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# SLP-76 Expression Is Restricted to Hemopoietic Cells of Monocyte, Granulocyte, and T Lymphocyte Lineage and Is Regulated During T Cell Maturation and Activation[1]

**James L. Clements,[2]* Susan E. Ross-Barta,[2]* Lorraine T. Tygrett,† Thomas J. Waldschmidt,† and Gary A. Koretzky[3]*‡**

**The leukocyte-specific adapter protein SLP-76 is known to augment the transcriptional activity of nuclear factor of activated T cells and AP-1 following TCR ligation. A role for SLP-76 in additional receptor-mediated signaling events is less clear. To define the pattern of SLP-76 expression during murine hemopoiesis, we stained cells isolated from various tissues with a combination of surface markers followed by intracellular staining with a fluorochrome-labeled SLP-76-specific Ab. In the bone marrow, SLP-76 expression is largely restricted to cells of granulocyte and monocyte lineage. Heterogeneous SLP-76 expression is first detected in the $CD44^+CD25^-$ subset within the $CD3^-CD4^-CD8^-$ thymocyte population. Interestingly, SLP-76 expression increases as thymocyte maturation progresses within the $CD4^-CD8^-$ compartment but decreases as cells mature to a $CD4^+CD8^+$ phenotype. SLP-76 expression is then up-regulated following selection and concomitant with maturation to a $CD4^+$ or $CD8^+$ phenotype. In the periphery, SLP-76 is expressed in T lymphocytes with no detectable expression in the B cell compartment. Exposure to the superantigen staphylococcal enterotoxin B augments SLP-76 expression in the reactive T cell subset. Furthermore, in vitro stimulation with TCR-specific Abs augments the existing levels of SLP-76. These data reveal that SLP-76 expression is coordinately regulated with surface expression of a pre-TCR or mature TCR complex during thymocyte development and that TCR ligation elicits signals that result in increased expression of SLP-76.** ***The Journal of Immunology*****, 1998, 161: 3880–3889.**

Adapter proteins are gaining attention as important modulators of receptor-mediated signal transduction. Perhaps the best example of adapter protein function to date is the role of Grb2 in coupling growth factor receptor signaling with Ras activation (1). While a role for adapter protein function in TCR signal transduction is less clear, several potential TCR coupled adapter proteins have been described, including Cbl, Grb2, Crk, Shc, and the recently described SLP-76 (2–7). SLP-76 was identified as a Grb2-associated protein, which is rapidly phosphorylated following TCR ligation (7). When transiently overexpressed in Jurkat T cells, SLP-76 augments TCR-induced transcriptional activity of AP-1 and the nuclear factor of activated T cells (NF-AT)[4] (8, 9). This effect requires several distinct domains of SLP-76, which include an amino-terminal acidic domain, a proline-rich central region, and a carboxyl-terminal SH2 domain (9). A number of potential effector molecules that associate with SLP-76 either constitutively or in an activation-dependent manner have been identified and include Vav, SLAP-130 (also designated FYB), an unidentified phosphoprotein of 62 kDa (pp62), and an unidentified serine/threonine kinase (8, 10–12). Thus, SLP-76 may function to bridge TCR-coupled effector molecules with potential downstream substrates in a manner analogous to the function of Grb2 in growth factor receptor-mediated signaling.

Given the demonstrated role for SLP-76 in signaling through the TCR in T cell lines, it is possible that SLP-76 may also function to mediate TCR signals during thymocyte development. The first level of selection in the thymus occurs before the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition and is mediated by a pre-TCR complex consisting of a TCR$\beta$-chain coupled to a surrogate TCR$\alpha$ chain (pT$\alpha$) (13, 14). Many of the early biochemical signals generated via pre-TCR ligation are similar to those initiated following engagement of the mature TCR complex, implying that the pre-TCR complex may recruit signaling intermediates in a manner similar to the mature TCR complex (15, 16). Furthermore, the process of positive selection within the $CD4^+CD8^+$ subset is thought to rely on signals mediated by the mature TCR complex (reviewed in Ref. 17). Thus, SLP-76 may function to couple the pre-TCR and/or the mature TCR with downstream signaling events at the appropriate stages of thymocyte development.

In addition to T cells, SLP-76 mRNA or protein expression has been detected in several B cell lines, monocyte lines, and a rat basophilic leukemia (RBL) cell line (7, 18). To understand better which hemopoeitic cell types naturally express SLP-76 and how SLP-76 expression might be regulated during hemopoietic cell development, we surveyed various murine cell lineages for SLP-76 expression using a fluorochrome-labeled SLP-76 Ab and flow cytometry. Given the observation that SLP-76 functions as a TCR-signaling intermediate, we addressed a potential role for SLP-76 in T cell maturation by assaying various thymocyte developmental intermediates for SLP-76 expression. SLP-76 expression following TCR-dependent stimulation

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[4] Abbreviations used in this paper: NF-AT, nuclear factor of activated T cells; RBL, rat basophilic leukemia; PE, phycoerythrin; WGA, wheat germ agglutinin; SEB, staphylococcal enterotoxin B; MFI, mean fluorescence intensity; GST, glutathione-*S*-transferase; BSS, balanced salt solution.



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of mature T cells was also determined. The data presented reveal that SLP-76 expression in the adult mouse is restricted to cells of granulocyte, monocyte, and T lymphocyte lineage with no detectable expression in any of the B cell compartments analyzed. SLP-76 expression is regulated during thymocyte development, with highest expression detected early and late during thymocyte maturation at developmental stages that coincide with pre-TCR signaling and exit from the selection process which occurs within the $CD4^+CD8^+$ compartment. Finally, SLP-76 expression is enhanced following TCR engagement and remains high in those cells that acquire a memory phenotype. Together, these data suggest that SLP-76 expression is coupled with surface expression of a pre-TCR or mature TCR complex and that TCR-mediated signals augment the existing levels of SLP-76 expression.

## Materials and Methods

### *Mice*

Mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) or from the National Cancer Institute (Frederick, MD) and were housed under viral Ag-free conditions at the University of Iowa Animal Care Facility. Seven- to 10-wk-old female BALB/c mice were used for all experiments.

### *Abs*

Hamster anti-mouse CD3$\varepsilon$ (145-2C11), biotin-conjugated hamster anti-murine CD69 (H1.2F3), biotin-conjugated rat anti-mouse V$\beta$6 TCR (RR4-7), and FITC-conjugated rat anti-mouse Ly-6G (Gr-1, RB6-8C5) were purchased form PharMingen (San Diego, CA). Phycoerythrin (PE)-conjugated goat IgG was purchased from Caltag (Burlingame, CA), Texas Red-conjugated ultra-avidin was purchased from Leinco Technologies (Ballwin, MO), and Texas Red-conjugated wheat germ agglutinin (WGA) was purchased from Molecular Probes (Eugene, OR). Rat anti-mouse CD11b (Mac-1, M1/70), Ly-76 (Ter-119), CD4 (GK1.5), CD8 (53.6.72), CD44 (9F3), V$\beta$8 TCR (F23.1), Ly-6C (15.1), B220 (6B2), CD25 (7D4), CD62L (Mel-14), CD16/32 (2.4G2), CD43 (S7), Ly-51 (BP-1), CD24 (J11d/HSA), and hamster anti-mouse CD3 (145-2C11) were partially purified by 50% saturated ammonium sulfate precipitation from serum-free supernatants (HB101) and were conjugated where indicated with FITC, biotin, Cyanine 5-18, or PE using standard procedures.

