). Thus SAP-deficient Treg cells have normal suppressive functions. However, the proliferation of XLP Tresp cells was not inhibited by either Treg cells from healthy donors or patients with XLP, thereby showing that Tresp cells from patients with XLP are resistant to *in vitro* suppression by Treg cells (Fig 6, *A* and *C*). Tresp cells from patients with XLP were hyperresponsive to stimulation because 47% of Tresp cells from patients with XLP had undergone 4 divisions after 4.5 days in culture versus only 17% of Tresp cells from healthy control subjects (Fig 6, *B*). This increased proliferation of SAP-deficient Tresp cells was linked to increased concentrations of cytokines, such as IL-2, IFN-γ, TNF-α, and GM-CSF, which were detected in the supernatants of these *in vitro* cultures in the presence of Treg cells from either healthy donors or patients with XLP (Fig 6, *D*). These cytokines originate from Tresp cells themselves, as shown by the increased production of the T_H1 cytokines IL-2, IFN-γ, TNF-α, and GM-CSF, as well as the T_H2 cytokine IL-13, by *in vitro*–stimulated Tresp cells from patients with XLP compared with healthy donor control cells (Fig 6, *E*). Increased production of IFN-γ and TNF-α by Tresp cells from patients with XLP activated for 4.5 days *in vitro* was further evidenced by intracytoplasmic detection of these cytokines by means of flow cytometry (see Fig E5 in this article's Online Repository at www.jacionline.org). Sera of patients with XLP displayed increased concentrations of several cytokines, further reflecting the hyperactive T-cell phenotype of SAP deficiency. Although we did not detect increased serum IL-2 and IFN-γ levels, TNF-α and IL-7 concentrations were higher in patients with XLP (Fig 7). In addition, the sera of patients with XLP were especially enriched in IL-4, IL-6, IL-10, and IL-21, which favor B-cell survival and activation (Fig 7, *A*). Moreover, we found that IL-4, IL-7, and IL-21 and, to a lesser extent, IL-6 and TNF-α blocked *in vitro* Treg cell suppression by themselves (Fig 7, *B* and *C*). When all the cytokines with levels found to be increased in patients with XLP (XLP cytokine mix) were added to the Treg cell assay, we observed an inability for Treg cells to suppress refractory Tresp cell proliferation similarly to Treg-Tresp cells isolated from patients with XLP

