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SAP Is Required for Th Cell Function and for Immunity to Influenza

Cris Kamperschroer, John P. Dibble, Dana L. Meents, Pamela L. Schwartzberg, and Susan L. Swain

Ab is a crucial component of protective immunity to infection, but Ab responses do not proceed normally when defects occur in a protein called signaling lymphocytic activation molecule-associated protein (SAP). To explain this Ab defect, we analyzed B cell and plasma cell responses under conditions of SAP deficiency. Our results demonstrate that SAP-deficient (SAP knockout (KO)) mice have a profound CD4 T cell-intrinsic defect in generating Ag-specific plasma cells following challenge with model Ags or influenza virus, resulting in low Ag-specific Ab titers. We also show that SAP is required in CD4 T cells for normal division and expansion of B cells. These B cell and plasma cell defects were observed during the expansion phase of the primary immune response, indicating early defects in Th cell activity. In fact, additional experiments revealed a nearly complete lack of T cell help for B cells in SAP KO mice. Our work suggests that the ability of SAP to promote T-dependent humoral immune responses is important for antiviral immunity because mice lacking SAP are unable to prevent high dose secondary influenza infection, and because passive transfer of IgG in immune serum from wild-type, but not SAP KO mice can protect mice from an otherwise lethal influenza infection. Overall, our results demonstrate that SAP is required in CD4 T cells for their ability to help B cell responses and promote influenza-specific immunity. The Journal of Immunology, 2006, 177: 5317–5327.

Circulating Ab is arguably the most effective immune component of protection against secondary infections with pathogens. In rare instances, however, genetic lesions lead to defects in Ab responses. One such instance results when inactivating mutations prevent the function of a protein called signaling lymphocytic activation molecule (SLAM)–associated protein (SAP). In humans, nonfunctional SAP results in X-linked lymphoproliferative disease (XLP) (1–3), which is characterized by the following: 1) an uncontrolled and often fatal lymphoproliferative response to EBV infection; 2) increased lymphomas; and 3) hypogammaglobulinemia (4–6). Consistent with the low serum IgG observed in XLP patients, SAP-deficient (SAP knockout (KO)) mice have low basal serum levels of IgG and IgE and have lower titers of Ag-specific IgG or IgE following protein Ag challenge or following infection with Leishmania major, lymphocytic choriomeningitis virus (LCMV), Toxoplasma gondii, or gammaherpesvirus-68 (7–11).

We currently do not know why defective SAP function leads to defective Ab responses. SAP is a cytoplasmic signaling adapter protein expressed by T cells, NK cells, NKT cells, and possibly B cells that functions by binding to kinases and to members of the SLAM family of transmembrane receptors, including SLAM, CD84, CD229/Ly-9, CD244/2B4, and NTB-A/Ly-108 (4–6). In the best studied of these interactions, SAP binds to SLAM and also to Fyn, allowing Fyn to phosphorylate tyrosine residues in the cytoplasmic tail of SLAM and initiate signal transduction (12–14). SLAM receptors are thought to be self ligands and are expressed on both CD4 T cells and B cells (4–6), so cognate interactions of B cells with Th cells may allow for SAP-dependent signaling through SLAM receptors either in the T cell or the B cell. In SAP KO mice, there are normal proportions of developmental B cell subsets in the bone marrow (BM) (15) and developmental T cell subsets in the thymus (7, 9). Moreover, mature B cell and CD4 T cell subsets and the total numbers of cells in lymphoid organs are generally normal (7–9, 15). Thus, SAP appears to be important for the function, not the development, of cells that generate Ab responses.

Although certain B cell subsets reportedly express SAP, this is controversial (15–18) and it remains unclear whether SAP is required within B cells for normal Ab responses (17–20). By contrast, all evidence indicates a role of CD4 T cells in SAP-dependent Ab responses. CD4 T cells express SAP (2, 3, 21–23), and SAP is required within CD4 T cells for normal humoral immune responses (11, 17, 19). Furthermore, SAP deficiency diminishes Ab responses against T-dependent, but not T-independent Ags (10, 11). Together, these findings suggest that SAP enables Th cells to promote B cell responses.

The generation of a T cell-dependent Ab response is a complex process involving several steps (24). Following Ag encounter and critical interactions with CD4 T cells, B cells undergo rapid expansion, class switching, and differentiation into short-term Ab-secreting plasma cells. A subset of Ag-specific B cells forms germinal centers (GCs), in which B cells that receive T cell help undergo affinity maturation and develop into memory B cells and into long-lived plasma cells, which are selectively maintained in the BM (24). Although the production of Ab, the functional end
product of this process, is impaired in SAP KO mice (7–10), it is not known what specific B cell functions are compromised due to SAP inactivation. GCs in SAP KO mice are smaller and less frequent than in normal mice (10, 17, 19); however, it is unclear whether humoral defects occur before GC development. Although some groups have found depressed IgG Ab titers early in the immune response (11, 17), others have reported normal early IgG Ab titers and nearly normal numbers of Ag-specific IgG1 plasma cells (7, 19), which led to the conclusion that early B cell help does not require SAP (19).

To determine whether the primary humoral immune response is normal during SAP deficiency, we measured the early production of B cells and plasma cells in mice with SAP deficiency in B cells and/or T cells following challenge with model Ags or influenza. In doing so, we have demonstrated that SAP is required in CD4 T cells and not in B cells for generating normal numbers of Ag-specific plasma cells and normal titers of circulating Ab. Furthermore, our results indicate that SAP-deficient CD4 T cells are nearly devoid of B cell helper activity. Finally, our results suggest that this defect in Th cell function compromises immunity to influenza infection.

