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SAP Enables T Cells to Help B Cells by a Mechanism Distinct from Th Cell Programming or CD40 Ligand Regulation

Cris Kamperschroer, Deborah M. Roberts, Yongqing Zhang, Nan-ping Weng, and Susan L. Swain

Genetic mutations disrupting the function of signaling lymphocytic activation molecule-associated protein (SAP) lead to T cell intrinsic defects in T cell-dependent Ab responses. To better understand how SAP enables Th cells to help B cells, we first assessed whether molecules important for B cell help are dysregulated in SAP-deficient (SAP knockout (KO)) mice. CD40 ligand (CD40L) expression was enhanced on unpolarized SAP KO T cells; however, Th2 polarization returned their CD40L expression to wild-type levels without rescuing their ability to help B cells. CD40L also localized normally to the site of contact between SAP KO T cells and Ag-bearing B cells. Finally, CD40L-deficient Th cells and SAP KO Th cells differed in their abilities to help B cells in vitro. These data argue that Ab defects caused by SAP deficiency do not result from a loss of CD40L regulation or CD40L function on CD4 T cells. SAP KO Th cells additionally displayed normal patterns of migration and expression of ICOS and CXCR5. Global gene expression was remarkably similar in activated SAP KO vs wild-type T cells, prompting us to investigate whether SAP is necessary for “programming” T cells to become B cell helpers. By restricting SAP expression during differentiation, we determined that SAP is not required during the first 5 days of T cell activation/differentiation to generate Th cells capable of helping B cells. Instead, SAP is necessary for very late stages of differentiation or, most likely, for allowing Th cells to communicate during cognate T:B interactions. The Journal of Immunology, 2008, 181: 3994–4003.
and initiate signaling (17–19). SLAM family members expressed on CD4 T cells include SLAM itself, CD84, CD229/Ly9, Ly108 (NTB-A in humans), and CRACC, although SAP may not associate with CRACC in mouse (17–19). These SLAM receptors are self-ligands and are therefore expected to be bound during interactions with APC expressing the same receptors. Those expressed by B cells are SLAM, Ly108, CD229, CD84, and CRACC and those expressed by dendritic cells (DC) are SLAM, CD84 (in humans, not known for mice), and CRACC. This provides for the possibility that SAP could act via SLAM family receptor signaling within T cells during their initial Ag encounters on APC (such as DC) to “program” T cells to undergo subsequent differentiation such that they acquire the capacity to help B cells. Alternatively, SAP could be promoting the B cell helper capacity of Th at the time when Th cells deliver helper signals to B cells.

What “helper” molecules are regulated by SAP-dependent signaling? It has been reported that CD40L expression is enhanced and sustained on SAP-deficient (SAP KO) CD4 T cells (21). This result seems counterintuitive because CD40L is required for T cells to help B cells; however, there are reports of excessive CD40L signaling impairing Ab responses (32, 33). Lower ICOS expression has been observed on SAP KO CD4 T cells after stimulation in vitro (21, 31) and, to a lesser degree, in vivo (21), and ICOS deficiency prevents the normal generation of CXCR5 expressing Tfh, impairing migration, and T cell help (14–16). These ICOS deficiency prevents the normal generation of CXCR5 expressing Tfh, impairing migration, and T cell help (14–16). These data suggest that CD40L, ICOS, and CXCR5 are candidate receptors that SAP might regulate, but whether these molecules mediate SAP-dependent helper signals has not been directly tested. There is evidence suggesting that IL-10 may play a role in SAP-dependent T cell help, at least in humans (31), but IL-10 expression can be rescued by Th2 polarization of mouse T cells (34, 35) without rescuing their helper function (21); therefore, we have not focused on IL-10 in our studies.

In this report, we have investigated whether candidate molecules are involved in SAP-dependent T cell helper activity and whether SAP programs T cells to help B cells. Our results argue that SAP promotes helper activity independently of regulating CD40L expression, localization, or function. The results also show that the loss of helper activity does not result from altered ICOS or CXCR5 expression on CD4 T cells. Finally, our data show that SAP is required to promote B cell helper activity only after CD4 T cells have become activated and differentiated substantially. Overall, our findings indicate that SAP promotes B cell help independently of CD40L regulation and of programming during early helper T cell differentiation. From this latter finding, SAP likely promotes helper activity at very late stages of differentiation or at the time helper signals are delivered.

### Materials and Methods

#### Mice

C57BL/6 (B6, referred to as wild type (WT)) mice, congenic B6.CD45.1 mice, and mice deficient in CD4 (CD4 KO) (36) or CD40L (5) were originally purchased from The Jackson Laboratory. We also used OT-II TCR-transgenic mice with or without Thy1.1 (or CD90.1), SAP-deficient (SAP KO) mice (23) N10–12 onto B6, SAP-OT-II mice generated by breeding SAP KO mice to OT-II mice, and VDJhel-Ki (also named anti-hen egg lysozyme (HEL) knockin homozygous, or HEL.KI) mice (37) made doubly mutant by crossing to Rag-1-deficient mice (38), which we refer to as VDJhel-Ki.RAG mice. All strains were bred, maintained, and used at the Trudeau Institute according to institutional regulations.