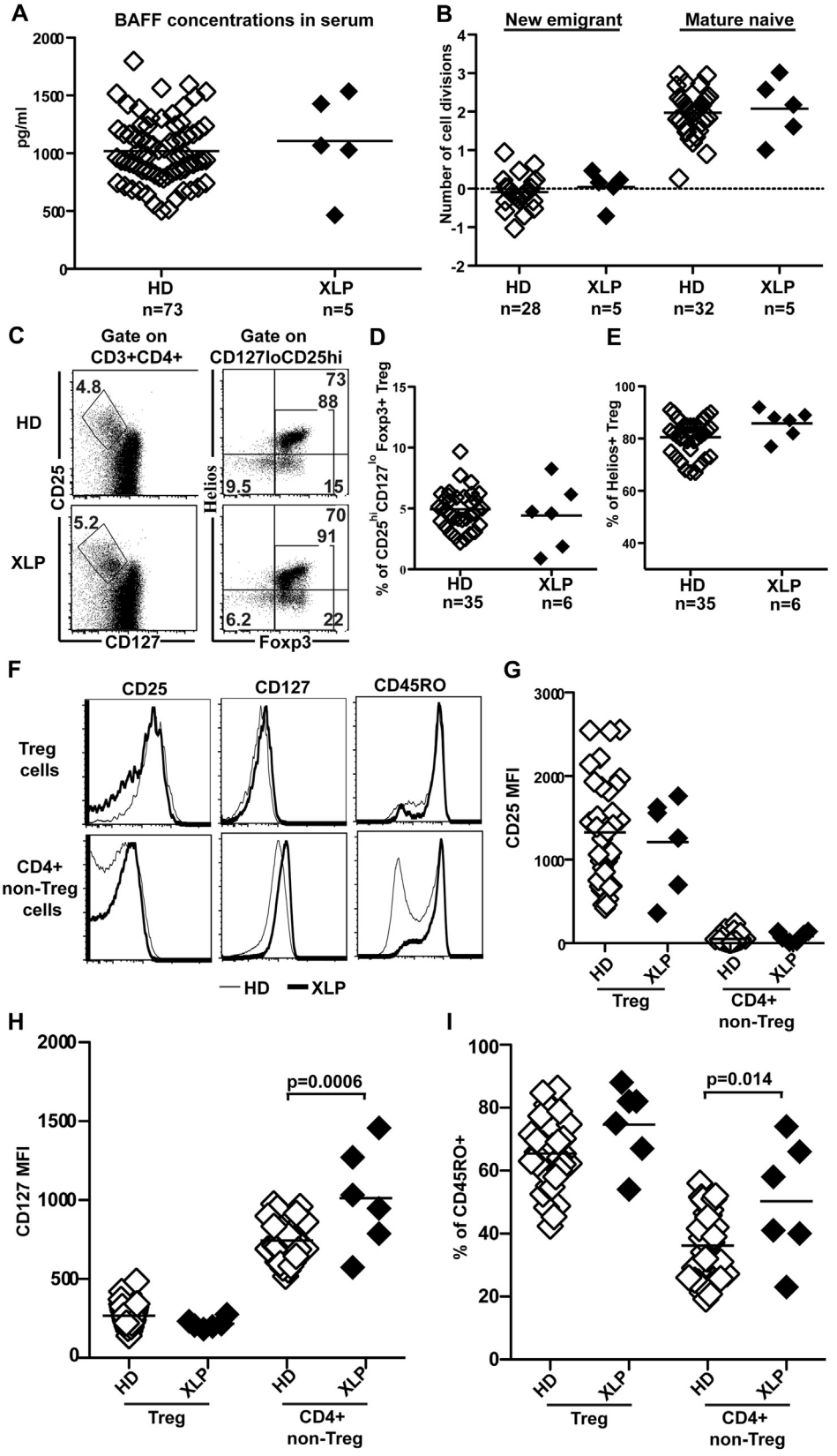

FIG 5. Normal serum BAFF concentrations and Treg cell frequencies in patients with XLP. **A,** Serum BAFF concentrations (in picograms per milliliter) in healthy donors (n = 73) and patients with XLP (n = 5). **B,** Evaluation of the number of cell divisions undergone *in vivo* by using κ-deleting recombination excision circle analysis on new emigrant and mature naive B cells of healthy donors (n = 28 and n = 32, respectively)

(Figs 6, *C*, and 7, *C*). Hence SAP deficiency affects peripheral $CD4^+$ T cells, which are hyperresponsive to T-cell receptor (TCR) stimulation and prone to secrete many T_H1 and T_H2/T_{FH} cytokines, rendering these T cells resistant to Treg cell suppression.

DISCUSSION

We report here that SAP/SLAMs play a major role in the establishment of central and peripheral B-cell tolerance in human subjects. Many transitional B cells that recently emigrated from the bone marrow of patients with XLP expressed BCRs characterized by an abnormal immunoglobulin repertoire associated with self-reactivity, thereby demonstrating a defective central B-cell tolerance checkpoint in the absence of functional SAP. Central B-cell tolerance is normally regulated by intrinsic B-cell pathways involved in sensing the binding of self-antigens and inducing tolerance mechanisms, such as receptor editing, anergy, or deletion.[11] Defective BCR and TLR signaling and function result in a failure to remove developing autoreactive B cells in the bone marrow.[10-12] We identified SAP expression in a discrete population of B-cell precursors that expressed both heavy and light chains and therefore belonged to the immature B-cell compartment in which the reactivity of newly synthesized BCRs is assessed for binding to self-antigens. In addition, the preferential expression of SAP in immature B cells displaying aggregated BCRs likely binding self-antigens suggests that SAP expression might be mostly restricted to autoreactive clones. SAP expression in immature B cells might therefore be important for the establishment of central B-cell tolerance by potentially regulating the BCR signaling threshold and modifying SLAM functions. In T cells the SAP/Ly108 pathway enhances the signaling strength of the TCR to reach the threshold required for restimulation-induced apoptosis and cytolytic function.[25,26] Indeed, in the absence of SAP, many SLAMs bind SHP-1, which is a negative regulator of TCR signaling.[27] When SAP is expressed, SAP competes with and displaces SHP-1 binding to SLAMs and switches off Ly108/SLAMF6 inhibitory function.[27,28] Interestingly, we found that SLAMF6 is expressed in immature B cells and colocalized with SAP in BCR clusters. Moreover, Ly108, the mouse homologue to SLAMF6, regulates mechanisms of central B-cell tolerance by modulating the strength of the BCR signaling.[6] Hence SAP expression at the immature B-cell stage might allow SLAM proteins, including SLAMF6, to modulate BCR signaling strength and thereby participate in the counterselection of autoreactive B cells. We conclude that SLAM/SAP play an essential role in mediating central B-cell tolerance in human subjects, potentially by favoring the induction of apoptosis in immature autoreactive B cells, as reported in B cell–derived cell lines.[29]