Materials and Methods

Mice

C57BL/6 (B6, referred to as wild-type (WT)) mice, congenic CD45.1+ mice, and mice deficient in CD4 (CD4 KO), I-Aα (I-Aα KO), and RAG-2 (RAG KO) were originally purchased from The Jackson Laboratory. We also used OT-II TCR transgenic mice, SAP-deficient (SAP KO) mice (7), backcrossed 8–10 generations onto B6, and SAP-OT-II mice generated by breeding SAP KO mice to OT-II mice. We used two mouse strains carrying a transgenic Igs L chain and a transgenic IgH chain knocked in to the endogenous locus that when paired confer specificity to HEL-VDJ IgH, mice (25), a gift from J. Cyster (University of California, San Francisco, CA), and SWI IgH mice (26), a gift from R. Brink (Centenary Institute, Newtown, Australia). All strains were bred, maintained, and used at the Trudeau Institute according to institutional regulations.

Infections and immunizations

The influenza A virus A/Puerto Rico/8 (PR8) and the recombinant influenza A/WSN virus expressing aa 323–339 of OVA (WSN-OVA39) (27) were grown in the allantoic fluid of embryonated hens’ eggs, as previously described (28). For infections, mice were anesthetized by injecting 2,2,2-trichloroethanol and forced to aspirate virus in 50 μl of PBS. The 50% egg ldi (EID50) is indicated in the figure legends or the text, as appropriate. Ld50 values given apply to C57BL/6 WT mice and are provided throughout the text primarily as a reference point, particularly for survival studies. For immunization, 4-hydroxy-3-nitrophenylacetyl (NP; Biosearch Technologies) conjugated to OVA (Sigma-Aldrich) was precipitated in alum, and 50 μg per mouse was injected i.p. Alternatively, hen egg lysozyme (HEL) (Sigma-Aldrich) was coupled to OVA using glutaraldehyde and purified by size exclusion chromatography using a HiPrep 26/60 Sephacryl S-100 high resolution column (Amersham Biosciences). HEL-OVA conjugate (50 μg) was mixed with 20 μg of LPS and injected i.v.

Cell preparations and adoptive transfer

Single-cell suspensions were prepared from spleens, mediastinal lymph nodes (MLN), or BM in complete medium consisting of RPMI 1640 (Invitrogen Life Technologies) containing 9% FBS (HyClone), 10 mM HEPES (Research Organics), 2 mM l-glutamine (Invitrogen Life Technologies), 100 IU penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), and 50 μM 2-ME (Sigma-Aldrich). After osmotic removal of RBC, cells were used in assays.

For adoptive transfer experiments, CD4 T cells from OT-II 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 purified from spleens and lymph nodes of HEL-specific IgH knock-in mice by negative selection using MACS 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 from BD Pharmingen) and with anti-CD4 magnetic beads, followed by streptavidin magnetic beads (Miltenyi Biotec).

Where indicated, purified cells were labeled for 10 min at room temperature with 1.7 μM CFSE in RPMI 1640 containing 1–2% FBS. Before transfer, cells were stained to determine the proportion of transgenic cells. Equal numbers of OT-II or SAP-OT-II transgenic CD4 T cells with or without the same number of B cells (generally 1–3 million for dual transfers and 1 million for transfers, as in Fig. 5, A and B) were then injected i.v. in 200 μl of PBS into CD4 KO or CD45.1+ hosts.

For experiments involving reconstitution of RAG KO mice, CD4 T cells and B cells were purified from SAP KO mice and WT B6.CD45.1+ mice (to distinguish WT vs SAP KO cells) using MACS, as described above; then 2 × 107 B cells and 1 × 107 CD4 T cells were transferred i.v. into RAG KO mice. All possible combinations of WT vs SAP KO cells were transferred. Host mice were rested for 30 days to avoid effects of homeostatic proliferation, and a subset of mice was then used to confirm reconstitution. The remaining mice were used for experiments. By flow cytometry, cell populations were >99% pure.

CD4 depletion

Mice were injected i.p. with 1.0 mg of anti-CD4 (GK1.5) or with 1.0 mg of a rat IgG2b isotype control Ab (LT-F2) in PBS on days −1 and 5 relative to influenza infection. Effectiveness of depletion was assessed by staining with the anti-CD4 Ab RM4-4 (BD Pharmingen) 10 days after infection.

BrdU labeling

On days 8 and 9 after infection with influenza PR8, 0.8 mg of BrdU (Sigma-Aldrich) was injected i.p. in PBS. BrdU incorporation was analyzed by flow cytometry 10 days after infection.

ELISPOT and intracellular cytokine staining

The ELISPOT and intracellular cytokine-staining assays to detect Ag-specific T cells have been previously described in detail (29). Peptides used for stimulation corresponded to aa residues 261–275 of the influenza PR8 nucleoprotein (NP261–275) or to residues 323 to 339 of OVA (OVA323–339). To detect Ab-forming cells (AFC), HA ELISPOT plates (Millipore) were coated overnight at room temperature with UV-irradiated influenza strain PR8 or strain WSN-OVA110, HEL, or NP110–BSA. After blocking wells with complete medium, dilutions of test cells were incubated overnight. After washing, HRP-conjugated Abs specific for mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA (Southern Biotechnology Associates) 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

Plates (Nunc) coated overnight at room temperature with UV-irradiated influenza PR8 or with NP110–BSA in PBS were washed and blocked with PBS containing 2% FBS. Sample serum samples diluted in PBS-Tween 20 with 1% BSA were incubated 2 h at room temperature. After washing, HRP-conjugated Abs specific for mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA (Southern Biotechnology Associates) were added at 0.2 μg/ml in PBS-Tween 20 with 1% BSA, and plates were incubated 1 h at room temperature. After washing, the HRP substrate o-phenylenediamine dihydrochloride was added, and the OD of the color reaction was measured at 492 nm. Endpoint serum titers were defined by the last serum dilution that gave an OD reading that was above 2 SD of the mean of negative control values giving baseline OD readings.

