Src-Like Adaptor Protein Regulates B Cell Development and Function

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Regulated immune responses to foreign and self-Ags are critical in protecting the host from diseases such as infection, malignancy, and autoimmunity, but the balance of the immune system can be disrupted by intrinsic defects in lymphocyte signaling pathways (1, 2). Understanding the factors that regulate these signaling pathways could lead to greater insight into pathogenesis and/or treatment of disease.

One protein that regulates signaling pathways is the Src-like adaptor protein (SLAP) (3, 4). SLAP has sequence and domain similarity to Src-family protein tyrosine kinases containing highly homologous SH3 and SH2 domains and has the ability to complex with activated components of the TCR complex (5). However, instead of a kinase domain, it has a unique C terminus with a poorly defined function.

Initial functional analysis identified SLAP as a negative regulator of cellular signaling in cell culture (5–7). In vivo studies have shown that SLAP deficiency leads to increased TCR expression selectively on double-positive (DP) thymocytes, the cells in which SLAP is most highly expressed (8). This increased expression level was associated with increased TCR-mediated signaling, as evidenced by increased CD5 and CD69 levels, and enhanced positive selection (8). These data support a role for SLAP in regulating TCR levels during a specific stage of T cell development where selection events occur, and its absence leads to alterations in development and maturation (8).

Recently, in addition to being highly expressed in thymocytes, SLAP mRNA was found to be expressed in naïve peripheral mouse B cells (9). Moreover, SLAP mRNA expression is highly regulated during B cell activation (10, 11). SLAP mRNA is down-regulated in naïve B cells following BCR activation but is maintained in anergized B cells (10, 11). Similar to mouse B cells, SLAP mRNA also is highly regulated in human B cells and, as determined by the serial analysis of gene expression technique, is highly expressed in pre-, naïve, and germinal-center B cells (9). This regulated expression suggests that differential expression of SLAP may be required for normal B cell development and maturation, analogous to its role in mouse thymocytes.

Like T cells, B cells have a highly ordered and well-described developmental program (12). The earliest bone marrow pro-B cells are defined by their expression of B220, CD43, BP-1, and AA4.1 (fractions A–C) (13) and are selected based on their productive rearrangement of Ig H chain, when they lose CD43 expression and advance to the pre-B cell stage (fractions C and D). In addition, immature bone marrow B cells (fraction E) expressing IgM are negatively selected based on their avidity for self-Ag before exiting the bone marrow. These newly formed (NF) transitional type 1 (T1) B cells traffic to the spleen for continued selection/maturation, where they encounter selection signals. They then advance into the follicle, where they are defined as transitional type 2 (T2) cells. These T2 cells are further selected and either die by apoptosis or develop into marginal zone (MZ) or IgM- and IgD-expressing follicular mature (M) B cells (14). Ligand-dependent and ligand-independent signals through the BCR complex are important in determining the fate of a developing B cell and shape the mature B cell repertoire (15). In addition, modifications in intracellular signaling proteins that alter signal strength through the BCR complex change the developmental fate of B cells (15). Therefore, modifications in SLAP expression during development of B cells also may influence BCR signal strength and lead to alterations in development of BCR.

The avidity of BCRs and TCRs influences signal strength during processes of lymphocyte development. Avidity is determined by both the intrinsic affinity for Ag and surface levels of the Ag receptor. The Src-like adaptor protein (SLAP) is a regulator of TCR levels on thymocytes, and its deficiency alters thymocyte development. We hypothesized that SLAP, which is expressed in B cells, also is important in regulating BCR levels, signal strength, and B cell development. To test this hypothesis, we analyzed the B cell compartment in SLAP-deficient mice. We found increased splenic B cell numbers and decreased surface IgM levels on mature, splenic B cells deficient in SLAP. Immature bone marrow and splenic B cells from BCR-transgenic, SLAP-deficient mice were found to express higher surface levels of IgM. In contrast, mature splenic B cells from BCR-transgenic mice expressed decreased levels of surface BCR associated with decreased calcium flux and activation-induced markers, compared with controls. These data suggest that SLAP regulates BCR levels and signal strength during lymphocyte development. The Journal of Immunology, 2006, 176: 335–345.