### *Purification of sheep anti-murine SLP-76*

Glutathione-*S*-transferase (GST) and a fusion protein consisting of GST in frame with murine SLP-76 amino acids 136–235 (GST/SLP-76) were expressed in bacteria and purifed from bacterial lysates with glutathione agarose (Sigma, St. Louis, MO). Ten milliliters of sheep serum raised against the GST-murine SLP-76 fusion protein were precleared with Sepharose beads coupled to GST alone to remove Abs directed against the GST component of the immunogen. Precleared serum was then passed through an affinity column prepared by coupling GST-SLP-76 fusion protein to cyanogen bromide (CNBr)-activated Sepharose 4B beads that had been prepared according to manufacturer's suggestions (Pharmacia Biotech, Piscataway, NJ) and washed with 10% FBS in PBS to block any remaining active sites. Following sample application, the column was washed with PBS, and purified Ab was eluted with 200 mM acetic acid. Eluted fractions were monitored for protein content (OD) and samples with peak activity were dialyzed against PBS and concentrated. The purified SLP-76 specific Ab preparation was then conjugated directly to PE using standard protocols.

### *Cell preparation and stimulation*

Thymus, spleen, or lymph nodes (axillary and inguinal) were removed and homogenized using frosted glass slides. Bone marrow was flushed from the femur and a single cell suspension prepared using a 22-gauge needle. Cells were washed once in balanced salt solution (BSS) and isolated by density gradient centrifugation (Ficoll-Lite, Atlanta, GA) prior to staining. For in vivo stimulation, 8-wk-old mice were injected i.p. with 30 $\mu$g of staphylococcal enterotoxin B (SEB) (Sigma) or with an equal volume of PBS. The spleen was harvested and cells analyzed at days 2, 5, and 7 posttreatment. For in vitro stimulation, cells were stimulated with plate-bound CD3$\varepsilon$-specific Ab (145-2C11, preincubated at 8 $\mu$g/ml) for the indicated times.

### *Flow cytometric analysis*

Cells were incubated in staining buffer (1× BSS/5% FBS/0.1% sodium azide) with the appropriate biotin, FITC, PE, or Cyanine 5-18-conjugated Abs for 30 min at 4°C in the presence of normal rat serum (Pel-Freez, Rogers, AR) and rat anti-CD16/32 (2.4G2) to eliminate FcR-mediated background staining. Following two washes with staining buffer, samples were incubated with the appropriate avidin conjugate (Texas Red, FITC, or Cyanine 5-18) for 30 min at 4°C. After two more washes with staining buffer, cells were fixed and permeabilized using Permeafix (Ortho Diagnostic Systems, Raritan, NJ). Cytoplasmic staining was then carried out using either PE-conjugated goat IgG or PE-conjugated murine SLP-76 in wash buffer (PBS/5% FBS/1.5% BSA/0.005% EDTA) at room temperature. Normal goat serum (Jackson ImmunoResearch, West Grove, PA) was included at a 20% final concentration to reduce nonspecific binding. Cells were washed once in wash buffer and analyzed using a Coulter EPICS 753 instrument at the University of Iowa Flow Cytometry Facility. Approximately 10,000 events were analyzed for each sample unless indicated otherwise and spectral overlap was corrected by electronic compensation when necessary. Data analysis and display were performed using Flowjo version 2.3.3 software (Treestar, San Carlos, CA).

## Results

### *Purification and specificity of the murine SLP-76-specific PE-conjugated Ab*

To prepare a fluorochrome-conjugated SLP-76-specific Ab for use in flow cytometric analysis of murine hemopoietic cell subsets, we affinity purified an existing polyclonal Ab raised in sheep against a GST-SLP-76 fusion protein. This serum was precleared against GST alone to remove any Abs specific for the GST component of the immunogen followed by affinity column purification using immobilized GST-SLP-76 fusion protein. Purified SLP-76-specific Abs were then eluted from the column, concentrated, and directly conjugated to PE. SLP-76 expression has been detected by Northern blot analysis in virtually all T cell lines tested and in several B cell lines (7). As shown in Figure 1, the murine T cell line 2B4 displays specific intracellular staining with the affinity-purified PE-conjugated SLP-76-specific Ab (SLP-76-PE). The murine B cell line CH12 exhibits heterogeneous SLP-76 staining, while the murine B cell line WEHI-231 does not express detectable levels of SLP-76 (Fig. 1*A*). These data agree with our observation that RT-PCR analysis reveals expression of SLP-76 mRNA in the 2B4 and CH12 cell lines but not in our WEHI-231 cell line (data not shown). We confirmed the specificity of the affinity-purified SLP-76 antiserum by using this reagent for immunoblot analysis of lysates obtained from the above cell lines. The affinity-purified SLP-76-specific antisera clearly reacts with a 76-kDa protein in lysates obtained from the 2B4 and CH12 cell lines, but not from the WEHI cell line (Fig. 1*B*). In addition, our murine SLP-76-specific antiserum demonstrates limited cross-reactivity with human SLP-76 by immunoblot analysis (Fig. 1*B*) and, as such, does not significantly stain the human T cell line Jurkat (Fig. 1*A*).

### *SLP-76 expression is restricted to myeloid cells of granulocyte and monocyte lineage in adult murine bone marrow*

A number of Abs that recognize surface markers expressed during hemopoiesis are currently available which facilitate the identification of various cell lineages in the adult bone marrow. By using a combination of fluorochrome-labeled Ly-6C and B220-specific Abs with WGA, it is possible to identify at least six distinct lineage subsets within the bone marrow (19, 20). A number of additional surface markers have been used to confirm the identity of these populations (S. Bhatia, P. de Vries, and T. Waldschmidt, manuscript in preparation). When SLP-76-PE is used in conjunction with Ly-6C, B220, and WGA to stain adult murine bone marrow cells, it is apparent that the predominant population that expresses SLP-76 is of myeloid derivation (population 5, Fig. 2*A*). This observation agrees with the reported expression of SLP-76 in several

