

SAP Regulation of Follicular Helper CD4 T Cell Development and Humoral Immunity Is Independent of SLAM and Fyn Kinase¹

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Mutations in *SH2D1A* resulting in lack of SLAM-associated protein (SAP) expression cause the human genetic immunodeficiency X-linked lymphoproliferative disease. A severe block in germinal center development and lack of long-term humoral immunity is one of the most prominent phenotypes of *SAP*^{-/-} mice. We show, in this study, that the germinal center block is due to an essential requirement for SAP expression in Ag-specific CD4 T cells to develop appropriate follicular helper T cell functions. It is unknown what signaling molecules are involved in regulation of SAP-dependent CD4 T cell help functions. SAP binds to the cytoplasmic tail of SLAM, and we show that SLAM is expressed on resting and activated CD4 T cells, as well as germinal center B cells. In addition, SAP can recruit Fyn kinase to SLAM. We have now examined the role(s) of the SLAM-SAP-Fyn signaling axis in *in vivo* CD4 T cell function and germinal center development. We observed normal germinal center development, long-lived plasma cell development, and Ab responses in *SLAM*^{-/-} mice after a viral infection (lymphocytic choriomeningitis virus). In a separate series of experiments, we show that SAP is absolutely required in CD4 T cells to drive germinal center development, and that requirement does not depend on SAP-Fyn interactions, because CD4 T cells expressing SAP R78A are capable of supporting normal germinal center development. Therefore, a distinct SAP signaling pathway regulates follicular helper CD4 T cell differentiation, separate from the SLAM-SAP-Fyn signaling pathway regulating Th1/Th2 differentiation. *The Journal of Immunology*, 2007, 178: 817–828.

The gene *SH2D1A* (*SAP/DSHP*) encodes SLAM-associated protein (SAP).³ Mutations in *SH2D1A* resulting in lack of SAP expression or destabilized SAP protein are the cause of the human genetic immunodeficiency X-linked lymphoproliferative disease (XLP) (1–3). XLP patients exhibit a variety of immunological defects. The most notable and problematic symptoms of SAP-deficient humans are the inability to control EBV infection (as well as difficulties with other infections, including measles virus, *Neisseria meningitidis*, and vaccinia virus), the development of progressive hypogammaglobulinemia, and a heightened occurrence of B cell lymphomas (4–7). Before recent improvements in diagnosis and treatment, most XLP patients died before reaching adulthood.

SAP is a small SH2-domain protein expressed in CD4 T cells, CD8 T cells, NK cells, NKT cells, and some B cells (3, 8, 9). SAP is now known to play a role in an impressive array of lymphocyte functions. Examination of SAP-deficient mice revealed roles for SAP in CD8 and CD4 T cell proliferation (10–14), T cell IFN- γ production (10, 11, 15), CD4 T cell Th1/Th2 differentiation (10, 11, 16, 17), and germinal center formation, and the generation of memory B cells (12, 18). Recent work has also shown that SAP is essential for the development of NKT cells, both in mice and humans (19–21). Work in XLP humans has confirmed the memory B cell defect and germinal center defect observed in *SAP*^{-/-} mice (22–24). Separately, a collection of studies in humans have shown that SAP plays multifaceted roles in the regulation of NK cell killing (25–27). How does SAP regulate or influence such a wide range of lymphocyte functions? SAP binds to the cytoplasmic tail of SLAM (CD150) via an ITAM/ITIM-related motif termed an immunotyrosine switch motif (ITSM) (3, 28, 29). SLAM plays roles in cell adhesion (via homotypic interactions), costimulation, and cytokine synthesis (28, 30–33). SLAM is also the primary receptor for measles virus, a highly lymphotropic virus (34–36). A cluster of seven receptors related to SLAM are closely grouped together on chromosome 1 of both mice and humans. These genes are termed the SLAM family of receptors: *CD150* (SLAM), *CD244* (2B4), *CD229* (Ly9), *CS1* (CRACC), *CD48*, *CD84*, and *LY108* (NTB-A) (4, 5, 28). All of these genes encode surface receptors with at least two ITSM motifs in their cytoplasmic tail, except CD48, which has no cytoplasmic tail (28). Intriguingly, a major systemic lupus erythematosus genetic susceptibility locus maps to the SLAM family receptors in both human and mice (37, 38). SAP is known to bind to SLAM, CD244, CD229, CD84, and Ly108/NTB-A (3, 26, 39, 40). SAP binding to these receptors is not identical, because SAP can bind to SLAM in the presence or

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³ Abbreviations used in this paper: SAP, SLAM-associated protein; XLP, X-linked lymphoproliferative disease; ITSM, immunotyrosine switch motif; MHCII, MHC class II; LCMV, lymphocytic choriomeningitis virus; PNA, peanut lectin agglutinin; QPCR, quantitative real-time PCR; WT, wild type.

absence of tyrosine phosphorylation (41). SAP binding to other SLAM family receptors appears to be fully dependent on tyrosine phosphorylation.

The initial observation that SAP is a small SH2 domain protein without any additional adaptor or enzymatic domains led to the observation that SAP can function as a negative inhibitor, preventing other SH2 domain containing proteins from binding the ITSM phosphotyrosines of SLAM family receptors (3). Subsequently, it was shown that SAP recruits Fyn kinase to SLAM (and other SLAM family receptors) by direct binding via an unconventional SH3 domain binding surface (15, 42, 43). That surprising recruitment and activation of Fyn is critical for SAP regulation of Th2 differentiation in CD4 T cells (16, 17) and controlling IFN- γ production (17, 42). SAP-Fyn interactions also appear to be essential for NKT cell development, because NKT cells are absent in *Fyn*^{-/-} and *SAP*^{-/-} mouse strains (19–21, 44). Therefore, data currently in the literature indicate that SAP's major signaling contributions in T cells may occur primarily via recruitment of Fyn kinase to different SLAM family receptors.

A severe block in germinal center development is one of the most prominent phenotypes of *SAP*^{-/-} mice. That block results in a failure to generate memory B cells and long-lived plasma cells, and thereby results in a failure to establish long-term humoral immunity (4, 12). This role for SAP in humoral immunity and immunological memory is conserved in humans (4, 22–24). We show in this study that the germinal center block is due to an essential requirement for SAP expression in Ag-specific CD4 T cells to develop appropriate follicular helper CD4 T cell functions. It is unknown what signaling molecules are involved in SAP-dependent regulation of CD4 T cell help functions and humoral immunity. Our studies reported in this study have focused on dissecting the role(s) of the SLAM-SAP-Fyn signaling axis in germinal center development, because SLAM is the prototypic member of the SLAM family of receptors and Fyn is the primary downstream kinase activated by SAP. We observed normal germinal center development in SLAM-deficient mice. In a separate series of experiments, we demonstrate that whereas SAP is absolutely required in CD4 T cells to drive germinal center development, germinal center T cell help is not dependent on SAP-Fyn interaction. Therefore, a distinct SAP signaling pathway regulates follicular helper CD4 T cell differentiation, separate from the SLAM-SAP-Fyn signaling pathway regulating Th1/Th2 differentiation.

Materials and Methods

Mice

C57BL/6J (B6), B6 *CD4*^{-/-}, and B6 SJL (Ly5.1^{+/+}) mice were purchased from The Jackson Laboratory. *SLAM*^{-/-} mice were generated at Kyushu University by replacing exon 2 of the *CD150* gene with a neo cassette in a 129 ES cell line (17). SLAM-deficient 129 mice were backcrossed two generations to C57BL/6, and *SLAM*^{-/-} and *SLAM*^{+/-} progeny from that F₂ cross were interbred and used in all experiments. *SAP*^{-/-} mice backcrossed nine generations to B6 were provided by Dr. P. Schwartzberg (National Human Genome Research Institute, National Institutes of Health, Bethesda, MD) (10), and then backcrossed to B6 for one additional generation and bred at La Jolla Institute for Allergy and Immunology (LIAI). Because the *SAP* gene is X-linked, knockout mice were either - or -/-. SMARTA-2 TCR transgenic mice (SMtg⁺) are specific for the immunodominant I-A^b MHC class II (MHCII) lymphocytic choriomeningitis virus (LCMV) epitope gp61–80 (45), and SMtg⁺ mice fully backcrossed to B6 were obtained from Dr. C. Surh (The Scripps Research Institute, San Diego, CA), courtesy of Dr. H. Hengartner (Zurich, Switzerland) and Dr. R. Zinkernagel (Zurich, Switzerland), and then bred at LIAI and backcrossed to Ly5.1⁺ congenics. *SAP*⁻SMtg⁺Ly5.1^{+/+} B6 and SMtg⁺Ly5.1^{+/+} B6 mice were generated in-house. Sex-matched, 6- to 12-wk-old mice were used for all LCMV_{arm} infection experiments. Animals were maintained in an accredited facility at the LIAI, and the studies reported in this study conform to the principles outlined by the Animal Welfare Act and the