In contrast to central tolerance, the establishment of peripheral B-cell tolerance seems to rely on B cell–extrinsic factors. Indeed, defects in the expression of functional CD40L and FOXP3 mostly restricted to the T-cell lineage result in a specific abnormal peripheral B-cell tolerance checkpoint.[15,16] The failure in patients with XLP to counterselect autoreactive B cells between the new emigrant/transitional and mature naive B-cell stages at which SAP is no longer expressed in B cells further supports the B cell–extrinsic regulation of peripheral B-cell tolerance. Defects in the peripheral B-cell tolerance checkpoint often correlate with decreased Treg cell frequencies and increased serum BAFF concentrations.[15] Recently, we identified similar peripheral B-cell tolerance abnormalities in FOXP3-deficient patients with IPEX syndrome, who do not have functional Treg cells and display normal serum BAFF concentrations. This observation further suggests that defective Treg cell functions in these patients, but not serum BAFF concentrations, are responsible for the impairment in preventing the accumulation of autoreactive B cells in the mature naive B-cell compartment.[16] The normal serum BAFF concentrations in most patients with XLP who display defective peripheral B-cell tolerance also suggest that BAFF is not responsible for the impaired peripheral selection of autoreactive B cells. However, we did not observe any decrease in Treg cell frequencies in SAP-deficient patients or any unusual phenotype of these T cells that could infer compromised functions. In addition, we found that Treg cells retained their *in vitro* suppressive function in the absence of functional SAP. It has been proposed that Treg cells can directly interact with autoreactive B cells by recognizing self-antigens presented by these B cells through MHC class II, leading to their elimination.[30] Interestingly, examination of SAP-deficient mice revealed that functional interactions between B and T cells do not form in the absence of SAP, whereas T cell–dendritic cell interactions are not affected.[27,31-33] Hence the defective peripheral B-cell tolerance checkpoint in patients with XLP might be due to the inability of T cells and potentially Treg cells to interact directly with autoreactive B cells and mediate their removal in the absence of functional SAP independently of dendritic cells and other antigen-presenting cells.

Alternatively, increased cytokine secretion by SAP-deficient T cells might be responsible for a break in peripheral B-cell tolerance. Enhanced cytokine secretion in the absence of functional SAP detected *in vitro* in activation experiments, as well as *in vivo* directly in the patients' sera, contrasted with the hyporesponsive phenotype reported in SAP-deficient T-cell lines obtained from 2 patients with XLP or in engineered Jurkat cells.[34,35] However, these artificial models might not be representative of polyclonal responses induced in freshly isolated T cells from patients with XLP. Indeed, enhanced T-cell responses in patients with XLP were recently reported in 2 asymptomatic

and patients with XLP (n = 5). **C,** Representative CD25 and CD127 *(left)* and Helios and FOXP3 *(right)* staining on $CD3^+CD4^+$ T cells from a healthy donor and a patient with XLP. **D,** $CD3^+CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ Treg cell frequencies in 35 healthy donors and 6 patients with XLP. **E,** Frequencies of $FOXP3^+Helios^+$ cells in the $CD3^+CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ population in 35 healthy donors and 6 patients with XLP. **F,** Representative staining of CD25, CD127, and CD45RO expression in a patient with XLP *(bold line)* and a healthy donor *(thin line)* for $FOXP3^+Helios^+$ Treg cells and $FOXP3^-Helios^-$ $CD4^+$ T cells. **G-I,** Mean fluorescence intensity *(MFI)* of CD25 (Fig 5, *G*) and CD127 (Fig 5, *H*) and frequency of $CD45RO^+$ cells (Fig 5, *I*) for $Helios^+FOXP3^+$ Treg cells and $Helios^-FOXP3^-CD4^+$ non–Treg cells from healthy donors and patients with XLP. *HD*, Healthy donors.