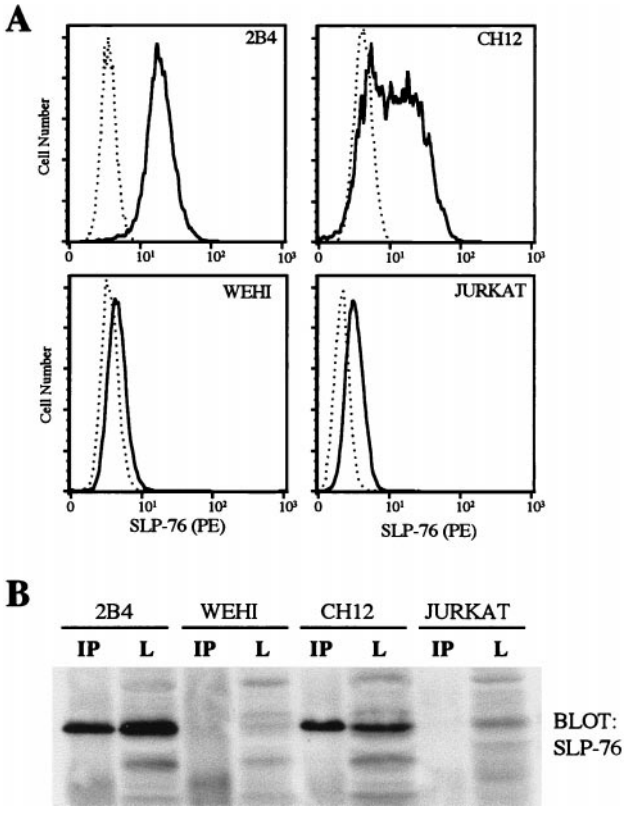


**FIGURE 1.** The PE-conjugated SLP-76-specific Ab (SLP-76-PE) reacts differentially with several T and B lymphocyte cell lines. *A*, The murine T cell line 2B4, B cell lines CH12 and WEHI-231, and the human T cell line Jurkat were permeabilized and fixed prior to staining with PE-conjugated goat Ig-PE (dashed line) or PE-conjugated SLP-76-specific Ab (solid line). Samples were then analyzed by flow cytometry. *B*, Lysates were prepared from $5 \times 10^6$ cells for immunoprecipitation (IP) with the affinity-purified SLP-76-specific antiserum or from $2 \times 10^6$ cells for Western blot analysis of lysates (L). In each case, samples were resolved by SDS-PAGE and transferred to nitrocellulose followed by immunoblot analysis with the affinity-purified SLP-76-specific antiserum and detection by ECL.

monocytic and in a rat basophilic cell line (7, 18). Monocytic precursors (population 6) also display appreciable SLP-76 expression, although the level of background staining in this subset is substantial compared with the other major developmental subsets in the bone marrow. A population of cells with a phenotype consistent with stem cell precursors (population 4) express low to intermediate levels of SLP-76. Neither the early (population 2) or recirculating mature (population 3) B cell subsets express detectable levels of SLP-76. In addition, erythroid precursors (population 1) also exhibit minimal SLP-76 staining.

While the population denoted as early or immature B cells (population 2) displays no appreciable SLP-76 expression, it is possible that SLP-76 may be expressed at more discrete stages of B cell development within this subset. Several surface markers, including HSA, CD43, and BP-1, have been described, which facilitate the identification of several specific stages of early B cell maturation (21). One of the most immature B cell subsets in the bone marrow is $HSA^{low}B220^{int}$. These cells then progress to a $HSA^{high}B220^{int}$ phenotype prior to down-regulation of HSA and up-regulation of B220 (population 3 above). Within the $HSA^{high}B220^{int}$ subset, maturation can be further monitored by assaying for CD43 and BP-1 expression, with BP-1 being transiently expressed on the surface of $HSA^{high}B220^{int}$ pre-B cells as CD43 expression is gradually lost (21). We detect no appreciable SLP-76 expression in any of these subsets, with the possible exception of the very immature $HSA^{low}B220^{int}$ $CD43^+$ population (Fig. 2*C*). Note that a subset of this heterogeneous population displays very low levels of SLP-76, although this level of expression is not significantly above background staining. SLP-76 is not expressed in any of the $HSA^{high}B220^{int}$ susbets identified based on CD43 or BP-1 expression. If SLP-76 is expressed in a multipotent lymphocyte precursor, expression is down-regulated prior to the $HSA^{low}B220^{int}$ stage of B lymphocyte development and is not detectable at any subsequent stage of B cell maturation.

To confirm the myeloid-restricted expression of SLP-76 in the bone marrow, the SLP-76-specific reagent was used in combination with various surface markers specific for cells of the monocyte (Mac-1), granulocyte (Gr-1), or erythroid (Ter-119) lineage. When bone marrow cells are analyzed for SLP-76 expression based solely on side-scatter phenotype, it is apparent that the more granular subset contains SLP-76-positive cells, consistent with the predominance of myeloid lineage cells in this subset (data not shown). Indeed, SLP-76 expression is restricted to the granulocyte ($Gr\text{-}1^+$) and monocyte ($Mac\text{-}1^+$) subsets (Fig. 2*B*). The erythroid compartment ($Ter\text{-}119^+$) does not express SLP-76. Consistent with the data obtained from bone marrow, SLP-76 expression is also detected in $Gr\text{-}1^+$ and $Mac\text{-}1^+$ cells in the peripheral blood but not in the $Ter\text{-}119^+$ population (data not shown).

### *SLP-76 expression is regulated during thymic development*

The process of T cell maturation in the thymus can be followed closely by monitoring the surface expression levels of several markers, including the CD4 and CD8 coreceptors as well as CD3. Using these markers in conjunction with SLP-76, we assayed distinct thymocyte maturational subsets for SLP-76 expression. As shown in Figure 3*A*, the $CD4^-CD8^-$ thymocyte compartment displays a heterogenous level of SLP-76 expression. The $CD4^+CD8^+$ thymocytes express a uniform albeit intermediate level of SLP-76. Within this population, the small subset of $CD3^{high}$ cells (approximately 5%) express higher levels of SLP-76 than the predominant $CD3^{-/low}$ subset (see arrow, Fig. 3*B*). This $CD3^{high}$ population may represent those cells that have been recently selected for further maturation. This notion is supported by the observation that the level of SLP-76 expression in the mature thymocytes ($CD4^+$ or $CD8^+$) is approximately twofold higher than that observed for the majority of $CD4^+CD8^+$ thymocytes and more comparable with the level of SLP-76 expression seen in the $CD3^+CD4^+CD8^+$ subset.

To address more definitively the correlation between SLP-76 expression and thymic selection, SLP-76 expression was analyzed more closely in the $CD4^+CD8^+$, $CD4^+$, and $CD8^+$ compartments. CD69 is an early activation marker and has been reported to be expressed transiently on the subset of $CD3^+CD4^+CD8^+$ thymocytes that are undergoing TCR-mediated positive selection (22). When the $CD4^+CD8^+$ compartment was analyzed for CD69 expression, approximately 3% of these cells were CD69 positive. It is evident that the $CD69^+$ subset expresses higher levels of SLP-76 than the majority of the $CD69^-CD4^+CD8^+$ thymocytes (Fig. 3*B*). In addition, SLP-76 expression remains high in the $CD69^+CD4^+$ and $CD69^+CD8^+$ thymocytes. Interestingly, SLP-76 expression is slightly down-regulated in both the $CD4^+$ and $CD8^+$ compartments, with a more pronounced effect in the $CD8^+$ T cells, as CD69 expression is lost and these cells are prepared for export from the thymus to the peripheral lymphocyte compartments (Fig. 3*B*).