Abbreviations used in this paper: SLAP, Src-like adaptor protein; DP, double positive; NF, newly formed; T1, transitional type 1; T2, transitional type 2; MZ, marginal zone; M, mature; HEL, hen egg lysozyme; IP, immunoprecipitation; KLH, keyhole limpet hemocyanin; p-ERK, phospho-ERK; PFA, paraformaldehyde; SHP-1, Src homology region 2 domain-containing phosphatase 1; WT, wild type; Q-PCR, quantitative PCR.
The observation that the expression of SLAP mRNA is regulated in B cells, together with an identified role for SLAP as a regulator of thymocyte development, led us to hypothesize that SLAP expression in B cells may be important in regulating BCR signal strength and may be important for normal B cell development. To test this hypothesis, we analyzed B cell composition and function in SLAP-deficient mice and bred them into two transgenic lines, one expressing a BCR specific for hen egg lysozyme (HEL) and the other also expressing HEL as a soluble neo-self-Ag (16–18). The HEL/BCR-transgenic system was used to study how SLAP deficiency affects B cell development, maturation, and tolerance induction in vivo (19). The generation of SLAP-deficient mouse strains that were transgenic for the BCR alone or the BCR and HEL transgenes allowed us to assess the influence of SLAP on a population of Ag-stimulated and potentially tolerized B cells. The phenotype of the B cells and efficiency of tolerance induction was determined. In addition, the HEL/BCR-transgenic mice were the source of a homogenous population of B cells with single Ag specificity that were used to define how SLAP deficiency affected BCR signal transduction. In the experiments described here, we show that SLAP is expressed in B cells and that its deficiency leads to alterations in B cell development and to a peripheral B cell pool with decreased BCR levels that is hyporesponsive to Ag stimulation.

Materials and Methods
Mice
The SLAP-deficient mice have been described previously (8). The mice were backcrossed to C57BL/6 (B6) for eight generations and crossed into MD4 HEL-Ig and ML5 sHEL Tg mouse lines. The MD4 and ML5 lines were obtained from Dr. J. Cyster (University of California, San Francisco, CA) and were genotyped as described (20). All mice were maintained under specific pathogen-free conditions and were analyzed between 8 and 12 wk of age for all studies performed. The Animal Use and Care Committee at the University of California, San Francisco, approved all of the animal research methods used in this study.

Flow cytometry
Single-cell suspensions from bone marrow and spleen were stained in FACS buffer (PBS supplemented with 2% FCS, 2 mM L-glutamine (Invitrogen Life Technologies), 100 IU/ml penicillin/streptomycin (Invitrogen Life Technologies), 2 mM EDTA) with mAbs conjugated with FITC, PE, TriColor, PerCP, PerCP-Cy5.5, allophycocyanin, or biotin-conjugated mAbs as described previously (8). Biotinylated mAbs were visualized with streptavidin F(ab')2 IgM (concentrations ranging from 0.5 to 60 μg/ml; Jackson ImmunoResearch Laboratories), or anti-Mac-1, anti-NK1.1, anti-CD3, anti-CD19, anti-CD21, anti-CD23, anti-CD24, and anti-CD43 (BD Pharmingen or Biocytex). Data were collected on a FACScalibur (BD Biosciences) system and analyzed using FlowJo software (Tree Star). Data analysis included determination of mean, SEM, and statistical significance using a two-tailed Student t test.

Cell purification, RNA isolation, and real-time quantitative PCR (Q-PCR) analysis
Single-cell suspensions from bone marrow were stained for anti-CD24, anti-CD24, anti-CD24, anti-CD24, anti-CD24, anti-IgM, Pro-B cells (fractions A–C), pre-B cells (fraction D), immature B cells (fraction E), and recycling B cells (fraction F) were sorted as described previously (13). Single-cell suspensions from spleens of MD4−/− and B6 mice as described previously (25). For quantitative analysis of IgM expression, 2-fold serial dilutions of serum were added to plates coated with mouse anti-mouse IgM (BD Pharmingen). Serum from SLAP−/− or B6 mice either expressing the MD4 or MD4/bHL-5 transgenes, as well as serum from pooled BALB/c mice, were used for the study. Plates were washed three times with distilled water and blocked with blocking buffer described previously (25). IgM expression was determined using biotinylated mouse anti-mouse IgM (BD Pharmingen), followed by streptavidin-HRP (Jackson Immunoresearch Laboratories). In addition, 3,3′,5,5′-tetramethylbenzidine slow kinetic substrate (Sigma-Aldrich) was added, the reaction was stopped with 1 N sulfuric acid, and the plates were read on a TiterTek Multiscan MC ELISA reader (MP Biomedical) using a 405-nm filter. The amount of IgM in each experimental serum sample was determined by comparison to a known amount of serum IgM in the pooled BALB/c control serum.