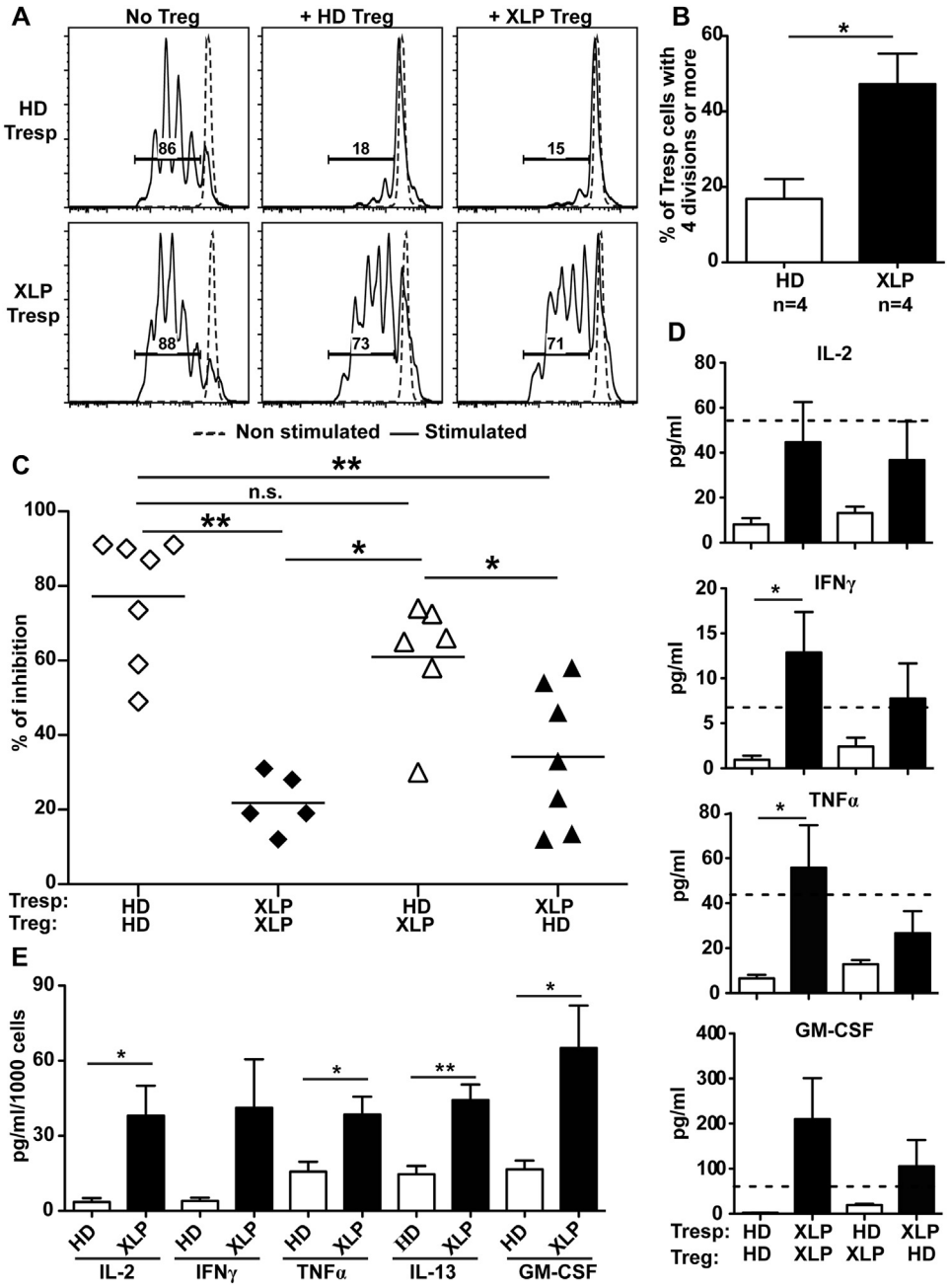

FIG 6. Increased resistance of SAP-deficient responder $CD4^+$ T cells to suppression by Treg cells. **A,** Representative histograms of Treg cell–mediated suppression of autologous and heterologous CFSE-labeled Tresp cells on day 4.5 from a patient with XLP compared with a healthy donor. The *dashed line* displays nonstimulated Tresp cells. **B,** Increased percentage of Tresp cells that divided 4 times or more in patients with XLP compared with healthy donors in the "Tresp only" culture after 4.5 days. **C,** Autologous and heterologous suppressive activities of Treg cells from healthy donors and patients with XLP. **D,** Increased concentrations of IL-2, IFN-γ, TNF-α, and GM-CSF in supernatants of XLP "Tresp+Treg" cocultures. The *dotted line* represents the level of each cytokine in the supernatant of the stimulated "HD Tresp only" well. **E,** Concentrations of the indicated cytokines in stimulated healthy donors or patients with XLP $CD4^+$ T-cell monocultures after 4 days. $*P < .05$ and $**P < .01$. *HD,* Healthy donors.

patients with XLP.[36] In addition, increased IFN-γ secretion has been reported during acute EBV infection in patients with XLP and in SAP-deficient mice infected with lymphocytic choriomeningitis virus or *Toxoplasma gondii*.[37-39] The increased IL-2, IFN-γ, and TNF-α secretion might account for the ineffective suppression of Tresp cell proliferation by Treg cells, as suggested by several reports.[40-42] In agreement with this hypothesis, we found that cytokines, such as IL-4, IL-7, and IL-21 and, to a lesser extent, IL-6 and TNF-α, were able to inhibit by themselves *in vitro* Treg cell suppression. SAP deficiency recapitulates defective suppressive scenarios observed in patients with type 1 diabetes and multiple sclerosis. Indeed, patients with