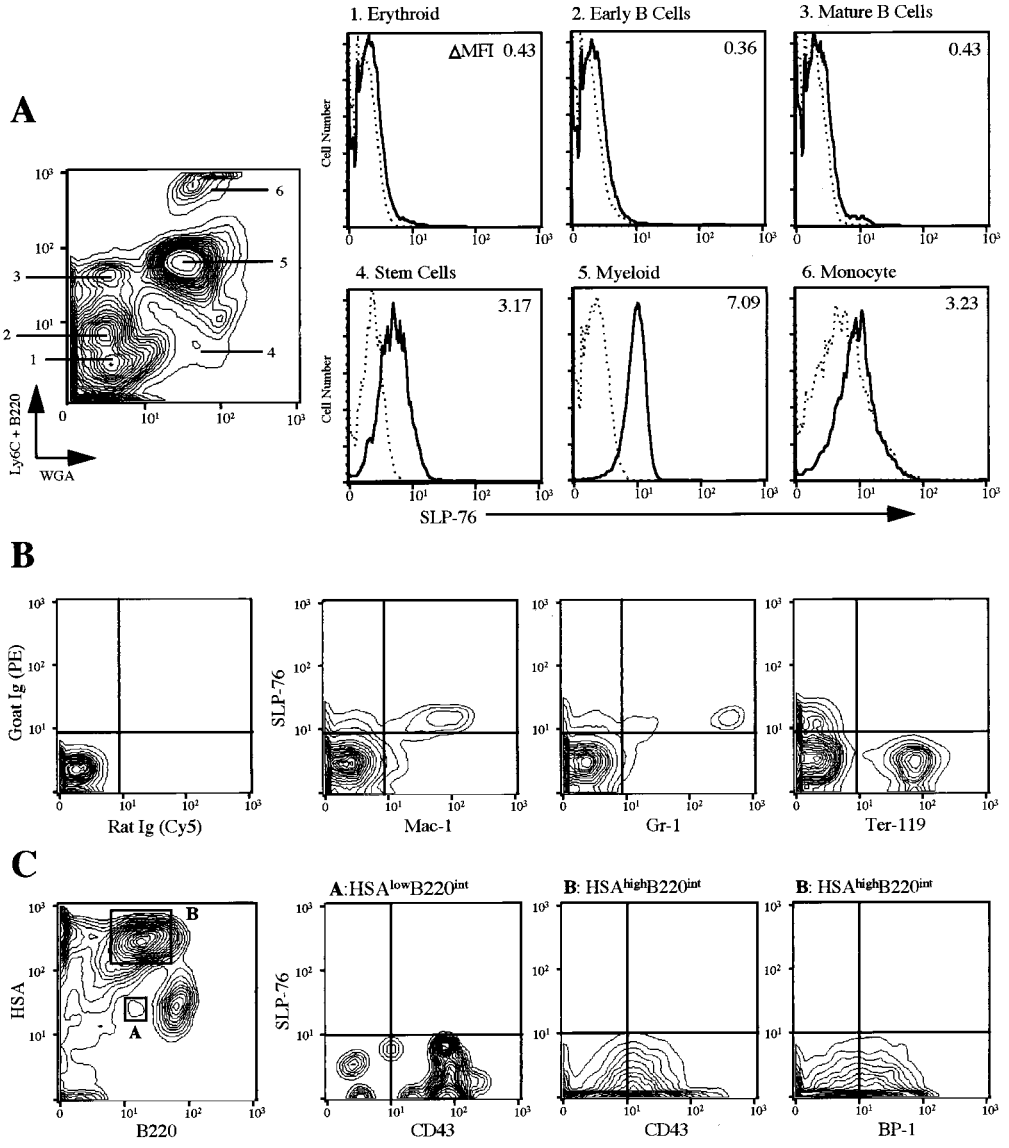


**FIGURE 2.** SLP-76 expression is restricted to hemopoietic cells of monocyte and granulocyte lineage in adult murine bone marrow. Bone marrow was removed from the femurs of adult BALB/c mice and prepared as a single cell suspension. Surface staining was performed with the indicated Abs followed by cell permeabilization and intracellular staining with PE-conjugated goat IgG (dashed line) or PE-conjugated SLP-76-specific Ab (solid line). *A*, The different lineages of hemopoietic cells in the bone marrow were identified using a combination of FITC-conjugated Ly-6C and B220-specific Abs and Texas Red-conjugated WGA. SLP-76 expression in each of these populations was then analyzed. The change in mean fluorescence intensity (ΔMFI) for each population is displayed in the *upper right hand corner* of each histogram and was calculated by subtracting the MFI obtained with goat IgG-PE from the SLP-76-specific signal. *B*, *C*, Bone marrow cells were isolated as described and stained with the indicated surface stains followed by cytoplasmic staining with PE-conjugated goat IgG or PE-conjugated SLP-76-specific Ab. For *B*, cells with scatter characteristics of both lymphocytes and myeloid lineage cells were analyzed. For *C*, analysis was restricted to only those cells with scatter characteristics of small, resting lymphocytes. The gates used to identify and analyze the $HSA^{low}B220^{int}$ and $HSA^{high}B220^{int}$ subsets are shown.

*SLP-76 is expressed most abundantly at the proT2 and proT3 stages of development within the $CD3^-CD4^-CD8^-$ thymocyte compartment*

The heterogeneous nature of SLP-76 expression within the $CD4^-CD8^-$ thymocyte subset prompted us to explore in more detail the expression pattern of SLP-76 in this compartment. Within the $CD3^-CD4^-CD8^-$ (triple-negative) thymocyte subset, the maturation process can be delineated further based on surface expression of CD44 and CD25 (23). The earliest thymic immigrants are $CD44^+CD25^-$ (proT1). These cells then acquire CD25 ($CD44^+CD25^+$ or proT2) and mature further to a $CD44^-CD25^+$ stage (proT3). The generation and expression of a productive TCR$\beta$-chain in a complex with the surrogate pT$\alpha$-chain triggers cell division, expansion, and further maturation to a $CD44^-CD25^-$ phenotype (ProT4) (24, 25). In the adult thymus, the proT3 and T4 populations are readily identified based on CD44 and CD25 expression when those cells that express CD3, CD4, or CD8 are excluded from analysis (Fig. 4*A*). The proT2 and proT3 subsets can also be visualized, but represent a small fraction of the total triple-negative compartment (Fig. 4*A*). SLP-76 expression is detectable in all four early thymocyte developmental subsets, but subtle differences in expression are worth noting. SLP-76 expression is heterogenous in the proT1 subset, but uniformly high in the proT2 and proT3 cells (Fig. 4). Interestingly, the level of SLP-76

