Abstract

Intracellular signaling from the T cell receptor (TCR) is important in the development of immature T cells in the thymus before they migrate to the peripheral tissues as mature T cells. TCR-mediated signaling events continue to be crucial in the activation of mature T cells and in immune responses against foreign pathogens. In T cells, a cytosolic adapter protein, SLP-76 (SH2 domain-containing leukocyte phosphoprotein of 76 kDa), is critical in the assembly of the TCR signaling complex following T cell activation. Adaptors are signaling proteins that have no enzymatic or transcriptional activities. These proteins serve as scaffolds to nucleate multiple proteins in close proximity to form signaling complexes. SLP-76 contains four structural domains – three of the domains have been well-studied, but the function of the N-terminal sterile α-motif (SAM) domain at the N-terminus has yet to be characterized.

Unlike other frequently encountered protein domains, the SAM domain has not been associated with any unifying function. SAM domain-containing proteins bind to a wide variety of proteins and are involved in numerous biological processes. The SAM domain was deleted from SLP-76 in mice to probe its role in SLP-76. Thymocyte development in the mutant mice was partially blocked, leading to reduced output of mature T cells. TCR-mediated calcium mobilization was reduced in the thymocytes. Calcium mobilization, MAP kinase activation and IL-2 production were reduced in mature mutant T cells. These results suggest that the SAM domain in SLP-76 plays an important role in the TCR-mediated signaling that is necessary for thymocyte development and activation of mature T cells.
Introduction

T cells are a type of white blood cells that play an important role in cell-mediated immunity. T cells express membrane-bound T cell receptors (TCRs) on their cell surface to recognize antigens in the form of short peptides presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells, such as dendritic cells and macrophages (figure 1) (1). Antigen stimulation through the TCR will trigger downstream mechanisms such as cell proliferation and differentiation, cytokine production, and direct killing of the pathogen-infected cells (2).

Figure 1. Engagement of the TCR with peptide/MHC complex on antigen-presenting cells. Modified from http://library.med.utah.edu/kw/ms/mml/ms_etiology01.html

T cells originate in the bone marrow as hematopoietic stem cells that migrate to the thymus where they undergo differentiation (3, 4). These precursor cells undergo a series of arrangements of the TCR α and β gene fragments to generate a large diversity of antigen-specific TCRs that recognize a unique antigen (5, 6). However, amongst the large pool of immature thymocytes produced, only about 2% of them survive the stringent selection processes described below to become mature T cells that are released into peripheral tissues, such as the spleen and lymph nodes (7, 8).

The T cell population and the specific stages of their development can be identified by the presence of specific cell surface marker proteins. In the early stages, pre-T cells
express neither CD4 nor CD8 markers (9, 10). This population of cells is termed the double-negative or ‘DN’ cells. The DN thymocytes can be further subdivided into four subpopulations by surface expression of cell markers CD44 and CD25 (11, 12). Maturation within the DN population follows the sequence CD25−CD44+ → CD25+CD44+ → CD25−CD44− → CD25−CD44− (figure 2) (11, 12). At the CD25+CD44− DN3 stage, the TCRβ chain is first expressed in the thymocytes. TCRβ chain associates with the surrogate α chain and its accessory chains to form the pre-TCR complex (13). Signaling through the pre-TCR drives the development of these cells into CD4+CD8+ immature double positive (DP) T cells, during which the TCRα chain is expressed and replaces the surrogate α chain (14, 15).

**Figure 2. Maturation of the subsets of DN population.** The precursor T cells (yellow) develop following the sequence DN1 to DN4 (green), upregulating or downregulating the cell markers, CD44 and CD25. TCRβ chain is first expressed at the DN3 stage and the α chain is then expressed in the immature T cells (blue).

The DP immature T cells expressing functional TCRs will undergo selection processes (figure 3), after which they downregulate the expression of one of the cell surface markers, CD4 or CD8 (4, 16). The cells then differentiate to either CD4+ or CD8+ cells. These mature T cells then leave the thymus to the peripheral lymphoid organs such as the spleen and lymph nodes (figure 3) (16).
**Figure 3. Stages of T cell maturation.** Modified from Kuby, Immunology (W.H. Freeman), 2006. Pre-T cells from the bone marrow are DN cells (yellow). After rearrangement of TCR genes, immature DP cells (pink) express both CD4 and CD8 markers alongside with the functional TCR and associated CD3 chains (figure 5). CD4 and CD8 are also the co-receptors that are part of the TCR complex. After selection (figure 4), the cells mature into CD4+ or CD8+ T cell populations (red).

T cells are an integral part of the immune system to protect the host and therefore it is crucial that T cells are able to distinguish self from foreign antigens (16, 17). The selection processes in the thymus ensures that only T cells with TCRs that are able to recognize foreign peptides to survive and mature, while eliminating those which are reactive to self-antigens (4, 16, 17).

There are two selection processes – positive and negative selection – in thymocyte development (figure 4) (16). In positive selection, T cells which bind with low or intermediate affinity to peptides presented by an MHC molecule are protected from cell death (18, 19). This also ensures that the thymocyte receptors are capable of binding self-MHC molecules. Cells which fail to recognize self-MHC are eliminated by apoptosis (19, 20).

Following positive selection, the population of MHC-restricted thymocytes is subjected to negative selection (21). The cells express TCRs recognizing self antigens presented by self-MHC molecules with varying affinities from low to high (8, 16). Thymocytes with high-affinity receptors will be directed towards apoptosis, resulting in self-
tolerance, while the rest of the thymocytes are selected for expansion and differentiation towards mature T cells (16, 21).