Flow cytometry

Cells were stained and washed in PBS containing 1% BSA and 0.1% NaN3. After blocking Fe receptors with 2.4G2 supernatant, B cells were stained sequentially with anti-Ig reagents (Southern Biotechnology Associates), and then with Abs specific for other surface Ags, and with biotinylated HEL or allophycocyanin-conjugated NP. B-cell staining was done according to instructions of the manufacturer (BD Pharmingen). For determining GC phenotype, cells were stained with anti-CD19, anti-GL7, anti-Fas (BD Pharmingen), and biotinylated peanut lectin agglutinin (PNA; Sigma-Aldrich). After revealing biotinylated reagents with fluorescent streptavidin, data were collected using a FACSCalibur flow cytometer and analyzed using FlowJo software (Tree Star).

Staining for apoptotic B cells

After staining for cell surface markers, samples (2 × 106 cells) were washed with annexin binding buffer (0.01M HEPES, 140 mM NaCl, 2.5
nM CaCl2) and then stained 15 min at room temperature with 2 μl of annexin V FITC (BD Pharmingen) in annexin binding buffer. Just before flow cytometry, 3 μl of 7-aminoactinomycin D (BD Pharmingen) was added to each sample.

**Immunofluorescence**

MLN were frozen in OCT (Tissue Tek) using liquid N2, and then 0.7-μm-thick sections were cut and transferred to slides. After acetone fixation, slides were stained with anti-B220 FITC (BD Pharmingen) and PNA biotin (Sigma-Aldrich), followed by SA-Alexafluor 594 (Molecular Probes). Slides were analyzed using a Zeiss Axioshot fluorescence microscope (Zeiss).

**Quantitative RT-PCR**

RNA was prepared from whole lung homogenates using TRIzol (Sigma-Aldrich), and 2.5 μg of RNA was reverse transcribed into cDNA using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen Life Technologies). Quantitative PCR to amplify the polymerase (PA) gene of influenza PR8 was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems) with 50 ng of cDNA per reaction and the following primers and probe: forward primer, 5'-CCGTTCAAATTTCTGCTGTA-3'; reverse primer, 5'-CATTTGCTTCTCCTCCATCA-3'; probe, 5'-6-FAM-CCAAAGTCATGAGGAGAGGAAATACCCGCT-3'. Data were analyzed using Sequence Detector v1.7a software (Applied Biosystems), and the number of copies of the PA gene per 50 ng of cDNA was calculated using a PA-containing plasmid (a gift from R. Webster, St. Jude Children’s Hospital, Memphis, TN) of known concentration as a standard. Multiplying by dilution factors allows an estimation of the number of copies of PA per lung.

**Serum transfer**

Blood was collected from immune mice at least 30 days after sublethal influenza infection and incubated 2 h at room temperature. Samples were spun at high speed for 6 min in a microfuge and serum was collected. Naive mice received an i.v. injection of 50 μl of serum before lethal influenza infection. Some serum samples were diluted 1/100 in naive mouse serum before transfer. IgG was depleted from other samples before transfer by incubating serum with prewashed (in PBS) and drained recombinant protein G-agarose beads (Exalpha Biologicals) overnight at 4°C in a spin column with a 0.45-μm filter (Millipore). After centrifugation, all serum volume was recovered. Removal of influenza-specific IgG was confirmed by ELISA (data not shown), as described above.

**Statistical analyses**

Unless indicated otherwise, statistical comparisons of data sets were done using an unpaired two-tailed Student's t test.

**Results**

**Primary expansion of plasma cells and B cells in SAP KO mice**

Ab responses are depressed in SAP KO mice (7–10), but the specific B or Th cell functions leading to Ab defects are not clear. We began to address this issue by asking whether there are B cell or plasma cell malfunctions in SAP KO mice during the primary expansion phase of the B cell response, which has been reported to be nearly normal in SAP KO mice by some (7, 19), but not by others (11, 17). After immunization with the hapten conjugate NP-OVA precipitated in alum, SAP KO mice generated anti-NP IgM serum titers (Fig. 1A) and numbers of IgM+ AFC (Fig. 1B) similar to or modestly lower than C57BL/6 WT controls (not statistically different). However, anti-NP IgG1, IgG2a, and IgG2b titers (Fig. 1A) and AFC numbers (Fig. 1B) were >10-fold lower in SAP KO mice. These results were observed both at day 8 (data not shown) and day 11 (Fig. 1) after immunization. Because cell numbers in spleens of WT and SAP KO mice are similar before (7–9) and after (data not shown) immunization, AFC frequencies are directly proportional to AFC numbers. We therefore conclude that SAP is necessary for generating normal numbers of IgG+ plasma cells and normal IgG Ab titers during a primary immune response.

We next tested whether SAP KO mice have defects in generating IgG+ B cells, which would result in reduced numbers of cells available to differentiate into IgG-secreting plasma cells. By flow cytometry, we found that ~0.2% of spleen cells from WT mice bind to fluorescent NP 11 days after NP-OVA challenge (Fig. 1C). By contrast, we detected few, if any, NP-binding cells in SAP KO mice. Furthermore, fewer NP-binding IgG1+ B cells (p < 0.03) and a trend toward fewer IgM+ B cells (not statistically significant) were observed in spleens of SAP KO mice at 8, 9, and 11 days after challenge (Fig. 1D and data not shown). Therefore, SAP is required for generating normal numbers of Ag-specific B cells during a primary immune response.