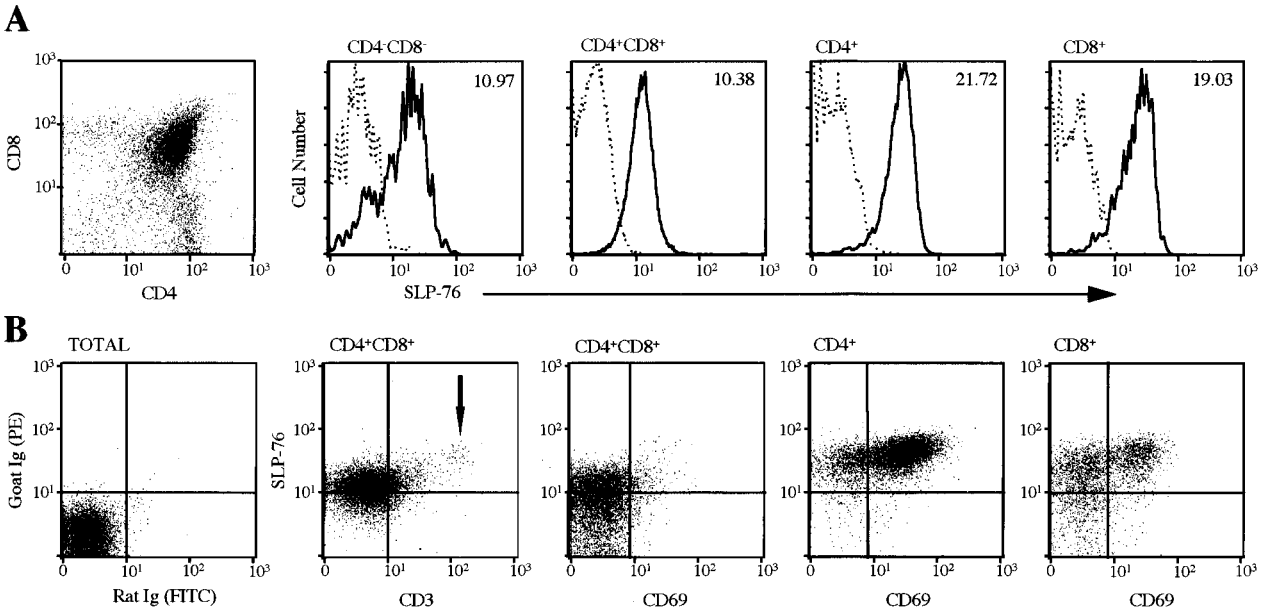


**FIGURE 3.** SLP-76 expression is regulated during thymocyte development. Thymocytes were isolated from adult BALB/c mice (7 to 8 wk) and stained with the indicated Abs followed by cytoplasmic staining with PE-conjugated goat IgG (dashed line) or PE-conjugated SLP-76-specific Ab (solid line). *A*, The CD4 (Texas Red) and CD8 (Cyanine 5-18) profile (*first panel*) was used to gate on the four main developmental subsets defined by CD4 and CD8 expression for subsequent SLP-76 expression analysis. The ΔMFI for each histogram is displayed in the *upper right-hand corner*. *B*, Using the CD4 and CD8 expression profile for gating, the indicated populations were analyzed for CD3 (FITC), CD69 (Texas Red), and SLP-76 (PE) expression. The level of background staining obtained with a biotin-conjugated rat IgG followed with avidin-conjugated Texas Red was comparable with the FITC-conjugated rat IgG control and is not shown.

expression in the proT4 subset is less than that observed in the proT2 and proT3 population and more comparable with that found in the $CD4^+CD8^+$ compartment.

The transition from the proT3 to proT4 stage of thymocyte development is characterized by the induction of cell cycling, presumably as a consequence of preTCR-mediated signaling (25). A small fraction (5–10%) of $CD25^+$ triple-negative cells display forward scatter characteristics indicative of cycling cells, consistent with the induction of cell cycling within the proT3 compartment (25) (Fig. 4*B*). A significantly larger fraction of the $CD25^-$ cells, which contain mostly proT4 thymocytes, exhibit large forward scatter characteristics (Fig. 4*B*). Within the $CD25^+$ population, the level of SLP-76 expression is comparable in both the small resting cells and large cycling cells, suggesting that SLP-76 expression remains elevated as cells transit from the proT2 to proT3 stage of development and does not appreciably change as these cells enter the cell cycle (Fig. 4*B*). In contrast, SLP-76 expression is higher in the large $CD25^-$ triple-negative thymocytes when compared with $CD25^-$ cells with a smaller phenotype. In this case, SLP-76 expression may decrease concomitant with loss of surface CD25 expression and increase again as proT4 thymocytes enter the cell cycle. Alternatively, SLP-76 expression may remain elevated as thymocytes transit from a proT3 phenotype to the proT4 stage of development and gradually falls as thymocytes lose CD25 and attain a quiescent state prior to acquisition of CD4 and CD8.

### *SLP-76 expression is restricted to T cells in the peripheral lymphoid organs and is highest in T cells bearing a memory cell phenotype*

In the bone marrow, no SLP-76 expression is detected in either the immature or mature recirculating B cell subsets (Fig. 2). In agreement with this observation, SLP-76 expression in lymph node and spleen is restricted to T lymphocytes ($CD3^+$) with no appreciable levels observed in the B cell ($B220^+$) compartment (Fig. 5*A*). The T cell-restricted expression of SLP-76 is supported by the observation that no SLP-76 mRNA was detected via RT-PCR in sorted $B220^+CD23^+$ and $B220^+CD23^-$ splenic populations (M. Musci and G. Koretzky, unpublished observations). When the T cell compartment is divided into $CD4^+$ and $CD8^+$ subsets, no obvious difference in SLP-76 expression is seen (Fig. 5*B*). However, it is evident that the memory cell subset within the $CD4^+$ compartment as defined by CD62L (Mel-14) and CD44 expression contains more SLP-76 than the naive population, with the Mel-$14^{low}$ subset displaying approximately twofold higher SLP-76 expression than the Mel-$14^{high}$ subset (see ΔMFI for each population, Fig. 5*C*). The elevated expression of SLP-76 might therefore relate to the lower threshold of TCR-mediated activation observed in $CD4^+$ memory T cells (26). In our experiments with BALB/c mice, it was difficult to identify a memory population in the $CD8^+$ subset based on Mel-14 and CD44 expression, preventing the stringent analysis of SLP-76 expression in this population.

### *SLP-76 expression increases following TCR ligation in vitro*

To address SLP-76 regulation following TCR stimulation, we used a plate-bound CD3ε-specific Ab (145-2C11) as a means to stimulate freshly isolated murine T lymphocytes. The early activation marker CD69 was used to identify activated T cells following 14 h of culture in the absence or presence of anti-CD3ε-specific Ab. As demonstrated in Figure 6, SLP-76 expression is augmented approximately three- to fourfold within 14 h after stimulation via the TCR. This effect correlates with an activated phenotype as increased SLP-76 levels are found in cells that express CD69 (*lower panels*) and have internalized surface TCR (*upper panels*). This increased level of SLP-76 expression is characterized by a loss of the diffuse staining pattern of SLP-76 observed in freshly isolated T cells. TCR induced augmentation of SLP-76 expression has also been observed within 4 h following exposure to plate-bound 2C11, suggesting that the activation-dependent up-regulation of SLP-76

