Immunity, Vol. 21, 707–717, November, 2004, Copyright ©2004 by Cell Press

Genetic Evidence Linking SAP, the X-Linked Lymphoproliferative Gene Product, to Src-Related Kinase FynT in T_H2 Cytokine Regulation

Dominique Davidson,[1] Xiaochu Shi,[1] Shaohua Zhang,[1] Hao Wang,[2] Mona Nemer,[2,4,5] Nobuyuki Ono,[6] Shinji Ohno,[6] Yusuke Yanagi,[6] and André Veillette[1,3,4,5,*]
[1]Laboratory of Molecular Oncology
[2]Laboratory of Cardiac Growth and Differentiation
Clinical Research Institute of Montreal
Montréal, Québec H2W 1R7
Canada
[3]Departments of Microbiology and Immunology
[4]Department of Medicine
McGill University
Montréal, Québec H3G 1Y6
Canada
[5]Department of Medicine
University of Montréal
Montréal, Québec H3C 3J7
Canada
[6]Department of Virology
Faculty of Medicine
Kyushu University
Fukuoka 812-8582
Japan

Summary

SAP is an adaptor mutated in X-linked lymphoproliferative disease. It plays a critical role in T helper 2 (T_H2) cytokine production. This function was suggested to reflect the capacity of SAP to associate with SLAM family receptors and enable tyrosine phosphorylation signaling by these receptors through SAP-mediated recruitment of Src-related kinase FynT. Here, we addressed by genetic means the importance of the SAP-FynT interaction in normal T cell functions. By creating a mouse in which the FynT binding site of SAP was inactivated in the germ line (*sap*^{R78A} mouse) and by analyzing mice lacking SAP, FynT or SLAM, evidence was obtained that the SAP-FynT cascade is indeed crucial for normal T_H2 functions in vitro and in vivo. These data imply that SAP is necessary for T_H2 cytokine regulation primarily as a result of its capacity to recruit FynT. They also establish a previously unappreciated role for FynT in SAP-dependent T_H2 cytokine regulation.

Introduction

SAP (also named SH2D1A) is composed of an Src homology 2 (SH2) domain and a short carboxyl-terminal tail of unknown function (Latour and Veillette, 2003; Engel et al., 2003). It is expressed in T cells, natural killer (NK) cells and, possibly, B cells. The *sap* gene is mutated or deleted in X-linked lymphoproliferation (XLP), a fatal human immune dysfunction characterized by a deregulated response to Epstein-Barr virus (EBV) infection, hypogammaglobulinemia and malignant lymphomas. Whereas the precise pathophysiology of XLP has been difficult to establish, studies using *sap*$^-$ mice revealed that SAP deficiency leads to reduced CD4$^+$ T cell helper 2 (T_H2) functions in vitro, in particular T cell antigen receptor (TCR)-induced IL-4 and IL-13 production (Wu et al., 2001; Crotty et al., 2003; Czar et al., 2001; Yin et al., 2003). These defects result in immunoglobulin (Ig) deficiency and a reduction of memory B cells. Increased T_H1 function and decreased CD8$^+$ T cell-dependent antiviral responses were also identified in *sap*$^-$ animals.

Through its SH2 domain, SAP associates with tyrosines in the cytoplasmic domain of signaling lymphocyte activation molecule (SLAM) family receptors (Veillette and Latour, 2003; Engel et al., 2003; Veillette, 2004). This receptor family includes SLAM, 2B4, Ly-9, CD84, NK-T-B antigen (NTB-A)/Ly-108 and CD2-like receptor activating cytotoxic cells (CRACC). Although the function of most of these receptors is not elucidated, characterization of *slam*$^{-/-}$ mice showed that, like SAP, SLAM promotes IL-4 production by activated T cells (Wang et al., 2004).

We previously proposed that SAP is critical for SLAM-like receptor functions as a result of its capacity to enable tyrosine phosphorylation signaling by these receptors (Veillette and Latour, 2003; Engel et al., 2003). This effect correlates with the aptitude of SAP to associate selectively with the Src-related protein tyrosine kinase (PTK) FynT (Latour et al., 2001). Biochemical and crystallographic data demonstrated that SAP interacts with FynT via a second binding surface within the SAP SH2 domain and via the FynT SH3 domain (Latour et al., 2003; Chan et al., 2003). The binding region in SAP is centered on arginine 78 (R78), which is opposite the phosphotyrosine binding fold of the SH2 domain. This feature enables a single SAP molecule to bind SLAM and FynT simultaneously. Such an activity was postulated to be crucial for SAP function as a mutant in which R78 was replaced by alanine was incapable of recruiting FynT and promoting SLAM-like receptor-induced protein tyrosine phosphorylation in a T cell line (Latour et al., 2003; Chen et al., 2004).

Here, we wanted to determine the functional importance of the SAP-FynT interaction during T_H2 cytokine regulation in normal T cells. By analyzing mice lacking SAP, FynT or SLAM, and by engineering mice in which SAP R78 is mutated in the germ line, we obtained firm genetic evidence that the SLAM-SAP-FynT cascade is pivotal in T_H2 cytokine production.

Results

fyn$^{-/-}$ T Cells Have Global TCR-Induced Activation Defects

If FynT were to play a critical and specific role in SAP-mediated functions, one would expect that the T_H2 defects observed in *sap*$^-$ T cells would exist in *fyn*$^{-/-}$ T cells. Along these lines, *fyn*$^{-/-}$ T cells display reduced proliferation and IL-2 secretion in response to anti-CD3 antibodies (anti-CD3) plus phorbol myristate acetate

*Correspondence: veillea@ircm.qc.ca

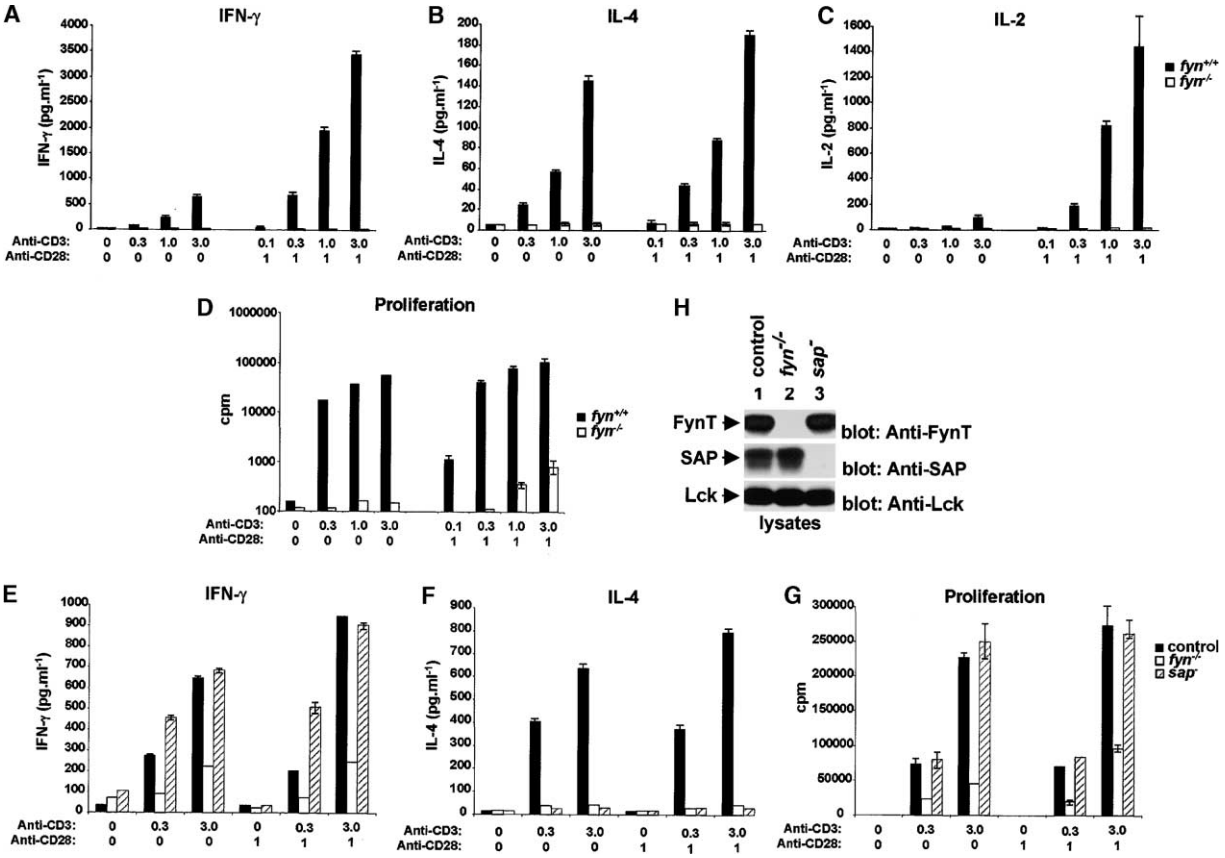

Figure 1. TCR-Mediated Responses in *fyn*$^{-/-}$ and *sap*$^{-}$ T Cells

(A–D) Comparison of *fyn*$^{+/+}$ and *fyn*$^{-/-}$ T cells. Purified CD4^{+} T cells were stimulated with the indicated concentrations of anti-CD3 alone or in combination with anti-CD28. After 48 hr, cytokine production (A–C) was determined by ELISA, whereas proliferation (D) was monitored by measuring tritiated thymidine incorporation. Assays were done in duplicate and repeated at least five times. Error bars are shown.

(E–G) Comparison of *fyn*$^{-/-}$ and *sap*$^{-}$ T cells. CD4^{+} T cells were stimulated with anti-CD3 with or without anti-CD28, in the presence of exogenous IL-2 (100 U ml^{-1}). Cytokine production (E and F) and proliferation (G) were assessed as for Figures 1A–1D.