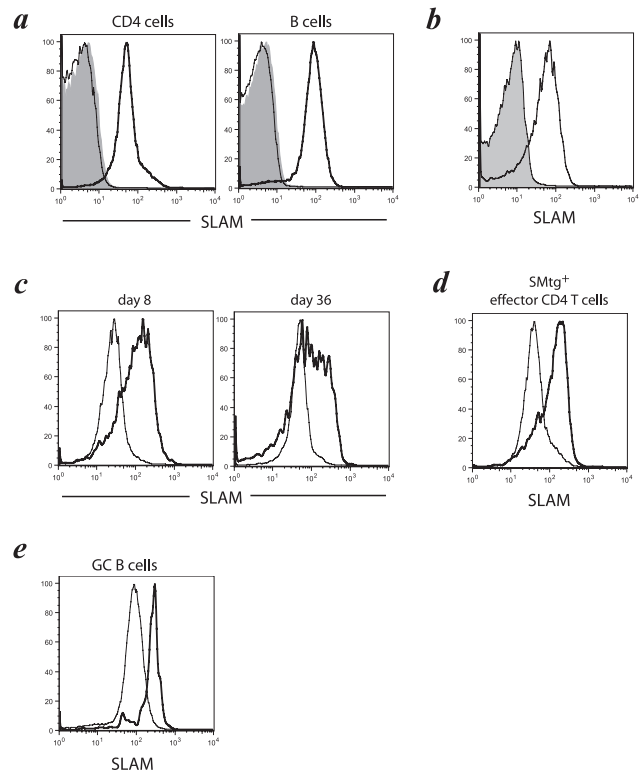


FIGURE 1. SLAM expression modulation. *a*, SLAM is constitutively expressed on the surface of splenic CD4 T cells and B cells. *SLAM*^{+/-}, thick black line; *SLAM*^{-/-}, thin black line; isotype control, filled line. *b*, SLAM is constitutively expressed on murine peripheral blood leukocytes (SLAM⁺, black line). No SLAM expression is detectable in *SLAM*^{-/-} mice (filled line). *c*, SLAM is up-regulated on activated effector CD4 T cells in vivo (CD62L^{low}CD44^{high}, thick line), day 8 postinfection with LCMV, stained directly ex vivo. Naive CD4 T cells (CD44^{low}), thin line. SLAM is also up-regulated on memory CD4 T cells (CD44^{high}), thick line, day 36 postinfection, stained directly ex vivo. *d*, SLAM is up-regulated on Ag-specific effector CD4 T cells in vivo. Adoptively transferred congenic SMARTA TCR transgenic (SMtg⁺) LCMV-specific CD4 T cells (thick line) were analyzed at day 8 after LCMV infection. Naive CD4 T cells, thin line. *e*, SLAM is up-regulated on germinal center B cells (GC B cells) in vivo. Germinal center B cells (B220⁺IgD⁻PNA⁺Fas^{high}) were stained for surface expression of SLAM, day 30 after LCMV infection (thick line). B220⁺Fas⁻ B cells, thin line.

National Institutes of Health guidelines for the care and use of animals in biomedical research.

Viruses

Plaque-purified clones of the Armstrong strain of LCMV (LCMV_{arm}) were propagated in BHK-21 cells (American Type Culture Collection) (46), and tested for biological activity in vitro and in vivo (M. M. McCausland and S. Crotty, unpublished data). A second passage stock of subclone SC3 (LCMV_{arm-sc3}) was used for all LCMV_{arm} experiments. For acute infections, mice received 1×10^5 PFU LCMV_{arm} in a volume of 0.5 ml (suspended in RPMI 1640) by bilateral i.p. inoculation.

Lymphocyte isolation and purification

Single-cell suspensions of spleen were prepared by standard gentle mechanical disruption through a 70- μ m nylon mesh screen (BD Falcon) using a 3-ml syringe plunger (BD Biosciences), followed by removal of RBC with ACK Lysis Solution (BioSource International).

Untouched SMtg⁺ CD4 T cells were magnetically purified from spleen and lymph node preparations (MACS CD4 T cell Isolation Kit; Miltenyi Biotec). A total of 95% purity and naive status was confirmed by surface Ab staining (CD4, V α 2, V β 8.3, CD44, and CD62L) and flow cytometry.

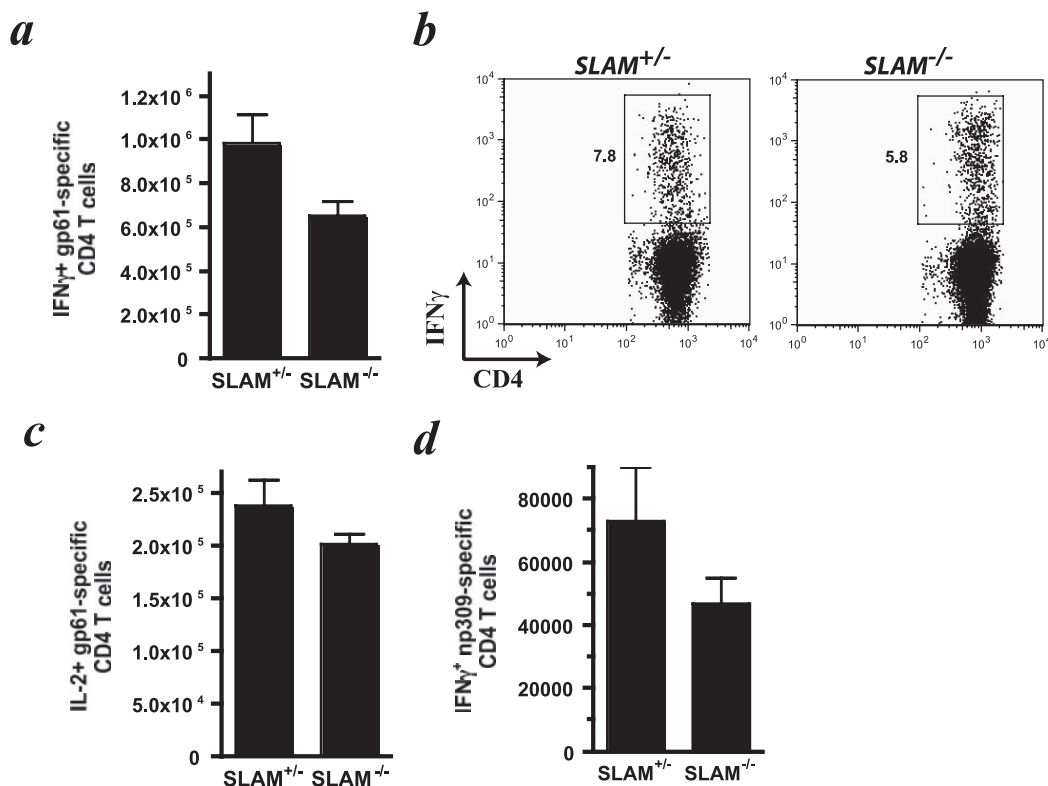


FIGURE 2. Function of SLAM-deficient CD4 T cells in vivo. Effector CD4 T cell responses were analyzed at day 8 after LCMV_{arm} infection. *a*, Splenocytes were stimulated for 5 h with the immunodominant LCMV gp61–80 MHCII peptide in the presence of brefeldin A, then analyzed by intracellular cytokine staining. IFN- γ ⁺ gp61-specific CD4 T cells per spleen is shown ($n = 4$ /group). *b*, Flow cytometric analysis of IFN- γ production by gp61-specific SLAM^{+/+} vs SLAM^{-/-} effector CD4 T cells. *c*, IL-2⁺ gp61-specific CD4 T cells. *d*, Effector CD4 T cell response to the subdominant np309 I-A^b epitope. Data are representative of three independent experiments.