**Figure 4. Positive and negative selection of DP T cells.** Modified from Kuby, Immunology (W.H. Freeman), 2006. The color schemes of the cells are as previous. The epithelial cells, macrophages and dendritic cells (colored blue) are antigen-presenting cells displaying the antigens on MHC molecules.

Active signaling from mature TCR initiated by engagement with an intrathymic ligand has been found to be essential in the selection processes of T cell development and mature T cell activation and proliferation (22, 23). As shown in figure 5, the αβ TCR contains the antigen binding site to recognize the peptide/MHC complex (8). However, these chains have very short cytoplasmic tails and are not able to associate with intracellular molecules alone to mediate downstream signal transduction (23). The αβ TCR associates with CD3 chains to form the TCR complex. CD3 is a complex of 5 polypeptide chains that
form 3 dimers, as shown in figure 5 (23). The CD3 chains have short extracellular regions
and long cytoplasmic chains containing immunoreceptor tyrosine-based activation motifs
(ITAMs) that are initiators of the downstream signal transduction (24).

Figure 5. T cell receptor with its associated CD3 chains. From Janeway, Immunobiology Fig. 6.8,
Garland Pub, 2001. The ITAMs on the associated CD3 chains (εδ and γε heterodimers and ζζ homodimer) are represented by the yellow bars. The horizontal black linkers between α and β or ζ and ζ subunits indicate the disulphide bonds between the subunits. The cell membrane is represented as a gray layer.

Figure 6 summarizes the TCR-initiated signal transduction cascades. Following TCR-
peptide/MHC engagement, Src kinase Lck is activated and phosphorylates tyrosine residues
within the ITAMs of CD3 chains (25, 26). Protein tyrosine kinase ZAP-70 is recruited to the
TCR via its interaction with ITAMs (25, 27). ZAP-70 is phosphorylated by Lck and in turn
phosphorylates LAT, a membrane adaptor protein (27-30). LAT interaction with Grb2/Sos
(an adaptor protein-enzyme complex) activates G-protein Ras which induces the Ras-Raf-
MEK-ERK MAP kinase cascade (31, 32), while LAT interaction with Gads and SLP-76, two
other adaptor proteins (33-38), activates Vav (a guanine nucleotide exchange factor) which
further activates the Rac/JNK and the p38 MAP kinase cascades (39-43). SLP-76, the focus
of my work (see below), also recruits tyrosine kinase Itk to the LAT complex, and the
complex binds to intracellular enzyme PLCγ1 (44, 45). Upon phosphorylation, PLCγ1
enzyme is activated and cleaves phosphatidylinositol biphosphosphate (PIP$_2$) into diacylglycerol (DAG) and second messenger inositol triphosphate (IP$_3$). IP$_3$ induces downstream calcium mobilization, while DAG activates enzyme protein kinase C (PKC) which initiates IκB kinase activation of transcription factor NFκB (46-48). Activation of three MAP kinase cascades, the calcium mobilization pathway and the IκB/ NFκB pathway leads to production of cytokine IL-2 which stimulates the survival, proliferation and differentiation of T cells (25, 49, 50).

Figure 6. Downstream TCR signaling pathways of SLP-76 and LAT. Modified from http://www.med.uni-magdeburg.de/fme/institute/iim/imd30302.html. TCR with its associated CD3 chains are shown at the top of the diagram. The vertical blue bars represent the ITAMs. Certain phosphorylation events are indicated by black circles with “P”. As shown, phosphorylated SLP-76 and LAT serve as major docking scaffolds for several downstream signaling molecules to form a large complex at the membrane. Dotted arrows represent signaling events. Perpendicular cross arrows from PIP$_2$ indicate cleavage of the molecule. Solid arrows at the bottom of the diagram represent the activation of IL-2 promotor to initiate IL-2 gene transcription. Details in text.
The molecule of interest in my thesis is SH2 domain-containing leukocyte-specific phosphoprotein of 76 kDa (SLP-76). SLP-76 is a cytosolic adaptor protein that is critical in the nucleation of the TCR signaling complex (29). Adaptor proteins are signaling proteins that have no enzymatic or transcriptional activities (25). These proteins serve as scaffolds to nucleate multiple proteins in close proximity to form signaling complexes (25).

Previous studies have shown that SLP-76 is essential for thymocyte development and mature T cell activation (51). SLP-76 deficient mice have a profound block in thymic development, leading to a lack of DP cells and mature SP T cells (30, 52-54). Jurkat T cell lines deficient in SLP-76 also exhibit defects in TCR-induced ERK activation, PLC\(\gamma_1\) phosphorylation, and calcium influx, resulting in a defect in IL-2 production (55). Other experiments have also shown that overexpression of SLP-76 results in increased TCR-mediated production of IL-2 (56, 57). SLP-76 is therefore speculated to act as a common downstream target for upstream TCR signals that are important to drive the development and expansion of DP thymocytes, as well as the activation of mature T cells to lead to their expansion and proliferation (3).