#### Infections, immunizations, and Ags

The influenza A virus A/Puerto Rico/8 (PR8) was grown in the allantoic fluid of embryonated hen eggs as previously described (39). For infections, mice were anesthetized by injecting 2,2,2-tribromoethanol i.p. and forced to aspirate a PR8 50% egg infectious dose of 500 in 30–50 μl of PBS. For immunization, 4-hydroxy-3-nitrophenylacetyl (NP; Biosearch Technologies) was conjugated to OVA (Sigma-Aldrich), then precipitated in alum and 50 μg per mouse was injected i.p. in a volume of 200 μl. For use in the vitro B cell helper assay, HEL (Sigma-Aldrich) covalently coupled to OVA (HEL-OVA) using glutaraldehyde was purified by HPLC using a HiPrep 26/60 Sephacyr S-100 High Resolution Column (Amerham Biosciences). Peptide Ag consisted of amino acid residues 323–339 (ISQAVHAAHAEINEAGR) of OVA, referred to as OVA323.

#### Cell preparations

Single-cell suspensions were prepared from disrupted spleens or lymph nodes (LN) in complete medium consisting of RPMI 1640 (Invitrogen) containing 9% FBS (HyClone), 10 mM HEPES (Research Organics), 2 mM L-glutamine (Invitrogen), 100 IU penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 50 μM 2-ME (Sigma-Aldrich), and RBC were removed by osmotic lysis. CD4 T cells from OT-II, OT-II.CD90.1, or SAP-OT-II mice were purified using MACS with anti-CD4 magnetic beads (Miltenyi Biotec) according to the manufacturer’s instructions. B cells were MACS purified from spleens and LN of VDJhel-Ki.RAG mice either by positive selection after labeling with anti-CD19 beads or by negative selection after labeling with a mixture of biotinylated Abs (anti-CD3, anti-CD5, anti-CD8, anti-CD11b, anti-CD11c, anti-Gr-1, and anti-NK1.1; BD Biosciences) plus anti-CD4 magnetic beads followed by streptavidin (SA)-conjugated magnetic beads (Miltenyi Biotec). Where indicated, cells were labeled for 10 min at room temperature with 1.7 μM CFSE in RPMI 1640 medium containing 1–2% FBS. In adoptive transfer experiments, cells were stained to determine the proportion of transgenic cells and equal numbers of OT-II or SAP-OT-II transgenic CD4 T cells were then injected i.v. in 200 μl of PBS.

#### T cell cultures

Purified naive CD4 T cells were activated in the presence of 11 ng/ml IL-2 either on plates precoated with anti-CD3 (2C11) plus anti-CD28 (37N5) Abs or were incubated with Ag-pulsed splenic blasts that had previously been cultured for 2 days with 25 μg/ml LPS and 25 μg/ml dextran sulfate (used as APC unless indicated otherwise). To pulse APC with Ag, APC at 5 × 10^5/ml in complete medium were incubated with OVA323 peptide at a concentration of 5 μM for 45 min at 37°C in a shaking water bath. For microarray experiments, T cells were cultured with Ag-pulsed bone marrow-derived DC that were prepared by 7-day culture with GM-CSF, which was added to 5 ng/ml on day 0 and 2.5 ng/ml GM-CSF on days 3 and 5 of culture. Where indicated, T cells were polarized toward a Th2 phenotype by adding 15 ng/ml IL-4 and 2 μg/ml anti-IFN-γ clone XM1.2 (both produced in our laboratory) to the cultures. Resulting effector T cells (Teff) were used in the experiments.

#### In vitro B cell helper assay

In wells of 2-well plates, 0.5–1 × 10^6 CD4 T and 1 × 10^5 purified VDJhel-Ki.RAG B cells were cultured in complete medium along with 5 μg/ml HEL-OVA or 5 μg/ml HEL in a total volume of 3 ml/well. In some experiments, B plus T plus HEL cultures (upper chambers) were separated from B plus HEL cultures (no T cells, lower chambers) using Transwell inserts (Nalge Nunc). Between 5 and 7 days later, cells were collected and HEL-specific IgG Ab-forming cells (AFC) were enumerated using ELISPOT.

#### ELISPOT

ELISPOT was performed by the method previously described (25). To detect AFC, HA ELISPOT plates (Millipore) were coated overnight at room temperature with HEL or NP<sub>6</sub>BSA. After blocking wells with complete medium, dilutions of test cells were incubated overnight. After washing, HRP-conjugated Abs specific for mouse IgG1 (Southern Biotechnological Associates) or IgG2c (Jackson ImmunoResearch Laboratories) were added at 1 μg/ml and plates were incubated overnight at 4°C. After washing, the HRP substrate 3-amin-9-ethylcarbazole was added until spots were observed. Spots were counted using a stereo microscope.

#### ELISA

Dilutions of IL-4 and IFN-γ standards of known concentration and dilutions of supernatants from T cell cultures were added to 96-well plates precoated with anti-cytokine Abs and preblocked with PBS containing 1% BSA. Samples were incubated at room temperature for 2 h. After washing, photometrically anti-cytokine Abs were diluted in PBS with 0.05% Tween 20 (PBS/Tween 20) containing 1% BSA and added for 1 h at room temperature. After washing, HRP-conjugated SA in PBS/Tween 20 containing 1% BSA was added for 30 min at room temp. After final washes, the HRP...
substrate o-phenylenediamine dihydrochloride was added and the OD of the color reaction was measured at 492 nm. Abs were generated in house. Concentrations of cytokine in the culture supernatant were calculated using the standard curves as references.

Flow cytometry
Cells were stained and washed in PBS containing 1% BSA and 0.1% NaN₃. After blocking Fc receptors with 2.4G2 supernatant, cells were stained with Abs specific for the indicated surface Ags and washed. Data were collected using a FACS Calibur flow cytometer and analyzed using FlowJo software (Tree Star).