**Proliferation of B cells in the presence of SAP KO CD4 T cells**

One possible reason for why SAP KO mice generate fewer B cells is that the B cells divide less. To determine whether B cells divide less in the presence of SAP KO CD4 T cells than in the presence of WT CD4 T cells, we used an adoptive transfer model. We labeled transgenic HEL-specific knock-in B cells (CD45.2+) with CFSE and transferred them into CD45.1+ congenic hosts along with either WT OT-II or SAP-deficient OT-II (SAP−/−) CD4 T cells. Hosts were then challenged with covalently conjugated HEL-OVA so that the B and T cells would recognize the same Ag, allowing for B cell help. As early as 4 days after transfer and challenge, defects in generating AFC (Fig. 2A) and B cells (Fig. 2B) specific for HEL are observed when CD4 T cells lack SAP, indicating that SAP is necessary in CD4 T cells early in the primary response for generating normal plasma cell and B cell responses. We then analyzed CFSE dilution by transferred B cells expressing either IgM or IgG2a, the dominant IgG isotype in this system (25), by designating high (CFSEhigh) and low (CFSElow) CFSE intensities shown in Fig. 2C. We found that SAP KO CD4
etry was used to detect HEL-binding CD45.2 the indicated isotypes were enumerated using ELISPOT (i.v. with HEL-OVA plus LPS. Four days after challenge, splenic AFC of right panels either IgM or IgG2a (

B cells, plasma cells, and Ab during a primary response against a viral infection. We therefore infected WT and SAP KO mice with influenza virus and measured anti-influenza Ab titers and plasma cells at various time points after infection. We found that, compared with WT, splenic anti-influenza IgM AFC were either similar or lower in SAP KO mice. IgM Ab titers and frequencies of IgM BM AFC were generally lower in SAP KO mice, particularly at late time points (Fig. 3, A–C). Therefore, SAP deficiency results in reductions in the magnitude of IgM responses against influenza.

When anti-influenza IgG responses were analyzed, we observed that between days 0 and 8 after infection, SAP KO mice generate antiviral serum titers of IgG1, IgG2a, IgG2b, and IgG3 that are similar to WT (Fig. 3A). After this time, serum IgG titers continue to increase in WT mice, but do not increase in SAP KO mice, resulting in persisting serum Ab titers that are on the order of 10- to 100-fold lower in SAP KO mice. Corresponding plasma cell frequencies of all IgG isotypes are similar in spleens of SAP KO mice and WT mice until day 8 and continue to increase in WT mice until day 10 or 11, but decrease or fail to increase after day 8 in SAP KO mice (Fig. 3B). Because SAP KO mice have similar numbers of total spleen cells to WT before (7–9) and after (data not shown) influenza infection, splenic plasma cell frequencies are directly proportional to numbers. This leaves SAP KO mice with 10- to 40-fold fewer IgG-secretion plasma cells, depending on the IgG isotype. Similarly, lower peak frequencies of IgG\(^{+}\) plasma cells were also observed in the draining MLN of SAP KO mice (data not shown). These data demonstrate that, as for model Ags (Figs. 1 and 2), SAP is required for initially generating normal serum titers of antiviral IgG Ab and normal numbers of IgG\(^{+}\) antiviral plasma cells. Beyond the peak of the Ab response, anti-influenza titers of IgG1, IgG2a, IgG2b, and IgG3 were maintained in both SAP KO mice and WT mice (Fig. 3A). Corresponding splenic IgG\(^{+}\) plasma cell frequencies of isotypes that remained detectable (IgG1 and IgG2b) decreased at rates in SAP KO mice that were not distinguishable from WT (Fig. 3B). Therefore, our data show an early defect in generating, but not in maintaining influenza-specific plasma cells in the spleen. In the BM, the site in which long-lived plasma cells are preferentially maintained (24), there was a progressive accumulation over time of plasma cells secreting IgG1, IgG2a, and IgG2b in WT mice, but not in SAP KO mice (Fig. 3C). These results are consistent with previous findings (7, 19) and suggest that SAP promotes the formation of BM-resident long-lived IgG\(^{+}\) plasma cells.

As expected (30), little IgA was detected in the serum (Fig. 3A). The differences in frequencies of IgA-secreting antiviral plasma cells between SAP KO and WT mice were generally lower in magnitude than the differences in IgG\(^{+}\) plasma cells (Fig. 3B). Also, IgA was the only class-switched isotype for which we detected plasma cells in the BM, albeit at lower frequencies than in WT (Fig. 3C). IgA\(^{+}\) plasma cells are therefore less dependent upon SAP for their generation than IgG\(^{+}\) plasma cells. Because a defect in GC formation has been described in SAP KO mice using other experimental models (10, 17, 19), we assessed the ability of SAP KO mice to form GCs after influenza infection. By histology, we detected very few GCs in MLN tissue sections from either group 8 days after infection (data not shown). By day 11, 81% of MLN B cell follicles in WT mice contained GCs, whereas 17% of B cell follicles in SAP KO mice contained GCs (Fig. 3D). GCs in SAP KO mice were generally smaller, and PNA\(^{-}\) cells were often more diffusely scattered throughout the follicle (data not shown). Flow cytometric staining of B cells (CD19\(^{+}\)) with a GC phenotype (GL7\(^{+}\)Fas\(^{high}\)) revealed that 10 specific class-switched plasma cells is nearly normal (19). This discrepancy prompted us to determine whether defects would also arise during a primary immune response against a viral infection. We therefore infected WT and SAP KO mice with influenza virus and measured anti-influenza Ab titers and plasma cells at various time points after infection. We found that, compared with WT, splenic anti-influenza IgM AFC were either similar or lower in SAP KO mice. IgM Ab titers and frequencies of IgM BM AFC were generally lower in SAP KO mice, particularly at late time points (Fig. 3, A–C). Therefore, SAP deficiency results in reductions in the magnitude of IgM responses against influenza.

FIGURE 2. SAP KO CD4 T cells promote less B cell division than WT CD4 T cells. OVA-specific OT-II (WT, □) or SAP-OT-II (KO, □) or no (none, □) CD4 T cells were transferred along with CFSE-labeled HEL-specific knock-in B cells (CD45.2\(^{+}\)) into WT CD45.1\(^{+}\) mice challenged i.v. with HEL-OVA plus LPS. Four days after challenge, splenic AFC of the indicated isotypes were enumerated using ELISPOT (A). Flow cytometry was used to detect HEL-binding CD45.2\(^{+}\) donor B cells expressing either IgM or IgG2a (B) and the proportion of those cells that had divided and diluted CFSE (C, C). Left panels, Show representative histograms of CFSE dilution by B cells; right panels, graphically represent the mean (±SD) percentage of CFSE\(^{low}\) (left of center) and CFSE\(^{high}\) (right of center) cells defined by the gates shown in the histograms and derived from five mice per group. *, p ≤ 0.001. The experiment was repeated and gave similar results.