Four structural domains of SLP-76 have been identified (figure 7): (1) a sterile \(\alpha\)-motif (SAM) domain at the N-terminus; (2) an acidic region with three tyrosine residues within consensus motifs for SH2 (Src homology 2) binding with phosphorylated tyrosine residues in proteins (29, 45, 58). Phosphorylated SLP-76 binds to Vav and Itk in this region to activate the JNK and PLC\(\gamma_1\) downstream pathways (figure 6) (58-60); (3) a central proline-rich region that binds the SH3 (Src homology 3) domain of Gads (35, 55, 61, 62); and (4) a carboxy-terminal SH2 domain that interacts with adaptor protein SLAP, a negative regulator of TCR signaling and kinase HPK1 (63).
In B cells, the homologous cytosolic adaptor protein of SLP-76, named BLNK (or SLP-65), has been identified as a signaling element important in the nucleation of the B-cell receptor (BCR) signaling complex (64-66). BLNK has a strikingly similar domain structure as SLP-76 despite only 33% protein sequence homology (64, 66). Like SLP-76, BLNK contains an amino-terminal acidic region with five (instead of three) tyrosine phosphorylation motifs for SH2 binding, a central proline-rich domain and a carboxy-terminal SH2 domain (64, 66) (figure 7). The functions of the acidic SH2 domain, proline-rich region and the C-terminal SH2 domain have been studied in both SLP-76 and BLNK. However, the additional SAM domain at the N-terminus of SLP-76 (absent in BLNK) has yet to be characterized.

**Figure 7. Comparison of structural domains of SLP-76 and BLNK.** Modified from SLP76 and SLP65: complex regulation of signaling in lymphocytes and beyond, Gary A. Koretzky, Farhad Abtahian and Michael A. Silverman, Nature reviews, 2006. As shown, SLP-76 and BLNK share very similar domain structure, represented by the different colored regions (cyan, blue and green). SLP-76 has an additional SAM domain at the N-terminus (colored orange) that is absent in BLNK. See text for details.

The SAM domain is known to be a protein-protein interaction motif and it has been reported that there are over 1300 SAM-containing proteins (67). This number is comparable to that of the common SH2 domain (68, 69). However, unlike SH2 and SH3 (70, 71), no common function is known for SAM domains, as they have several binding properties (72). SAM domains have been found to interact with each other to form homodimers or polymers. They can also bind to other related SAM domains or non-SAM domain-containing proteins,
and have been also reported to bind to RNA (73-75). This versatility accounts for the wide range of roles the SAM domain plays in biological processes, from signal transduction to transcriptional and translational regulation (67, 72). For example, SAM domains can serve as kinase docking sites in the ETS family of transcription activators that are involved in cell growth control, differentiation and embryonic development, leading to the activation of the Ras/MAP kinase pathway (76, 77). The SAM domain is also found in several Drosophila Polycomb group proteins and their mammalian relatives (78, 79). These proteins are transcriptional repressors that are essential for restricting Hox gene expression along the anterior-posterior axis. An example is the Polyhomeotic (Ph) and Sex comb on midleg (Scm) gene, both containing C-terminal SAM domains (79, 80). The SAM domains of these two proteins interact with themselves, as well as with each other, each forming a polymer that has been shown to be crucial in transcriptional repression (80).

Given the diverse functions of the SAM domain, I was curious to investigate the function of the SAM domain in SLP-76 in mediating T cell development and mature T cell activation. To elucidate the role of the SAM domain of SLP-76, we generated knock-in mice homozygous for SLP-76 with the SAM domain deleted (SLP76<sup>m/m</sup>).

We showed that SLP76<sup>m/m</sup> mice exhibited a partial block in T-cell development. In the thymocytes, there was a decreased ratio of CD4<sup>+</sup>:CD8<sup>+</sup> thymocytes and significant reduction in the absolute thymocyte cell count compared to SLP76<sup>m/+</sup> mice. In the mature peripheral T cells, there was a corresponding decrease in the proportion of mature SP T cells in the spleen of SLP76<sup>m/m</sup> mice. TCR-mediated MAP kinase activation, calcium flux, and IL-2 production were decreased in mature T cells from SLP76<sup>m/m</sup> mice, suggesting that the SAM
domain in SLP-76 plays an important role in the TCR-mediated signaling that is crucial in T cell development and activation of mature T cells.

**Results**

*Generation of SLP\(\Delta\)SAM mutant mice*

To generate knock-in mice expressing SLP-76 lacking the SAM domain, the genomic fragment that contains exons 1 to 3 of the SLP-76 gene (encoding SAM amino acids 1-80 region of murine SLP-76) was replaced by a PGK-neo cassette flanked by two loxP sites in embryonic stem (ES) cells (figure 8). The Pgk-1 promoter was used to drive strong ubiquitous expression of neo (neomycin resistance gene) (81). For negative selection of random recombinants, the diphtheria toxin-A fragment (DTA) gene was added to the upstream of the homologous recombination sites. The DTA gene will be lost upon homologous recombination with the genomic fragment but may be maintained in the non-homologous recombinants (82, 83). When the neomycin analog G418 was added, cells which underwent random integration and contained the DTA gene would not survive the negative selection process.

The short arm and long arm areas indicate the homologous recombination sites to delete exons 1, 2 and 3, which encode the SAM domain. Successful homologous recombinant ES cells were injected into blastocysts and implanted into pseudopregnant female recipient mice to establish chimeric mice. The neo cassette, flanked by loxP sites, was subsequently removed by crossing the chimeric mice with \(\beta\)-actin-Cre transgenic mice, which expresses the Cre recombinase enzyme in all cells following the ubiquitous expression of the housekeeping gene \(\beta\)-actin. Heterozygous mice with deletion of exons 1, 2 and 3 (SLP76\(^{m/+}\)) were inbred to obtain mice homozygous for the SAM deletion in SLP-76.
SLP76<sup>m/m</sup> mice were generated at the expected Mendelian ratio and appeared healthy under specific pathogen-free conditions. The deletion of exons 1, 2 and 3 was confirmed by PCR and sequencing (data not shown).