Immunofluorescence
Immunofluorescence was performed as previously described (25). Briefly, 0.7-μm frozen sections cut from spleens or draining LNs were fixed and then stained with anti-CD45.2-Alexa Fluor 488 (eBioscience) and anti-Thy1.1-allophycocyanin (BD Biosciences), then with biotinylated peanut agglutinin (PNA) followed by SA-PE (BD Biosciences). All staining reagents were diluted in PBS/Tween 20 containing 1% BSA.

Live cell imaging
MACS-purified CD4 T cells from OT-II, SAP-OT-II, or CD40L-OT-II mice were cultured 1:1 with Ag-pulsed APC for 4 days under Th2-polarizing conditions. Splenic B cells from naïve CD40 KO mice were isolated using positive MACS selection for CD19⁺ cells. B cells were pulsed with 5 μM OVA α and then labeled with CFSE as described in Cell preparations. T cells and B cells were cultured in a 1T:1B ratio in imaging dishes (MatTek) for 2–4 h. Time-lapse images were captured using AxiosVision 4.5 software on a Zeiss Axiosvert 200M microscope fitted with a humidified chamber at 37°C containing 5% CO₂.

Illumina oligonucleotide microarray
Total RNA (0.5 μg) from each sample was labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion). RNA was first converted into single-stranded cDNA using reverse transcription with an oligo(dT) primer containing the T7 RNA polymerase promoter and then the single-stranded cDNA was copied to produce double-stranded cDNA molecules. The double-stranded cDNA was used in an overnight in vitro transcription reaction where single-stranded RNA (cRNA) was generated and labeled by incorporating biotin-16-UTP. Biotin-labeled cRNA (0.85 μg) was hybridized for 16 h to Illumina Sentrix MouseRef-8 Expression BeadChips. Each BeadChip has 24,000 well-annotated RefSeq transcripts per sample with ~30-fold redundancy. The arrays were washed and blocked. Biotinylated cRNA was detected with SA-Cy3 and quantitated using an Illumina BeadStation 500GX Genetic Analysis Systems scanner. The image data were extracted using BeadStudio software version 1.5. (Illumina).

Microarray data were analyzed using DIANE 6.0, a spreadsheet-based microarray analysis program. Raw microarray data were subjected to Z normalization and tested for significant changes as previously described. Genes were determined to be differentially expressed after calculating the Z ratio, which indicates the fold difference between experimental groups, and false discovery rate, which controls for the expected proportion of falsely rejected hypotheses (31). Individual genes with Z ratio >2 and false discovery rate <0.3 were considered significantly changed. Principal components analysis was performed to identify clustering within groups. Array data for each experimental animal were hierarchically clustered in Illumina BeadStudio version 1.5.

Retroviral transduction
Methods for retroviral transduction were adapted from those made available by the laboratory of G. Nolan at www.stanford.edu/group/nolan/protocols. The Sh2d1α cDNA was cloned into the multiple cloning site of a previously described retroviral construct (40) upstream of an IRES-GFP segment, generating SAP-RV-GFP, SAP-RV-GFP or empty RV-GFP constructs were introduced into the Phoenix packaging cell line by Ca²⁺PO₄ segment, generating SAP-RV-GFP. SAP-RV-GFP or empty RV-GFP constructs were introduced into the Phoenix packaging cell line by Ca²⁺PO₄ transfection. After 48 h, culture supernatants containing replication-deficient viral particles were collected and used for transductions. For transduction, 1.5 ml of viral supernatant containing 6.7 μg/ml polybrene (Sigma-Aldrich) and 11 ng/ml IL-2 was added to each well of 6-well plates along with Th2-polarized Tₐg that had been previously stimulated for 5 days in culture. Plates were centrifuged for 2.5 h at 2600 rpm at 30°C, incubated for 8 h, then the medium was removed and replaced with fresh medium containing 11 ng/ml IL-2. After incubation overnight, live, transduced T cells (GFP⁺ CD4⁺ CD44⁺ 7AAD⁻) were sorted using FACS and transferred to NP-OVA-immunized CD4 KO hosts.

FIGURE 1. Effect of Th2 polarization on CD40L expression and on B cell help. A, CD40L expression. CD4 T cells purified from WT (filled histogram) and SAP KO (solid black lines) mice were stimulated on plate-bound anti-CD3 plus anti-CD28 under Th0- or Th2-polarizing conditions. Histograms show flow cytometry plots of CD40L expression after culture for the time periods indicated at the left of the histograms. The legend in the top left plot applies to all histograms. B–F, B cell help. OT-II (WT, □) or SAP-OT-II (SAP KO, ■)-transgenic T cells were stimulated with APC pulsed with antigenic peptide under Th1- or Th2-polarizing conditions. After 4 days in culture, cells were restimulated on anti-CD3 plus anti-CD28 without cytokines and the concentrations of IL-4 (B) and IFN-γ (C) in the supernatants were measured by ELISA. Th1-polarized cells were also included as a control for IFN-γ detection. D–F, The remaining day 4 WT Th2 cells (□) or SAP KO Th2 cells (SAP– □) or no cells (☐) were then transferred into SAP KO host mice (to prevent endogenous T cell help) that were then immunized with NP-OVA in alum. Seven days after transfer, NP-specific IgG1 AFC were enumerated by ELISPOT (D). Also, CD19 gated GL7⁺ Fas⁺ GC B cells (E) and CD4 gated Vα2⁺ Vγ5⁺ transgenic donor T cells (F) were detected using flow cytometry. Data show means ± SD. Data in A are representative of four independent experiments and data in B–F are representative of three independent experiments (n = 3/group). Differences between WT and SAP– were observed in C (*, p = 0.0025) and D (**, p = 0.001), but not in E (p = 0.05).