T cells promoted less CFSE dilution and therefore less division by B cells than WT CD4 T cells did in the first 4 days of the response (Fig. 2C). Similar results were obtained when dividing cells were labeled with BrdU (data not shown). Therefore, less early cell division among B cells may contribute to the lower numbers of B cells generated in SAP KO mice.

Another possible reason for the lower numbers of B cells in SAP KO mice is that more cell death occurs. However, in the adoptive transfer model described above, the proportions of transgenic B cells that are apoptotic (annexinV\(^{+}\) 7-aminoactinomycin D\(^{+}\)) are similar regardless of whether cotransferred CD4 T cells express SAP. Therefore, we detect no evidence of increased B cell apoptosis when CD4 T cells are SAP deficient (data not shown).

Humoral immune responses against influenza infection in SAP KO mice

We have shown defects in the ability of SAP KO mice to generate B cells, plasma cells, and Ab during a primary response against influenza and protein Ags (Figs. 1 and 2). This was unexpected given a previous report showing that the primary generation of LCMV-
FIGURE 3. Early defects in the humoral immune response to influenza in SAP KO mice. WT mice (WT, blue circles or bars) or SAP KO mice (KO, red squares or bars) were infected intranasally (i.n.) with 500 EID<sub>50</sub> (0.25 LD<sub>50</sub>) of influenza PR8. At various times after infection, influenza-specific endpoint serum titers of the indicated Ab isotypes were determined by ELISA (A). Also, AFC of the indicated isotypes in the spleen (B) and in the BM (C) were enumerated by ELISPOT. Data points (±SD) represent three to five mice per group per time point. D, GC formation was assessed by histology and by flow cytometry. For histology (left-hand panels and graph), MLN sections taken from mice 11 days after infection or from noninfected mice (NI, ill) were stained with anti-B220 (green) and PNA (red). Representative images are shown on the left, and the graph shows the percentage of B cell follicles that contained GC (PNA<sup>+</sup>). Data are compiled from two separate experiments with four mice per group. Total numbers of follicles counted were 32 for WT, 53 for KO, and 15 for NI. For flow cytometry (right-hand panels and graph), pooled spleen and MLN cells 10 days after infection were stained with anti-CD19 to identify B cells and with anti-GL7 along with PNA to identify GC. Representative plots are gated on CD19<sup>+</sup> cells, and numbers on plots indicate the percentage in the region shown. The graph shows the means (±SD) of five to six mice per group (*, p < 0.0001). The experiment was performed three times. E, Spleen cells were prepared 0, 9, or 84 days after infection, as indicated, and CD4 T cells reactive against the NP261–275 class II-restricted influenza epitope were measured by IFN-γ ELISPOT. Bars represent the means (±SD) of three to five mice per group per time point.
days after infection, 2.2% of B cells in WT mice and 0.16% of B cells in SAP KO mice acquired a GC phenotype (Fig. 3D). Similar results were obtained using GL7 and PNA to identify GC phenotype. Taken together, these results indicate a profound defect in GC formation in SAP KO mice responding to influenza infection.

The humoral defects we have identified in SAP KO mice occur despite the fact that normal numbers of CD4 T cells specific for influenza are generated and maintained. Thus, at days 9 and 84 after infection, the numbers of cells reactive to the class II-restricted NP261–275 influenza epitope were similar between WT and SAP KO mice (Fig. 3E).

Interestingly, multiple successive high dose challenges with influenza, which results in reinforcement of SAP KO mice (see Fig. 8) and increases in antiviral CD4 T cell numbers, did not restore IgG Ab or IgG\textsuperscript{+} plasma cell numbers in SAP KO mice to levels found in WT mice (data not shown). Therefore, multiple antigenic challenges cannot overcome the humoral defects in SAP KO mice.

**New plasma cell generation in SAP KO mice early after influenza infection**

Because the numbers of antiviral IgG\textsuperscript{+} plasma cells continue to expand beyond 8 days after influenza infection in WT mice, but not in SAP KO mice, we were interested to determine whether there are new plasma cells being recruited in SAP KO mice during that time. Plasma cells are terminally differentiated and do not divide. Therefore, if BrdU is made available, plasma cells that become BrdU\textsuperscript{+} represent B cells that divided and then differentiated into plasma cells. To measure new plasma cell generation, we injected BrdU on days 8 and 9 after influenza infection. Then, 10 days after infection, we stained cells from pooled spleen and MLN with Abs against CD138 (syndecan-1) and BrdU. As expected, we found that influenza infection increased the number of CD138\textsuperscript{+} plasma cells present in WT mice, and SAP KO mice generated fewer plasma cells than WT mice (Fig. 4A). Although ~35% of CD138\textsuperscript{+} cells in WT mice were BrdU\textsuperscript{+} 10 days after infection, <20% of CD138\textsuperscript{+} cells were BrdU\textsuperscript{+} in SAP KO mice (Fig. 4, B and C). This percentage of BrdU\textsuperscript{+}CD138\textsuperscript{+} cells in SAP KO mice was similar to the percentages detected in naïve mice. This suggests that SAP KO mice generate few new plasma cells between 8 and 10 days after influenza infection.