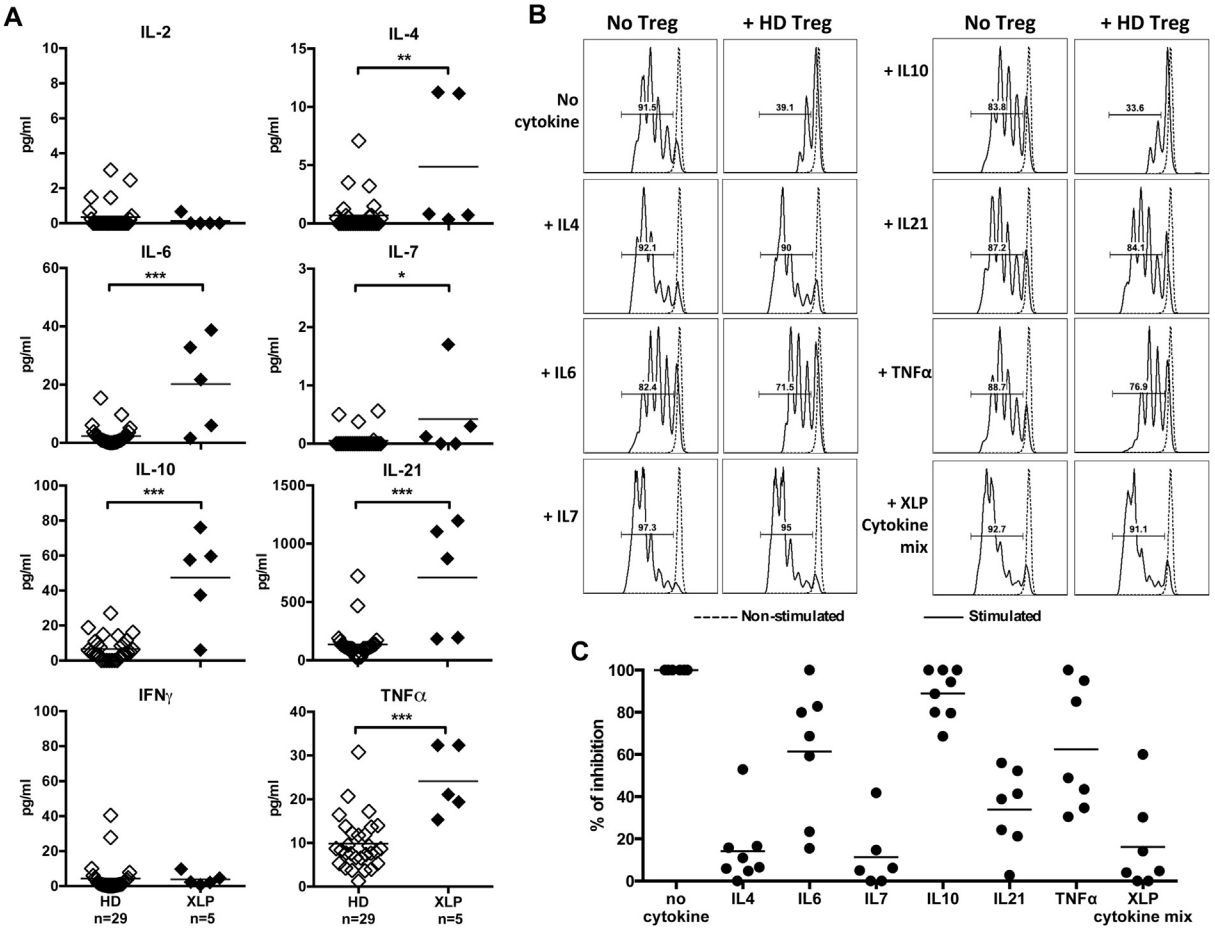

FIG 7. Increased T_H2/T_{FH} cytokine concentrations in sera of patients with XLP. **A,** IL-2, IFN-γ, TNF-α, IL-7, IL-4, IL-6, IL-10, and IL-21 cytokine concentrations in sera of 5 patients with XLP and 29 healthy donors were measured by using Luminex or ELISA. $*P < .05$, $**P < .01$, and $***P < .0001$. **B,** Representative histograms of Treg cell–mediated suppression of autologous CFSE-labeled Tresp cells on day 4.5 from a healthy donor in the presence of the indicated cytokines. The *dashed line* displays nonstimulated Tresp cells. **C,** Suppressive activities of Treg cells in the presence of diverse cytokines. The XLP cytokine mix includes IL-4, IL-6, IL-7, IL-10, IL-21, and TNF-α, levels of which were found to be increased in patients with XLP.

type 1 diabetes also display Tresp cells that are refractory to Treg cell suppression *in vitro* associated with a defective peripheral B-cell tolerance checkpoint.[12,43] IFN-γ secretion by Treg cells from patients with multiple sclerosis results in a loss of *in vitro* suppressive activity and correlates with an abnormal peripheral B-cell tolerance checkpoint in these patients.[19,40] A defective suppression of T cells by Treg cell is also consistent with the overwhelming and uncontrolled B-cell and T-cell responses to EBV infection in patients with XLP.[39] In addition to rendering Tresp cells refractory to Treg cells and perhaps directly affecting Treg cell suppressive function, T_H2/T_{FH} cytokines, including IL-4, IL-6, IL-10, and IL-21, favor B-cell activation, isotype switch, and plasma cell development.[42,44] Paradoxically, patients with XLP display severely decreased isotype-switched B-cell frequencies, likely resulting from defective interactions with $CD4^+$ T cells.[23,24,45] Nonetheless, SAP deficiency could also interfere with the establishment of peripheral B-cell tolerance by favoring the survival of autoreactive B cells through enhanced serum cytokine concentrations.