Statistical analyses
All statistical comparisons of data sets reported were done using an unpaired two-tailed Student’s t test.
were taken every 20 s for 4 min. Along with CFSE-labeled B cells (green) pulsed with antigenic peptide and PE-conjugated anti-CD40L Ab (red) for 3 h, then time-lapse images of cultures enable staining (data not shown). These data demonstrate that enmary APC lacking CD40 to prevent CD40L internalization and was labeled with anti-CD3 plus anti-CD28 or with peptide-pulsed primary APC lacking CD40 to prevent CD40L internalization and enable staining (data not shown). These data demonstrate that enhanced CD40L expression (observed under nonpolarizing conditions) does not result directly from SAP deficiency, but instead results from a lack of Th2 polarization of SAP KO CD4 T cells.

To determine whether restoring normal CD40L expression via Th2 polarization would also restore the ability of SAP KO CD4 T cells to help B cells, OT-II or SAP-OT-II T cells were effectively Th2 polarized (Fig. 1, B and C), then were adoptively transferred into SAP KO hosts that were challenged with NP-OVA in alum. Within 7 days after transfer and challenge, recipients of OT-II T cells generated robust responses of NP-specific IgG1 plasma cells (Fig. 1D) and GC B cells (Fig. 1E). Transfer of SAP-OT-II T cells, by contrast, led to almost no IgG1 plasma cells or GC B cells specific for NP. Similar or slightly lower proportions of SAP-OT-II T cells were recovered after 7 days when compared with OT-II T cells (not statistically significant; Fig. 1F). These data agree with a previous report (21) and demonstrate that Th2 polarization of SAP KO T cells cannot rescue their ability to help B cells. Importantly, because Th2 polarization restores normal CD40L expression on SAP KO T cells without restoring their ability to help B cells (Fig. 1), it is unlikely that the B cell helper defect results from an increase in CD40L expression on SAP KO CD4 T cells.

Results

SAP KO CD4 T cells: CD40L expression and B cell helper activity

To investigate what molecules may mediate SAP-dependent B cell helper functions of CD4 T cells, we began by measuring expression of molecules known to be involved in B cell help on SAP KO CD4 T cells compared with WT CD4 T cells. CD40L is an obvious potential candidate downstream of SAP function because, like SAP, CD40L is critical for generating T cell-dependent B cell responses (1, 3, 5, 7) and because altered expression of CD40L has been reported on SAP KO CD4 T cells (21). We therefore stimulated SAP KO CD4 T cells and WT CD4 T cells with anti-CD3 plus anti-CD28 in vitro and measured CD40L expression at various time points by flow cytometry. We first detected CD40L up-regulation after 2–3 h of stimulation under all culture conditions and there was no difference in expression between WT and SAP KO T cells in the first several hours (Fig. 1A). This indicates that SAP function does not influence expression of CD40L early following CD4 T cell activation. By 1–2 days of stimulation, we consistently detected more CD40L on SAP KO CD4 T cells than on WT CD4 T cells if they were cultured under nonpolarizing (Th0) cytokine conditions (Fig. 1A). This agrees with a previous report (21) and indicates that SAP restrains expression of CD40L at these times in unpolarized CD4 T cells.

SAP KO CD4 T cells show B cell helper defects regardless of Th1 or Th2 polarization (21). However, SAP KO CD4 T cells fail to produce Th2 type cytokines under neutral conditions (23, 27, 34, 35) and CD40L expression can be down-regulated by Th2 cytokines (41). Thus, increased CD40L expression could simply reflect the Th2 defect in SAP KO CD4 T cells. As predicted (41), Th2-polarizing conditions decreased CD40L expression on WT T cells at these later time points. On SAP KO T cells, Th2 polarization also decreased CD40L expression to levels that were comparable to those on WT T cells (Fig. 1A). We observed similar results with OT-II-transgenic T cells compared with SAP-deficient OT-II (SAP-OT-II) T cells regardless of whether the T cells were stimulated with anti-CD3 plus anti-CD28 or with peptide-pulsed primary APC lacking CD40 to prevent CD40L internalization and enable staining (data not shown). These data demonstrate that enhanced CD40L expression (observed under nonpolarizing conditions) does not result directly from SAP deficiency, but instead results from a lack of Th2 polarization of SAP KO CD4 T cells.