Flow cytometry staining and analysis

Staining for flow cytometry used mAbs to CD4, B220, CD44, CD45.1, CD45.2, CD62L, CD19, CD3, IFN- γ , TNF, IL-2 (eBioscience); polyclonal goat anti-mouse IgG γ (Caltag Laboratories); Fas, CD138, V α 2, V β 8.3 (BD Pharmingen); CD150 (SLAM) (clone TC15-12F12.2; Biologend); biotinylated anti-IgD (Southern Biotechnology Associates); streptavidin-allophycocyanin, streptavidin-PE, and FITC-labeled peanut lectin agglutinin (PNA) (Vector Laboratories). MHCII tetramer of D^b loaded with LCMV GP_{33–41} was provided by Dr. H. Cheroutre (LIAI, La Jolla, CA). Cells were fixed in 2% ultrapure formaldehyde (Polysciences) before acquisition. Flow cytometry samples were acquired on a FACSCalibur instrument (BD Biosciences) and analyzed using FlowJo software (Tree Star). For intracellular cytokine staining, 1×10^6 cells were cultured in the absence or presence of 2 μ g/ml MHCII peptide (LCMV gp61–80 or np309–328) or 0.2 μ g/ml MHCII peptide (LCMV gp33–41 or gp276–286) and brefeldin A for 5–6 h at 37°C. Following staining for surface Ags, cells were fixed and permeabilized with 2% (w/v) paraformaldehyde and 0.1% saponin for 15 min, then stained for the intracellular cytokine of interest in the presence of 0.1% saponin and 2% Newborn calf serum for 30 min. Cells were washed and then fixed in 2% ultrapure formaldehyde before acquisition.

Ig ELISA

Anti-LCMV Ab was measured by ELISA using sonicated cell lysate from LCMV-infected BHK-21 cells as capture Ag. Ninety-six-well Polysorp microtiter plates (Nunc) were coated overnight with lysate in PBS and then UV inactivated (300 mJ in Stratilinker 1800; Stratagene). All samples were run in duplicate. HRP-conjugated goat anti-mouse Ig secondary Abs directed against IgG γ or the isotype or IgG subclass of interest (Caltag Laboratories) were used.

Duplicate 2-fold serial dilutions of mAb 1–1.3 were run in each LCMV ELISA as a calibrated standard. Mouse mAb 1–1.3 directed against LCMV nucleoprotein was obtained from Dr. M. Buchmeier (The Scripps Research Institute, La Jolla, CA) (47). Absolute concentration of stock mAb 1–1.3 was determined by total IgG γ ELISA, titrating against a 0.5 mg/ml IgG2a standard Ab (Southern Biotechnology Associates). Standard curve r^2 regularly exceeded 0.98, and the sensitivity was ≤ 3 ng/ml in all assays.

LCMV viral load quantitative real-time PCR (QPCR)

A detailed version of this assay will be published elsewhere (M. M. McCausland and S. Crotty, unpublished data) (14). Briefly, RNA was isolated from 50 μ l of serum using RNAqueous (Ambion). All serum samples were frozen at -80°C until time for RNA extraction. RNA was eluted in a volume of 20 μ l and purified RNA was frozen at -80°C until use. A total of 10 μ l of RNA was used in a 20 μ l cDNA reaction with SuperScript III Reverse Transcriptase (Invitrogen Life Technologies) and a gene specific primer (GP-R, GCAACTGCTGTGTTCCCGAAAC). A total of 5 μ l of cDNA was then used as a template for 25 μ l of QPCR reaction on a GeneAmp 5700 (ABI), using primers GP-R and GP-F (CATTACCTGG ACTTTGTCAGACTC). A standard curve was generated using the pSG5-GP plasmid (48), a gift from Dr. J. C. de la Torre (The Scripps Research Institute, San Diego, CA). All QPCR samples were run in duplicate. Standard curves were log-linear over $>10^5$ range with high r^2 values ($\sim 98\%$).

Immunofluorescence

The 7- μ m OCT-embedded frozen spleen sections were fixed in acetone and stained for germinal centers with monoclonal anti-B220 IgG labeled with Alexa647 (Caltag Laboratories) and PNA-FITC (Vector Laboratories), or rabbit anti-Bcl6 (Santa Cruz Biotechnology) and PE-labeled anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories). Sections were visualized using a Marianas fluorescence microscope with deconvolution software.

Retrovirus molecular biology, production, and transductions

SAP cDNA was cloned from B6 thymus RNA and sequenced. SAP R78A mutation was introduced using the QuickChange II mutagenesis kit (Stratagene), and the mutation was confirmed by DNA sequencing. Genes were cloned into the retroviral expression vector pMIGR-GFP, upstream of the internal ribosome entry site-driven GFP. Retrovirus production was done by transfection of PLAT-E cells with GeneJammer (Stratagene) plus the retroviral construct of interest (pMIG-GFP, pMIG-SAP, pMIG-SAP R78A) and collection and filtration of culture supernatant at 48 h post-transfection. Before transduction, SMTg⁺ CD4 T cells were activated for

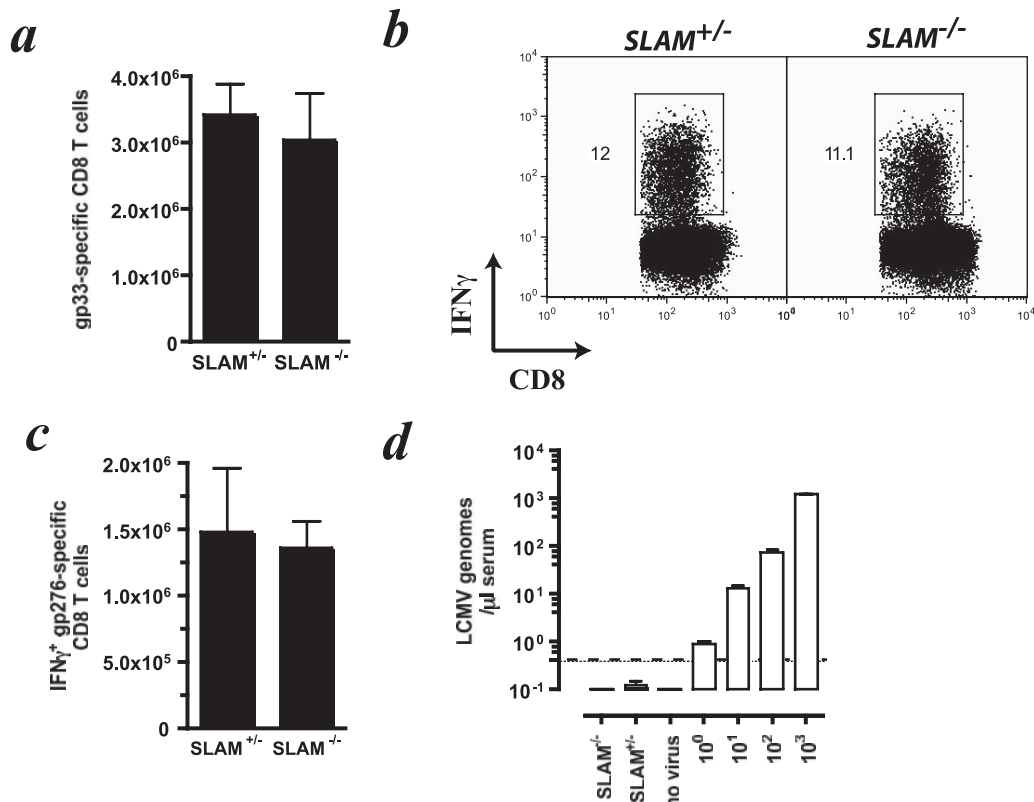


FIGURE 3. Function of SLAM-deficient CD8 T cells in vivo. Effector CD8 T cell responses were analyzed at day 8 after LCMV_{arm} infection. *a*, D^b gp33-tetramer binding CD8 T cell numbers per spleen ($n = 4/\text{group}$). *b*, Flow cytometric analysis of IFN- γ production by LCMV gp33-specific SLAM^{+/+} vs SLAM^{-/-} effector CD8 T cells. Splenocytes were stimulated for 5 h with gp33–41 peptide in the presence of brefeldin A, then analyzed by intracellular cytokine staining. *c*, IFN- γ ⁺ effector CD8 T cells per spleen specific for the subdominant gp276 LCMV epitope. Data are representative of three independent experiments. *d*, Viral clearance. Both SLAM^{+/+} and SLAM^{-/-} mice efficiently clear LCMV_{arm} by day 8 postinfection, as determined by QPCR for LCMV viral genomes in serum. An uninfected mouse (“no virus”) is shown as negative control. Standard curve LCMV genome samples are shown as positive controls (□). Limit of detection of the assay is indicated by the dashed line.

48 h (2.25×10^6 SMtg⁺ CD4 T cells with 3.75×10^6 mitomycin C treated splenocytes as APCs plus 1 $\mu\text{g}/\text{ml}$ peptide and 10 ng/ml IL-2 in a 24-well dish well). Transduction was done by centrifugation of viral medium plus 2×10^6 activated SMtg⁺ cells plus 8 $\mu\text{g}/\text{ml}$ polybrene and 10 ng/ml IL-2 at 1200 rpm for 90 min at room temperature. Medium was then replaced with DMEM with 10% FCS (D-10) plus 10 ng/ml IL-2, and cells were incubated for two additional days. Cells were then washed and replated at 5×10^6 cells/ml in plain D-10 in a 96-well dish. After 72-h rest, cells were collected and live CD4⁺GFP⁺7AAD⁻ cells were obtained by cell sorting using a FACSDiva (BD Biosciences).