SAP is essential for natural killer T (NKT) cell development.[46,47] Consequently, patients with XLP lack NKT cells. NKT cells express an invariant TCR that recognizes lipid self-antigens and are able to provide cognate help to B cells in a SAP-dependent manner potentially, preventing the activation of autoreactive B cells.[48] Lower NKT numbers and function have been linked to the development of type 1 diabetes and lupus in NOD mice with genetic defects of *slamf1* and *slamf6* combined with altered SLAM expression.[49] Therefore we cannot exclude the involvement of NKT cells in the control of human peripheral B-cell tolerance. The absence of NKT cells might contribute to the improper removal of autoreactive clones from the mature naive B-cell compartment of SAP-deficient patients with XLP.

In conclusion, we found that SAP is required for the establishment of B-cell tolerance in human subjects. Transient SAP expression in bone marrow–developing B cells is required for functional central B-cell tolerance, whereas in the periphery SAP expression in T cells is likely necessary for preventing the accumulation of autoreactive mature naive B cells by ensuring

stable B-cell/T-cell interactions and repressing uncontrolled cytokine secretion by human T cells.

We thank Dr S. Rudchenko, L. Devine, and C. Wang for cell sorting.

Key messages

- **SAP expression in human autoreactive immature B cells is required for the establishment of central B-cell tolerance in bone marrow.**
- **SAP expression by T cells prevents the accumulation of autoreactive mature naive B cells in the periphery.**

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