**SAP requirement in CD4 T cells or B cells for antiviral plasma cell responses**

Fig. 2 shows that SAP is required in CD4 T cells for normal plasma cell and B cell responses against protein Ag, To determine whether SAP is required within CD4 T cells for plasma cell responses against influenza, we transferred OVA-specific OT-II or SAP-OT-II transgenic CD4 T cells into CD4-deficient (CD4 KO) mice, which do not have endogenous Th cell activity (Fig. 5B controls and data not shown), and infected them with WSN-OVA\textsubscript{\textit{pr}}\textsubscript{p}, a recombinant influenza virus that contains the epitope that OT-II T cells recognize (27). Following infection, similar numbers of CD4 T cells reacted against cognate OT-II peptide by secreting IFN-\gamma, TNF-\alpha, and IL-2 (Fig. 5A). Despite this, mice receiving WT OT-II CD4 T cells generated virus-specific IgG1\textsuperscript{+}, IgG2a\textsuperscript{+}, and IgG2b\textsuperscript{+} AFC, but mice receiving SAP-OT-II CD4 T cells and mice receiving no cells did not (Fig. 5B). As observed in intact SAP KO mice (Fig. 3, B and C), the defect in generating IgM\textsuperscript{+} and IgA\textsuperscript{+} plasma cells was less pronounced than that in generating IgG\textsuperscript{+} plasma cells (Fig. 5B). These data demonstrate that, as for protein Ag, SAP is required within CD4 T cells for generating normal numbers of IgG\textsuperscript{+} plasma cells following influenza infection.

To further examine which cell types require SAP, we adoptively transferred CD4 T cells and B cells into RAG KO mice. By controlling whether the transferred cells were SAP deficient, we could assess whether CD4 T cells or B cells require SAP for normal Ab responses. RAG KO hosts were infected with influenza 30 days after reconstitution with all combinations of T and B cells, and the ability of these chimeras to generate Ab and plasma cell responses to influenza was analyzed 10 days after infection. To control for purity of the transferred populations, the experiment was set up such that WT cells expressed CD45.1 and SAP KO cells expressed CD45.2. This allowed us to rule out the possibility of contaminating cells in the reciprocal population. For example, in the group receiving WT T cells and SAP KO B cells (T\textsuperscript{+} B\textsuperscript{−}), 99.5% of the B cells are CD45.1 negative and thus SAP KO 10 days following influenza infection (data not shown). This excludes the possibility that plasma cell responses from this group result from WT B cells contaminating the WT CD4 T cell preparation. Mice reconstituted with all combinations of cells generated virus-specific plasma cells of the IgM isotype (Fig. 5C). When WT CD4 T cells were transferred,
antiviral plasma cells of all IgG isotypes were generated, regardless of whether the B cells expressed SAP (Fig. 5C). If the CD4 T cells were SAP deficient, however, there were almost no IgG plasma cells detected (Fig. 5C). Corresponding virus-specific Ab titers showed similar results (data not shown). The ability of the transferred T cells to provide help for an endogenous plasma cell response against WSN-OVA323–339 was assessed by AFC ELISPOT on spleen cells 21 days after infection (B). Graphs in A and B show means (±SD) of three to six mice per group. C–E, RAG KO hosts were reconstituted with the indicated combinations of purified CD4 T cells (T) and B cells (B) isolated from either WT SAP-sufficient (+) or SAP-deficient (−) mice, or were not reconstituted (none). After 30 days, reconstituted hosts were infected with 250 EID$_{50}$ (0.125 LD$_{50}$) influenza PR8. Ten days after infection, AFC in pooled spleen and MLN cells were enumerated by ELISPOT (C). For all IgG isotypes, T$^{+}$B$^{−}$ and T$^{−}$B$^{−}$ were not different from each other, but were different from all other groups ($p < 0.03$). D, B cells (CD19$^{+}$) with a GC phenotype (GL7$^{+}$ PNA$^{hi}$Fas$^{hi}$) were measured using flow cytometry. Numbers on representative flow cytometry plots show the percentage of CD19-gated cells that fall into the indicated GL7$^{+}$ PNA$^{hi}$Fas$^{hi}$ region. The bar graph shows the mean percentages (±SD) of CD19-gated cells that are GL7$^{+}$ PNA$^{hi}$Fas$^{hi}$, and statistical comparisons are shown below. E, T cells specific for the NP261–275 influenza epitope were enumerated by IFN-$\gamma$ ELISPOT, and means (±SD) are shown. C–E, $n = 5–6$ per group.

**Lack of B cell help in SAP KO mice**

Our observations that SAP KO mice have a profound early CD4 T cell-intrinsic defect in generating primary Ab responses prompted us to test whether CD4 T cells in SAP KO mice provide any B cell help during the early stages of the response. If the small numbers of plasma cells in SAP KO mice are generated independently of help from T cells, then plasma cell responses in SAP KO mice should be similar to those in mice that lack CD4 T cells entirely. We therefore compared plasma cell numbers in SAP KO mice with those in CD4 KO mice and MHC class II (I-Ab$^{b}$) KO mice over the first 36 days following influenza infection. The numbers of antiviral IgG$^{+}$ plasma cells in pooled spleen and MLN of SAP KO mice were similar to those generated in CD4 KO and I-Ab$^{b}$ KO mice, but, as expected, were much lower than the numbers in WT mice (Fig. 6A). Only WT mice generated significant numbers of IgG$^{+}$ plasma cells in the BM (Fig. 6B). As observed in our other
experiments, the IgM and IgA isotypes were less affected by SAP deficiency (Fig. 6, A and B). Antiviral CD4 T cell responses were similar between WT and SAP KO mice, not detectable in I-Ab KO mice, and detected only occasionally and at low frequencies in CD4 KO mice (Fig. 6C). These data suggest that the virus-specific plasma cells we observe in SAP KO mice are generated independently of help from T cells.

To determine whether SAP KO mice can generate any measurable T cell help, we depleted CD4 T cells before influenza infection. In WT mice, removal of CD4 T cells (Fig. 7A) had little effect on the generation of IgM⁺ or IgA⁺ plasma cells, but reduced the numbers of IgG⁺ plasma cells present 10 days after infection by 20- to 80-fold, depending on IgG isotype (Fig. 7B). By contrast, depletion of CD4 T cells from SAP KO mice did not measurably change the numbers of plasma cells generated (p > 0.05). Similarly, 10 days after infection, CD4 depletion reduced the proportion of B cells with a GC phenotype in WT mice from 1.68 to 0.03%, but had a modest effect on GC formation in SAP KO cells (Fig. 7C). Similar results were obtained 8 days after infection (data not shown). Therefore, SAP KO mice almost completely lack T cell help for B cells.