Adoptive transfers

For adoptive transfers into CD4^{-/-} mice (see Figs. 5 and 6), purified Ly5.1⁺SMtg⁺ CD4 T cells were i.v. injected via the retro-orbital sinus. A total of 4×10^3 , 2×10^4 , and 5×10^4 SMtg⁺ cells was used in preliminary experiments, with similar outcomes (data not shown); 2×10^4 SMtg⁺ cell transfers were used in all experiments shown. Mice were given LCMV_{arm} i.p. immediately thereafter.

For adoptive transfers into CD4-depleted mice (see Fig. 7), 5×10^4 purified Ly5.1⁺SMtg⁺ CD4 T cells were i.v. injected via the retro-orbital sinus. Wild-type (WT) B6 mice were first depleted of endogenous CD4 T cells by a single i.v. injection of 150 μg of mAb GK1.5 7 days before the SMtg⁺ CD4 T cell adoptive transfer (day -7). In a series of preliminary experiments, we established that a single i.v. 150- μg dose of GK1.5 fully depleted the endogenous CD4 T cells, and the GK1.5 was cleared by day 0 such that the adoptively transferred CD4 T cells survived normally (data not shown).

Protein biochemistry

CD4 T cells were magnetically purified from spleen and lymph node preparation of SAP⁻ SMtg⁺ mice. Purified CD4s were then stimulated with 200 ng/well anti-CD3 and anti-CD28 (eBioscience) in plates precoated with 30 $\mu\text{g}/\text{ml}$ anti-hamster IgG (H+L) (Vector Laboratories) and retro-

virally transduced at 24 and 36 h poststimulation with either vector alone (SAP⁻), WT SAP (SAP⁺), or mutant SAP (SAP-R78A⁺). Cells were stimulated for 4 days total and rested in 20 ng/ml IL-2 (R&D Systems) for 3 days. After 3 days in IL-2, cells were restimulated for 15 min with plate-bound anti-CD3 and anti-CD28. A total of 10×10^6 cells was harvested and lysed in 500 μl of 1 \times TNE buffer (50 mM Tris (pH 8.0), 1% Nonidet P-40, and 2 mM EDTA) supplemented with protease and phosphatase inhibitors. Lysates were incubated with 2 μl of anti-SAP (a gift from P. Schwartzberg, National Institutes of Health, Bethesda, MD) overnight at 4°C followed by immunoprecipitation with protein G agarose (Promega) for 2 h at 4°C. Lysates were resolved in a 4–12% gradient SDS-PAGE gel and immunoblotted with anti-Fyn (FYN3) and anti-SAP (FL-128) Abs (Santa Cruz Biotechnology). Secondary Abs used were anti-rabbit IgG, L chain-specific HRP (Jackson ImmunoResearch Laboratories), and anti-rabbit IgG (H+L) HRP (Promega).

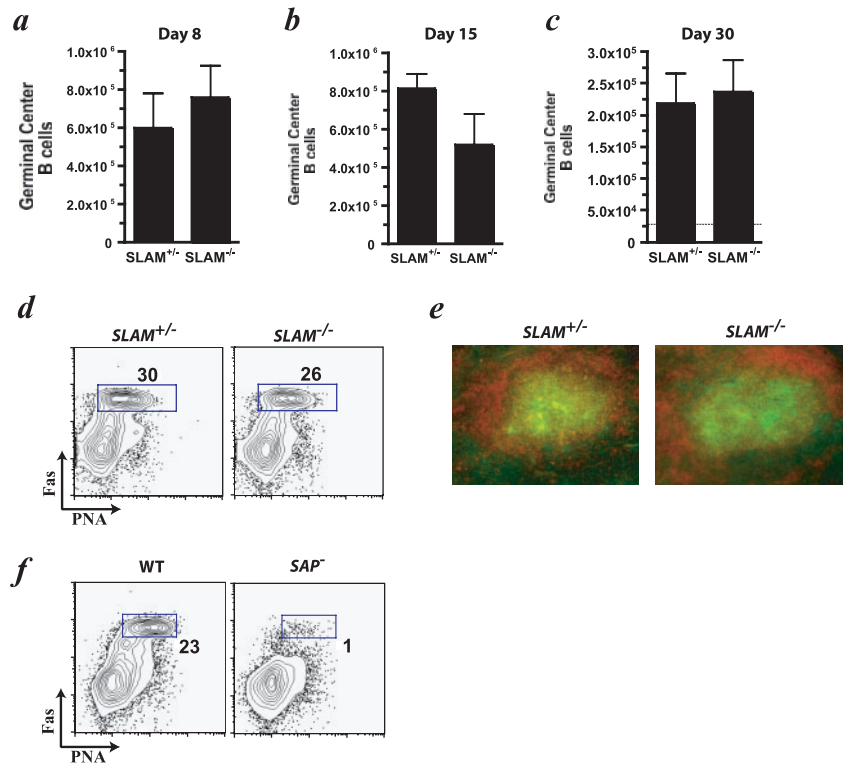
Statistical analysis

Tests were performed using Prism 4.0 (GraphPad). Statistics were done using two-tailed, unpaired Student's *t* test with 95% confidence bounds unless otherwise indicated. Data involving multiple time points or groups were analyzed by two-way ANOVA. Error bars are ± 1 SEM unless otherwise indicated. Arithmetic means were used for all analyses.

Results

SLAM is constitutively expressed on the surface of resting CD4 T cells, CD8 T cells, and B cells (Fig. 1). No SLAM protein is detectable on these cell populations in SLAM^{-/-} mice (Fig. 1, *a* and *b*, and data not shown). SLAM is substantially up-regulated on the surface of WT CD4 T cells after TCR-mediated activation in vitro (17, 32). SLAM is also up-regulated after CD4 T cell activation in vivo (Fig. 1). Effector CD4 T cells responding to an LCMV infection express 5-fold higher levels of SLAM than naive

FIGURE 4. Germinal center development in the absence of SLAM. Germinal centers in SLAM⁺ and SLAM⁻ mice after LCMV infection. Germinal center B cells (PNA⁺Fas^{high}IgD⁻B220⁺) were quantified by flow cytometry at day 8 (a), day 15 (b), and day 30 (c) postinfection (n = 4/group). d, Flow cytometric analysis of germinal center B cells, day 15 postinfection. B220⁺IgD⁻-gated B cells are shown. Box demarcates the germinal center B cells (PNA⁺Fas^{high}). Data for each time point are representative of two independent experiments. e, Immunofluorescence histology of spleen germinal centers. PNA, green; B220, red. f, Flow cytometric analysis of germinal center B cells in SAP⁻ mice and WT C57BL/6 after LCMV infection, gated as above.



CD4 T cells (Fig. 1c). Increased SLAM expression is not only present on activated CD4 T cells, but is also maintained at elevated levels on memory CD4 T cells (Fig. 1c). SLAM up-regulation on effector CD4 T cells in vivo was also shown by adoptive transfer of SMARTA TCR transgenic CD4 T cells, specific for the immunodominant gp61–80 I-A^b MHCII epitope. At day 8 after LCMV infection, SMARTA CD4 T cells had strongly up-regulated SLAM expression (Fig. 1d). SLAM is also up-regulated on effector and memory CD8 T cells (data not shown).

SLAM is constitutively expressed on murine B cells (Fig. 1a). Much like CD4 T cells, B cells increase SLAM expression after activation, with uniformly higher levels of SLAM receptor present on the surface of germinal center B cells (Fig. 1e).