(H) Expression of FynT and SAP. Purified CD4^{+} T cells were lysed, and the abundance of FynT (top), SAP (middle), and Lck (bottom) was ascertained by immunoblotting total cellular proteins with anti-FynT, anti-SAP and anti-Lck, respectively.

(PMA) (Appleby et al., 1992; Stein et al., 1992). Unfortunately, few additional analyses have been performed on *fyn*$^{-/-}$ T cells. In the light of this, the impact of FynT deficiency on TCR-mediated responses was reexamined (Figure 1).

CD4^{+} T cells from *fyn*$^{-/-}$ and *fyn*$^{+/+}$ mice were stimulated with anti-CD3 alone or in combination with anti-CD28. After 48 hr, cytokine production (Figures 1A–1C) and proliferation (Figure 1D) were determined. Compared to wild-type T cells, *fyn*$^{-/-}$ T cells had pronounced defects in TCR-initiated proliferation (Figure 1D), and production of IFN-γ (Figure 1A), IL-4 (Figure 1B), and IL-2 (Figure 1C). Some of these responses, in particular IFN-γ secretion and proliferation, were partially corrected by addition of exogenous IL-2 (Figures 1E–1G; Supplemental Table S1, available online at http://www.immunity.com/cgi/content/full/21/5/707/DC1/). However, the block in IL-4 was not relieved (Figure 1F).

These defects were compared to those seen in *sap*$^{-}$ T cells (Figures 1E–1G). In agreement with earlier reports (Wu et al., 2001; Czar et al., 2001), CD4^{+} T cells from *sap*$^{-}$ mice demonstrated a defect in IL-4 production (Figure 1F). As was the case for *fyn*$^{-/-}$ T cells, this deficit was not rectified by addition of IL-2 (data not shown). However, contrary to *fyn*$^{-/-}$ T cells, no reduction of IFN-γ production (Figure 1E) or T cell proliferation (Figure 1G) was seen in *sap*$^{-}$ T cells. In some experiments, a small increase in IFN-γ release was actually observed (Figure 1E). Thus, FynT deficiency yielded pronounced activation defects involving T_H2 and, to a lesser extent, T_H1 cytokines, whereas SAP deficiency led to a more selective deficit implicating T_H2 cytokines.

Generation of *sap*^{R78A} Knockin Mice

While these data were consistent with the idea that SAP is utilizing FynT for T_H2 priming, this interpretation was complicated by the fact that the defects in *fyn*$^{-/-}$ T cells were more severe than those in *sap*$^{-}$ T cells. To address more clearly the role of FynT in SAP function, we created a mouse in which the FynT binding site of SAP, R78, was replaced by an alanine in the germ line. This knockin mutation was expected to preserve the capacity of SAP to associate with SLAM receptors, while eliminating its ability to recruit FynT. After transfection of embryonic stem (ES) cells with the DNA construct depicted in Figure 2A, ES cells showing evidence of homologous recombination were identified. Mice carrying the *sap*^{R78A} mutation were obtained as detailed in the Experimental Proce-

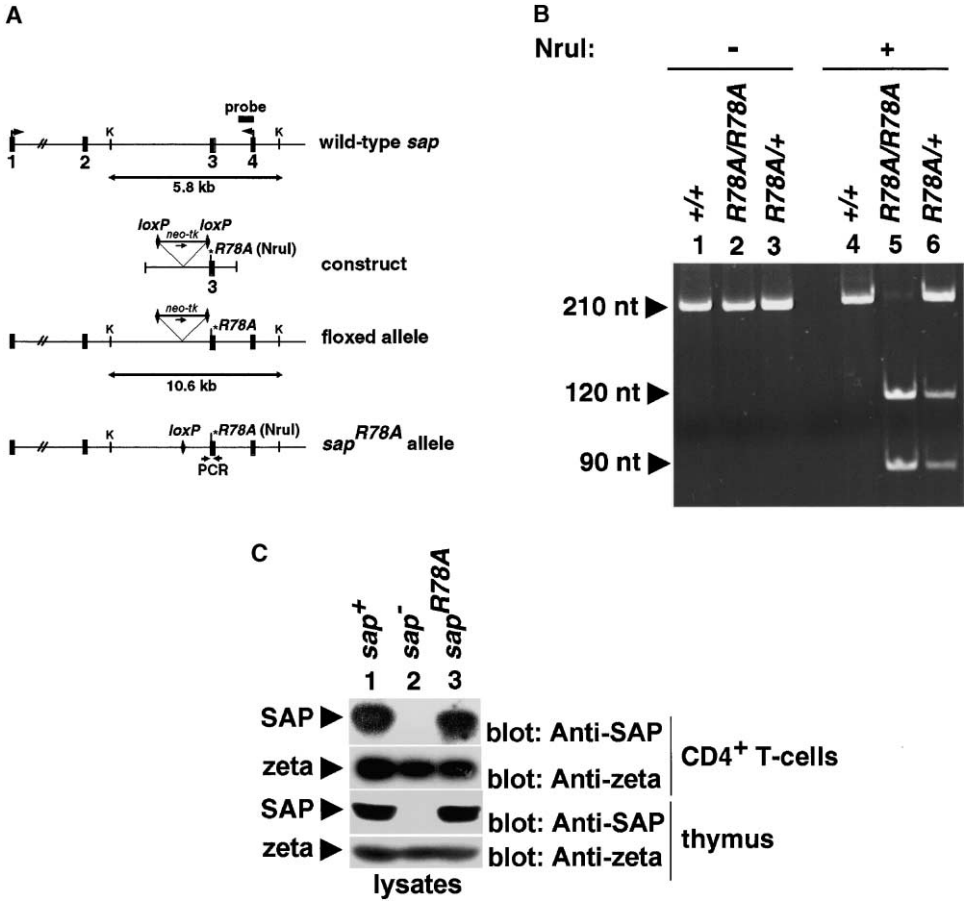

Figure 2. Generation of sap^{R78A} Mice

(A) Genomic structure of mouse *sap* gene and construct. The wild-type *sap* gene is shown at the top. The four exons are indicated. The first exon contains the initiating ATG (right-sided arrow), whereas the fourth exon bears the stop codon (left-sided arrow). The R78 codon is in exon 3. The positions of the KpnI (K) sites and of the probe used for screening recombinants by Southern blotting are also indicated. The construct used for homologous recombination (second from the top) contains a *neo-tk* cassette flanked by two *loxP* sites. The left arm is 2.2 kilobases (kb) long, whereas the right arm, which contains exon 3 where the R78 codon is mutated to an alanine codon, is 2.9 kb. Introduction of the *R78A* mutation creates an NruI site. The floxed and Cre recombinase-deleted sap^{R78A} alleles are depicted as the two lower schematics. The positions of the oligonucleotides used for PCR-based screening are shown at the bottom.

(B) PCR screening for the sap^{R78A} mutation. Genomic DNA was prepared from mouse tail and amplified by PCR using the oligonucleotides depicted in Figure 2A. The DNA was then digested or not with NruI and the products were resolved in polyacrylamide gels. In the absence of NruI, a 210 nucleotide product exists for both wild-type (sap^{+}) and sap^{R78A} DNA. In the presence of NruI, wild-type DNA migrates as a single 210 nucleotide fragment, while sap^{R78A} DNA resolves as two products of 120 and 90 nucleotides. DNAs from three female mice ($sap^{+/+}$, $sap^{R78A/+}$ and $sap^{R78A/R78A}$) were analyzed in this experiment.

(C) Levels of SAP protein. The abundance of SAP was studied by immunoblotting of total cell proteins with anti-SAP (first and third panels). Immunoblots were reprobed with anti-ζ to confirm equal loading (second and fourth panels).

dures. The sap^{R78A} mutation was easily recognized by digestion of PCR-amplified genomic DNA with NruI (Figure 2B). This mutation had no effect on the abundance of SAP (Figure 2C).

As the *sap* gene is on the X chromosome, biochemical and functional analyses were performed using hemizygous sap^{R78A} males or homozygous $sap^{R78A/R78A}$ females. The two types of mice (termed sap^{R78A}) showed normal T cell development and unaltered proportions of T cells in peripheral lymphoid organs. Moreover, they displayed the same alterations in mature T cell functions (see below).

SAP R78A Binds to SLAM, but Is Unable to Mediate SLAM Signaling and T_H2 Cytokine Regulation

The effect of the R78A mutation on the ability of SAP to bind SLAM-like receptors and recruit FynT was examined. Since SLAM seems to be the predominant SLAM family member involved in IL-4 regulation (Wang et al., 2004; Veillette, 2004), we focused our attention on this receptor. To study the ability of SAP R78A to associate with SLAM, SLAM was immunoprecipitated from thymocytes using anti-SLAM mAb 12F12, and immunoprecipitates were probed by immunoblotting with anti-SAP (Figure 3A, first panel). SAP R78A (lane 2) was able to associate with SLAM to the same extent as wild-type SAP (lane 1), supporting the idea that R78 is not a critical component of the phosphotyrosine binding fold of the SAP SH2 domain (Chan et al., 2003). Since the SAP SH2 domain has the peculiarity of interacting with SLAM in a phosphotyrosine-independent manner (Sayos et al., 1998), the SLAM-SAP complex formed even in the absence of SLAM tyrosine phosphorylation (Figure 3B) (Li et al., 1999; Latour et al., 2001).