In vitro B cell cultures and proliferation assays
Pure B cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine (Invitrogen Life Technologies), 100 IU/ml penicillin/streptomycin (Invitrogen Life Technologies), and 100 mM 2-ME (Sigma-Aldrich). Cells were stimulated with 2-fold serial dilutions of goat anti-mouse F(ab')2 IgM (concentrations ranging from 0.5 to 60 μg/ml; Jackson Immunoresearch Laboratories), or anti-CD40 (1 μg/ml; BD Pharmingen), at a fixed concentration plus goat anti-mouse F(ab')2 IgM (concentrations ranging from 0.5 to 60 μg/ml), or LPS (Sigma-Aldrich; 2.5 μg/ml, starting concentration). For thymidine uptake assays, 2 × 105 cells were cultured in duplicate for 48 h in 96-well flat-bottom plates, pulsed with 1 μCi of 3Hthyidine for 12 h, and harvested using a Harvester 96 (Tomtec). [3H]Thymidine uptake was detected in a MicroBeta Trilux liquid scintillation counter (PerkinElmer).

Immunoassay with keyhole limpet hemocyanin (KLH)
Sera were obtained from SLAP−/− and B6 mice 3 days before and 3, 5, 7, 10, 14, 21, and 28 days after primary immunization with 200 μg of KLH (Sigma-Aldrich) in CFA (Difco). The same mice then received a secondary immunization with 100 μg of KLH in IFA on day 35 after primary immunization, and sera were collected on days 3, 5, 7, 10, 14, 21, and 28 after secondary immunization. Nine mice per genotype were immunized, and each serum sample was analyzed separately. For each immunization, KLH-specific ELISAs were performed as described previously (25).

Intracellular phospho-ERK (p-ERK) staining
Single-cell suspensions from spleens of MD4−/−, SLAP-deficient, and wild-type (WT) mice were harvested and either immediately fixed in 1.6% paraformaldehyde (PFA) in FACS buffer (Electron Microscopy Sciences) or rested for 30 min at 37°C in PBS containing magnesium and calcium and...
then stimulated with PMA (50 ng/ml) (Sigma-Aldrich) for 2 min before fixation with 1.6% PFA. Following fixation, cells were permeabilized in ice-cold 90% methanol for 10 min and washed in FACS buffer. The fixed cells were stained for 30 min with either IgM, unconjugated rabbit anti-mouse p-ERK (Cell Signaling Technology), followed with donkey anti-rabbit F(ab')2 (Jackson ImmunoResearch Laboratories), anti-CD45R (B220), and anti-IgG, or anti-IgM, unconjugated purified rabbit IgG, followed with donkey anti-rabbit F(ab')2, anti-CD45R(B220), and anti-IgG as a control. The cells were washed with FACS buffer twice after staining, and the data were collected on a FACS Calibur system and analyzed using FlowJo software.

Calcium flux

Spleen-cell suspensions from B6 MD4 mice and SLAP-deficient MD4 mice were adjusted to 10 × 10^6/ml in RPMI 1640 (Invitrogen Life Technologies) and incubated with 2 μg of Fluor-3-AM (Invitrogen Life Technologies) for 30 min at 37°C. Cells were stained with allophycocyanin-conjugated anti-Mac-1, anti-Gr-1, anti-NK1.1, anti-TER-119, and anti-CD3 to gate all non-B cells and all positive cells excluded from the calcium flux analysis. To demonstrate that the cells in our negative gate were primarily B cells, a separate aliquot of splenocytes was stained with anti-B220, and the percentage of B cells was determined. The B220^− cells were equal in percentage to the cells in the allophycocyanin-negative gate and, hence, were B cells (data not shown). Calcium flux was measured by determining the change of fluorescence of Fluor-3 on the allophycocyanin-negative cells on a FACS system. A baseline reading was collected for 60 s before cross-linking the BCR with HEL (Sigma-Aldrich) at either 0.1 or 1 μg/ml. As a positive control, cells were stimulated with ionomycin (1 μM). All data analysis was performed using FlowJo software.