To determine whether restoring normal CD40L expression via Th2 polarization would also restore the ability of SAP KO CD4 T cells to help B cells, OT-II or SAP-OT-II T cells were effectively Th2 polarized (Fig. 1, B and C), then were adoptively transferred into SAP KO hosts that were challenged with NP-OVA in alum. Within 7 days after transfer and challenge, recipients of OT-II T cells generated robust responses of NP-specific IgG1 plasma cells (Fig. 1D) and GC B cells (Fig. 1E). Transfer of SAP-OT-II T cells, by contrast, led to almost no IgG1 plasma cells or GC B cells specific for NP. Similar or slightly lower proportions of SAP-OT-II T cells were recovered after 7 days when compared with OT-II T cells (not statistically significant; Fig. 1F). These data agree with a previous report (21) and demonstrate that Th2 polarization of SAP KO T cells cannot rescue their ability to help B cells. Importantly, because Th2 polarization restores normal CD40L expression on SAP KO T cells without restoring their ability to help B cells (Fig. 1), it is unlikely that the B cell helper defect results from an increase in CD40L expression on SAP KO CD4 T cells.
CFSE-labeled B cells when visualized, the CD40L signal was concentrated mainly at the site of contact between the two cells (Fig. 2A, turquoise arrow). To further illustrate this, D–F of Fig. 2 show images (time moving left to right) “zoomed in” on such a representative interaction, where the red CD40L signal on the WT T cell (yellow arrows) localized to the dynamic site of contact as the green B cell (white arrows) moved back and forth along the surface of the WT T cell. If WT T cells were not actively engaged by a B cell, the CD40L localized primarily to the uropod at the pole of the cell opposite the direction of movement (Fig. 2A, pink arrow), a localization pattern that has been observed for other molecules involved in T cell activation (43). Such events likely correspond to T cells that have recently interacted with B cells and displayed CD40L but are no longer in contact. When we imaged SAP.OT-II T cells in this experimental system, we did not observe any differences in CD40L localization compared with WT OT-II T cells and the same two patterns of CD40L expression emerged (Fig. 2B). We observed no staining on either WT OT-II or SAP.OT-II cells using a labeled isotype control Ab (data not shown). Furthermore, imaging of control CD40L.OT-II T cells in the presence of the anti-CD40L-PE Ab did not result in any PE signal (Fig. 2C), confirming that the observed red fluorescence corresponded to CD40L. These data indicate that SAP is not required for CD40L to localize to the site of cognate interaction between T cells and B cells.

**B cell helper activity of SAP KO CD4 T cells and CD40L KO CD4 T cells in vitro**

In an effort to facilitate our studies, we set up an in vitro assay for measuring B cell helper function of Th cells, which yielded unexpected findings related to the role of CD40L in SAP-dependent B cell help. For this assay, we have primarily used OT-II or SAP.OT-II Th2 effector cells (to focus on helper activity and minimize effects related to Th2 cytokines). As an indicator population of B cells, we used CD19+ cells purified from naïve VDJHEL-KI mice (37) on a RAG-deficient background. All B cells were specific for HEL and can class switch from IgM to other Ig isotypes.

**FIGURE 3.** In vitro B cell helper activity of SAP KO CD4 T cells. OVA-specific Th2-polarized OT-II T eff (WT, □), SAP.OT-II T eff (SAP−/−, ■), or CD40L.OT-II T eff (CD40L−/−, □) taken on day 4 of primary T cell culture were added to triplicate secondary cultures containing HEL-specific B cells purified from VDJHEL-KL.RAG mice and HEL-OVA conjugate or HEL alone as control. T cells were added at a 1T:2B ratio. A, In a Transwell format, WT T eff plus B cells or SAP−/− T eff plus B cells in the upper chambers (U) were separated from B cells alone in the lower chambers (L). Separate wells with B cells alone without Transwells served as negative controls (NO T). HEL-OVA Ag was added to both chambers of all wells. After 6 days, IgG1 AFC were enumerated by ELISPOT. B, WT T eff, SAP−/− T eff, and CD40L−/− T eff were cultured 5 days with HEL-specific B cells and HEL-OVA or with HEL alone. Shown is the mean (±SD) number of IgG1 AFC per triplicate culture. Data are representative of two independent experiments.

**FIGURE 4.** ICOS and CXCR5 expression on CD4 T cells from SAP KO mice. WT mice and SAP KO mice were infected intranasally with influenza PR8 (INF) and a group of WT mice was not infected (WT NI). On day 7 (A), day 9 (B), and day 14 (C and D only) following infection, draining LN cells were analyzed by flow cytometry. A and B, upper plots show ICOS and CXCR5 expression on gated CD4+ T cells and lower plots show GL7 and Fas expression on gated CD19+ cells. C and D, Shown are the mean (±SD) percentages of CD4 gated ICOS+CXCR5+ cells (C) and CD19 gated GL7+Fas+ cells (D) from five mice per group as indicated by gates in A and B. Legend applies to both C and D. Data are representative of two independent experiments. C, No statistically significant differences were observed between WT influenza (INF) and SAP INF at any time point. D, Statistically significant differences were observed between WT influenza and SAP influenza at day 7 (p = 0.0054), day 9 (p < 0.0001), and day 14 (p = 0.0038).
upon activation (37). To enable T cell help, we used HEL covalently linked to OVA as an Ag (HEL-OVA). As a stringent measure of T cell help, we enumerated HEL-specific IgG1 AFC by ELISPOT after 5–7 days of culture. To generate IgG1 AFC, the B cells require Th cells (NO T control, Fig. 3A) and linked Th cell Ag (HEL alone controls, Fig. 3B). Physical contact with the Th cells is also required since in Transwell experiments, B cells alone in a lower chamber did not generate IgG1 AFC while B plus T cells placed in the upper chamber led to 40,000–50,000 IgG1 AFC per culture (Fig. 3A). When we used this assay to test helper function of SAP KO CD4 T cells, we were surprised to find that Th2 SAP.OT-II cells promoted generation of HEL-specific IgG1 AFC as well as Th2 OT-II T cells did (Fig. 3B). This was the case with SAP.OT-II T_{eff} derived from either in vitro cultures or from Ag-challenged mice (data not shown) and helper activity of SAP.OT-II T cells was also contact dependent (Fig. 3A). We observed no difference in helper activity of WT- vs SAP KO Th1-polarized cells in this assay (data not shown). This unexpected finding provided an opportunity to test whether B cell helper defects related to SAP deficiency are linked to defects in CD40L function. If the inability of SAP KO T cells to help B cells results from defective CD40L function, then CD40L KO T cells should show the same...
phenotype as SAP KO T cells with regard to B cell help. We found that in our in vitro helper assay, there was a profound decrease in the number of HEL-specific IgG1 AFC generated in cultures where the T cells lacked CD40L (CD40L.OT-II) (Fig. 3B). Therefore, in this assay, CD40L is required in CD4 T cells for B cell help, whereas SAP is not. This finding uncouples SAP-related helper defects from defects in CD40L and indicates that the B cell helper defect in SAP KO CD4 T cells is not due to a loss of CD40L function.