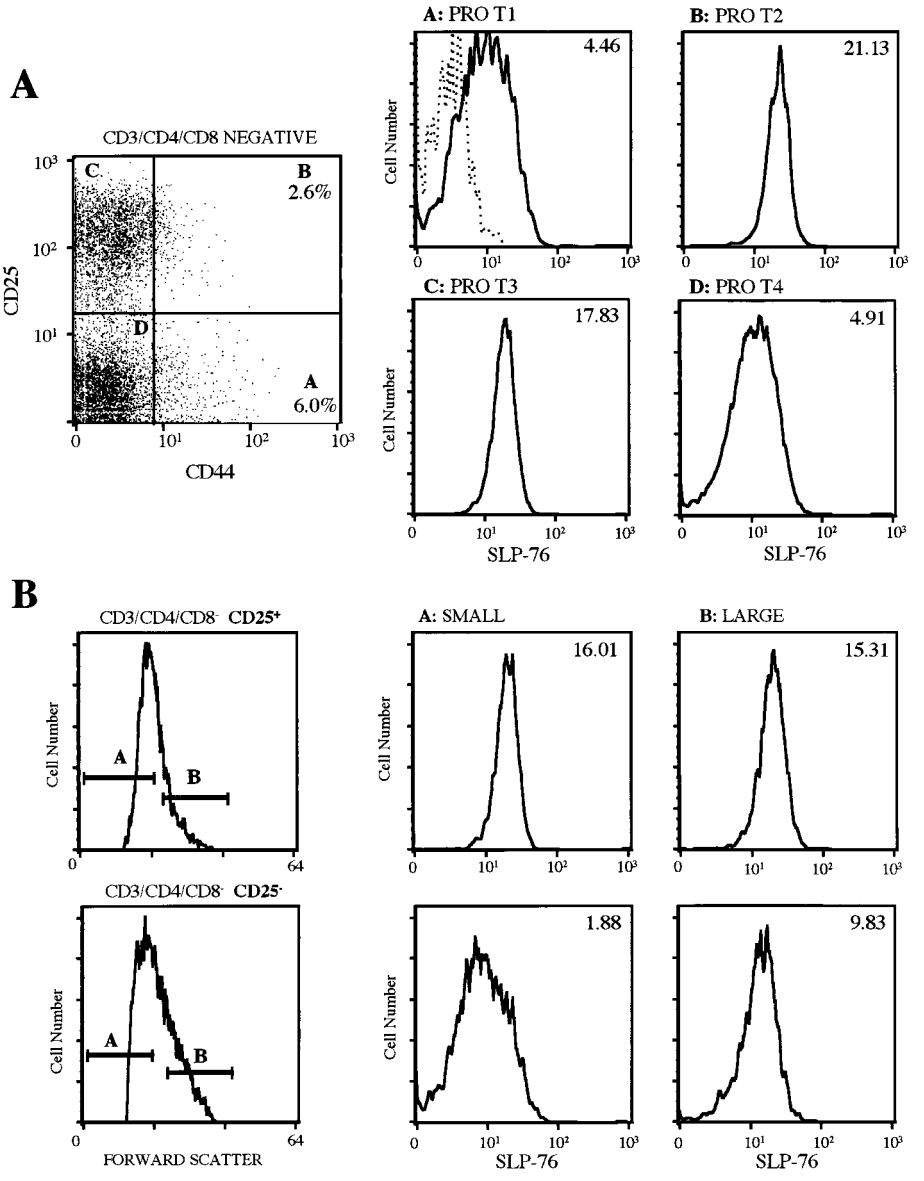


**FIGURE 4.** SLP-76 expression increases as immature thymocytes develop but is down-regulated at a point prior to acquisition of CD4 and CD8. Total thymocytes were isolated and surface stained with a combination of FITC-conjugated CD3-, CD4-, and CD8-specific Abs. Approximately $1 \times 10^6$ events were collected and FITC-negative cells ($CD3^-CD4^-CD8^-$) were further analyzed for CD44 (Texas Red) and CD25 (Cyanine 5-18) expression. *A*, SLP-76 expression in each of the four developmental subsets defined by CD44 and CD25 expression was then analyzed. The background staining (goat-IgG PE, MFI value = 2.97) for the total $CD3^-CD4^-CD8^-$ thymocyte compartment is shown in the proT1 panel and has been subtracted from each MFI shown. *B*, $CD25^+$ or $CD25^-$ triple-negative (CD3/CD4/$CD8^-$) thymocytes were analyzed for forward scatter, and SLP-76 expression in small and large cells was determined. Background staining (goat-Ig-PE) was determined for each subset and the ΔMFI is displayed in the *upper right-hand corner* of each histogram.

is rapid (data not shown). It should be noted that these experiments were performed in the presence of reduced serum levels (1% FBS). Culture in higher levels of serum alone results in significant augmentation of SLP-76 expression, suggesting that factors present in serum are also capable of activation-dependent augmentation of SLP-76 expression (data not shown).

### *SLP-76 expression is elevated following in vivo superantigen stimulation*

The observations that SLP-76 is expressed more abundantly in $CD4^+$ T cells bearing a memory phenotype and that TCR ligation augments SLP-76 expression led us to hypothesize that SLP-76 might be up-regulated following antigenic exposure. To address further the activation-dependent expression of SLP-76, mice were treated with the superantigen SEB. Exposure to SEB in the appropriate genetic background leads to the specific stimulation of T cells bearing a TCR with a V$\beta$8 component (27). These SEB-reactive cells expand to roughly twice the original number followed by a rapid deletion phase that is characterized by apoptosis of the SEB-reactive cells (28). In our experiments, we also observed a dramatic increase in the $V\beta8^+$ T cell compartment with maximum expansion at approximately 2 days post-SEB treatment (Table I). This expansion phase was followed by a gradual decrease in $V\beta8^+$ T cell frequency, consistent with apoptosis of the SEB-reactive subset. Interestingly, SLP-76 expression was substantially higher in the $V\beta8^+$ T cell subset compared with the non-SEB-reactive $V\beta6^+$ control population at day 2 posttreatment (Table I), suggesting that SLP-76 expression is augmented following ligation of the TCR. However, SLP-76 expression decreased to a level comparable with naive T cells as the SEB-reactive subset was deleted.

## Discussion

TCR-mediated activation is a highly ordered process that requires the orchestrated phosphorylation, activation, and recruitment of many signaling intermediates. The physical or functional loss of a single signaling molecule in a biochemical pathway can effectively short-circuit the TCR-signaling process. This notion has been supported by numerous transgenic and gene knockout studies in which key TCR-coupled signaling intermediates have been functionally altered or disrupted at the genomic level. Adapter proteins serve as