Activation-induced markers

Total splenocytes (1 × 10^6/well) were cultured in duplicate in 96-well flat-bottom plates, as above, in the presence of HEL at a starting concentration of 10 μg/ml, and 2-fold serial dilutions were performed. After 24 h in culture, the splenocytes were stained with anti-CD19-, anti-IgM-, anti-CD68-, and anti-CD86 (B7-2)- (BD Pharmingen or eBioscience) specific Abs and analyzed by flow cytometry. The level of Ag expressed on the B cells was expressed as the geometric mean fluorescence intensity and was plotted as a function of HEL Ag concentration (nanograms/milliliter).

Results

SLAP is expressed in B cells

To determine whether SLAP protein is expressed in B cells, we examined levels in lysates obtained from total splenocytes and purified splenic B cells (Fig. 1). SLAP was immunoprecipitated from lysates using a rabbit anti-SLAP polyclonal anti-serum and detected by immunoblot analysis (Fig. 1A). As demonstrated in Fig. 1A, SLAP protein is expressed in total splenocytes and purified total splenic B cells (>95% pure). Because SLAP protein is highly expressed in thymocytes, they served as a positive control (Fig. 1A). In the thymus, SLAP expression is highly regulated during T cell development. Therefore, we determined whether SLAP expression is also regulated during B cell development. We performed real-time PCR on mRNA isolated from bone marrow and splenic B cell subsets from B6 mice (Fig. 1B). SLAP mRNA was expressed at low levels in pro- and pre-B cells (fractions A–D). There was a 2- to 3-fold increase in SLAP mRNA levels in Ig-expressing immature B cells, both in the bone marrow (fractions E and F) and the spleen (T1, T2/MZ, and M) (Fig. 1B). Thymocytes express >4-fold more SLAP mRNA than mature B cells, and they served as a positive control (Fig. 1B). These data demonstrate that SLAP protein is expressed in splenic B cells and that SLAP mRNA is developmentally regulated with its highest levels in the BCR-expressing B cell subsets.

SLAP-deficient mice have increased splenic B cell numbers that are hyporesponsive to BCR stimulation

To examine the effects of SLAP deficiency on B cell numbers and function, the total number of splenocytes in SLAP-deficient and WT mice was determined. The number of total splenocytes was increased by 31% (p = 0.005) in the absence of SLAP, compared with B6 controls (Fig. 2C) as a result of a 43% increase (p = 0.002) in absolute B cell numbers in the SLAP-deficient mice, compared with controls (Fig. 2, A–C). However, resting serum Ig levels (IgM, total IgG, IgG1, IgG2a, and IgA) in SLAP-deficient mice were found to be similar to those in B6 controls despite the increased B cell numbers in the SLAP-deficient mice (Fig. 2D).

To further assess B cell function in the absence of SLAP, purified B cells were stimulated with an anti-IgM Ab with or without anti-CD40 costimulation (Fig. 3, A–C). Surprisingly, SLAP-deficient B cells were hypoproliferative in response to BCR stimulation, compared with B6 controls (Fig. 3, A and B). The SLAP-deficient B cells were less hyporesponsive to LPS and were more similar to the B6 control (Fig. 3C). These data suggest that SLAP-deficient B cells are hyporesponsive to BCR-mediated stimulation.

To extend our functional studies, we immunized mice with a T-dependent Ag KLH. Despite increased splenic B cell numbers, the magnitude of the primary IgM response was impaired (Fig. 3, D and E). However, the secondary IgM and total IgG immune responses to KLH were normal, compared with WT controls (Fig. 3, D and E). These data demonstrate that SLAP deficiency leads to B cells that are relatively hyporesponsive in vitro and to primary Ag challenge but that, on secondary Ag challenge, are able to mount a robust immune response.