Role of SLAM in T cell expansion

Given the roles of SLAM in SAP function, and the prominent expression of SLAM on B and T lymphocytes, we probed the roles of SLAM in vivo by examining SLAM-deficient mice. Excessive

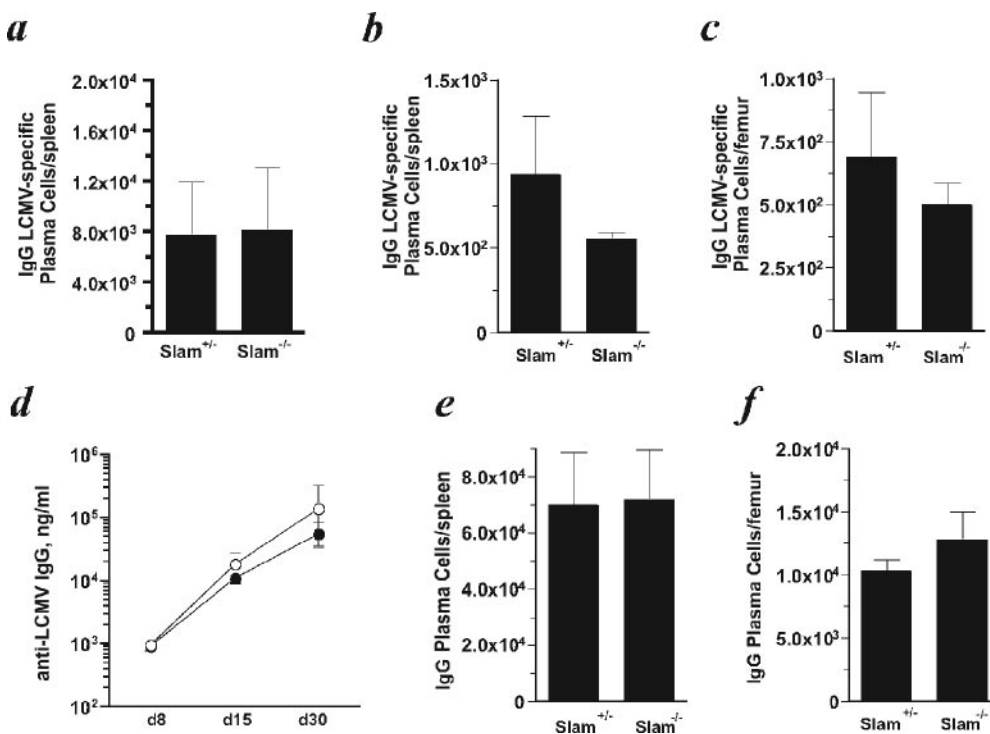


FIGURE 5. Anti-viral Ab response in the absence of SLAM. Anti-LCMV plasma cells and serum IgG in SLAM^{+/-} and SLAM^{-/-} mice after LCMV infection. a, ELISPOT detected anti-LCMV IgG plasma cells at day 8 postinfection in spleen and (b) day 30 postinfection in spleen and (c) bone marrow. No significant difference was observed (p > 0.05; n = 4/group). d, Anti-LCMV IgG levels were quantified in the serum at day 8, 15, and 30 postinfection. SLAM^{+/-}, ●; SLAM^{-/-}, ○; n = 4–6/group. e, Total IgG plasma cells in spleen and (f) bone marrow were quantified at day 30 postinfection as described above. Data are representative of 2–3 independent experiments.

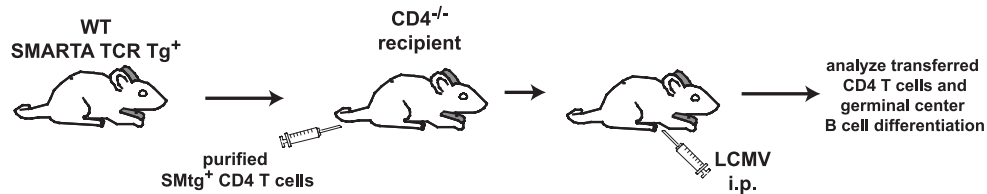
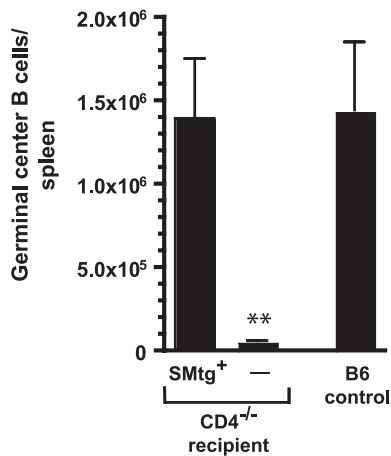
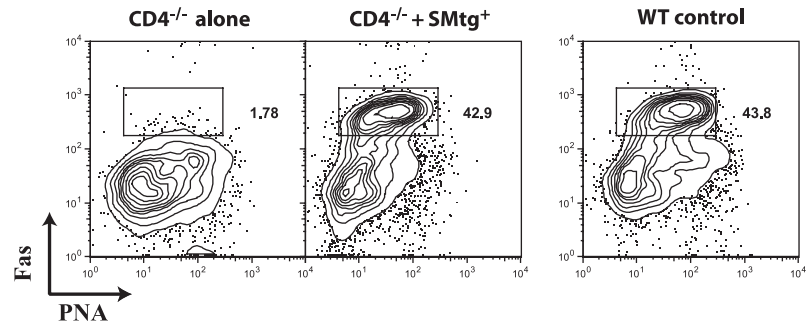
a**b****c**

FIGURE 6. Rescue of germinal center development in $CD4^{-/-}$ mice. *a*, Germinal center T cell help experimental design. SMARTA TCR transgenic (SMtg⁺) CD4 T cells specific for the immunodominant LCMV gp61–80 I-A^b MHCII epitope were isolated. Small numbers of SMtg⁺ CD4 T cells (5,000–20,000) were adoptively transferred into $CD4^{-/-}$ mice, which were then infected with LCMV. SMtg⁺ CD4 T cell expansion and germinal center B cell differentiation was subsequently analyzed. *b*, Germinal centers postimmunization. $CD4^{-/-}$ recipients receiving no cells (—) made no germinal centers. $CD4^{-/-}$ recipients receiving SMtg⁺ CD4 T cells (SMtg⁺) generated germinal center B cells with comparable efficiency to immunized WT B6 mice. Additional control groups included uninfected $CD4^{-/-}$ mice and uninfected $CD4^{-/-}$ mice that received SMtg⁺ cells; no germinal centers or SMtg⁺ cell expansion was observed in those negative controls (data not shown). Results are representative of five independent experiments. *c*, Flow cytometric analysis of germinal center B cells. B220⁺IgD⁻-gated B cells are shown. Box demarcates the germinal center B cells (PNA⁺Fas^{high}). **, $p < 0.01$.

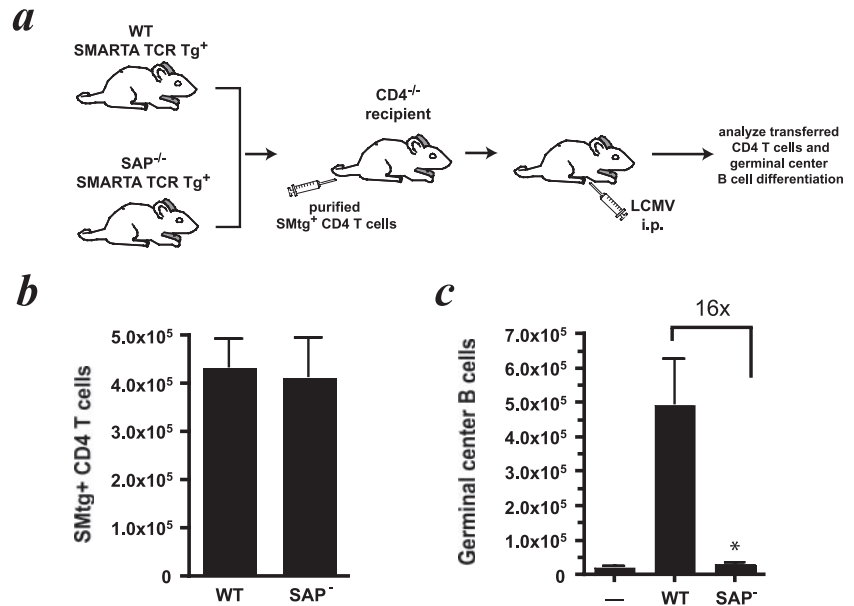
T cell proliferation is observed in SAP^{-} mice during acute infection with LCMV_{arm} (10–12, 14) or infection with murine γ -herpesvirus 68 (13). Excessive T cell proliferation is a phenotype observed in human XLP (4). Excessive SAP^{-} CD8 T cell proliferation causes severe XLP-like disease immunopathology in a mouse chronic viral infection model (14). We therefore examined T cell proliferation in $SLAM^{-/-}$ mice during an acute LCMV infection. Unlike SAP^{-} mice, excessive CD4 T cell expansion was not observed in $SLAM^{-/-}$ mice (Fig. 2). A trend of lower effector CD4 T cell responses was observed in $SLAM$ -deficient mice, both to the immunodominant LCMV MHCII gp61 epitope (Fig. 2*a*), and the subdominant np309 epitope (Fig. 2*d*), although these differences did not reach statistical significance. The $SLAM \rightarrow SAP$ signaling pathway has been shown to be important for suppression of IFN- γ production in vitro (15). Increased IFN- γ production by $SLAM^{-/-}$ virus-specific effector CD4 T cells was not observed (Fig. 2*b* and data not shown), nor were there any significant alterations in the number of $SLAM^{-/-}$ IL-2-producing LCMV-specific CD4 T cells (Fig. 2*c*; $p \gg 0.05$). Numbers of effector CD8 T cells to the immunodominant LCMV MHCII gp33 epitope were normal in $SLAM^{-/-}$ mice both by MHCII tetramer staining (Fig. 3*a*; $p \gg 0.05$) and IFN- γ production (Fig. 3*b*). Normal numbers of effector CD8 T cells to the subdominant gp276 epitope were also observed (Fig. 3*c*). Control and clearance of LCMV is primarily mediated by CD8 T cells, and viral clearance was normal in $SLAM^{-/-}$ mice (Fig. 3*d*).