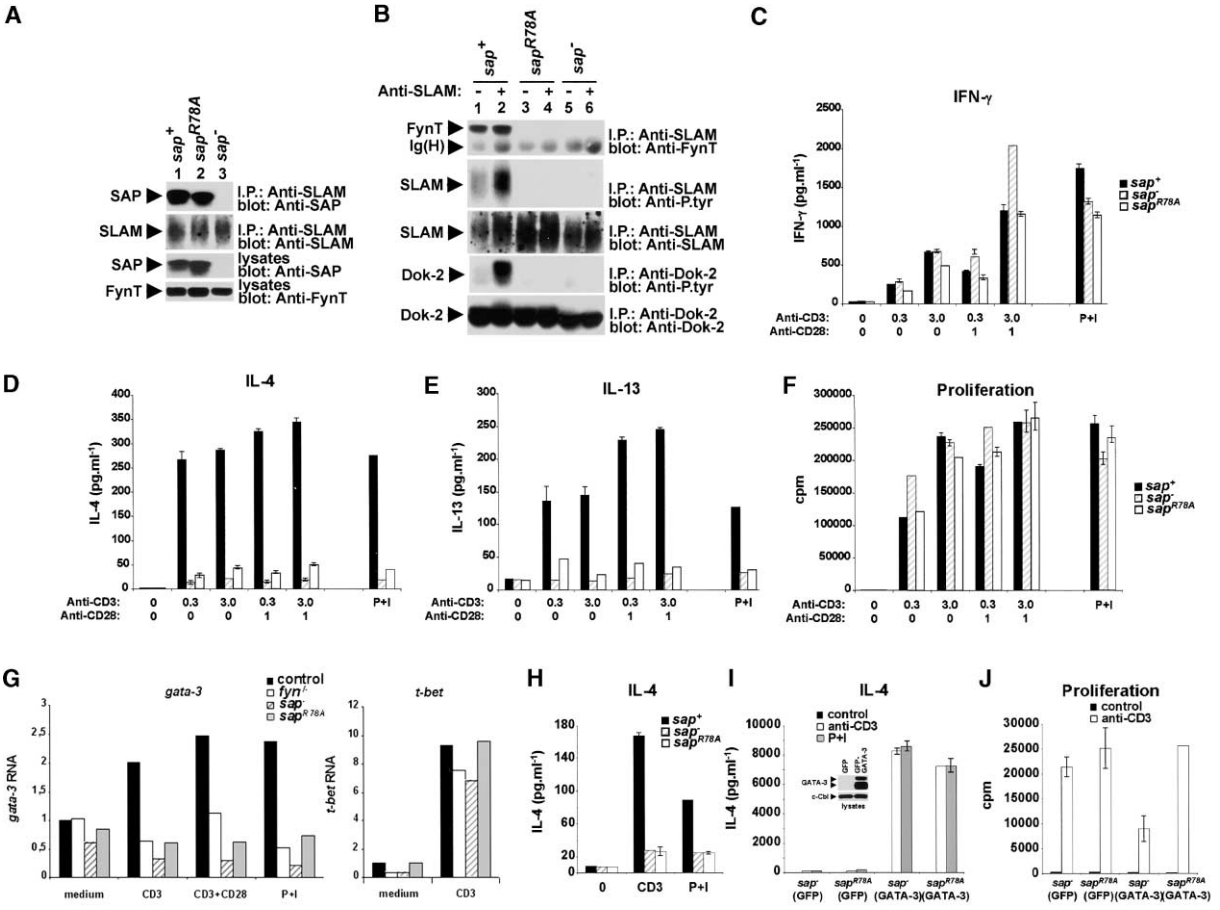

Figure 3. Biochemical and Functional Impact of *sap*^{R78A} Mutation

(A) Ability of SAP R78A to associate with SLAM. Thymocytes were lysed, and SLAM was immunoprecipitated using mAb 12F12 (first and second panels). The presence of associated SAP was revealed by immunoblotting with anti-SAP (first panel). The presence of SLAM was confirmed by reprobing with a rabbit anti-SLAM serum (second panel). The abundance of SAP (third panel) and FynT (fourth panel) was assessed by immunoblotting cell lysates with anti-SAP and anti-FynT, respectively.

(B) Ability of SAP R78A to recruit FynT and promote SLAM-dependent protein tyrosine phosphorylation. Thymocytes were stimulated or not for 5 min at 37°C with anti-SLAM and RAR IgG. After SLAM immunoprecipitation, FynT recruitment was ascertained by immunoblotting with anti-FynT (first panel). Tyrosine phosphorylation of SLAM was assessed by immunoblotting with anti-phosphotyrosine (P.tyr) (second panel). The presence of SLAM was confirmed by reprobing with anti-SLAM (third panel). Tyrosine phosphorylation of Dok-2 was ascertained by probing Dok-2 immunoprecipitates with anti-P.tyr (fourth panel), while the abundance of Dok-2 was verified by reprobing with anti-Dok-2 (fifth panel). Ig(H): heavy chain of Ig.

(C–F) T cell responses in *sap*^{R78A} mice. CD4$^+$ T cells were activated with the indicated concentrations of anti-CD3 or anti-CD3 plus anti-CD28 in the presence of IL-2, or with PMA and ionomycin (P+I). Cytokine secretion (C–E) and proliferation (F) were determined as outlined in the legend of Figure 1.

(G) *gata-3* and *t-bet* RNA induction. CD4$^+$ T cells were stimulated for 48 hr with anti-CD3 (3 μg ml^{-1}), anti-CD3 (3 μg ml^{-1}) plus anti-CD28 (1 μg ml^{-1}), or PMA plus ionomycin. All stimulations were done in the presence of IL-2. After RNA isolation, the abundance of *gata-3* and *t-bet* transcripts was assessed by real-time PCR.

(H–J) Retroviral rescue of IL-4 defect by GATA-3. (H) The IL-4 defect seen in *sap* mutant T cells is not affected by the retroviral infection protocol. CD4$^+$ T cells were subjected to a mock retroviral transduction. Once the protocol was completed, cells were stimulated with anti-CD3 or PMA plus ionomycin, and IL-4 production was measured by ELISA. (I and J) Rescue of IL-4 defect by GATA-3. Sorted cells expressing GFP alone or in combination with GATA-3 were stimulated for 48 hr with anti-CD3 or PMA plus ionomycin. IL-4 production (I) was monitored by ELISA, while proliferation (J) was assessed by measuring thymidine incorporation. The expression of GATA-3 (inset in [I]) was determined by immunoblotting of cell lysates with anti-GATA-3 (top). Equal loading was confirmed by immunoblotting with anti-c-Cbl (bottom). Note that the levels of IL-4 induced after retroviral transduction of GATA-3 in *sap* mutant T cells were much higher than those observed in nontransduced wild-type T cells (seen in Figure 3H). This is presumably because the levels of GATA-3 induced by retroviral transduction of GATA-3 were much higher, in comparison to those reached in non-transduced activated wild-type cells.

Next, the ability of SAP R78A to couple SLAM to FynT was examined (Figure 3B). Thymocytes were stimulated or not with anti-SLAM, and the ability of SLAM to interact with FynT was ascertained by immunoblotting of anti-SLAM immunoprecipitates with anti-FynT (first panel). Like SAP deficiency (lanes 5 and 6), mutation of R78 (lanes 3 and 4) abolished the capacity of SLAM to associate with FynT. The impact of the R78A mutation on SLAM-induced tyrosine phosphorylation events was also tested (second panel). The R78A mutation elimi-

nated both baseline (lane 3) and anti-SLAM-induced (lane 4) tyrosine phosphorylation of SLAM. A similar defect was seen in *sap*$^-$ T cells (lanes 5 and 6) (Chan et al., 2003). Likewise, the R78A substitution (fourth panel, lanes 3 and 4) eliminated the ability of anti-SLAM to stimulate tyrosine phosphorylation of the adaptor Dok-2, a downstream target of SLAM signaling (Latour et al., 2001, 2003). Thus, the R78A mutation preserved the ability of SAP to interact physically with SLAM, while eliminating its capacity to recruit FynT and promote SLAM-induced protein tyrosine phosphorylation.

Then, we assessed whether SAP R78A was able to promote T_H2 cytokine production (Figures 3C–3F). CD4$^+$ T cells from *sap*^{R78A}, *sap*$^-$, and *sap*$^+$ mice were triggered with anti-CD3, anti-CD3 and anti-CD28, or PMA plus ionomycin, and the production of cytokines (IFN-γ, IL-4, and IL-13), as well as T cell proliferation, was determined. *sap*^{R78A} T cells exhibited no reduction of IFN-γ production (Figure 3C) or proliferation (Figure 3F). However, like *sap*$^-$ T cells, they demonstrated a markedly reduced production of IL-4 (Figure 3D) and IL-13 (Figure 3E), in response to anti-CD3 or anti-CD3 plus anti-CD28. Surprisingly, defects in IL-4 and IL-13 were also seen in cells stimulated with PMA plus ionomycin (Figures 3D and 3E). Similarly defective responses to PMA plus ionomycin were noted in *fyn*$^{-/-}$ T cells (Supplemental Figure S1 available at above URL).

While the R78A mutation compromised the ability of SAP to enhance T_H2 cytokine secretion, slightly greater amounts of IL-4 (Figure 3D) and IL-13 (Figure 3E) were consistently released by activated *sap*^{R78A} T cells, in comparison to *sap*$^-$ T cells. As will be discussed, this finding suggests that a minor, possibly FynT-independent mechanism contributes to the capacity of SAP to regulate T_H2 cytokines.