**Figure 8. Cloning strategy for generating SLP76<sup>m/m</sup> knock-in mice.** Top panel shows the targeting construct with the mouse genomic fragment containing the SLP-76 gene. The symbols are as indicated in the legend. The two crosses 'X' indicate the homologous recombination sites at the short arm and long arm areas. Middle panel shows the successful homologous recombination event with exons 1, 2, and 3 replaced by the PGK-Neo cassette. Bottom panel shows the resulting genome fragment of the SLP76<sup>m/m</sup> mutant mice after crossing with the Cre transgenic mice to delete the cassette.

To confirm the deletion of the SAM domain in the SLP-76 protein in SLP76<sup>m/m</sup> mice, I analyzed the lysates of T cells by immunoblotting with an anti-SLP-76 antibody. As shown in figure 9, mutant SLP-76 indeed had a smaller molecular weight than wild-type SLP-76 on SDS-PAGE/Western analysis. Lysates of heterozygotes gave a double band showing both the wild-type and mutant copy of SLP-76.
Figure 9. Expression of SLP-76 in T cells from SLP76\(^{+/+}\), SLP76\(^{m/+}\) and SLP76\(^{m/m}\) mice. Cells were solubilized with RIPA lysis buffer and the extracted proteins were subjected to Western blot analysis with anti-SLP-76 antibodies. Calculated molecular weight of mutant SLP-76 should be 8.8 kiloDaltons (kD) smaller than wild-type SLP-76. The difference in protein size is indicated by the horizontal line markers at the sides.

**Analysis of Thymocytes in SLP76\(^{m/m}\) mice**

SLP76\(^{m/+}\) mice displayed a phenotype identical to wild-type mice. The thymi from SLP76\(^{m/+}\) mice did not show any significant differences in size and cellularity compared with SLP76\(^{+/+}\) mice. However, the thymi from SLP76\(^{m/m}\) mice showed a significant 30% decrease in thymocyte numbers from 1.07x10\(^8\) to 7.6x10\(^7\) cells.

**Partial Defect in Thymocyte Development**

To examine if the decrease in thymocyte numbers was a result of their defective development, thymocytes were isolated from SLP76\(^{m/+}\) and SLP76\(^{m/m}\) mice for flow cytometry analysis. Cells were stained with CD8 and CD4 fluorescent dyes to examine their expression levels and identify the various stages of the thymocyte development from DN → DP → SP stages. The analysis revealed that there was a partial defect in thymocyte development in SLP76\(^{m/m}\) mice, as the percentage of CD4\(^+\) cells decreased (7.39 to 4.96%) (figure 10, top panel). The thymocyte counts confirmed the decrease in CD4\(^+\) cell numbers, as shown below in figure 11. There was no significant difference in the CD8\(^+\) population.

To further determine if the defect in thymocyte development occurred at the early maturation stages from DN1 → DN4, we conducted further analysis on the DN subpopulations by examining the expression of cell surface markers CD44 and CD25. The results showed that the development of thymocytes in the DN stages was normal, as the
percentages of cells at each of the DN subsets were similar in the SLP76\textsuperscript{m/m} and SLP76\textsuperscript{m/+} thymocytes (figure 10, bottom panel). This suggests that the defect in T cell development in the SLP76\textsuperscript{m/m} mice occurred mainly at the DP to SP transition, where positive and negative selection takes place.

**Figure 10. Flow cytometric analysis of thymocytes in SLP76\textsuperscript{m/m} mice.** Direction of axes indicates increasing levels of cell marker expression. Numbers in quadrants represent percentages of cell populations. Top panel: Four quadrants boxed up in pink represent each of the four cell populations – clockwise from upper left: CD8\textsuperscript{+} SP cells, DP cells, CD4\textsuperscript{+} SP cells, DN immature T cells. There was a significant decrease in the CD4\textsuperscript{+} population in the SLP76\textsuperscript{m/m} thymocytes compared to the SLP76\textsuperscript{m/+} thymocytes. Bottom panel: Red arrows represent analysis on the DN population in the bottom left quadrant of the plots in top panel. Four quadrants represent each of the four DN subpopulations in developmental order – clockwise from upper left: DN1, DN2, DN3, DN4.
Figure 11. Thymocyte cell counts of CD4\(^+\) and CD8\(^+\) populations. The CD8\(^+\) cell numbers (left) were comparable in the SLP76\(^{m/+}\) mice (1.07 million cells) and the SLP76\(^{m/m}\) mice (0.829 million cells). The CD4\(^+\) cell numbers (right) were significantly lower in the SLP76\(^{m/m}\) mice (1.44 million cells) compared to the SLP76\(^{m/+}\) mice (3.88 million cells).