B cell development is altered in SLAP-deficient mice

To determine the effect of SLAP deficiency on B cell development, we performed multiparameter flow cytometric analysis on single-cell suspensions using spleen and bone marrow from SLAP-deficient and WT control mice (Fig. 4). Analysis of bone marrow
BCR levels were altered in SLAP-deficient MD4-transgenic mice

Our initial studies suggest that SLAP functions as a modulator of BCR levels. Because BCR avidity is dependent on affinity as well as BCR levels, SLAP deficiency may alter the overall cellular avidity for Ag. However, SLAP-deficient B cells may partially compensate for alterations in BCR levels in a native BCR repertoire by selecting for B cells with different BCR affinities. To test this hypothesis, we fixed BCR affinity by crossing the SLAP-deficient mice with mice expressing a BCR transgene specific for HEL (MD4). The MD4 BCR transgene is expressed in B cells at increased levels, compared with native BCR levels. This increase in BCR levels may alter the level of tonic signaling through the BCR during B cell development and contribute to the phenotype seen in the SLAP-deficient mice.

In addition, we sought to determine whether SLAP-deficient MD4-transgenic B cells have altered responses to self-Ag in vivo. To achieve this, we crossed MD4-expressing SLAP-deficient and B6-transgenic mice with mice expressing HEL (ML5), a soluble self-Ag, and monitored anergy induction. An analysis of bone marrow lymphocytes revealed equal numbers of pre- and pro-B cells in SLAP-deficient MD4- and MD4/ML5 double-transgenic mice, compared with B6 controls. (Fig. 5 and data not shown). Immature bone marrow B cells (fraction E) in SLAP-deficient MD4-expressing B cells were increased in number and had a 35% increase in BCR levels, compared with WT controls (p = 0.0085) (Fig. 5, A, B, and D). In contrast, there was a 32% decrease in IgM levels on fraction F recycling mature B cells in SLAP-deficient MD4-expressing B cells, compared with B6 controls (p = 0.019) (Fig. 5, A, C, and D). Notably, BCR down-modulation was intact in the SLAP-deficient MD4/ML5 double-transgenic mice, as indicated by normal composition and BCR levels on B cells from bone marrow and spleen (Figs. 5A and 6, A and B). These data demonstrate that SLAP deficiency in MD4-transgenic mice leads to increased BCR levels in immature bone marrow B cells (fraction E) with decreased BCR levels on the recycling B cell pool (fraction F). Furthermore, SLAP is not required for BCR down-modulation on immature B cells in MD4/ML5 double-transgenic mice, compared with controls.

We next analyzed splenic B cell subsets in the SLAP-deficient MD4-transgenic mice and found that the percentages of NF/T1, MZ, and M B cells were similar to that of B6 controls (Fig. 6). However, in the absence of SLAP, newly formed (NF/T1) B cells had a modest but reproducible 25% increase in BCR levels (p = 0.028), compared with B6 controls (Fig. 6C). More strikingly, SLAP-deficient MD4-transgenic mature B cells had normal composition but a 61% decrease in BCR levels (p = 0.00042), compared with B6 controls (Fig. 6, B and D).

Analysis of the double-transgenic MD4/ML5 SLAP-deficient mice revealed that anergy induction was intact, with normal percentages of splenic B cells and down-modulation of BCR levels in the SLAP-deficient mice, compared with controls (Fig. 6, A and B). Furthermore, whereas quantitative ELISA measurements of transgene-specific IgM levels in the serum of SLAP-deficient MD4-transgenic mice in the absence of HEL were slightly elevated in the absence of HEL Ag, in MD4/ML5 double-transgenic mice, they were similar to controls (Fig. 6E). These data demonstrate that SLAP deficiency may alter the overall cellular avidity for Ag.
deficiency leads to alterations in B cell development, even in the presence of a fixed BCR repertoire. However, the efficiency of anergy induction is not impaired in the SLAP-deficient mice.