Defects in *gata-3* Induction Caused by Mutations in the SAP-FynT Pathway

Transcription factor GATA-3 is critical for IL-4 and IL-13 production by activated T cells (Murphy and Reiner, 2002; Grogan and Locksley, 2002). To assess whether defects in the SAP-FynT pathway influenced GATA-3 induction, CD4$^+$ T cells from wild-type, *sap*$^-$, *sap*^{R78A}, and *fyn*$^{-/-}$ mice were stimulated with anti-CD3, anti-CD3 and anti-CD28, or PMA plus ionomycin, and the presence of *gata-3* RNA was monitored by real-time PCR (Figure 3G). *gata-3* RNA was induced in wild-type T cells treated with any of these activating stimuli. However, this response was nearly absent in *sap*$^-$, *sap*^{R78A}, and *fyn*$^{-/-}$ T cells. Such a result was observed in three experiments (data not shown). In comparison, levels of *t-bet*, which is implicated in T_H1 regulation (Murphy and Reiner, 2002; Grogan and Locksley, 2002), were maintained.

To ascertain whether the *gata-3* defect might contribute to the IL-4 deficit in *sap*$^-$ and *sap*^{R78A} T cells, a retroviral rescue experiment was performed (Figures 3H–3J). CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 and IL-2, in the presence of anti-IL-4 and anti-IFN-γ to prevent polarization. After 36 hr, cells were infected with retroviruses encoding green fluorescent protein (GFP) alone or in combination with GATA-3. After an additional 48 hr, GFP-positive cells were sorted and propagated for another 48 hour period with IL-2. In the absence of retroviral transduction, these manipulations did not alter the IL-4 defect in *sap*$^-$ and *sap*^{R78A} T cells (Figure 3H). However, after retroviral transduction (Figure 3I), expression of GATA-3, but not GFP alone, induced prominent IL-4 secretion in *sap*$^-$ and *sap*^{R78A} T cells. This difference was not caused by an increase in cell proliferation (Figure 3J; data not shown). Therefore, decreased *gata-3* induction may be involved in the T_H2 defect seen in *sap* mutant T cells.

Defect in IgE Production in *sap*^{R78A} Mice

sap$^-$ mice have low levels of serum IgE (Wu et al., 2001; Czar et al., 2001). While the basis of this deficit is not clearly established, it may result at least in part from the reduced production of IL-4 and IL-13 by CD4$^+$ T cells. These cytokines are critical for T-cell-dependent B cell maturation and production of antibodies, in particular IgE (Nelms et al., 1999; Finkelman et al., 1990). This notion was also supported by the finding that, in a viral infection model, adoptive transfer of *sap*$^-$ CD4$^+$ T cells conferred antibody production defects in a reconstituted host (Crotty et al., 2003).

Given these observations, we ascertained the relevance of the SAP-FynT interaction in vivo by measuring IgE levels in *sap*^{R78A}, *sap*$^-$, and *sap*$^+$ mice (Figures 4A–4C). *sap*$^+$ mice demonstrated appreciable IgE production, especially after immunization with ovalbumin (OVA) (Figures 4A–4C). This response was maximal after 14 days, and was promptly reinduced following a boost immunization (Figures 4A and 4B). By comparison, *sap*$^-$ animals had little or no IgE, even after boost immunization. A severe IgE deficit was also observed in *sap*^{R78A} mice, although, in keeping with the results of IL-4 and IL-13 production, IgE levels were slightly higher than those in *sap*$^-$ mice. These results were reproduced in three experiments and with at least seven mice in each group (data not shown). Contrary to the reduction of IgE, the abundance of IgG_1 (Figure 4D) was normal or minimally affected in *sap*$^-$ and *sap*^{R78A} mice, implying that the SAP-dependent Ig deficit did not affect all Ig subclasses. This may relate to the observation that IL-4 is more critical for IgE than IgG_1 synthesis in vivo (Kopf et al., 1993; Kuhn et al., 1991).

IgE levels were also examined in *fyn*$^{-/-}$ mice (Figure 4C). Like *sap*$^-$ mice and *sap*^{R78A} mice, mice devoid of FynT had dramatically reduced levels of IgE. However, IgG_1 levels were essentially normal (Figure 4D). While the interpretation of the IgE defect in *fyn*$^{-/-}$ mice is complicated by the fact that these animals have global T cell signaling defects, this finding supported the idea that SAP is linked to FynT in the regulation of IgE production in vivo.

Functional Deficits in Memory-Type and Naive CD4$^+$ T Cells Contribute to the T_H2 Defect Caused by Mutations in the SAP-FynT Pathway

The observation that the IL-4 and IL-13 defects in T cells from *sap*$^-$, *sap*^{R78A}, and *fyn*$^{-/-}$ mice were seen in cells stimulated with PMA plus ionomycin raised two possibilities. First, SAP and SAP-associated FynT may participate in distal T cell signaling events, occurring after the induction of overall protein tyrosine phosphorylation by

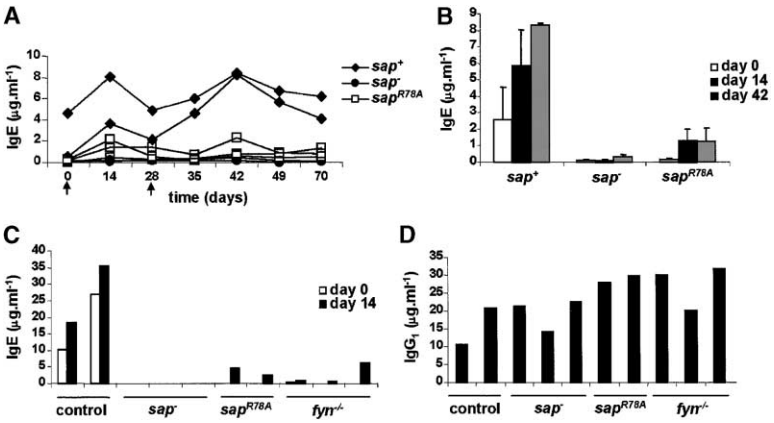

Figure 4. In Vivo Antibody Production in sap^{R78A} and $fyn^{-/-}$ Mice

(A) IgE levels in sap^{R78A} and sap^{-} mice. Mice were immunized with ovalbumin and complete Freund adjuvant (arrow on the left). A boost with ovalbumin and incomplete Freund adjuvant was given at day 28 (arrow on the right). At the indicated times after immunization, serum was obtained and total IgE was determined by ELISA. "Day 0" corresponds to nonimmunized animals. The results shown are for individual mice. This experiment was repeated three times (data not shown; see Figure 4C for results of another experiment).

(B) Average values of serum IgE. The average value of total serum IgE for each group of mice in the experiment of Figure 4A was determined at the indicated times. Standard deviations are shown.

(C) IgE levels in sap^{R78A}, sap^{-} and $fyn^{-/-}$ mice. Mice were immunized as detailed for Figure 4A. Serum IgE was measured prior (day 0) and after (day 14) immunization. Results for individual mice are shown.

(D) IgG_1 levels in sap^{R78A}, sap^{-} and $fyn^{-/-}$ mice. Ovalbumin-specific serum IgG_1 was measured in the mice from the experiment depicted in Figure 4C. Only the values observed after immunization (day 14) are shown. No ovalbumin-specific IgG_1 was detected prior to immunization (data not shown).

TCR stimulation and after activation of the presumed targets of PMA plus ionomycin, i.e., protein kinase Cs (PKCs) and calcium fluxes (Weiss and Littman, 1994; van Leeuwen and Samelson, 1999; Latour and Veillette, 2001). Second, SAP and SAP-associated FynT may be needed for the development and/or maintenance of one or more T cell subpopulation(s) involved in T_H2 priming.

To address these issues, we first determined the relative production of IL-4 by naive ($CD44^{lo}CD62L^{hi}$) and memory-type ($CD44^{hi}CD62L^{lo}$) T cells. The $CD4^+$ T cells used in our experiments contained conventional naive and memory-type T cells, and a small amount (1%–2%) of $NK1.1^+CD3^+$ natural killer (NK)-T cells (data not shown) (Woodland and Dutton, 2003; Brigl and Brenner, 2004). Hence, cells depleted of NK-T cells were first obtained, and then subjected or not to removal of memory-type cells using anti-CD44 (Figure 5A). Staining with anti-CD44 showed that, in the absence of depleting anti-CD44, approximately 10% of cells were $CD44^{hi}$ memory-type T cells. However, when anti-CD44 was added to the depletion cocktail, no $CD44^{hi}$ T cells remained. The two cell preparations were then stimulated with anti-CD3, anti-CD3 and anti-CD28, or PMA plus ionomycin, and IL-4 secretion was determined. Depletion of memory T cells markedly reduced IL-4 production by $CD4^+$ T cells. The remaining cells, i.e., naive $CD4^+$ T cells, produced only 2%–5% of the IL-4 released in preparations containing memory-type T cells.

To examine if mutations in the SAP-FynT pathway reduced T_H2 cytokine production by affecting the presence of memory-type T cells, NK-T-cell-depleted $CD4^+$ T cells from the various mice were stained with anti-CD44 and anti-CD62L (Figure 5B). There was no obvious difference in the abundance of memory-type ($CD44^{hi}CD62L^{lo}$) T cells between wild-type, sap^{-}, sap^{R78A}, and $fyn^{-/-}$ mice (left-side panels). Nevertheless, an IL-4 defect was clearly seen in NK-T-cell-depleted cells from sap^{-}, sap^{R78A}, and $fyn^{-/-}$ mice (right-side panel). Therefore, a functional deficit, rather than the absence, of memory-type $CD4^+$ T cells seemed involved in the T_H2 dysfunctions caused by mutations in the SAP-FynT pathway.

We also ascertained whether the IL-4 defect was present in naive $CD4^+$ T cells (Figure 5C). Naive cells were purified by negative selection as detailed above. Greater than 99.5% of cells obtained were $CD44^{lo}CD62L^{hi}$ (left-side panels). Cells were then stimulated for 72 hr with anti-CD3 or PMA plus ionomycin, and IL-4 secretion was monitored (right-side panel). Although the production of IL-4 by naive T cells was much lower than that observed in memory-type T cells, an IL-4 deficit clearly existed in cells from sap^{-}, sap^{R78A}, and $fyn^{-/-}$ mice. Thus, the T_H2 defect of *sap* and *fyn* mutant T cells was found not only in memory-type T cells, but also in naive T cells.