Expression of Tfh markers and T cell migration of SAP KO CD4 T cells

In addition to CD40L, T cells require ICOS and CXCR5 to help B cells (9–13), and recent evidence indicates that CXCR5 expression is regulated by ICOS (14–16). Given the profound defect in the ability of SAP KO T cells to help B cells (25), it was of interest to determine whether aberrant expression of either ICOS or CXCR5 correlated with the SAP-related helper defect. To test this, we infected WT and SAP KO mice with influenza and afterward analyzed cells from draining LNs using flow cytometry. CXCR5 and ICOS were expressed almost exclusively by activated (CD45R0high) CD4 T cells (data not shown), and frequencies of CXCR5+ICOS+ cells reached 20–30% in both WT and SAP KO mice at day 7 (Fig. 4A, top panels, and 4C), day 9 (Fig. 4B, top panels, and 4C) and day 14 (Fig. 4C) after infection, times when there were clear defects in generating GL7+PNA+ GC B cells in SAP KO mice (Fig. 4, A and B, bottom panels, and 4D). These data argue that the B cell helper defect in SAP mice does not result from an inability to generate CXCR5+ICOS+ Th cells.

This defect in B cell help is likely due to a failure of SAP KO T cells to migrate to T cell areas. To determine whether SAP KO T cells were capable of migrating to follicles and GC, we cotransferred OT-II-transgenic T cells along with SAP KO OT-II-transgenic T cells (SAP.OT-II) into host mice expressing CD45.1 to distinguish SAP KO donor cells from WT donor cells (Fig. 5). Large numbers of both OT-II (CD45.2+) and SAP.OT-II (SAP KO) T cells were stimulated with APC pulsed with antigenic peptide under Th2-polarizing conditions. After 5 days in culture, an aliquot of cells was analyzed by flow cytometry for the indicated markers of activation/differentiation (B). Solid black lines correspond to WT and gray-filled histograms correspond to SAP KO. The remaining cells were either left untreated (denoted ø in C, NONE in D), retrovirally transduced with a SAP-encoding retroviral vector (+ in C, SAP-RV-GFP in D), or transduced with an empty vector (− in C, RV-GFP in D), both of which encode GFP as a marker of transduction. GFP+ transduced T cells were sorted by FACS and transferred to CD4 KO host mice (to prevent endogenous T cell help) that were immunized with NP-OVA (A). Seven days after immunization, splenic NP-specific IgG1 AFC were measured by ELISPOT (C). Also, the percentage of B cells (CD19 gated) that are PNA+ Fas+ (D, top panels) and the percentage of CD4+ GFP+ donor cells (D, bottom panels) from spleens were measured using flow cytometry. Data are representative of two independent experiments with five to six mice per group. Statistically significant differences were observed between the following: WT plus RV-GFP vs SAP plus RV-GFP in C (p < 0.0001) and D, top panels (p < 0.0001); SAP KO plus RV-GFP vs SAP KO plus SAP-RV-GFP in C (p = 0.0073) and D, top panels (p = 0.0003); and WT plus RV-GFP vs SAP KO plus SAP-RV-GFP NS in C but significant in D, top panels (p = 0.0188).

Gene expression of activated WT vs SAP KO CD4 T cells

To identify candidate molecules regulated by SAP-dependent signaling, we have used high through put approaches (44). We performed microarray experiments comparing gene expression of...
live, activated (CD45.2+CD11b−CD11c−7AAD−) WT vs SAP KO CD4 T cells that were stimulated for 3 days in vitro using peptide-pulsed bone marrow-derived DC under Th2 conditions to minimize cytokine-related effects. A subset was also restimulated on anti-CD3- plus anti-CD28-coated plates. Results from our preliminary data using a 2-fold change cutoff indicate that only Glis1 (3- to 4-fold lower) and the gene encoding SAP (sh2d1a, 6- to 7-fold lower) were different in both stimulated and restimulated SAP KO T cells (data not shown). These data suggest that gene expression is very similar in WT and SAP KO CD4 T cells in the first few days after activation, which raises the possibility that SAP-dependent B cell helper functions do not manifest themselves at early times after activation. Instead, SAP may be required only later, when cognate interactions allow for T cells to communicate with B cells.