Immature splenic B cells have increased tonic signals

The increased levels of BCR observed on SLAP-deficient MD4-transgenic immature B cells could lead to increased tonic signaling via the BCR. To test this hypothesis, we determined levels of p-ERK in immature B cells as a surrogate marker for signaling activity downstream of the BCR (Fig. 7). Splenocytes from both SLAP-deficient MD4-transgenic and WT-transgenic control mice were isolated and immediately fixed in PFA or stimulated with PMA as a positive control. The cells were permeabilized and stained with a purified rabbit IgG specific for p-ERK or an equivalent amount of purified preimmune rabbit IgG as a control in combination with the appropriate markers to assess developmental status. Increased levels of p-ERK were observed in immature B cells, defined as IgM^+IgD^−, from SLAP-deficient MD4-transgenic mice, compared with WT controls (Fig. 7, C and E), whereas mature B cells (IgM^+IgD^+) had virtually equal levels of p-ERK and served as an internal control (Fig. 7D). As an additional control, splenocytes stained with other combinations of surface markers to define the immature B cells, including CD24 vs IgM and CD24 vs CD23, were examined for ERK activation. All stain combinations demonstrated increased p-ERK in immature B cells in SLAP-deficient MD5-transgenic mice, compared with controls (data not shown). Quantitation of four independent experiments demonstrated increased p-ERK in immature B cells in SLAP-deficient MD5-transgenic mice, compared with controls (p = 0.0028). Together, these results suggest that SLAP deficiency leads to increased tonic signaling, presumably due to the increased BCR levels on SLAP-deficient, immature, MD4-expressing B cells.

Mature splenic B cells have decreased responses to Ag in vitro

Increased signaling in immature B cells has been shown previously to lead to a hyporesponsive mature splenic B cell pool (15). To determine whether SLAP-deficient, MD4-transgenic, mature B cells...
cells are hyporesponsive to BCR stimulation, splenocytes from SLAP-deficient MD4-transgenic and WT controls were loaded with Fluo-3, AM, a calcium-sensitive dye, and calcium flux following BCR stimulation was assessed by flow cytometry (Fig. 8, A–C). SLAP-deficient MD4-transgenic B cells were hyporesponsive to HEL stimulation, especially at low doses of Ag (Fig. 8B). Splenocytes also were examined for the induction of activation markers following stimulation with HEL for 24 h (Fig. 8, D–F). Up-regulation of the activation markers B7-2 and CD69 in response to BCR cross-linking was decreased 5- to 6-fold in SLAP-deficient MD4-transgenic B cells, compared with B6 controls (Fig. 8, D and E). Furthermore, BCR levels were similarly down-modulated in response to HEL Ag in both SLAP-deficient MD4-expressing B cells and B6 controls, indicating that activation-induced BCR down-modulation is intact in the absence of SLAP (Fig. 8F). Surprisingly, despite decreased calcium flux and activation-induced markers in short-term signaling assays, proliferation of SLAP-deficient MD4-expressing B cells stimulated with an anti-IgM Ab for 48 h was similar to MD4-expressing B6 controls (data not shown). It is possible that the large differences in calcium flux and activation marker induction are mitigated by alterations in B cell development, leading to normal proliferative and immune responses. These data suggest that the integrated signals necessary for optimal B cell proliferation are complex, and that SLAP

**FIGURE 4.** B cells composition of bone marrow and spleen from SLAP-deficient and B6 littermate mice. Bone marrow B cell analysis, two-dimensional contour plots of pre-B cell subsets from B6 (A) or SLAP-deficient mice (C). B, D, and F, Splenic B cell analysis, two-dimensional contour plots of pre-B cell subsets from B6 (B) and SLAP-deficient mice (D). E, Mean IgM level on pre-B cell bone marrow subsets from B6 (●) or SLAP-deficient mice (■). F, Mean IgM level on splenic B cell subsets from B6 (●) or SLAP-deficient mice (■). Each experiment was performed using three mice per genotype, and each experiment was repeated three to four independent times with similar results. Results represent averages obtained from one experiment ± SEM.
deficiency may alter signaling through multiple ITAM-containing receptors, leading to no significant effect in B cell proliferation. These data demonstrate that similar to nontransgenic SLAP-deficient B cells, mature follicular MD4 SLAP-deficient B cells also are hyporesponsive to BCR stimulation. However, the hyporesponsive phenotype is more profound than the phenotype observed for SLAP-deficient B cells expressing a native diverse BCR repertoire.