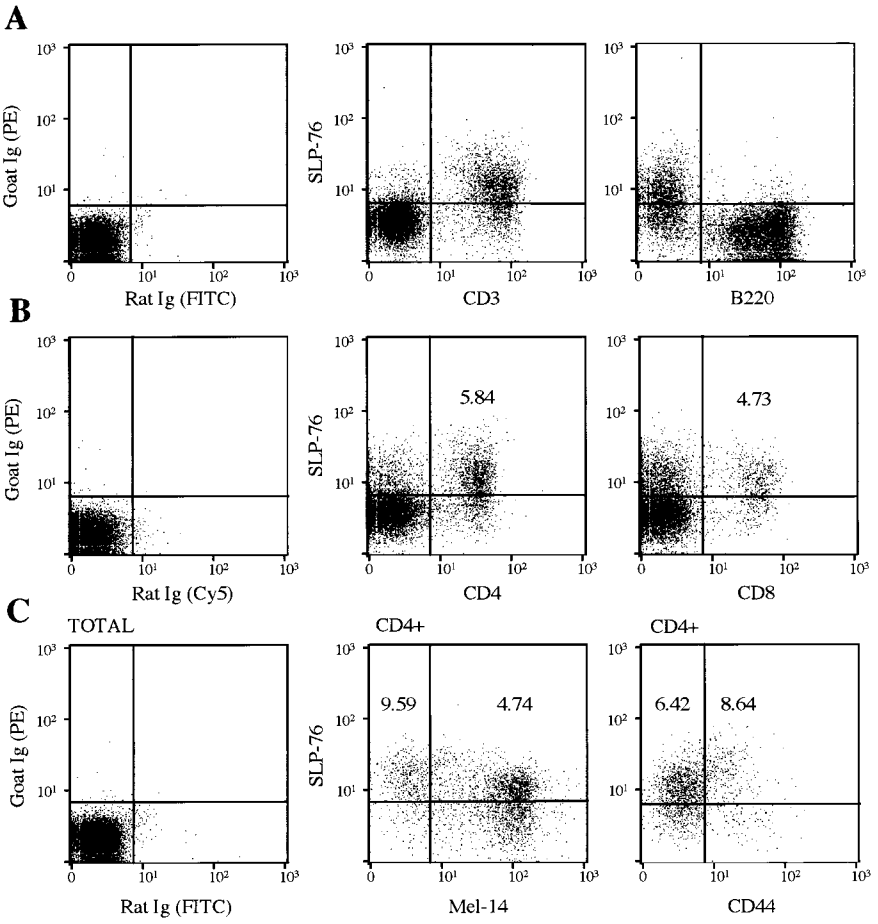


**FIGURE 5.** SLP-76 expression is restricted to T cells in the peripheral lymphocyte compartments and expressed most abundantly in the memory cell subset. Lymphocytes isolated from spleen and lymph nodes were subjected to surface staining with the indicated reagents (*horizontal axis*) followed by cytoplasmic staining with the indicated Abs (*vertical axis*). For each plot, quadrants were established based on nonspecific control staining (*left panels*). *A*, Total splenic/lymph node lymphocytes were stained with FITC-conjugated CD3 or B220-specific Abs and analyzed for SLP-76 expression in the T cell ($CD3^+$) compartment and B cell compartment ($B220^+$). *B*, Total splenic/lymph node lymphocytes were stained with Cyanine 5-18-conjugated CD4- or CD8-specific Abs and analyzed for SLP-76 expression. The ΔMFI for the CD4 and CD8 susbsets are shown. *C*, Total splenic/lymph node lymphocytes were stained with a combination of Cyanine 5-18-conjugated CD4-specific Ab and FITC-conjugated Mel-14 or CD44-specific Abs to define SLP-76 expression in the naive and memory cell subsets. The ΔMFI for the naive ($Mel\text{-}14^{high}$ or $CD44^-$) or memory ($Mel\text{-}14^{low}$ or $CD44^+$) $CD4^+$ T cell subsets are indicated above the appropriate populations.

important regulatory components in many receptor-mediated signal transduction pathways by coupling effector molecules with potential substrates in an activation-dependent manner. In this capacity, SLP-76 has been defined as a leukocyte-specific adapter protein that couples TCR-mediated signaling events with downstream transcriptional activation. How SLP-76 mediates these effects remains unclear, but the identification of several SLP-76-associated proteins has provided some potential clues. The carboxyl-terminus of SLP-76 contains a single SH2 domain that binds several molecules, including the recently cloned molecule SLAP-130 (Fyb) and an unidentified phosphoprotein of 62 kDa (8, 11, 12). The SH2 domain of SLP-76 also precipitates a protein with serine/threonine kinase activity from stimulated Jurkat lysates, although the significance of this finding remains unknown (8). The proline-rich region of SLP-76 mediates binding to one or both of the Grb2 SH3 domains and the SH2 domain of the proto-oncogene product Vav has been

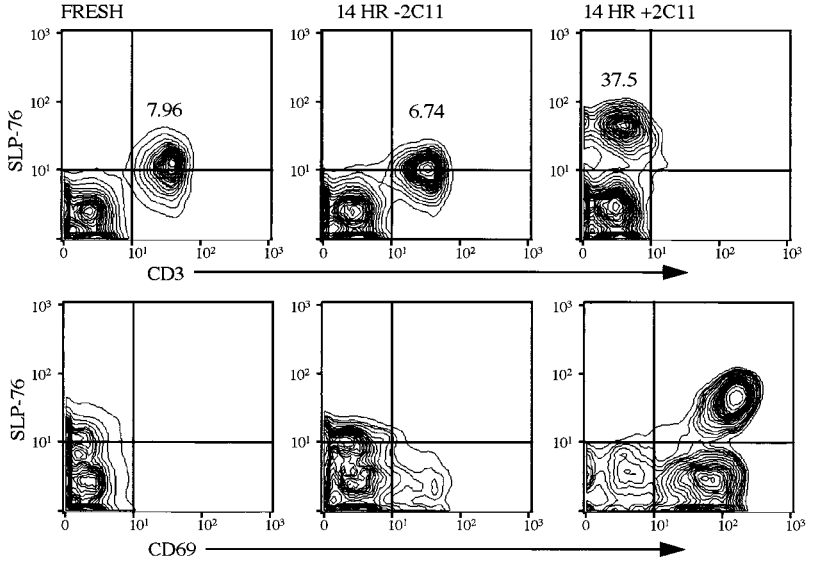


**FIGURE 6.** SLP-76 expression is augmented following TCR ligation in vitro. Purified splenic/lymph node lymphocytes were used fresh or cultured in 1% FBS in the absence or presence of plate-bound 2C11 for 14 h. Following culture, cells were scraped from the plate and stained with the indicated surface markers (*horizontal axis*) followed by cytoplasmic staining with the indicated reagents (*vertical axis*). Expression of CD69 (lower panels) was used to identify activated lymphocytes. Although a significant number of blast cells were present in the 14-h cultures, each plot is representative of cells with similar forward and side-scatter characteristics which were established based on the resting lymphocyte population. The ΔMFI of SLP-76 expression within the T lymphocyte subset is displayed above the appropriate population in the indicated plots.

Table I. *SLP-76 expression is up-regulated following in vivo stimulation with SEB*[a]

| | Experiment 1 | | | | Experiment 2 | | | |
|---|---|---|---|---|---|---|---|---|
| Day | % Vβ8 | MFI (SLP-76) | % Vβ6 | MFI (SLP-76) | % Vβ8 | MFI (SLP-76) | % Vβ6 | MFI (SLP-76) |
| 0 | 12 | 3.93 | 5.2 | 2.66 | 16.4 | 7.85 | 3.6 | 8.9 |
| 2 (PBS) | 11.1 | 4.06 | 3.8 | 3.02 | 15.4 | 5.6 | 3.7 | 6.31 |
| 2 (SEB) | 19.2 | 12.41 | 3.9 | 1.82 | 25.9 | 16.85 | 0.9 | 4.29 |
| 5 (PBS) | ND | ND | ND | ND | 14.6 | 9.78 | 3.8 | 8.08 |
| 5 (SEB) | 12.1 | 3.97 | 3.9 | 1.66 | 14.2 | 7.27 | 3.1 | 8.14 |
| 7 (PBS) | 14.4 | 2.91 | 4.9 | 3.82 | 13.5 | 7.08 | 3.2 | 7.46 |
| 7 (SEB) | 12.6 | 5.27 | 5.1 | 2.25 | 11.7 | 7.06 | 3.2 | 6.54 |

[a] Lymphocytes were isolated from spleens harvested at the indicated day posttreatment and analyzed for Vβ8, Vβ6, and SLP-76 expression by FACS. The mean SLP-76 fluorescence intensity (MFI) for the Vβ8 and Vβ6 populations was determined by subtracting background (goat Ig-PE) fluorescence from the SLP-76-specific signal. ND, not done.

demonstrated to bind the acidic amino-terminal domain of SLP-76 in a phosphotyrosine-dependent manner (8, 10, 29). While the physiologic relevance of each of these associations is unclear and remains the focus of intense investigation, it has been demonstrated that each domain of SLP-76 is required for augmentation of TCR-mediated signals (9).