Normal αβTCR and CD3 cell surface expression

A defect of thymocyte development in the selection processes might be due to reduced cell surface expression of TCR and its associated CD3 chains, resulting in insufficient TCR-mediated signaling. To test this possibility, we examined the αβTCR and CD3 surface expression by staining thymocytes with antibodies specific for TCRβ and CD3 chains. Analysis of the DP, CD4\(^+\) and CD8\(^+\) SP cells showed that SLP76\(^{m/m}\) thymocytes expressed similar levels of TCRβ and CD3 as SLP76\(^{m/+}\) thymocytes (figure 12). This suggests that SLP76\(^{m/m}\) thymocytes had similar numbers of TCRs and the associated CD3 chains to receive activation signals, compared to SLP76\(^{m/+}\) thymocytes.
Figure 12. Expression of TCRβ and CD3 in SLP76<sup>m/m</sup> thymocytes by flow cytometry analysis. Direction of horizontal axes indicates increasing levels of TCRβ or CD3 respectively. Direction of vertical axes indicates number of cells. In all three populations analyzed, TCRβ (top panel) and CD3 (bottom panel) expression levels have overlapping graphs (shaded area and red line), indicating that expression levels are similar in SLP76<sup>m/m</sup> and SLP76<sup>m/+</sup> thymocytes.

Defective Calcium Mobilization

The SAM domain could be involved in mediating the downstream signaling events from SLP-76; therefore, a deletion of the SAM domain might result in the defect in thymocyte development in the selection processes. To probe this possibility, TCR-mediated calcium flux that is at the downstream of SLP-76 was analyzed in the thymocytes. The thymocytes were loaded with calcium indicator Indo-1 AM fluorescence dye and were then stimulated by adding anti-CD3 antibody. An anti-CD3 antibody is often used to cross-link TCR-CD3 chains to mimic engagement of MHC-antigen complexes (84). The cells were analyzed by flow cytometry, where the ratio of calcium-bound Indo-1 AM dye to calcium-free dye is a measure of the calcium concentration, as the calcium-bound dye corresponds to
a higher energy violet fluorescence (405nm wavelength) which is normalized to the calcium-free dye at a lower energy blue fluorescence (510nm wavelength). As shown in figure 13, anti-CD3-induced calcium flux was reduced in the DP, CD4$^+$ and CD8$^+$ SP thymocyte populations of the SLP76$^{m/m}$ mutant mice, suggesting that the SAM domain indeed plays an important role in mediating TCR downstream signaling. Defective signaling in the thymocytes may therefore result in a defect in the selection processes during their maturation.

Figure 13. Flow cytometric analysis of calcium flux of DP, CD4$^+$ and CD8$^+$ thymocytes following stimulation with anti-CD3 antibody. Directions of axes indicate increasing calcium concentration (measured by ratio of fluorescence intensity at 405nm to 510nm) and progression of time. Vertical arrows mark the time point when anti-CD3 was added to initiate TCR stimulation. The peak (shown in red) represented the wave of calcium release after signaling pathway activation. SLP76$^{m/m}$ thymocytes showed a significant decrease in calcium flux.
Analysis of Peripheral Mature T cells in SLP76^{m/m} mice

Corresponding decreased output of mature T cells from maturing thymocytes

The flow cytometry analysis of thymocytes revealed a partial defect in development, resulting in a decreased CD4\(^+\) population. As mentioned earlier, mature T cells migrate to the peripheral tissues after differentiation. Thus, we analyzed the mature T cell populations in the spleen and lymph nodes of SLP76^{m/m} mutant mice to examine if the defect in thymocyte development is reflected in these mature T cells. We isolated the peripheral T cells and stained them as above. The analysis revealed that the mature T cell populations in both the lymph node and the spleen in the SLP76^{m/m} mutant mice had a significantly reduced CD4\(^+\) population compared to that of the SLP76^{m/+} mice (figure 14). These results are consistent with the results obtained from thymocytes.

Normal \(\alpha\beta\) TCR and CD3 cell surface expression

We examined the \(\alpha\beta\) TCR and CD3 surface expression by staining mature T cells, pooled together from spleen and lymph nodes, with antibodies specific for TCR\(\beta\) and CD3 chains. Analysis of the peripheral mature T cells showed that SLP76^{m/m} T cells expressed similar levels of TCR\(\beta\) and CD3 as those of SLP76^{m/+} (data not shown). This data is consistent with previous data from the thymocytes.
Figure 14. Flow cytometric analysis of peripheral mature T cells from spleen and lymph nodes in SLP76<sup>m/m</sup> mice. Direction of axes indicates increasing levels of cell marker expression. Numbers in quadrants represent percentages of cell populations. Horizontal rectangles enclose the CD<sup>8+</sup> SP cells and vertical rectangles enclose the CD<sup>4+</sup> SP cells. Cell population in bottom left corner of figures represent non-T-cells such as B cells and dendritic cells and are irrelevant in this context. Note that the CD<sup>4+</sup> SP cell populations are significantly smaller in the SLP76<sup>m/m</sup> samples compared to SLP76<sup>m/+</sup>.

Normal upregulation of CD25 and CD69 early activation markers

Upon T cell activation via TCR stimulation, a characteristic downstream event is the upregulation of expression of T cell early activation markers on the cell surface. Two of these cell surface antigen proteins expressed are CD69 and CD25 (85-87). To investigate if the deletion of the SAM domain in SLP-76 affects the upregulation of these activation markers, CD<sup>4+</sup> T cells were isolated and purified from the lymph nodes of SLP76<sup>m/m</sup> and SLP76<sup>m/+</sup> mice and analyzed for the expression of these proteins following stimulation with anti-CD3. As shown in figure 15, T cells from both mice demonstrated normal upregulation of these
two markers with anti-CD3 stimulation at 6 hours, suggesting that this process is not affected by the deletion of the SAM domain of SLP-76.