Discussion

The studies presented here demonstrate that expression of SLAP in B cells is required to optimally regulate BCR levels, signaling through the BCR and B cell development. SLAP is expressed in B cells, and its deficiency in mice with a native BCR repertoire leads to increased numbers of splenic B cells, as well as the development of MZ B cells that express increased BCR levels. SLAP-deficient MD4-expressing B cells with single Ag specificity also have increased BCR levels on immature bone marrow B cells (fraction E) and immature NF/T splenic B cells but decreased BCR on mature B cells. The immature NF/T B cell had increased tonic signaling, as evidenced by increased intracellular p-ERK levels. The mature B cells had a substantially reduced calcium flux in response to signaling through the BCR and decreased expression of activation-induced surface markers. However, SLAP deficiency in the MD4/ML5 double-transgenic system does not alter anergy induction, because the numbers of peripheral B cells, BCR expression levels, and serum IgM levels are similar in the presence or absence of SLAP. Our data suggest that SLAP deficiency results in altered B cell development, leading to a pool of mature B cells with altered B cell function.

These data indicate that SLAP has a conserved role in regulating Ag receptor levels and signal strength during T cell and B cell development. Signaling via the TCR and BCR is required for normal lymphocyte development (15, 26, 27). SLAP-deficient immature DP thymocytes have a 300% increase in steady-state surface TCR levels, compared with WT controls and increased positive selection (8). DP thymocytes at steady state have a large intracellular pool of TCR, with 75–80% of the assembled receptor internalized (28, 29). Immature B cells, unlike DP thymocytes, have a much smaller intracellular BCR pool, with 35–45% of the total BCR in an intracellular compartment (30). In our current studies, immature bone marrow B cells have a 35% increase in steady-state surface BCR levels. Therefore, the greater effect of SLAP deficiency on the TCR levels on DP thymocytes vs BCR levels on immature B cells simply could be due to differences in the steady-state intracellular pools. It is likely that the alteration in BCR levels in the immature B cells leads to increased tonic signaling and contributes to the defects seen in the mature splenic B cell pool.

FIGURE 5. Compositional analysis of SLAP-deficient MD4 and MD4/ML5-transgenic bone marrow pre-B cells. A, Two-dimensional contour plots of composition of pre-B cell subsets (fractions D–F) from B6 MD4, B6 MD4/ML5, SLAP-deficient MD4, and SLAP-deficient MD4/ML5 mice. B, Overlay of flow histograms from B6 MD4 (shaded gray) and SLAP-deficient MD4 (black line) immature B cells. C, Histograms of mean IgM levels on immature B cells (fraction E) from B6 MD4 (□) and SLAP-deficient MD4 mice (■). D, Overlay of flow histograms from B6 MD4 (shaded gray) and SLAP-deficient MD4 (black line) recycling B cells. E, Histograms of mean IgM level on recycling B cells (fraction F) from B6 MD4 (□) and SLAP-deficient MD4 mice (■). Each experiment was performed on three mice per genotype, and each experiment was performed three to four independent times with similar results. Results represent averages obtained from one experiment ± SEM.
is possible that SLAP regulates signals via the TCR and BCR during a parallel process occurring in both B cells and T cells during ontogeny.