Control of influenza infection by SAP mice

Given that the Th cell defect in SAP KO mice results in less anti-influenza Ab following infection, we were interested in whether
SAP KO mice can control influenza infection as efficiently as WT mice. To test this, we first infected SAP KO and WT mice with a sublethal dose of influenza PR8 and afterward measured the number of copies of the influenza PA gene per lung by quantitative PCR. In SAP KO mice, the kinetics of viral replication and clearance following primary infection were indistinguishable from those in WT mice (Fig. 8A), demonstrating that SAP is not required for control of primary influenza infection. Because Ab is important for secondary immunity to influenza (31), we suspected that SAP KO mice would be more susceptible to secondary infection. Indeed, when immune mice that had recovered from primary influenza infection were given a high-dose (100 LD_{50}) secondary influenza infection, WT mice showed no evidence of reinfection, whereas SAP KO mice and nonimmune mice had large amounts of virus in the lungs (Fig. 8B). Similar data were obtained when a dose of 25 LD_{50} was used for secondary challenge (data not shown). These results show that SAP is required for preventing secondary influenza infection. If SAP KO mice fail to prevent secondary influenza infection because of the anti-influenza Ab defect we have observed, then immune serum from SAP KO mice should be less efficient than immune serum from WT mice at transferring protection to naive mice. To test this, we collected serum from SAP KO and WT mice 30 days after a sublethal primary influenza infection and transferred it into naive mice that were then challenged with 10 LD_{50} of influenza. Following infection, naive mice receiving immune serum from WT mice all survived, whereas 9 of 10 naive mice receiving SAP KO immune serum died (Fig. 8C). Removal of IgG before transfer of WT immune serum reversed protection, as did dilution of the WT serum 100-fold to mimic IgG concentrations in SAP KO serum. As expected, mice receiving serum from naive mice died. Together, these data indicate that the protection afforded by immune serum requires anti-influenza IgG Ab, and that the inability of SAP KO immune serum to protect can be accounted for by a quantitative defect in IgG. Overall, our results suggest that SAP KO mice do not control secondary influenza infection because of the defects we have observed in IgG Ab responses.

Discussion

Our results reveal that SAP-deficient CD4 T cells almost completely lack the ability to help B cells. The resulting defect in the primary expansion of B cells and plasma cells leaves animals with a paucity of circulating Ab that compromises immunity to infection. Thus, Th cell function is more severely affected by SAP deficiency than previously thought. In CD4 T cells, SAP acts as a signaling adaptor for SLAM, CD84, NTB-A/Ly-108, and CD229. These SLAM family receptors are thought to act as self ligands and are also expressed on B cells (4–6). Based on these and our findings, we speculate that SAP primarily promotes B cell responses by allowing signaling through SLAM family receptors in T cells during cognate interactions of activated CD4 T cells and B cells, where homotypic interaction of SLAM family receptors could occur. We presume that these signals induce changes within the T cell, allowing it to acquire the complement of cell surface receptors and/or cytokines that enables it to effectively deliver help to B cells.

Our finding that SAP KO mice are nearly devoid of T cell help from the beginning of the immune response was surprising given previous reports (7, 19). In particular, a publication by Crotty et al. (19) observed normal early antiviral IgG titers and modestly reduced (~5-fold lower) peak numbers of antiviral IgG-secreting plasma cells in the spleens of SAP KO mice 8 days after LCMV infection. These data led to the conclusion that SAP is not required for early B cell help. By contrast, at the peak of the response 10 days after influenza infection, we find Ab titers and plasma cell numbers that are, depending on the IgG isotype, ~10- to 100-fold lower than those in WT mice. Although we detect small numbers class-switched antiviral plasma cells in SAP KO mice, our experiments show that these are generated independently of T cells. Influenza infection generates class-switched T-independent Ab (32), and may do so because the virus induces tissue damage and inflammatory signals in the lung (33, 34), and because influenza can activate the innate immune system via TLRs (35). These viral effects may partially compensate for the lack of SAP-dependent helper function and may explain why the early Ab defects we observe are more pronounced with inert Ags, particularly when using alum as an adjuvant. Such virus-associated signals may also contribute to the hypergammaglobulinemia observed after LCMV infection (36), and could potentially explain why the early IgG response against systemic LCMV infection is less affected by SAP deficiency than the IgG response against influenza. Another difference between our findings and those of Crotty et al. (19) is these authors show that after LCMV-specific IgG plasma cell numbers peak, there is a 10-fold greater decline in their numbers in SAP KO.
mice than in WT mice over the next several days. After the peak of the response against influenza, we find no consistent differences in the rate of decay of either IgG Ab or plasma cell numbers (where frequencies remained high enough) in SAP KO mice. The reason for this discrepancy is not clear, but might also be related to differences intrinsic to the viruses.

The early defects in B cell and plasma cell responses are consistent with the lack of Th cell function we observe. First, we find that B cells proliferate slower and expand less in the presence of SAP KO CD4 T cells than they do in the presence of WT CD4 T cells. This is consistent with data from a recent report (11). Over 10 cell generations, the 20–30% difference we observe in dividing cells could result in a 9- to 35-fold difference in the cell numbers generated. Thus, slower proliferation of B cells most likely contributes to the defect in B cell expansion, which leaves fewer B cells available to generate into plasma cells. Second, our data indicate that many fewer plasma cells are generated in the presence of SAP KO CD4 T cells. When CD4 T cells lack SAP, we find that the defect in generating B cells is considerably less pronounced than the defect in generating plasma cells, suggesting a prominent defect in plasma cell differentiation. Third, we show that SAP is required in CD4 T cells and not in B cells for GC development, which may also explain the absence of plasma cells from the BM of SAP KO mice. All these defects could be attributed to the defective Th cell function resulting from SAP deficiency.