Role of $SLAM$ in germinal center development and the anti-viral Ab response

One of the major immunological defects observed in SAP^{-} mice is the inability to make germinal centers and thereby generate memory B cells and long-lived plasma cells (4, 12, 18, 49), resulting in hypogammaglobulinemia (14, 50, 51). We therefore examined whether this requirement for SAP for the development of long-term humoral immunity occurs via $SLAM$ receptor signaling. $SLAM^{-/-}$ mice were infected with LCMV, and germinal center B cells were quantified at 1, 2, and 4 wk postinfection (Fig. 4, *a–e*). Germinal centers were normal in the absence of $SLAM$ (Fig. 4, *a–e*). In contrast, in SAP -deficient mice there were virtually no germinal center B cells (Fig. 4*f*). Therefore, SAP control of germinal center formation is not dependent on $SLAM$ receptor signaling.

We also examined the impact of $SLAM$ -deficiency on the anti-viral Ab response, because both SAP and the $SLAM$ family receptors locus have striking impacts on Ab responses (4, 12, 37, 52). Normal numbers of anti-viral IgG plasma cells were present in spleen at day 8 postinfection, the peak of the acute response to LCMV (Fig. 5*a*). At 1 mo postinfection, long-lived plasma cell numbers were normal in bone marrow and spleen (Fig. 5, *b, c, e*, and *f*). Examination of the kinetics and levels of anti-LCMV serum IgG levels in $SLAM^{-/-}$ and $SLAM^{+/+}$ mice postinfection revealed no significant changes in the acute (day 8, 15) or long-term (day

FIGURE 7. SAP^{-} CD4 T cells are defective for follicular help function. Examination of germinal center help capabilities of WT vs SAP^{-} SMtg⁺ CD4 T cells. *a*, Germinal center T cell help experimental design. Twenty thousand WT or SAP^{-} SMtg⁺ CD4 T cells were adoptively transferred into immunized $CD4^{-/-}$ hosts, as per Fig. 5. *b*, Efficient WT and SAP^{-} SMtg⁺ CD4 T cell expansion, day 8 after LCMV infection of $CD4^{-/-}$ hosts, determined by FACS quantitation of $CD4^{+}$ TCR transgenic T cells. *c*, Germinal centers. WT, $CD4^{-/-}$ mice receiving WT SMtg⁺ CD4 T cells; SAP^{-} , $CD4^{-/-}$ mice receiving SAP^{-} SMtg⁺ CD4 T cells; —, control LCMV-infected $CD4^{-/-}$ mice receiving no donor SMtg⁺ cells. Results are representative of five independent experiments. *, $p < 0.05$.



30) anti-viral Ab response (Fig. 5*d*; $p > 0.05$), corroborating the $SLAM^{-/-}$ plasma cell data obtained above.

Roles of Fyn-SAP interaction in CD4 T cell functions

SAP recruits Fyn kinase to SLAM family receptors via an unconventional SH3 domain binding surface (15, 42, 43). That recruitment of Fyn is critical for SAP regulation of Th2 differentiation (16, 17), T cell IFN- γ production (15, 17), and NKT cell development (19–21). Therefore, it appears that SAP's major signaling contributions may occur primarily via recruitment of Fyn kinase to different SLAM family receptors. Our main interest is SAP-dependent regulation of humoral immunity and germinal center development. Does Fyn regulate SAP-dependent CD4 T cell help to B cells and germinal center development via recruitment by SAP to SLAM family receptors?

To address this issue, we attempted to rescue germinal center T help functions of SAP^{-} CD4 T cells, in both Fyn-dependent and

independent manners. Germinal center development is a complex process in vivo that is heavily dependent on CD4 T cells. $CD4^{-/-}$ mice fail to make germinal centers or virus-specific plasma cells after infection with LCMV (Fig. 6, *b* and *c*, and data not shown). Adoptive transfer of SMARTA TCR transgenic (SMtg⁺) CD4 T cells specific for the immunodominant LCMV gp61–80 I-A^b MH-CII epitope rescued germinal center B cell differentiation in $CD4^{-/-}$ mice, stimulating the development of over one million germinal center B cells in spleen after an acute LCMV infection (Fig. 6, *a–c*). This rescue was accomplished efficiently with adoptive transfer of as few as 4,000 naive SMtg⁺ CD4 T cells and, under optimal conditions, the number of germinal center B cells reached levels comparable to that present in intact WT mice immunized with LCMV (Fig. 6*b*), demonstrating that this experimental system efficiently recapitulates normal physiological development of Ag-specific T and B cell interactions and differentiation.

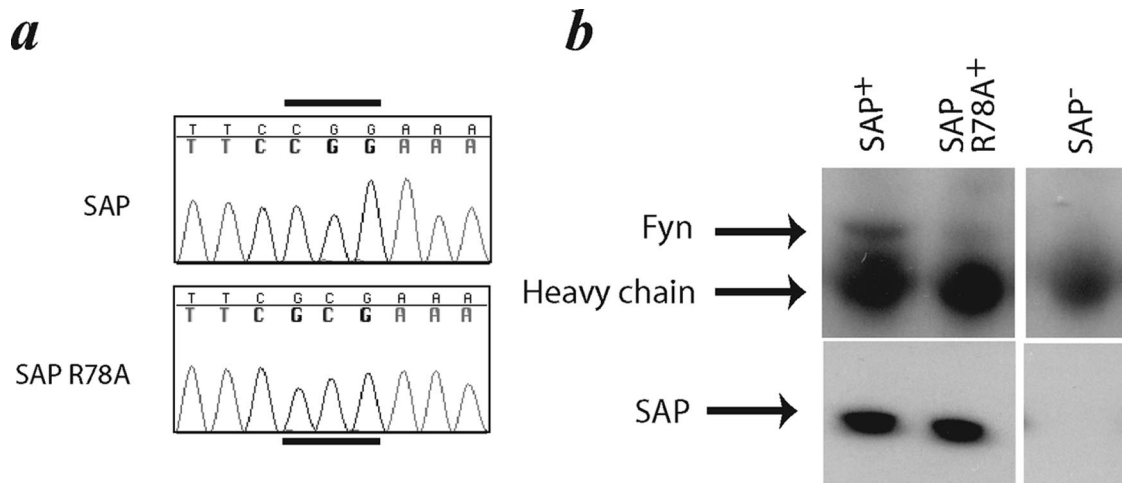
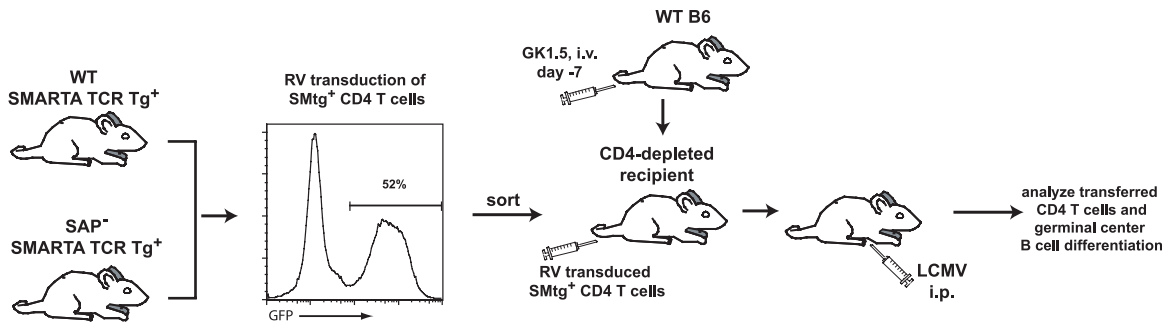
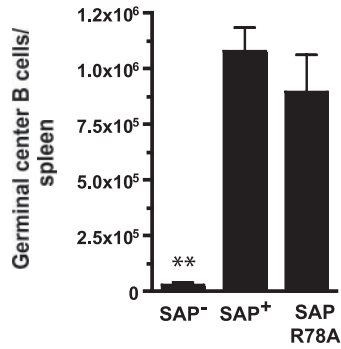


FIGURE 8. Retroviral constructs. *a*, Chromatogram of WT and mutant SAP retroviral construct DNA sequences, highlighting the R78A mutation. Arginine at residue 78 (codon indicated by black line above) was changed to an alanine in the mutant form (indicated by black line below). *b*, Immunoprecipitation showing expression of SAP and SAP R78A protein in transduced SAP^{-} SMtg⁺ CD4 T cells, bottom (SAP^{-} , SAP^{-} SMtg⁺ CD4 T cells plus GFPonly-RV; SAP^{+} , SAP^{-} SMtg⁺ CD4 T cells plus SAP-RV; SAP R78A⁺, SAP^{-} SMtg⁺ CD4 T cells plus SAP R78A-RV.). Immunoblotting for Fyn demonstrated SAP-Fyn interaction in SAP-RV⁺ SAP^{-} SMtg⁺ CD4 T cells after T cell activation and immunoprecipitation of SAP. Fyn binding was ablated in SAP R78A-expressing cells. GFP-RV-transduced SAP^{-} SMtg⁺ CD4 T cells were used as a control.