**SAP deficiency during T cell differentiation: effect on B cell helper function**

One hypothesis to explain why SAP KO T cells cannot help B cells is that SAP promotes a process of differentiation resulting in a subset of Th cells programmed to help B cells. An alternative hypothesis is that SAP is not critical for programming the Th, but is instead critical for helper functions when Th cells are communicating with B cells. To test whether SAP is required during activation or early differentiation for generating Teff with the capacity to help B cells, we activated CD4 T cells and allowed them to differentiate without SAP, then replaced SAP just before assessing their ability to help B cells (Fig. 6A). To achieve this, transgenic OT-II or SAP-OT-II T cells were activated in vitro for 5 days using peptide-pulsed APC. By this time, the cells all had divided extensively (data not shown) and were uniformly CD44high, CD25+, peptide-pulsed APC. By this time, the cells all had divided extensively (data not shown) and were uniformly CD44high, CD25+, CD27+, ICOS+, CD84+, and the majority of the cells were CD62Llow (Fig. 6B). This phenotype indicates an activated and highly differentiated cell population (45). These Teff were then transduced with a GFP-encoding retroviral vector with or without the SAP gene, and GFP+ transduced T cells were sorted and transferred into CD4 KO hosts (to prevent endogenous T cell help) that were then challenged with NP-OVA precipitated in alum (Fig. 6Aa). Seven days after challenge, spleens of host mice receiving OT-II T cells (WT) contained NP-specific IgG1-secreting plasma cells at frequencies of ~1 of 1400 total cells (Fig. 6C). Also, nearly 1% (0.8% in plot shown) of splenic B cells were Ag-induced PNA+Fas+ GC B cells (Fig. 6D, top panels). The process of retroviral transduction did not have a negative impact on helper activity, since transduction with the empty RV-GFP retroviral vector did not reduce IgG1 AFC or GC B cells promoted by donor T cells (Fig. 6, C and D). As expected, SAP-OT-II T cells (SAP KO) transduced with the empty RV-GFP control vector promoted the generation of few if any NP-specific plasma cells or GC B cells. However, replacement of SAP in SAP-OT-II T cells with the SAP-encoding vector before transfer restored their ability to promote plasma cell and GC B cell generation (Fig. 6, C and D). This is not due to selective survival of SAP-expressing T cells because in all cases where the T cells were transduced before transfer, GFP+ transgenic T cells were recovered in equivalent frequencies in host mice (Fig. 6D, bottom panels). These data show that SAP is not required during T cell activation or early differentiation for generating T cells capable of helping B cells, but rather is required either at very late phases of differentiation or during the time when helper signals are delivered to B cells. Thus, for promoting help, SAP likely is necessary within CD4 T cells when they are again stimulated through cognate interactions with Ag-bearing B cells.

**Discussion**

CD4 T cells are needed for B cells specific for most Ags to expand, undergo Ig class switching, and differentiate into Ab-secreting plasma cells. In recent years, it has become clear that SAP is critical for the ability of CD4 T cells to send the necessary helper signals for these processes to occur (21, 22, 25, 29, 31). Results of our work presented here indicate that SAP promotes the ability of T cells to help B cells independently of CD40L regulation, localization, and function. Our data also show that SAP does not promote B cell helper activity of CD4 T cells by regulating the expression of ICOS or CXCR5 or by allowing for T cell entry into B cell follicles or GC. Finally, our work demonstrates that SAP is critical for helper activity of CD4 T cells only after they have already become activated and undergone extensive differentiation.

Our work and a previous study (21) revealed CD40L as a candidate due to its altered expression on SAP KO T cells. Lee et al. (41) have shown that the Th2 cytokine IL-4 inhibits CD40L expression on WT T cells beyond 24 h of stimulation, which is similar to the kinetics of CD40L down-regulation in our studies. This article also showed that Th2 cytokines decreased CD40L on WT T cells regardless of whether the cells were stimulated with anti-CD3 or with APC pulsed with antigenic peptide, and our results confirm their work. Our experiments with SAP KO CD4 T cells show that Th2 polarization returns CD40L expression to normal without rescuing B cell helper activity. In the experiment shown, the mean percentage of SAP.OT-II T cells recovered 7 days after adoptive transfer was ~1.8-fold lower than the mean percentage of WT OT-II T cells recovered. This difference was not statistically significant (p = 0.05) and was not consistently observed; therefore, it cannot explain the nearly complete absence of plasma cells and GC B cells we consistently observed in these experiments. Therefore, we conclude that CD40L dysregulation in SAP KO T cells results from defects in Th2 cytokine production, not directly from SAP deficiency. This finding implies that SAP promotes B cell help by a mechanism independent of CD40L down-regulation.

In experiments using T and B cell lines, it has been shown that fluorescently tagged CD40 on B cells and, by inference, CD40L on T cells localizes to the central portion of the “immunological synapse” formed between T:B conjugates (42), a finding that is confirmed here by our work using primary transgenic T cells and primary B cells. We further show that SAP is not required for CD40L to localize to the contact site between activated T cells and B cells. Still, at the resolution examined, we cannot rule out the possibility that SAP-dependent signaling influences fine spatial distribution of CD40L within the immunological synapse, which could theoretically alter its function. Regardless, our results show that CD40L KO T cells are defective at B cell help in vitro while SAP KO T cells are not. This genetic evidence uncouples SAP function from CD40L function and suggests that SAP does not promote B cell helper function of Th cells via promoting CD40L function, at least not exclusively.

The fact that SAP KO CD4 T cells can help B cells in vitro was unexpected because they cannot do so in vivo. Interestingly, we have found that SAP KO T eff cells generated in vitro can help B cells in vitro but not when transferred in vivo. Conversely, SAP KO T eff cells generated in vivo do not help in vivo but can help when placed in culture with B cells in vitro (C. Kampscherro, unpublished results). These results could be explained either by altered in vivo regulation or function of putative helper molecules that are not necessary in the environment in vitro. Another possibility is that SAP KO CD4 T cells cannot migrate properly in vivo. CXCR5 and ICOS are both critical for normal Th cell migration (11–16), but at times when GC defects were apparent in SAP KO
mice, we detected no difference in expression of either CXCR5 or ICOS ex vivo. This indicates that SAP does not promote help by regulating expression of CXCR5 or ICOS. It is possible that a subpopulation of CXCR5^+ ICOS^+ cells or other critical homing molecules are deficient in SAP KO mice. A prior publication reported the presence of CD4 T cells of undefined specificity in and around the rarely underdeveloped GC that develop in SAP KO mice after infection (22), raising the possibility that CD4 T cells may migrate normally in SAP KO mice. The results from our experiments comparing migration of Ag-specific SAP KO T cells and WT T cells in the same host environment show that SAP is not required for migration of CD4 T cells to B cell follicles or GC. This suggests that the primary SAP-dependent mediators of helper function are not molecules enabling T cell migration.