Figure 15. Flow cytometric analysis of upregulation of early activation markers in CD4\(^+\) lymph node T cells at 6 hr after stimulation. Direction of horizontal axes indicates increasing levels of CD25 or CD69 respectively. Direction of vertical axes indicates number of cells. In unstimulated cells, there was no upregulation of either marker as shown by the shaded area. Stimulation using PMA and ionomycin (P+I) acts as a positive control, as indicated by the dotted blue lines. PMA and ionomycin are IP\(_3\) and DAG analogs and mimic their function to result in T cell stimulation which bypasses the TCR. Stimulation via the TCR using anti-CD3 antibody results in similar levels of CD25 and CD69 expression, as indicated by the red solid lines.

**Defective TCR-mediated Calcium Mobilization in mutant T cells**

As shown in figure 13, the deletion of the SAM domain of SLP-76 affected TCR-mediated calcium mobilization pathway that is downstream of SLP-76 in thymocytes, resulting in a defect in thymocyte development. TCR-mediated signaling is also important in the activation of mature T cells to drive their expansion and proliferation upon ligand engagement. Thus, we wanted to examine if TCR-mediated downstream signaling events were also affected by the deletion of the SAM domain in SLP-76. We analyzed the ability of SLP76\(^{m/m}\) lymph node T cells to mobilize calcium upon anti-CD3 stimulation, following the
same procedure as before. As shown in figure 16, anti-CD3-induced calcium flux was reduced in both the CD4\(^+\) and CD8\(^+\) SP mature T cell populations of the SLP76\(^{m/m}\) mutant mice, suggesting that the SAM domain also plays an important role in activating downstream calcium mobilization in the mature T cells.

![Figure 16. Calcium flux of CD4\(^+\) and CD8\(^+\) lymph node mature T cells following TCR stimulation with anti-CD3 treatment.](image)

**Defective Phosphorylation of Downstream Signaling Molecules**

The above results suggest that the deletion of the SAM domain of SLP-76 affects TCR-mediated signaling in mature peripheral T cells. To explore this further, we examined the phosphorylation of various TCR downstream signaling molecules (see outline in figure 6). We isolated lymph node T cells from SLP76\(^{m/m}\) and SLP76\(^{m/+}\) mice and expanded the T cell population *in vitro* to obtain sufficient numbers of T cells for biochemical studies. After activation with anti-CD3 for two days and expansion in IL-2 medium, the cells were then analyzed for their ability to activate various signaling pathways following TCR stimulation.
We analyzed TCR-mediated tyrosine phosphorylation by western blotting with phosphotyrosine antibodies. Figure 17a shows that there was some reduction in the tyrosine phosphorylation of cellular proteins of SLP76\textsuperscript{m/m} T cells upon TCR stimulation. Some of the protein bands were similar in intensity for both samples after stimulation (dashed arrows), while some bands in the SLP76\textsuperscript{m/m} samples appear to be weaker than the SLP76\textsuperscript{m/+} samples (asterisks). It is likely that the former represents the proteins upstream of SLP-76 and the phosphorylation of these proteins is not affected, while the latter are the proteins downstream of SLP-76 whose phosphorylation was affected by the deletion of SAM.

Immunoblotting of pPLC\textsubscript{γ1} (figure 17b), when adjusted for uneven loading, showed only a slight decrease in phosphorylated PLC\textsubscript{γ1} protein levels in SLP76\textsuperscript{m/m} T cells following TCR stimulation as compared to SLP76\textsuperscript{m/m} T cells. This result is consistent with the decrease in calcium flux as shown above, as pPLC\textsubscript{γ1} is directly upstream of the calcium mobilization pathway.

Immunoblotting of pERK and pp38 also showed significant decrease in ERK and p38 phosphorylation in SLP76\textsuperscript{m/m} mice (figure 17c, d), indicating that SAM is involved in the activation of these two MAP kinase signaling cascades downstream of SLP-76.
Figure 17. Western blot analysis of phosphorylation of downstream signaling molecules of SLP-76 in response to TCR engagement. (a) Analysis of protein tyrosine kinase phosphorylation activity using phosphotyrosine 4G10 antibody. Asterisks on the right of the figure indicate protein bands whose phosphorylation is reduced in SLP76\textsuperscript{mm} samples 1.5 minutes after stimulation (lanes 2 and 4). (b) Analysis of PLC\textsubscript{γ}1 phosphorylation level. Upper panel: In SLP76\textsuperscript{mm} cells, phosphorylation of PLC\textsubscript{γ}1 is slightly reduced (lane 2). Lower panel: Blotting with anti-PLC\textsubscript{γ}1 shows that more SLP76\textsuperscript{mm} lysates were loaded (lanes 3 and 4) than SLP76\textsuperscript{m/+} cells (lanes 1 and 2). Bottom graph shows normalized intensity of PLC\textsubscript{γ}1 phosphorylation band after dividing the intensities by the intensities of the corresponding loading control. (c), (d) Analysis of Erk and p38 phosphorylation levels. Upper panels: SLP76\textsuperscript{mm} T cells (lane 4) show weaker phosphorylation compared to SLP76\textsuperscript{m/+} T cells. Lower panels: Protein loading controls show that equal amounts of lysates were loaded.