Our observation that SLP-76 is expressed in cells of myeloid lineage suggests that SLP-76 may also function to couple receptor-mediated signaling in granulocytes and monocytes. In support of this notion, SLP-76 is tyrosine phosphorylated following ligation of the high affinity IgE receptor in a RBL cell line and associates with phosphoproteins that display m.w. consistent with those of SLAP-130 and pp62 (18). A role for the adapter proteins Shc and Grb2 in the regulation of respiratory burst following FcγRI ligation has also been demonstrated (30). Thus, adapter proteins such as SLP-76 may dictate the activation-dependent formation of one or more distinct signaling complexes within several hemopoietic cell lineages. These signaling complexes may vary depending on the cell type, and may promote positive or negative signals depending on the molecular composition of the complex.

Our data document that SLP-76 expression is first detected in the T cell lineage at the proT1 stage of thymic development. The heterogeneous nature of SLP-76 expression within the proT1 subset may reflect the continuum of maturation within this population. The population of proT1 cells with the lowest SLP-76 expression may represent those cells that are the most immature and have recently immigrated to the thymus from the bone marrow. As cells mature within the proT1 subset, SLP-76 expression may gradually increase until it reaches a level comparable with that observed in the proT2 susbset. Those cells with the highest SLP-76 expression within the proT1 subset would presumably represent the most mature cells in this subset at a stage of development just prior to aquisition of CD25. Alternatively, the SLP-76-negative cells within the proT1 subset may represent a previously described population of cells that are not true T lymphocyte precursors and exhibit the potential to give rise to B cells or thymic dendritic cells (31, 32). Thus, it is possible that SLP-76 expression may serve as a useful marker for the identification of cells that have recently homed to the thymus and are committed to the T cell lineage.

Given the ability of SLP-76 to promote TCR-mediated signaling events, it is intriguing that SLP-76 expression is highest in those thymocytes that express significant levels of pre-TCR or mature TCR-signaling complexes. During thymocyte development, there are several developmental checkpoints that require a pre-TCR or mature TCR-dependent signal. Within the triple-negative population, SLP-76 expression is highest within the $CD25^+$ subset, which contains thymocytes at the proT2 and proT3 stages of maturation. Expression of a competent pre-TCR complex at the proT3 developmental stage is required for signaling cell cycle initiation and further maturation (24, 25). Within the $CD25^+$ triple-negative thymocyte compartment, SLP-76 expression is elevated in both the small resting and large cycling cells, suggesting that SLP-76 levels rise prior to initiation of cycling. However, SLP-76 expression is higher in the large $CD25^-$ thymocyte subset compared with the small $CD25^-$ cells. Given the low level of SLP-76 expression observed at the next stage of maturation ($CD4^+CD8^+$), we favor the hypothesis that SLP-76 expression is gradually down-regulated as cells transit from the proT3 to proT4 stage of development and acquire a resting phenotype. Taken together, these data suggest that SLP-76 expression increases as cells approach a stage of thymocyte development (proT3) in which pre-TCR-mediated signaling events are required for further maturation and down-regulated once cells have passed through this pre-TCR-dependent checkpoint.

Engagement of the pre-TCR is known to activate several signaling cascades similar to those elicited by ligation of the mature TCR complex, including activation of tyrosine kinases (15). To date, the best candidate for the tyrosine kinase responsible for the TCR-dependent phosphorylation of SLP-76 is the Syk kinase family member ZAP-70 (33). The requirement for Syk family tyrosine kinases in pre-TCR-driven thymocyte development has beeen demonstrated by the generation of mutant strains of mice that lack both ZAP-70 and Syk (16). These mice manifest a severe block in the transition from the proT3 to proT4 stage of thymic development. If SLP-76 is required to translate ZAP-70 and/or Syk activation into downstream signaling events following ligation of the pre-TCR, one might predict that a SLP-76 deficiency would result in a phenotype similar to ZAP-70/Syk-deficient mice. It is also possible that additional adapter-like proteins are present, which serve a role similar to that of SLP-76 during thymocyte development. The generation of SLP-76-deficient and transgenic strains will facilitate the determination of a more definitive role for SLP-76 in these processes.

The second round of selection in the thymus is thought to be dictated by the mature TCR complex and is required to eliminate potentially autoreactive T cell clones and perpetuate those thymocytes that express a TCR complex capable of conferring a protective role. Again, the involvement of Syk family tyrosine kinases in this process has been demonstrated by the observation that ZAP-70 deficiency in mice prevents maturation from a $CD4^+CD8^+$ phenotype to a mature $CD4^+$ or $CD8^+$ phenotype (34). The processes of negative and positive selection are thought to be mediated by the affinity of the TCR complex for self-MHC expressed on accessory cells in the thymus and the subsequent "strength" of the TCR signal initiated by this interaction (reviewed in Refs. 17 and 35). Those TCRs with a high affinity for self MHC mediate signals

that lead to apoptosis (negative selection) while those with an intermediate affinity promote survival (positive selection). Thymocytes that contain a TCR with low or no affinity for self-MHC are proposed to die by "neglect." As a result, relatively few thymocytes mature to a functional, immunocompetent T cell. Several markers, including CD3 and CD69, have been identified that correlate with the onset of positive selection (22). Interestingly, $CD3^+$ or $CD69^+$ $CD4^+CD8^+$ thymocytes express a higher level of SLP-76 than their $CD3^-$ or $CD69^-$ counterparts. Given the effect of TCR ligation on SLP-76 expression, it is possible that TCR ligation during selection augments SLP-76 expression. Alternatively, it may be necessary to maintain a low level of SLP-76 expression at the $CD3^{low}$ $CD4^+CD8^+$ stage of development to prevent the conversion of low affinity TCR interactions into high affinity signals. The low expression of SLP-76 in the majority of $CD4^+CD8^+$ thymocytes correlates well with the reduced signaling potential of additional TCR-coupled signaling molecules, including the transcription factors NF-AT and AP-1. Both NF-AT and AP-1 are inducible in $CD4^-CD8^-$ and mature $CD4^+$ or $CD8^+$ thymocytes, but inducible DNA-binding activity is lost in the $CD4^+CD8^+$ subset (36). While SLP-76 demonstrates the capacity to augment both NF-AT and AP-1 when overexpressed in Jurkat T cells, a causal relationship between low SLP-76 expression and reduced NF-AT and AP-1-binding activity in the $CD4^+CD8^+$ population remains to be determined.

While it is clear that TCR ligation augments SLP-76 expression, the actual mechanism by which SLP-76 protein expression is regulated during thymic development and following TCR-dependent activation is not known. We have made the observation that SLP-76 contains a single, amino-terminal proline-glutamic acid-serine-threonine rich (PEST) domain (amino acid residues 99–163), a motif that has been implicated in the metabolic stability of many proteins, including members of the I-$\kappa$B family and specific components of the cell cycle control machinery (37–40). Interestingly, the SLP-76 PEST domain contains the tyrosine residues implicated in Vav recruitment. It is possible that the rapid recruitment of Vav or additional molecules to SLP-76 following TCR ligation masks the PEST domain and stabilizes SLP-76 expression. The diffuse nature of SLP-76 expression in freshly isolated T cells and the rapid increase in SLP-76 levels following TCR ligation support the hypothesis that existing SLP-76 protein is stabilized following activation. Alternatively, increased SLP-76 expression following TCR ligation may be a function of increased mRNA production or transcript stability. Detailed kinetic studies of SLP-76 mRNA and protein expression following TCR ligation will be required to determine the mechanism by which SLP-76 expression is regulated.

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