Our data suggested no involvement of CD40L or molecules critical for migration such as ICOS and CXCR5 in SAP-dependent helper activity; therefore, we also sought molecules affected by SAP-dependent signaling using an unbiased microarray approach. Gene expression analyses showed that in the first few days of activation, there are very few differences in WT vs SAP KO T cells. The SAP gene Sh2d1a served as an internal control and the consistent large differences observed in its expression rule out a global inability to detect differences. If we consider differences present both at 3 days of stimulation and in restimulated cells, the only gene other than Sh2d1a differentially expressed was Glis1, which was consistently less abundant in SAP KO T cells. Glis1 encodes a Kruppel-like zinc finger protein involved in embryonic development with no defined function in the immune system, and it will be interesting to determine whether Glis1 plays a role in regulating genes downstream of SAP-dependent signaling.

Because SAP did not appear to regulate many genes early during T cell differentiation, we were curious to determine whether SAP is necessary during differentiation to generate T cells capable of helping B cells. SLAM family members are expressed by CD4 T cells, B cells, and DC, allowing for the possibility that CD4 T cells are programmed to help B cells as a result of interactions culminating in T cell activation (presumably with DC) and subsequent differentiation or, alternatively, as a result of interactions with B cells. When SAP was deficient for the first 5 days of T cell activation/differentiation but then replaced just before adoptive transfer to Ag-challenged hosts, the ability of T cells to help B cells was restored. At the time SAP was replaced, SAP KO CD4 T cells consistently showed phenotypic characteristics of highly differentiated T_{eff}, although we cannot rule out that further differentiation of T cells could occur after adoptive transfer to assay helper activity. Our results thus demonstrate that SAP is not required during early differentiation for CD4 T cells to acquire B cell helper function and instead suggest that SAP is required for B cell helper activity of T cells either at late stages of differentiation or at the time when helper signals are delivered. A likely scenario is that SAP functions during cognate interactions of Th cells with B cells, allowing for a two-way helper communication. In this scenario, SAP may allow Th cells to receive signals from B cells via SLAM family members. Such signals could induce Th cells to produce key cell surface costimulators or cytokines (or to make them available), allowing the Th cells to reciprocate and provide helper signals to the B cells. Consistent with this idea, SAP-associated SLAM family members such as CD84 are expressed on both activated T cells and activated B cells, making such a SAP-dependent cell-cell communication possible.

The above results also have general implications regarding how Th cells gain the ability to help B cells. Depending on signals they receive from their environment, activated CD4 T cells are driven toward various paths of differentiation, but the extent to which various T cell functions are related to separate differentiation pathways, different stages along a linear progression, or multifunctional potential is not well understood (45). Of relevance here, it is not known whether B cell helper T cells represent a separate differentiation pathway from other functional CD4 T cell subsets. Although our experiments do not address whether B cell helper potential is restricted to distinct pathways of differentiation, they do suggest that B cell helper function is not restricted to a specific temporal stage along the Th2 differentiation pathway. Instead, helper potential may be present throughout the differentiation of Th2 cells. Also consistent with this idea, we have found that when Th2 cells are activated in vitro and then transferred to immunized hosts to assess B cell responses, T cells at all stages (days 0, 1, 2, 3, 4, 5, and 6) are able to help for B cell responses (C. Kampperschroer, unpublished results).

Which SLAM members does SAP promote signaling through to allow T cells to help B cells? Mice deficient in the genes encoding SLAM, CD229/Ly9, and Ly108 have been generated. Interestingly, SLAM KO and CD229 KO mice do not have defective GC B cell or plasma cell responses (46, 47) and no Ab defect has been reported for Ly108 KO mice (48). Therefore, SAP may promote B cell helper activity via signaling through one of the remaining SLAM family members. CD84 seems the most likely remaining candidate in mice since CRACC does not appear to associate with SAP, at least in NK cells (49, 50). Other possibilities are that some SLAM family members are redundant with respect to promoting helper activity or that the critical SLAM family member has not yet been identified. A final formal possibility is that a SLAM family member itself delivers helper signals. Further work is needed to determine which SLAM family members deliver critical signals to T cells to allow them to help B cells.

Results from the work presented here provide evidence that SAP functions to enable helper activity of CD4 T cells independently of the regulation of CD40L expression, localization, or function. Our results also show that SAP is not critical for regulation of ICOS or CXCR5 or for migration of Th cells into B cell follicles of GC, suggesting that SAP does not promote helper activity by affecting migration. Finally, SAP is necessary for T cell help only after substantial T cell differentiation has already taken place. Therefore, we conclude that SAP performs its critical functions in T cells either at very late stages of differentiation or, more likely, during the cell-cell communications that allow B cells to receive helper signals from T cells. It is our hope that future analyses of SAP functions within CD4 T cells will identify key effector molecules that deliver SAP-dependent helper signals to B cells and may lead to the discovery of novel players in the process of B cell help.

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References