Impaired IL-2 Production in SLP76\textsuperscript{mm} T cells

Activation of TCR-mediated signaling pathways leads to the production of cytokines, such as IL-2 (figure 6). As shown above, the calcium mobilization and MAP kinase pathways are affected by the deletion of the SAM domain. Therefore, we continued to investigate if the production of IL-2 is affected as well. To examine this, CD4\textsuperscript{+} lymph node T cells were purified from SLP76\textsuperscript{m/+} and SLP76\textsuperscript{mm} mice and stimulated with anti-CD3 and anti-CD28. IL-2 production was assayed by ELISA (enzyme-linked immunosorbent assay). As shown in
figure 18, SLP76<sup>m/m</sup> T cells showed significantly reduced IL-2 production as compared to that of SLP76<sup>m/+</sup>. In contrast, SLP76<sup>m/m</sup> CD4<sup>+</sup> T cells secreted IL-2 at levels comparable to that of SLP76<sup>m/+</sup> in response to PMA and ionomycin, which bypass TCR-mediated signaling. This result strongly suggests that the defective TCR-mediated signaling pathways due to the deletion of the SAM domain of SLP-76 also continue to affect IL-2 production.

![Figure 18](image-url) **Figure 18. Impaired IL-2 production in SLP76<sup>m/m</sup> CD4<sup>+</sup> lymph node T cells.** As indicated, cells were left unstimulated (negative control), stimulated with anti-CD3 and anti-CD28 or stimulated with PMA and ionomycin (P+I) (positive control). All cultures were tested in triplicates (error bars). Cytokine production was assayed by ELISA at 4h, 6h and 8h of culture. The brackets with the asterisks indicate that the difference between the amount of IL-2 produced by SLP76<sup>m/m</sup> and SLP76<sup>m/+</sup> cells is statistically significant. Unstimulated cells (left) show almost no IL-2 production. Stimulation with P+I (right) yields comparable levels of IL-2 production between SLP76<sup>m/m</sup> and SLP76<sup>m/+</sup> cells.
Discussion

Deletion of the SAM domain in SLP-76 resulted in a partial block in thymocyte development. In the thymocytes of SLP76<sup>m/m</sup> mice, there was a significant decrease in the CD4<sup>+</sup> SP population, decreased thymocyte cell counts and decreased TCR-mediated calcium mobilization compared to that of SLP76<sup>m/+</sup> mice. In the peripheral mature T cells of SLP76<sup>m/m</sup> mice, there was a corresponding decrease in the output of CD4<sup>+</sup> population and decreased T cell counts. Downstream TCR-mediated signaling pathways were also affected; there was decreased calcium mobilization, decreased phosphorylation of ERK and p38 in the MAP kinase pathways, and decreased IL-2 production. Altogether, these results suggest that the SAM domain in SLP-76 plays a crucial role in the selection processes of T cell development and TCR-mediated signaling in peripheral mature T cells.

The decreased output of mature CD4<sup>+</sup> SP T cells suggests that the deletion of SAM from SLP-76 results in defective positive selection processes during T cell development. The decision to downregulate either co-receptor to mature CD4<sup>+</sup> or CD8<sup>+</sup> SP T cells has been reported to depend on the strength of the activation signals that the thymocytes receive (88). CD4 co-receptors transduce stronger signals than the CD8 co-receptors. This accounts for the high CD4<sup>+</sup>: CD8<sup>+</sup> T cell ratio, as the development of CD8<sup>+</sup> T cells require a lower signal threshold of activation signals compared to the CD4<sup>+</sup> T cells to direct their commitment towards the respective lineages. The decreased CD4<sup>+</sup> cell population thus suggests that the TCR-mediated signaling events in SLP76<sup>m/m</sup> mice decrease in intensity.

The partial defect in ERK, p38 and PLCγ1/calcium mobilization pathways in SLP76<sup>m/m</sup> T cells suggests that there is a decrease in signal intensity downstream of SLP-76. How the SAM domain contributes to SLP-76 function is not clear and remains to be
determined. One possibility is that the SAM domain could be involved in the recruitment of SLP-76 to LAT or the membrane. SLP-76 associates with LAT in the signaling complex to initiate the downstream events. It is possible that SLP-76 interacts with the LAT adaptor protein via two ways: (i) via tethering of SLP-76 to LAT or the membrane via the SAM domain and (ii) via the adaptor protein Gads which is first recruited to LAT. Without the SAM domain, SLP-76 can still bind LAT although less efficiently. This hypothesis can be tested by conducting further experiments. Double-mutant mice of Gads and SAM of SLP-76 can be generated by crossing the current SLP76<sup>mim</sup> mice with Gads knockout mice. Previous studies have shown that Gads-deficient mice also exhibit an incomplete block in thymocyte development (89, 90). If SAM is indeed responsible for the tethering of SLP-76 to the membrane or LAT, there will be a complete disruption of signaling events downstream of SLP-76. As mentioned above, intracellular signaling from the TCR is crucial in thymocyte development in positive selection processes. Thus, it is expected that the mice will exhibit a complete block in thymocyte development and that the DP population will be absent.

The second possibility is that the SAM domain in SLP-76 mediates the binding of SLP-76 with other proteins in the signaling complex. The SAM domain has been shown to be involved in mediating protein-protein interactions (see introduction). I have previously tried GST pull-down assays in T cell lines using purified SAM domain fused with glutathione S-transferase (GST) to detect any potential binding partners of the SAM domain. However, the results did not reveal any proteins that bound to the SAM domain. One possibility of failing to detect any binding partners is that I had probed for phosphorylated proteins by immunoblotting with phosphotyrosine antibodies. The proteins interacting with the SAM domain in SLP-76 could have been proteins that are not phosphorylated and thus were not
detected. To investigate this possibility, metabolic labeling experiments can be conducted. J14 T cells (T cells with SLP-76 deleted) can be reconstituted with either wild-type SLP-76 protein or mutant SLP-76 with the SAM domain deleted. These cells can then be grown in radioactive $^{35}$S methionine or cysteine so that these amino acids will be incorporated into newly synthesized proteins. The proteins that are bound to SLP-76 in the signaling complex can then be co-immunoprecipitated and analyzed by SDS-PAGE. Autoradiography can be used to reveal all radioactive proteins. These proteins can be identified by mass spectrometry.