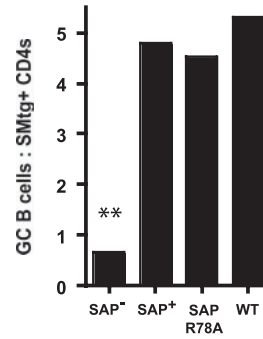
a



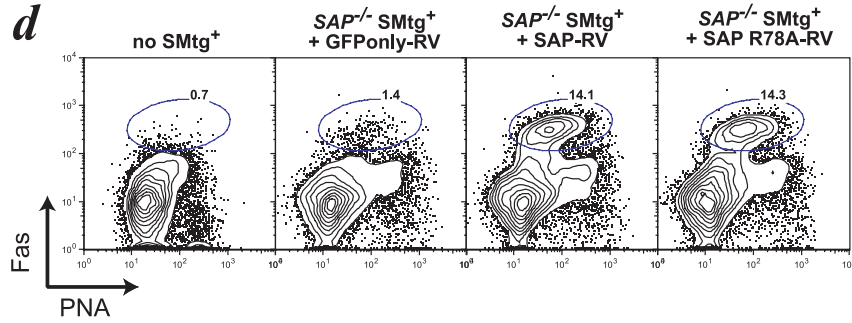
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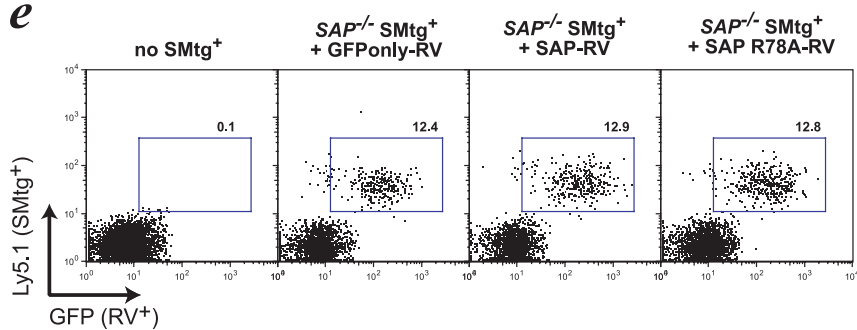
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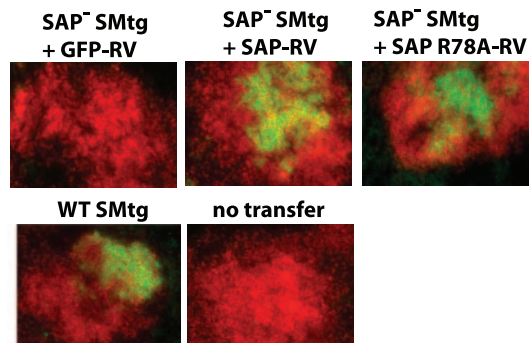
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e



f



Adoptively transferred WT SMtg⁺ CD4 T cells drive excellent germinal center development after LCMV infection (Fig. 6), but adoptively transferred SAP⁻ SMtg⁺ CD4 T cells are completely incapable of providing germinal center help (Fig. 7). Both SAP⁻ and WT transgenic CD4 T cells undergo efficient primary expansion after immunization (Fig. 7*b*). Therefore, the SAP⁻ help defect is not due to a proliferation defect but is due to a specific and absolute block in follicular helper CD4 T cell functions (Fig. 7*c*).

SAP protein requires arginine residue 78 on the backside of the SAP SH2 domain to interact with the SH3 domain of Fyn kinase (42, 43). Mutating that arginine (R78A) ablates SAP binding to Fyn, and thereby minimizes recruitment of Fyn to SLAM (16, 17, 42, 43) and other SLAM family receptors (53). It then follows that if recruitment of Fyn kinase is required for development of SAP-dependent CD4 T cell B cell help functions, CD4 T cells possessing the R78A SAP mutant will be defective for driving germinal center B cell development in vivo. Therefore, we reintroduced WT SAP or R78A mutant SAP into SAP⁻ SMtg⁺ CD4 T cells by retroviral transduction (SAP-RV and SAP R78A-RV) (Fig. 8), and tested the role of SAP-Fyn signaling in follicular help CD4 T cell functions (Fig. 9). Transduction was confirmed by coexpression of a GFP reporter from the bicistronic message (Fig. 9*a*). Expression of SAP and R78A mutant SAP was confirmed by immunoblot (Fig. 8*b*) and QPCR (data not shown). Protein function was confirmed in CD4 T cells by demonstrating via immunoprecipitation that binding of Fyn to SAP R78A was severely abrogated in comparison to Fyn binding to WT SAP after SMtg⁺ CD4 T cell activation (Fig. 8*b*).

In a variation on our earlier experimental strategy (Figs. 6–7), we adoptively transferred RV-transduced SMtg⁺ CD4 T cells into CD4-depleted mice, where we could track the ability of the transferred CD4 T cells to provide help to B cells and facilitate germinal center formation after immunization, in the absence of any contributions by endogenous CD4 T cells (Fig. 9, *b–f*). SAP-RV-transduced SAP⁻ SMtg⁺ CD4 T cells were highly efficient at driving germinal center B cell development in vivo (Fig. 9, *b–d*, and *f*), whereas SAP⁻ SMtg⁺ CD4 T cells transduced with an empty construct (expressing only the GFP reporter, “GFPonly-RV”) failed to drive germinal center B cell development (Fig. 9, *b–d*, and *f*), confirming that SAP is absolutely required in CD4 T cells for germinal center development. Transduced CD4 T cells survived and underwent efficient expansion after LCMV immunization (Fig. 9*e*). To control for any possible bias introduced by variability in CD4 T cell “take” and expansion between individual animals, the ratio of germinal center B cells generated per SMtg⁺ CD4 T cell was calculated as an independent metric of CD4 T cell-B cell help functional activity (Fig. 9*c*). Reconstitution of SAP protein expression resulted in highly efficient follicular helper CD4 T cell functions in transduced SAP⁻ SMtg⁺ CD4 T cells, because these SAP-expressing CD4 T cells were just as potent as WT SMtg⁺ CD4 T cells at driving germinal center B cell differentia-

tion (Fig. 9, *b* and *c*). Importantly, SAP⁻ CD4 T cells reconstituted with R78A mutant SAP became efficient germinal center helper CD4 T cells, and SAP R78A cells stimulated high levels of germinal center B cell development, comparable to reconstitution with WT SAP (Fig. 9, *b–d*, and *f*). Therefore, SAP regulates CD4 T cell helper functions in a Fyn-independent manner, demonstrating that there are distinct CD4 T cell signaling pathways that either regulate Th2 development in a SAP→Fyn-dependent manner or regulate germinal center helper functions in a SAP-dependent but Fyn-independent manner.

Discussion

SAP interacts with at least five different surface receptors of the SLAM family, making it complex to determine the mechanisms for SAP regulation of the many phenotypes observed in SAP⁻ mice and XLP humans. SLAM is the prototypic member of the SLAM receptor family. It is expressed on resting and activated CD4 T cells, CD8 T cells, and B cells (Fig. 1 and Refs. 17, 32, 54). Because SLAM is a self-ligand, its presence at elevated levels on activated lymphocytes presumably enhances Ag-specific T-B interactions during an immune response, and provides costimulatory signals to both the B cell and CD4 T cell (28, 55). It has been proposed that SLAM may be the critical SLAM family receptor controlling humoral immunity (55). Nevertheless, we observed normal germinal center development in *SLAM*^{-/-} mice, indicating that SLAM-SLAM interactions and SLAM receptor signaling are not essential for T-B interactions and humoral immune responses. Modestly lower CD4 T cell responses were observed in *SLAM*^{-/-} mice, indicating that SLAM may have T cell costimulatory function in vivo, consistent with reports of SLAM costimulatory activity on T cells in vitro using agonist anti-SLAM Abs (16, 32, 33), although no proliferative defect was observed in vitro in an earlier study with *SLAM*^{-/-} mice (54). One of two previous studies reported moderately increased IFN-γ production by SLAM-deficient T cells after in vitro stimulation (17, 54). We did not observe a change in T cell IFN-γ production after LCMV infection (Figs. 2–3).