Lack of SLAM Expression Reproduces the $CD4^+$ T Cell Defects Induced by Mutations in the SAP-FynT Pathway

Considering the report that $slam^{-/-}$ T cells displayed an IL-4 defect in response to anti-CD3 (Wang et al., 2004), it seemed reasonable to propose that SLAM, a self-associating receptor, might be the predominant trigger of the SAP-FynT pathway during T_H2 priming. To develop this further, we first tested the impact of anti-CD3 or PMA plus ionomycin on the expression of SLAM using purified splenic $CD4^+$ T cells (Figure 6A). As reported (Castro et al., 1999; Howie et al., 2002), SLAM expression was low on resting mature T cells. This was in contrast to thymocytes, which expressed high levels of SLAM (Figure 6C) (Castro et al., 1999). SLAM expression was strongly induced on splenic T cells within 12 hr of anti-CD3 stimulation (left panel). This upregulation was observed on ~100% of the cells, implying that it affected naive and memory-type T cells. Interestingly, expression of SLAM was also rapidly induced by PMA plus ionomycin (middle panel). With either stimulation, the increased SLAM expression persisted for at least 48 hr (right panel). Contrary to SLAM, expression of SAP (Figure 6B) was high in resting $CD4^+$ T cells (lane 1). Moreover, it was reduced by treatment with anti-CD3 (lanes 2, 4, and 6) or PMA plus ionomycin (lanes 3, 5, and 7), although some expression was maintained throughout the course of the stimulation. No change in FynT expression was seen under these conditions (data not shown).

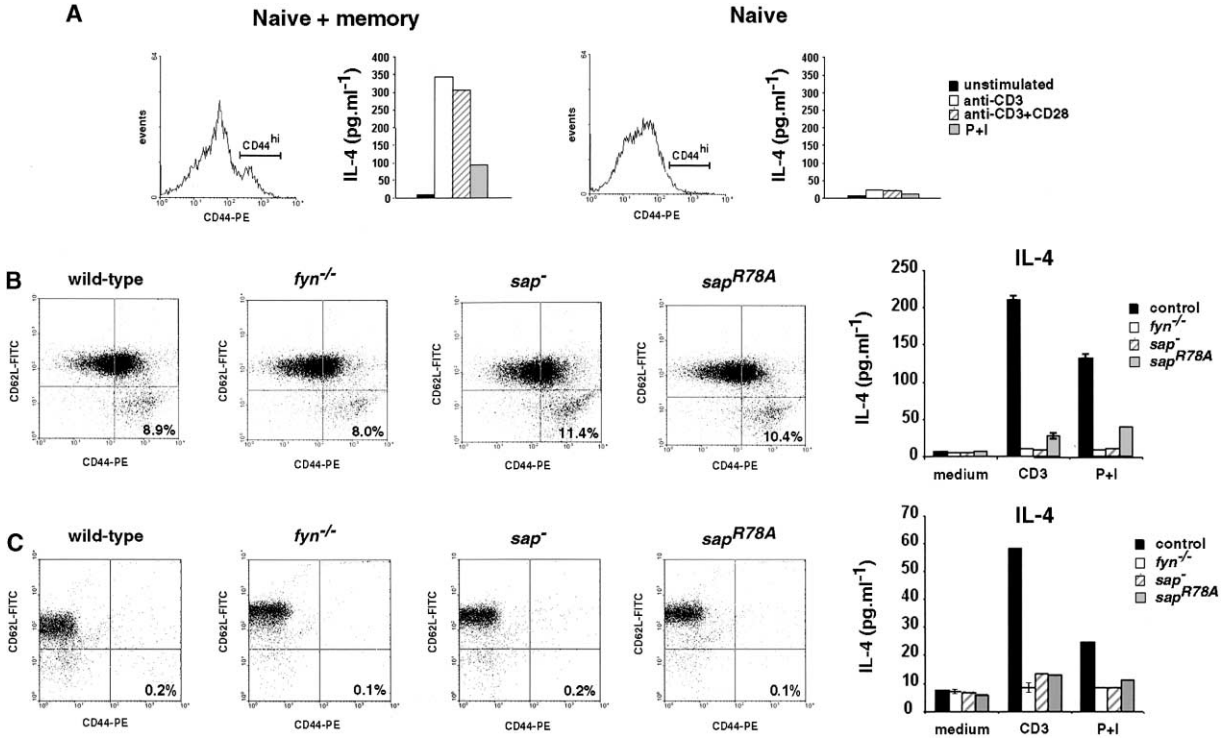

Figure 5. Characterization of T Cells Responsible for SAP-FynT-Dependent T_H2 Cytokine Regulation

(A) Differential T cell depletion. NK-T cell-depleted $CD4^+$ T cells were purified from the spleen of C57BL/6 mice in the absence (first and second panels) or the presence (third and fourth panels) of depleting anti-CD44 to remove memory-type T cells. Anti-CD49b, which removes NK cells, was also added during all purifications. Purified cells were stained with anti-CD44 and analyzed by flow cytometry (first and third panels). IL-4 production was determined by ELISA (second and fourth panels). Anti-CD3 was used at a concentration of 3 μg ml^{-1}, and all stimulations were done in the presence of IL-2.

(B) Detection and function of memory-type $CD4^+$ T cells. NK-T cell-depleted $CD4^+$ T cells were purified using the standard purification cocktail supplemented with anti-NK1.1 and anti-CD49b. Purified cells were stained with anti-CD44 and anti-CD62L to detect memory-type ($CD44^{hi}CD62L^{lo}$) and naive ($CD44^{lo}CD62L^{hi}$) $CD4^+$ T cells (first, second, third, and fourth panels from the left). The percentages of memory-type T cells are shown at the bottom right of each dot plot. Production of IL-4 is shown in the last panel on the right. Anti-CD3 was used as detailed for Figure 5A.

(C) Function of naive $CD4^+$ T cells. Naive $CD4^+$ T cells were purified by negative selection using the standard purification cocktail supplemented with anti-CD44 and anti-CD49b. To assess purity, cells were stained with anti-CD44 and anti-CD62L (first, second, third, and fourth panels from the left), as detailed for Figure 5B. Production of IL-4 is shown in the last panel on the right. Anti-CD3 was used as detailed for Figure 5A, except that cells were stimulated for 72 hr.

These results were compatible with the idea that induction of SLAM in response to TCR stimulation or PMA plus ionomycin might be needed for activation of the SAP-FynT pathway and upregulation of T_H2 cytokine production. To confirm this notion, $CD4^+$ T cells were obtained from *slam*$^{-/-}$ mice (Figure 6C) and subjected to several analyses (Figures 6D–6H). First, staining with anti-CD44 and anti-CD62L showed that SLAM deficiency had no impact on the abundance of memory-type ($CD44^{hi}$ $CD62L^{lo}$) T cells (Figure 6D), as documented above for mice with mutations in the SAP-FynT cascade. Second, functional studies demonstrated that lack of SLAM caused pronounced defects in IL-4 (Figure 6E) and IL-13 (Figure 6F) in response to anti-CD3 or PMA plus ionomycin, similar to those caused by mutations in the SAP-FynT pathway. In contrast, little or no effect was seen on T cell proliferation (Figure 6H). Furthermore, whereas a small decrease in IFN-γ release was noted in this assay (Figure 6G), this was not observed in other experiments (data not shown). Similar results were obtained with NK-T-cell-depleted $CD4^+$ T cells (data not shown) or naive $CD4^+$ T cells (Supplemental Figure S2, available online at http://www.immunity.com/cgi/content/full/21/5/707/DC1/). These findings supported the idea that SLAM is the major trigger of the SAP-FynT cascade needed for T_H2 cytokine production in vitro.

Discussion

Herein, we examined the role of FynT in SAP-mediated functions in T cells. We found that the ability of TCR stimulation to trigger T_H1 and T_H2 cytokines in $CD4^+$ T cells, as well as induce IL-2 secretion and proliferation, was markedly compromised in *fyn*$^{-/-}$ T cells. This was presumably because FynT is required in part to couple TCR to the proximal tyrosine phosphorylation cascade in T cells (Appleby et al., 1992; Stein et al., 1992). It is unlikely that SAP is involved in this aspect of FynT function, as TCR-triggered IFN-γ production, IL-2 secretion, and proliferation were not reduced in *sap*$^-$ T cells.