Mice deficient in key signaling molecules important in transmitting signals through the BCR show a variety of defects in BCR levels and lymphocyte development (15, 31–33). Signals through the BCR occur during B cell development in a ligand-dependent/independent manner (27, 34–37). SLAP appears to modulate the ligand-independent functions of the BCR during lymphocyte development, because it appears to do similarly during T cell development (8). In the absence of self-Ag, SLAP-deficient MD4-expressing immature B cells have increased BCR levels. This increase in BCR levels can be due to a direct effect of SLAP deficiency on BCR levels or an indirect effect of SLAP deficiency on B cell development leading to the selection of B cells with altered BCR levels. These alterations in B cell development lead to increased tonic signaling and down-modulation of BCR levels on mature follicular B cells. This phenotype is reminiscent of Src homology region 2 domain-containing phosphatase 1 (SHP-1)-deficient MD4-transgenic mice (38). SHP-1 forms a complex with CD22 and negatively regulates BCR signaling (39). Loss of SHP-1 alters the signaling threshold in immature B cells (38). SHP-1 deficiency leads to increased BCR signaling, which, in turn, leads to an anergic follicular B cell pool with decreased BCR levels and decreased response to Ag (38). The SLAP phenotype is similar to the SHP-1 phenotype, albeit quantitatively less robust, and is generated through a different mechanism. We suggest that SLAP deficiency leads to a phenotype similar to the SHP-1 deficiency by altering B cell development, BCR levels, and signaling thresholds in immature B cells. BCR tonic signaling thresholds are determined by the summation of signaling events occurring among the BCR and coexpressed positive and negative regulators. Increased BCR levels, such as those seen in SLAP-deficient immature B cells, will shift the balance of positive and negative regulators of BCR signaling and decrease the threshold for BCR tonic signaling. For example, increased BCR levels may lead to a relative deficiency of SHP-1 activity and increased permissive signaling via the BCR.

Insights into SLAP function were obtained from studies where SLAP was shown to associate with c-Cbl in vitro (3). Furthermore, mice deficient in c-Cbl and SLAP have similar thymic phenotypes, suggesting that they function in the same biochemical pathway (40, 41). c-Cbl is a 120-kDa protein that is widely expressed, with highest levels in hemopoietically derived cells (42). c-Cbl has been shown to have E3 ubiquitin ligase activity for activated receptor protein tyrosine kinases, including the PDGFR, the EGFR, and the TCR (43–45). c-Cbl is rapidly phosphorylated upon TCR activation and interacts with components of the proximal TCR signaling machinery, such as ZAP-70 (46, 47). In the many studies done to elucidate the role of c-Cbl inhibition of Ag receptor signaling, the function of SLAP has not been assessed. Recent work in our laboratory has shown that SLAP is required for c-Cbl-dependent down-modulation of the TCR in DP thymocytes (48). Furthermore, SLAP cooperates with c-Cbl in BCR down-modulation and
the SH2 domain of SLAP is required for this process (L. L. Dragone, M. D. Myers, C. White, S. Gadwal, T. Sosinowsk, H. Gu, and A. Weiss, manuscript in preparation). Therefore, we propose that SLAP functions as an adaptor of c-Cbl, bringing it to the activated components of the BCR complex and targeting them for degradation. This hypothesis does not exclude the possibility that, in addition to SLAP deficiency leading to alterations in Ag receptor signaling, it also affects signals through other signaling cascades. Developing lymphocytes express SLAP and have a conserved mechanism for regulating Ag receptor levels. SLAP appears to function as a putative adaptor of E3 ubiquitin ligases, controlling Ag receptor expression levels by targeting components of the Ag receptor for degradation. This process may act to dampen ligand-dependent/independent tonic signals from the Ag receptor, possibly allowing the selection of a broader range of Ag receptors with greater diversity of receptor affinity. Diversity of Ag receptor specificity is essential to generate a dynamic and plastic immune response to pathogens. Thus, SLAP may be necessary to generate Ag-receptor diversity and alterations in its activity may lead to a highly restricted or autoreactive repertoire.

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FIGURE 8. Mature splenic B cells from SLAP-deficient MD4-transgenic are hypo-responsive to Ag stimulation. A–C, Calcium flux of B6 MD4 and SLAP-deficient MD4-transgenic B cells stimulated with 100 ng of HEL (A), 1000 ng of HEL (B), and 1 μM ionomycin (C) as a positive control. SLAP MD4 (black line), B6 MD4 (gray line). Experiments performed on six mice per genotype with similar results. D–F, Total splenocytes stimulated with increasing concentrations of HEL for 24 h and B cells stained for the expression of activation markers B7-2 (D), CD69 (E), or for surface IgM expression (F). B6 MD4 B cells (gray line) and SLAP-deficient MD4 B cells (black line). Data represent the average of three mice per genotype ± SEM and three independent experiments.

Disclosures
The authors have no financial conflict of interest.

References