Of separate interest, the lethal immunopathology after EBV infection of XLP patients is thought to involve CD8 T cells (4, 7), and we have recently shown that SAP⁻ CD8 T cells cause severe immunopathology and illness during a chronic LCMV_{cl13} infection in mice (14). Excessive CD8 T cell expansion is observed after either acute (Armstrong strain) or chronic (clone 13 strain) LCMV infection of SAP-deficient mice (10–12, 14). That aberrant and pathological T cell expansion does not appear to occur via SLAM receptor signaling, because *SLAM*^{-/-} mice exhibited normal development of effector CD8 T cells to an acute LCMV infection (Fig. 3).

As discussed above, SLAM expression on CD4 T cells increases after activation, and it has recently been reported that SLAM expression can modulate CD40L expression (18). We observed no

FIGURE 9. Distinct Fyn-independent SAP signaling pathway for B cell help. Germinal center T cell help, functional rescue. *a*, Experimental design. WT or SAP⁻SMtg⁺ CD4 T cells were transduced with retroviral vectors expressing only GFP (GFPonly-RV), GFP and full-length SAP protein (SAP-RV), or GFP and a mutant of SAP (R78A) unable to bind Fyn (SAP R78A-RV). GFP⁺-transduced cells were sorted, and resting GFP⁺SMtg⁺ CD4 T cells were adoptively transferred into CD4-depleted WT B6 recipient mice. Mice were injected with LCMV, and the germinal centers and SMtg⁺ CD4 T cell expansion were analyzed 8 days postinfection. Experimental groups were SAP⁻ SMtg⁺ plus GFPonly-RV (SAP⁻), SAP⁻SMtg⁺ plus SAP-RV (SAP⁺), SAP⁻SMtg⁺ plus SAP R78A-RV (SAP R78A), and WT SMtg⁺ plus GFPonly-RV (WT). *b*, Germinal center B cell numbers. SAP-RV and SAP R78A-RV-transduced SAP⁻SMtg⁺ CD4 T cells both rescued germinal center development. *c*, Normalization of germinal center help activity. Ratio calculated of germinal center B cell numbers to SMtg⁺ CD4 T cells in each individual mouse, providing a quantitation of per cell T cell help activity. *d*, Flow cytometric analysis of germinal center B cells. B220⁺IgD⁻-gated B cells are shown. Oval demarcates the germinal center B cells (PNA⁺Fas^{high}). *e*, Flow cytometric analysis of SMtg⁺ CD4 T cells. CD4-gated cells are shown. Box demarcates GFP⁺SMtg⁺ CD4 T cells. TCR transgenic cells expressed the congenic marker Ly5.1 and were GFP⁺ from the retroviral expression vector (RV⁺). Results are representative of four independent experiments. **, *p* < 0.01. *e*, Immunofluorescence histology of spleen germinal centers. Bcl6, green; B220, red.

alterations in *SLAM*^{-/-} CD4 T cell help to B cells in vivo, demonstrating that SLAM is not required for SAP-dependent CD4 T cell help to B cells for germinal center formation and T-dependent Ab responses to a viral infection (Figs. 4–5). There are seven SLAM family receptors, and so it is possible that the functions of SLAM are compensated by another SLAM family receptor in *SLAM*^{-/-} mice. This seems somewhat unlikely for SAP-dependent signaling, because SLAM possesses unique SAP signaling modalities, in which SAP can bind to SLAM ITSMs in both a tyrosine phosphorylation-dependent and -independent manner (41). SAP SH2 domain binding to the other four known SAP-binding SLAM family receptors appears to be more conventional (tyrosine phosphorylation dependent). Therefore, our results indicate that SAP-dependent control of germinal center development is signaled by one of the other SLAM family receptors expressed on CD4 T cells. Recent work by Wakeland and colleagues (37) has shown that a major lupus susceptibility locus maps to the SLAM family receptor cluster. The *Sle1b* locus causes increased autoantibody production, suggesting that allelic variation in a SLAM family receptor can lead to heightened B cell help functions by CD4 T cells, leading to autoimmunity. That study reported allelic variations in the sequence or expression of CD229/Ly9, CD84, CD244, and Ly108/NTB-A (37). Ly108 appears to be responsible for B cell intrinsic defects resulting in susceptibility to autoantibody production (38). CD229, CD84, and Ly108/NTB-A are expressed on CD4 T cells and interact with SAP (4). Intriguingly, a recent study on human tonsillar CD4 T cells reported increased expression of CD84, CD229, and SAP on CXCR5⁺ follicular helper CD4 T cells (56). CD229 (Ly9) is not required for germinal center development in mice (57), and CD229^{-/-} mice exhibit normal Ab responses and long-lived plasma cell development after a viral infection (57). In aggregate, these data suggest that signaling through SLAM family receptors CD84 or NTB-A/Ly108 may be required for differentiation of follicular helper CD4 T cells and SAP-dependent germinal center formation.

SAP-Fyn and follicular helper differentiation

SAP recruits Fyn kinase to SLAM family receptors. That recruitment provides an obvious mechanism for mediating downstream signaling events (15, 16, 55). Recruitment of Fyn is critical for SAP regulation of Th2 differentiation, as well as SAP control of IFN- γ production. However, our earlier data indicated that the germinal center defect is independent of Th2 differentiation, because normal numbers of IL-4⁺ CD4 T cells were detected in *SAP*⁻ mice after LCMV infection (12). Normal germinal center development in *IL-4*^{-/-}, *IL-4R*^{-/-}, and *STAT6*^{-/-} mice after immunization also suggested that Th2 differentiation is dispensable for germinal center development (Ref. 58 and M. M. McCausland and S. Crotty, unpublished data). These and other results have led to the proposal that a distinct CD4 T cell differentiation lineage, termed follicular helper cells, is responsible for germinal center B cell help in vivo, independent of Th2 (59–63). Follicular helper CD4 T cell differentiation is poorly understood, particularly in mice, but appears to involve SAP, Roquin, and ICOS (12, 23, 56, 64–68). Of note, the CD4 T cell help block in *SAP*⁻ mice is specifically observed at the germinal center stage after LCMV infection, because the early T-dependent anti-viral IgG response is present in the absence of SAP (12), showing that SAP's primary role is to control germinal center CD4 T cell help and SAP can be dispensable for earlier CD4 T cell help to B cells (12).

We tested whether Fyn mediates SAP-dependent CD4 T cell help to B cells and germinal center development. We showed that germinal center T help functions of *SAP*⁻ CD4 T cells could be rescued in vivo by reconstituting SAP protein expression via ret-

roviral transduction, proving that SAP expression in CD4 T cells is required for germinal center T cell help (Figs. 7 and 9). Importantly, we also showed that a SAP mutant capable of binding to SLAM family receptors but defective for Fyn kinase binding is fully able to rescue germinal center CD4 T cell help functions. Therefore, there exist at least two distinct SAP signaling pathways in CD4 T cells: a Fyn-dependent Th2 differentiation pathway, and a Fyn-independent follicular helper differentiation pathway. The phenotypes of *Fyn*^{-/-} and *FynT*^{-/-} mice (69–71) are difficult to interpret, because both strains are present on mixed backgrounds (The Jackson Laboratory; strain no. 002385, <http://jaxmice.jax.org/strain/002385.html> and M. M. McCausland and S. Crotty, unpublished data). Nevertheless, *Fyn*^{-/-} and *FynT*^{-/-} mice are able to make germinal centers after immunization (Ref. 18 and M. M. McCausland and S. Crotty, unpublished data) in contrast to *SAP*⁻ mice, corroborating that SAP-dependent CD4 T cell help to B cells, and follicular helper CD4 T cell differentiation in particular, is independent of Fyn signaling.

In summary, SAP signaling regulates a variety of lymphocyte functions via at least five different surface receptors of the SLAM family. We have shown that two of the prominent phenotypes of *SAP*⁻ mice are not dependent on SLAM signaling: excessive T cell proliferation and failure to make germinal centers. These results further elucidate the complexities of SLAM receptor family roles in the array of biological phenotypes impacted by SAP. Our findings also clarify that SAP is essential in CD4 T cells for germinal center development and that a distinct SAP signaling pathway regulates follicular helper CD4 T cell differentiation, independent of the SAP signaling pathway regulating Th1/Th2. These experiments support the existence of follicular helper cells as a distinct CD4 T cell differentiation lineage.

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Disclosures

The authors have no financial conflict of interest.

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