The observation that the T_H2 defect encountered in *sap*$^-$ T cells was also seen in *fyn*$^{-/-}$ T cells supported the

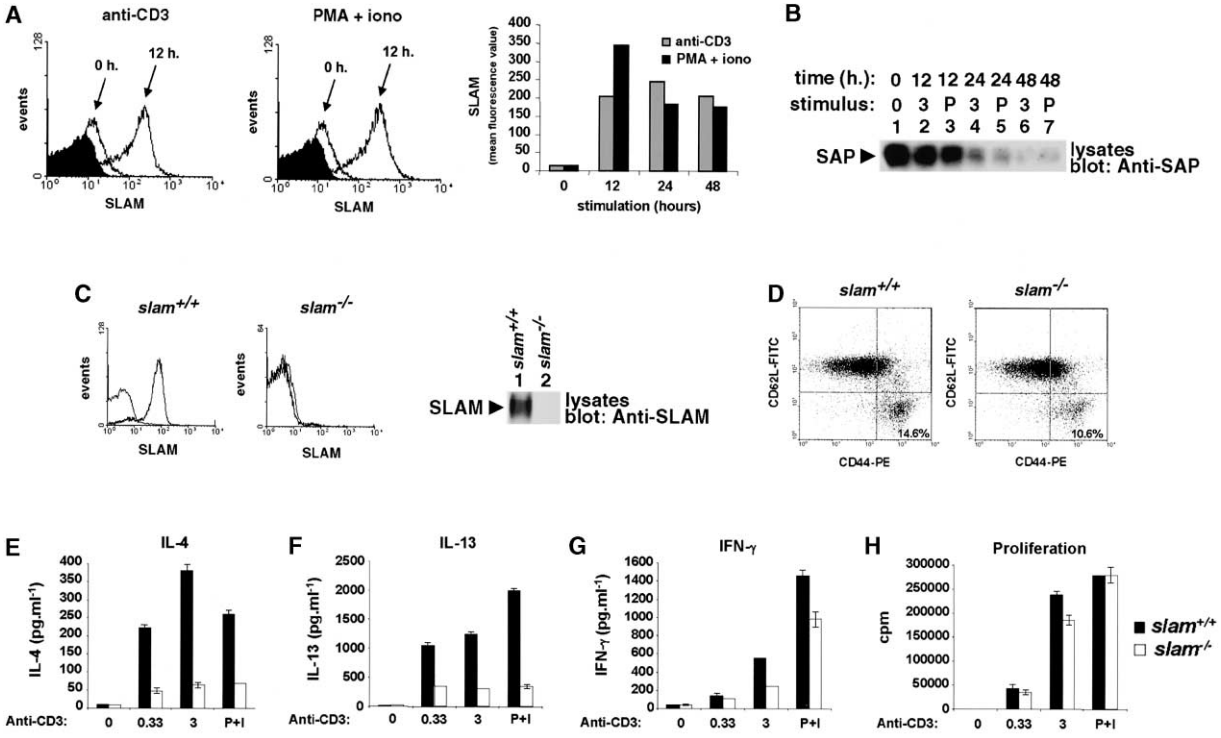

Figure 6. Evidence Supporting the Involvement of SLAM in SAP-FynT-Dependent T_H2 Priming

(A) Induction of SLAM expression. $CD4^+$ T cells from C57BL/6 mice were stimulated for 12 hr with anti-CD3 alone (3 μg ml^{-1}) (left panel) or PMA plus ionomycin (middle panel). SLAM expression was determined by flow cytometry. The expression of SLAM in untreated cells corresponds to "0 h." Nonspecific staining is shown as the filled curve. The impact of longer stimulations (24 hr or 48 hr) is shown in the right panel, where levels of SLAM are represented using the mean fluorescence value.

(B) SAP expression. Cells were stimulated as detailed for Figure 6A. SAP expression was measured by immunoblotting of total cell proteins with anti-SAP. 3: anti-CD3; P: PMA plus ionomycin.

(C–H) Immune defects in *slam*$^{-/-}$ mice. (C) Expression of SLAM. SLAM expression in *slam*$^{+/+}$ and *slam*$^{-/-}$ mice was determined by flow cytometry of thymocytes using anti-SLAM (left and middle panels). Thin line: secondary reagent alone; thick line: anti-SLAM plus secondary reagent. SLAM expression was also assessed by immunoblotting of total cellular proteins from thymus with anti-SLAM (right panel). (D) Detection of memory-type $CD4^+$ T cells in *slam*$^{-/-}$ mice. $CD4^+$ T cells were purified using the standard purification cocktail supplemented with anti-NK1.1 and anti-CD49b. Cells were stained with anti-CD44 and anti-CD62L. The percentages of memory-type T cells are shown at the bottom right of each dot plot. (E–H) $CD4^+$ T cells were stimulated with anti-CD3 alone or PMA plus ionomycin (P+I), in the presence of IL-2. Cytokine production and proliferation were assessed as for Figure 1.

idea that SAP was acting through FynT in T_H2 priming. However, since the deficits in *fyn*$^{-/-}$ T cells were more severe than those in *sap*$^-$ T cells, this interpretation was tenuous. It was conceivable that SAP and FynT were regulating T_H2 cytokines by acting in separate signaling cascades or distinct cell types. Therefore, we created a mouse strain in which the FynT binding site of SAP was mutated in the germ line. The mutation did not affect the levels of SAP or the aptitude of SAP to associate with SLAM, a receptor involved in T_H2 regulation. However, it eliminated the ability of SAP to recruit FynT and to promote SLAM-mediated protein tyrosine phosphorylation.

Our results showed that the SAP R78A substitution replicated the T_H2 defects caused by lack of SAP expression. *sap*^{R78A} $CD4^+$ T cells demonstrated a marked reduction of IL-4 and IL-13 release in response to anti-CD3 or anti-CD3 plus anti-CD28. Moreover, the ability of mice to produce IgE, a response dependent on IL-4 and, to a lesser extent, IL-13, was severely attenuated in *sap*^{R78A} mice. A similar defect was seen in *fyn*$^{-/-}$ mice. Hence, these results provided strong support for the concept that SAP regulates T_H2 cytokines by binding FynT.

Obviously, one cannot formally exclude that the R78A mutation also abrogated another as yet unknown function of SAP. This possibility is unlikely, however, as no other SH3 domain-containing molecule was found to interact with the R78-based motif of SAP (Latour et al., 2003). Furthermore, based on crystallographic studies, mutation of R78 is not expected to alter the capacity of SAP to interact with tyrosine phosphorylated SLAM-like receptors (Chan et al., 2003). This last idea is also supported by our finding that the ability of SAP to associate with SLAM and its relative 2B4 was not compromised by the R78A mutation (Chen et al., 2004; Latour et al., 2003).

Although the ability of SAP to promote T_H2 cytokine and IgE production was markedly reduced by the R78A mutation, a small residual function (~5%–10% of that of wild-type SAP) was observed. It is plausible that this residual activity reflected a remaining capacity of SAP R78A to bind FynT. This seems improbable, though, as R78 is the epicenter of the FynT binding site in SAP (Chan et al., 2003; Latour et al., 2003). Moreover, mutation of R78 abrogated all complex formation between

SAP and FynT, including in vitro binding assays using recombinant FynT SH3 domains (Latour et al., 2003). It appears more likely that SAP R78A utilized an alternative, albeit less efficient, mechanism for T_H2 cytokine regulation.

Among conventional $CD4^+$ T cells, the cells primarily responsible for SAP-FynT-dependent T_H2 cytokine production in vitro were memory-type T cells. A functional, rather than developmental, defect in these cells seemed responsible for most of the compromised T_H2 activity caused by mutations in the SAP-FynT pathway. Although the amount of T_H2 cytokines produced by naive $CD4^+$ T cells was much lower than that seen in memory-type cells, a defect in IL-4 production was also observed in naive cells. Obviously, future studies are needed to assess the contribution of these two cell types to the T cell-dependent abnormalities in B cell functions seen in *sap*$^{-/-}$ mice (Wu et al., 2001; Czar et al., 2001; Crotty et al., 2003). While not studied here, it is also conceivable that NK-T cells participate in SAP-FynT-dependent T_H2 regulation. Defects in NK-T cell development exist in *fyn*$^{-/-}$ mice and, possibly, *sap*$^{-/-}$ mice (Gadue et al., 1999; Eberl et al., 1999). However, we recently found that there is little correlation between the abundance of NK-T cells and T_H2 cytokine production in mice with mutations in the SAP-FynT cascade (our unpublished data).

The finding that *slam*$^{-/-}$ $CD4^+$ T cells exhibited T_H2 defects implied that altered SLAM function could explain the T_H2 cytokine dysfunction caused by mutations in the SAP-FynT pathway (Wang et al., 2004) (this report). This notion is in agreement with the finding that SLAM expression was strongly and rapidly induced on $CD4^+$ T cells treated with anti-CD3 or PMA plus ionomycin. Since SLAM is a self-ligand, this induction would presumably recruit SAP to the membrane, recruit and activate FynT in the vicinity of SLAM, and promote T_H2 cytokine production (Figure 7). In the absence of SLAM, SAP, or FynT, or when SAP is unable to bind FynT, this cascade would be defective, leading to the common T_H2 defect described herein.

Interestingly, we found that SLAM induction by PMA plus ionomycin was caused by ionomycin rather than PMA (Supplemental Figure S3A). This implied that SLAM expression in T cells may be triggered by the calcium-calcineurin pathway instead of the protein kinase C pathway. This notion is consistent with the observation that SLAM was also induced by thapsigargin, an agent causing an influx of calcium through the plasma membrane by depleting endoplasmic reticulum stores (Supplemental Figure S3B). Moreover, it is supported by the finding that SLAM induction by anti-CD3 or ionomycin was blocked by cyclosporin A, a calcineurin inhibitor (Supplemental Figure S3C). Since the induction of SLAM expression on activated T cells occurs at the transcriptional level (Cocks et al., 1995; Wang et al., 2001), it is likely that one or more transcription factors activated by the calcium-calcineurin pathway, such as nuclear factor of activated T cells (NFAT) and myocyte enhancer factor (MEF)-2 (Kuo and Leiden, 1999), regulate SLAM expression.