An additional possibility is that SAP deficiency prevents efficient class switching. Less efficient class switching to some IgG isotypes was reported when SAP KO B cells were stimulated in vitro (15). However, we detect no B cell-intrinsic requirement of SAP for class switching, suggesting that if SAP promotes class switching, it does so primarily by promoting T cell help. We most often observe reduced numbers of IgM^+ B cells and plasma cells in SAP KO mice or mice with SAP KO CD4 T cells, but these defects are not present in every experiment and are generally modest compared with the severe defects in the generation of IgG^+ B cells and plasma cells. The reason for this variability is not clear, but the overall trend toward decreased IgM responses suggest that SAP might promote B cell functions before class switching, at least under certain circumstances. Our finding of slower B cell proliferation in the presence of SAP KO CD4 T cells supports this notion. A final possibility is that B cells and plasma cells do not survive as well in SAP KO mice. We cannot exclude this possibility, but we have not observed any evidence of increased apoptosis of B cells in the presence of SAP KO CD4 T cells.

The issue of whether SAP is required in B cells for normal Ab responses has been controversial (17, 19, 20). A recent report by Morra et al. (17) presented data supporting a requirement of SAP in B cells for normal Ab responses and GC formation. In our experiments, WT, but not SAP KO CD4 T cells promoted generation of GC B cells and of anti-influenza Ab and plasma cells of the IgG1, IgG2a, and IgG2b isotypes. This occurred regardless of whether B cells expressed SAP. We therefore conclude that SAP is required in T cells, but not in B cells, for generating normal Ab, plasma cell, and GC responses. This argues that the plasma cell and GC defects result from a loss of Th function, not from a B cell-intrinsic defect. Our results agree with data from Crotty et al. (19) and with a paper by Cannons et al. (11) that was published while this manuscript was under review. Because Crotty et al. (19) used irradiated recipients as hosts for related experiments, Morra et al. (17) argued that they did not see a requirement for SAP in B cells because of the presence of endogenous WT B cells not removed by irradiation. However, our experiments and those of Cannons et al. (11) used the same experimental approach as Morra et al. (17) did, that is, reconstitution of RAG-2 KO recipients with different combinations of B and T cells from WT vs SAP KO mice. Under these conditions, the only possible source of WT B cells in transfers of SAP KO B cells is contaminating B cells in the WT CD4 T cell preparation (our T^"B" group). After this T^"B" transfer, we found that during the peak of the antiviral response, 99.5% of B cells were derived from SAP KO mice (CD45.2 ), making it unlikely that the plasma cell response we detect in the T^"B" group results from contamination by WT B cells. Although we cannot rule out a role of SAP in B cells, our data and a recent report (11) argue that SAP is not required in B cells, but is required in CD4 T cells for normal primary humoral immune responses.

What signals does SAP orchestrate within CD4 T cells to make them competent to help B cells? In CD4 T cells, SAP associates with several members of the SLAM family of transmembrane receptors, including CD84, CD229, and NTB-A (4–6), so it is possible that signaling through a combination of these eventually results in changes in the CD4 T cell that allow it to promote B cell functions. Further studies of mice deficient in these molecules or combinations of these molecules should determine which of these promotes helper functions. The expression of a number of protein species is altered in activated SAP-deficient CD4 T cells (37), but the molecular products of SLAM family receptor signaling that ultimately deliver SAP-dependent helper signals to B cells are unknown. SAP KO CD4 T cells show a striking defect in production of IL-4, IL-10, and IL-13 (8), but there is evidence that defects in these Th2 cytokines are not responsible for the Ab defect in SAP KO mice. In vitro polarization of SAP KO CD4 T cells toward a Th2 phenotype rescues production of IL-4, IL-10, and IL-13 (7, 8, 38), but Th2-polarized OVA-specific SAP KO CD4 T cells still do not help Ab or plasma cell responses upon transfer into helper-deficient hosts challenged with NP-OVA (11) or infected with an influenza recombinant expressing OVA peptide (C. Kamper- schroer, unpublished results). Furthermore, a SAP-dependent mutant that cannot recruit the kinase Fyn restores B cell helper activity of CD4 T cells without restoring their ability to produce IL-4, IL-10, or IL-13 (11). Whether other SAP-dependent cytokines play a role awaits further study.

Another possibility is that surface molecules on CD4 T cells are affected by SAP signaling. Activated SAP KO CD4 T cells have lower ICOS expression (11, 39) and higher and sustained CD40L expression (11) (C. Kamper- schroer, unpublished results) when compared with similarly activated WT CD4 T cells. Furthermore, these differences in ICOS and CD40L expression correlate with B cell helper defects, but not with Th2 cytokine defects (11). Although the expression pattern of CD40L is the opposite of what we expected, it is an attractive candidate given the similarity of the phenotype in SAP KO mice with the phenotype of CD40L KO mice (40, 41), and the possibility that too much available CD40L can be detrimental (42). It will be interesting to determine whether dysregulated ICOS and/or CD40L expression is responsible for B cell defects in SAP KO mice, and whether other helper molecules require SAP to exert their helper functions.

Given the Ab defect in SAP KO mice, we were interested to determine whether SAP KO mice are more susceptible to infection. One report found increased mortality of SAP KO mice during chronic LCMV infection (8), but whether this was related to the Ab defect was not investigated. Our data show that SAP is not required for control of primary influenza infection, which is consistent with another report showing that mice deficient in T cell help due to CD40 deficiency or MHC class II deficiency control primary influenza infection as WT mice do (32). Despite control of primary infection, our data demonstrate that SAP is required for preventing secondary high dose influenza infection, and that IgG in serum from WT, but not SAP KO mice can protect mice against
lethal infection. To either, these data strongly suggest that SAP KO mice do not control secondary influenza infection because they cannot generate sufficient amounts of circulating anti-influenza IgG Ab. Therefore, at least under certain circumstances, the Ab defect in SAP KO mice appears to be functionally important for immune control of infection and may at least partly explain the reports of increased susceptibility of XLP patients to infection (4).

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Disclosures

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