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