The precise mechanism linking the SLAM-SAP-FynT complex to transcriptional activation of GATA-3 and T_H2 cytokine production remains to be identified. Previously,

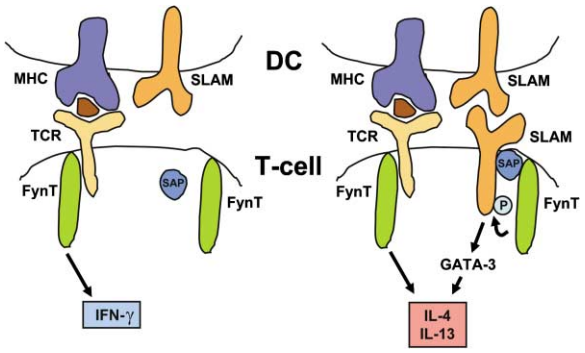

Figure 7. The SLAM-SAP-FynT Cascade in T_H2 Cytokine Regulation

Model of activation of SLAM-SAP-FynT pathway during T cell activation. Resting T cells express SAP and FynT, but express little SLAM. In response to TCR engagement by antigen/MHC (or the pharmacological agonists PMA and ionomycin), SLAM expression is rapidly induced. This triggers engagement of SLAM by SLAM molecules expressed on dendritic cells (DCs) or, alternatively, other T cells (not shown). This induces binding of SAP to SLAM, recruitment and activation of FynT, and tyrosine phosphorylation of SLAM and possible downstream targets such as SHIP-1 and Dok-related adaptors (not shown). This signal causes upregulation of GATA-3 and production of T_H2 cytokines like IL-4 and IL-13. In the absence of SLAM, SAP or FynT, or when SAP is unable to bind FynT (*sap*^{R78A} mutation), this pathway is not activated, leading to T_H2 defects. MHC: major histocompatibility complex.

we reported that SLAM engagement in the presence of SAP results in tyrosine phosphorylation and/or recruitment of the 5′ lipid phosphatase SHIP-1, the adaptors Dok-1, Dok-2 and Shc, and the negative regulator of Ras, Ras-GTPase-activating protein (GAP) (Latour et al., 2001). These events are eliminated by the SAP R78A mutation or by ablation of FynT expression, implying that they are true components of the SAP-FynT pathway (Latour et al., 2001, 2003) (this report). It should also be remembered that mutations in the SLAM-SAP-FynT pathway interfered with T_H2 cytokine production not only in response to TCR stimulation, but also following exposure to PMA and ionomycin. Therefore, the effectors of the SLAM-SAP-FynT pathway are probably not acting proximally in the TCR signaling cascade. Rather, their influence would likely take place after TCR-induced protein tyrosine phosphorylation and before triggering of T_H2 regulator GATA-3 (Figure 7).

Our data provide firm genetic evidence that SAP is involved in the positive regulation of T_H2-mediated functions in T cells as a result of its capacity to associate with and activate FynT. They also unravel that, in addition to coupling TCR stimulation to the proximal protein tyrosine phosphorylation cascade in T cells, FynT plays a previously unappreciated role in T_H2 cytokine regulation. Since 30%–50% of patients with XLP and XLP-like illnesses do not carry *sap* mutations (Latour and Veillette, 2003; Engel et al., 2003), the possibility that some of these individuals have mutations in other components of the SAP-FynT pathway deserves consideration.

Experimental Procedures

Mice

sap$^-$ and *fyn*$^{-/-}$ mice were described elsewhere (Czar et al., 2001; Stein et al., 1992). They were backcrossed up to 5 generations onto

a C57BL/6 background, and the phenotypes described herein were maintained throughout the backcrossing. As the *sap* gene is X linked, wild-type mice (denoted as sap^+) were either $^{+/+}$ or $^+$, whereas sap^- mice were either $^{-/-}$ or $^-$. Mice lacking SLAM ($slam^{-/-}$) were generated by replacing the exon 2 of the *slam* gene with a *neo* cassette (O.N. and Y.Y., unpublished data). These mice were found to lack detectable SLAM by flow cytometry and immunoblot, and to exhibit normal T cell development and mature T cell populations (Figures 6C and 6D; data not shown). To create a mouse in which R78 of SAP was mutated to alanine (sap^{R78A}), the *sap* gene was cloned from a 129/Sv genomic library. After subcloning the relevant genomic fragments, the R78 codon (CGG) located in the 3′ fragment was replaced by an alanine codon (GCG) by PCR. The resulting fragment was sequenced to identify the mutation and ensure that no unwanted mutation had been introduced. The 5′ and 3′ genomic fragments were cloned in the vector pcDNAII-loxP-frt (Figure 2A). After linearizing, the DNA was electroporated into the ES cell line R1, and transfected cells were selected with G418. Individual clones were screened by Southern blotting using KpnI, and positive clones were injected into blastocysts. Generation of chimeras and germ line transmission of the mutation were achieved according to standard procedures. Deletion of the *loxP*-flanked *neo-tk* cassette was performed by crossing mice with a transgenic mouse expressing the Cre recombinase under the control of an EIIa promoter (provided by P. Love, NIH, Bethesda, MD) (Sommers et al., 2002). Mice with an appropriately recombined sap^{R78A} allele were identified and bred with C57BL/6 mice to eliminate the *cre* transgene. In subsequent generations, mice carrying the sap^{R78A} mutation were identified by PCR amplification of genomic DNA and digestion with NruI. The presence of the sap^{R78A} mutation introduces an NruI site. Mice were backcrossed with C57BL/6 for at least three generations prior to experiments. Wild-type littermates were used as controls.

Antibodies and Reagents

Antibodies against SAP, SLAM, FynT, Dok-2, phosphotyrosine, and Lck were described (Latour et al., 2001; Davidson et al., 1992). Mouse anti-GATA-3 mAb HG3-31 was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. Purified and biotinylated rat anti-SLAM mAb 12F12, anti-CD3 mAb 145-2C11, anti-CD28 mAb 37.51, and anti-zeta mAb H146 were prepared in our laboratory. Biotinylated anti-CD69 mAb H1.2F3, biotinylated or phycoerythrin (PE)-conjugated anti-NK1.1 mAb PK136, biotinylated or PE-conjugated anti-CD44 mAb KM114, biotinylated anti-CD49b mAb DX-5, fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb 145-2C11, and FITC-conjugated anti-CD62L mAb MEL-14 were purchased from BD Biosciences, Mississauga, Ontario, Canada. Quantum Red-coupled streptavidin, PMA, ionomycin, ovalbumin (grade VI), Freund adjuvants, thapsigargin, and cyclosporin A were obtained from Sigma-Aldrich, Oakville, Ontario, Canada.

Cell Stimulation, Immunoprecipitations, and Immunoblots

Thymocytes (6.25×10^7 cells ml^{-1}) were incubated for 30 min on ice with mAb 12F12 (25 $\mu g\ ml^{-1}$). After washing unbound antibodies, cells were stimulated for 5 min at 37°C with rabbit anti-rat (RAR) IgG. Unstimulated cells were treated in the same manner, except that they were kept at 4°C. After lysis in 2× TNE buffer (1× TNE: 50 mM Tris [pH 8.0], 1% Nonidet P-40, and 2 mM EDTA) supplemented with protease and phosphatase inhibitors, SLAM was immunoprecipitated by addition of formalin-fixed *Staphylococcus aureus* (Calbiochem-Novobiochem Co., San Diego, CA). Dok-2 was immunoprecipitated from precleared cell lysates using affinity-purified anti-Dok-2. In experiments where cell stimulation was not necessary, cells were directly lysed in 1× TNE buffer. Immunoblots were performed using standard procedures.

Cytokine Production and T Cell Proliferation

$CD4^+$ T cells were purified from 6- to 12-week-old mouse spleens by negative selection, using StemSep magnetic columns (StemCell Technologies, Vancouver, British Columbia, Canada). Cell preparations were typically >95% $CD4^+$ and $CD3^+$ (data not shown). In some cases, additional depleting antibodies, including anti-CD44 and/or anti-CD49b (mAb DX-5), were used during cell purification. Depletion of NK-T cells was achieved by adding anti-NK1.1. Removal of NK-T cells was confirmed by staining with anti-NK1.1 and anti-CD3 (data not shown). To measure cytokine production or proliferation, T cells were stimulated with the indicated concentrations of anti-CD3 coated on plastic, with or without soluble anti-CD28 (1 $\mu g\ ml^{-1}$) and IL-2 (100 U ml^{-1}). In some cases, cells were stimulated with PMA (100 ng ml^{-1}) plus ionomycin (1 μM). After 48–72 hr, cytokine production was measured by ELISA, according to the protocols of the manufacturer (R&D Systems, Minneapolis, MN). Proliferation was assayed by labeling for 6 hr with tritiated thymidine and measuring thymidine incorporation. All assays were done in duplicate and repeated at least five times.

Expression of *gata-3* and *t-bet*

Purified $CD4^+$ T cells were stimulated with anti-CD3, anti-CD3 and anti-CD28, or PMA plus ionomycin, as detailed above. After 48 hr, cells were harvested and processed for RNA extraction according to standard protocols. Expression of *gata-3* and *t-bet* RNA was determined by real-time PCR, using the oligonucleotides 5′-CTTAT CAAGCCCAAGCGAAG-3′ and 5′-CAGGGATGACATGTGTCTGG-3′ for *gata-3*, and 5′-AATCGACAACAACCCCTTTG-3′ and 5′-AACTGT GTTCCCGAGGTGTC-3′ for *t-bet*. RNA levels were standardized by parallel measurements of *S16* RNA. All assays were specific for the indicated transcription factors and were done under linear conditions (data not shown).

Retroviral Infection

Purified $CD4^+$ T cells were activated with anti-CD3 (3 $\mu g\ ml^{-1}$) plus anti-CD28 (1 $\mu g\ ml^{-1}$), in the presence of IL-2 (100 U ml^{-1}), anti-IL-4 (10 $\mu g\ ml^{-1}$), and anti-IFN-γ (10 $\mu g\ ml^{-1}$). After 36 hr, they were infected twice with retroviral supernatant from Phoenix packaging cells transfected with empty pMIG (expressing GFP alone) or pMIG-mGATA-3 (encoding GFP and mouse GATA-3), in the presence of polybrene (5 $\mu g\ ml^{-1}$). After a rest period of 48 hr in IL-2-containing medium, GFP-positive cells were sorted (Mo-Flo, Cytomation, Fort Collins, CO). Following an additional 48 hr, cells were stimulated for 2 days with anti-CD3 alone or PMA plus ionomycin. IL-4 release and proliferation were assayed as outlined above.

Induction of SLAM Expression

$CD4^+$ T cells from normal C57BL/6 mice were stimulated for 18 hr with PMA (100 ng ml^{-1}), ionomycin (1 μM), PMA plus ionomycin, or thapsigargin (0.11 μM). In some experiments, cells were stimulated for 18 hr with anti-CD3 (3 $\mu g\ ml^{-1}$) or ionomycin, in the presence or the absence of cyclosporin A (300 ng ml^{-1}). Expression of SLAM or CD69 was measured by flow cytometry.

IgE Production

Groups of two to three 7- to 12-week-old mice were injected subcutaneously with 200 μg of ovalbumin (4 mg ml^{-1}; in phosphate-buffered saline) plus 50 μl of complete Freund adjuvant. In some cases, mice were boosted by subcutaneous injection of the same quantity of ovalbumin, mixed with incomplete adjuvant. After immunization, mice were bled on a weekly basis. Total serum IgE, ovalbumin-specific IgE and ovalbumin-specific IgG_1 were measured by ELISA, according to standard protocols.

Acknowledgments

We thank Sylvain Latour for useful discussions. We also thank Pamela Schwartzberg for sap^- mouse and useful discussions and Eric Massicotte and Martine Dupuis for cell sorting. This work was supported by grants from the Canadian Institutes of Health Research (to A.V. and M.N.), the National Cancer Institute of Canada (to A.V.), and the CANVAC National Centre of Excellence (to A.V.). H.W. holds a Fellowship of the Heart and Stroke Foundation of Canada. A.V. and M.N. are Senior Investigators of the Canadian Institutes of Health Research. M.N. holds the Canada Research Chair in Cardiac Differentiation and Growth Mechanism, while A.V. holds the Canada Research Chair in Signaling in the Immune System.

Received: June 8, 2004
Revised: September 29, 2004
Accepted: October 5, 2004
Published: November 16, 2004

References

Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X., and Perlmutter, R.M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. Cell *70*, 751–763.

Brigl, M., and Brenner, M.B. (2004). CD1: antigen presentation and T cell function. Annu. Rev. Immunol. *22*, 817–890.

Castro, A.G., Hauser, T.M., Cocks, B.G., Abrams, J., Zurawski, S., Churakova, T., Zonin, F., Robinson, D., Tangye, S.G., Aversa, G., et al. (1999). Molecular and functional characterization of mouse signaling lymphocytic activation molecule (SLAM): differential expression and responsiveness in Th1 and Th2 cells. J. Immunol. *163*, 5860–5870.

Chan, B., Lanyi, A., Song, H.K., Griesbach, J., Simarro-Grande, M., Poy, F., Howie, D., Sumegi, J., Terhorst, C., and Eck, M.J. (2003). SAP couples Fyn to SLAM immune receptors. Nat. Cell Biol. *5*, 155–160.

Chen, R., Relouzat, F., Roncagalli, R., Aoukaty, A., Tan, R., Latour, S., and Veillette, A. (2004). Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. Mol. Cell. Biol. *24*, 5144–5156.

Cocks, B.G., Chang, C.C., Carballido, J.M., Yssel, H., de Vries, J.E., and Aversa, G. (1995). A novel receptor involved in T-cell activation. Nature *376*, 260–263.

Crotty, S., Kersh, E.N., Cannons, J., Schwartzberg, P.L., and Ahmed, R. (2003). SAP is required for generating long-term humoral immunity. Nature *421*, 282–287.

Czar, M.J., Kersh, E.N., Mijares, L.A., Lanier, G., Lewis, J., Yap, G., Chen, A., Sher, A., Duckett, C.S., Ahmed, R., et al. (2001). Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. Proc. Natl. Acad. Sci. USA *98*, 7449–7454.

Davidson, D., Chow, L.M., Fournel, M., and Veillette, A. (1992). Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase p59fyn. J. Exp. Med. *175*, 1483–1492.

Eberl, G., Lowin-Kropf, B., and MacDonald, H.R. (1999). Cutting edge: NKT cell development is selectively impaired in Fyn-deficient mice. J. Immunol. *163*, 4091–4094.

Engel, P., Eck, M.J., and Terhorst, C. (2003). The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease. Nat. Rev. Immunol. *3*, 813–821.

Finkelman, F.D., Holmes, J., Katona, I.M., Urban, J.F., Jr., Beckmann, M.P., Park, L.S., Schooley, K.A., Coffman, R.L., Mosmann, T.R., and Paul, W.E. (1990). Lymphokine control of in vivo immunoglobulin isotype selection. Annu. Rev. Immunol. *8*, 303–333.

Gadue, P., Morton, N., and Stein, P.L. (1999). The Src family tyrosine kinase Fyn regulates natural killer T cell development. J. Exp. Med. *190*, 1189–1196.

Grogan, J.L., and Locksley, R.M. (2002). T helper cell differentiation: on again, off again. Curr. Opin. Immunol. *14*, 366–372.

Howie, D., Okamoto, S., Rietdijk, S., Clarke, K., Wang, N., Gullo, C., Bruggeman, J.P., Manning, S., Coyle, A.J., Greenfield, E., et al. (2002). The role of SAP in murine CD150 (SLAM)-mediated T-cell proliferation and interferon gamma production. Blood *100*, 2899–2907.

Kopf, M., Le Gros, G., Bachmann, M., Lamers, M.C., Bluethmann, H., and Kohler, G. (1993). Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature *362*, 245–248.

Kuhn, R., Rajewsky, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science *254*, 707–710.

Kuo, C.T., and Leiden, J.M. (1999). Transcriptional regulation of T lymphocyte development and function. Annu. Rev. Immunol. *17*, 149–187.

Latour, S., and Veillette, A. (2001). Proximal protein tyrosine kinases in immunoreceptor signaling. Curr. Opin. Immunol. *13*, 299–306.

Latour, S., and Veillette, A. (2003). Molecular and immunological basis of X-linked lymphoproliferative disease. Immunol. Rev. *192*, 212–224.

Latour, S., Gish, G., Helgason, C.D., Humphries, R.K., Pawson, T., and Veillette, A. (2001). Regulation of SLAM-mediated signal transduction by SAP, the X-linked lymphoproliferative gene product. Nat. Immunol. *2*, 681–690.

Latour, S., Roncagalli, R., Chen, R., Bakinowski, M., Shi, X., Schwartzberg, P.L., Davidson, D., and Veillette, A. (2003). Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signalling in immune regulation. Nat. Cell Biol. *5*, 149–154.

Li, S.C., Gish, G., Yang, D., Coffey, A.J., Forman-Kay, J.D., Ernberg, I., Kay, L.E., and Pawson, T. (1999). Novel mode of ligand binding by the SH2 domain of the human XLP disease gene product SAP/SH2D1A. Curr. Biol. *9*, 1355–1362.

Murphy, K.M., and Reiner, S.L. (2002). The lineage decisions of helper T cells. Nat. Rev. Immunol. *2*, 933–944.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., and Paul, W.E. (1999). The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol. *17*, 701–738.

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., et al. (1998). The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature *395*, 462–469.

Sommers, C.L., Park, C.S., Lee, J., Feng, C., Fuller, C.L., Grinberg, A., Hildebrand, J.A., Lacana, E., Menon, R.K., Shores, E.W., et al. (2002). A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science *296*, 2040–2043.

Stein, P.L., Lee, H.M., Rich, S., and Soriano, P. (1992). pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741–750.

van Leeuwen, J.E., and Samelson, L.E. (1999). T cell antigen-receptor signal transduction. Curr. Opin. Immunol. *11*, 242–248.

Veillette, A. (2004). SLAM family receptors regulate immunity with and without SAP-related adaptors. J. Exp. Med. *199*, 1175–1178.

Veillette, A., and Latour, S. (2003). The SLAM family of immune-cell receptors. Curr. Opin. Immunol. *15*, 277–285.

Wang, N., Morra, M., Wu, C., Gullo, C., Howie, D., Coyle, T., Engel, P., and Terhorst, C. (2001). CD150 is a member of a family of genes that encode glycoproteins on the surface of hematopoietic cells. Immunogenetics *53*, 382–394.

Wang, N., Satoskar, A., Faubion, W., Howie, D., Okamoto, S., Feske, S., Gullo, C., Clarke, K., Sosa, M.R., Sharpe, A.H., et al. (2004). The cell surface receptor SLAM controls T cell and macrophage functions. J. Exp. Med. *199*, 1255–1264.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Woodland, D.L., and Dutton, R.W. (2003). Heterogeneity of CD4(+) and CD8(+) T cells. Curr. Opin. Immunol. *15*, 336–342.

Wu, C., Nguyen, K.B., Pien, G.C., Wang, N., Gullo, C., Howie, D., Sosa, M.R., Edwards, M.J., Borrow, P., Satoskar, A.R., et al. (2001). SAP controls T cell responses to virus and terminal differentiation of TH2 cells. Nat. Immunol. *2*, 410–414.

Yin, L., Al Alem, U., Liang, J., Tong, W.M., Li, C., Badiali, M., Medard, J.J., Sumegi, J., Wang, Z.Q., and Romeo, G. (2003). Mice deficient in the X-linked lymphoproliferative disease gene sap exhibit increased susceptibility to murine gammaherpesvirus-68 and hypo-gammaglobulinemia. J. Med. Virol. *71*, 446–455.