In the phosphotyrosine western blot shown in figure 17a, the lowest protein band with the asterisk has a size that corresponds to the LAT protein. This result is puzzling as phosphorylation of LAT is upstream of SLP-76 and should therefore be unaffected. However, the SLP76$^{m/m}$ T cells shows a decrease in protein phosphorylation compared to SLP76$^{m/+}$ T cells. One possibility of this decreased LAT phosphorylation could be due to a positive feedback loop downstream of SLP-76 that maintains the phosphorylation of LAT. With the deletion in the SAM domain, the partial disruption of downstream signaling events may have reduced the strength of the feedback loop, therefore leading to decreased LAT phosphorylation.

There is an apparent discrepancy between the decrease in ERK phosphorylation (figure 17c) and the upregulation of the early activation markers CD69 and CD25 (figure 15). The upregulation of these markers CD69 and CD25 has been reported to be dependent on the Ras/MEK/ERK pathway activation (91, 92). However, we observe that the upregulation of the activation markers seem to be normal in the SLP76$^{m/m}$ mice, although there is an obvious decrease in ERK phosphorylation. One reasonable explanation would be that the level of phosphorylated ERK required for the activation of the marker expressions is rather low. Thus,
the decrease in ERK phosphorylation does not lead to a defective regulation of these cell surface markers.

To fully characterize the role of the SAM domain of SLP-76, more analysis of the downstream signaling pathways from SLP-76 is required. In the ERK MAP kinase pathway (figure 6), I have only examined the phosphorylation of ERK. Phosphorylation of the other members of the ERK MAP kinase cascade should be analyzed. It is expected that expression of Grb2/Sos, Ras, Raf and MEK1/2 will be lower in SLP76\textsuperscript{m/m} T cells compared to that of SLP76\textsuperscript{m/+}, as these proteins are all downstream of SLP-76 activation. In the same way, only phosphorylation of p38 has been examined and other members of the cascade should be examined to confirm the result. The third MAP kinase pathway – Rac/JNK cascade – has not been examined. It is likely that there will be defective signaling in the JNK cascade as well.

The decrease in phosphorylation of PLC\textsubscript{γ1} expression in SLP76\textsuperscript{m/m} T cell lysates compared to that of SLP76\textsuperscript{m/+} was only evident after adjusting for the differential loading of protein between the SLP76\textsuperscript{m/m} and SLP76\textsuperscript{m/+} samples. The experiment should to be repeated to assess whether decreased PLC\textsubscript{γ1} phosphorylation is real. PLC\textsubscript{γ1} phosphorylation activates calcium mobilization which in turn activates NFAT. NFAT is a transcription factor which regulates IL-2 transcription, along with MAP kinase cascades. The activity of NFAT could be measured by performing an NFAT-luciferase reporter assay. The activity of NFAT in SLP76\textsuperscript{m/m} T cells is expected to be lower compared to that of SLP76\textsuperscript{m/+} T cells.

Another downstream pathway not previously mentioned is the Akt pathway. In addition to activating Rac to lead to the downstream JNK pathway (figure 6), activated Vav also activates PI3 kinase. A major target of PI3 kinase is another kinase Akt, which activates the IκB kinase/ NFκB transcription factor downstream pathway (figure 6) (93-95). This is a
common downstream pathway that is also activated by the phosphorylation of PLCγ1. Further experiments to assess the role of the SAM domain in SLP-76 in the downstream signaling pathways include investigating the phosphorylation of Akt and members of the IκB kinase pathway by western blotting, and the activity of NFκB.

Examination of events upstream of SLP-76 is also important to confirm that these signaling events are not affected in the SLP76<sup>−/−</sup> mice. Figure 17a showed that the phosphorylation of some proteins are unaffected by the SAM deletion and it is likely that these proteins are those upstream of SLP-76. Examples of such molecules include ZAP-70, LAT and Gads. However, immunoblotting with specific antibodies to these molecules should be done to confirm the results of figure 17a. In addition, the phosphorylation of SLP-76 itself upon stimulation should be similar as well. However, experiments conducted on ZAP-70 and SLP-76 phosphorylation thus far have been unsuccessful as the antibodies available for immunoblotting are weak and failed to detect these proteins.

Detection of IL-2 production by ELISA was conducted using CD4<sup>+</sup> primary lymph node T cells, which are T cells obtained directly from the mice without expansion of the cell population. On the other hand, the immunoblotting data, which required more cells to obtain detectable levels of proteins, were carried out using activated T cells. Although the behavior of primary T cells is known to be similar to activated T cells at large, some differences may be present. The IL-2 production assay should be repeated using activated T cells and using total CD4<sup>+</sup> and CD8<sup>+</sup> populations instead of the isolated populations. Likewise, the calcium mobilization assays were conducted using primary T cells. The experiments need to be repeated using activated T cells.
The biochemistry results will also have to be repeated to obtain data using the primary T cells. However, as the number of primary T cells numbers from each mouse is low, these experiments can only be performed when the mouse colonies are larger. Cells from several mice of each genotype can be pooled together to obtain detectable levels of proteins.

Furthermore, I can examine the proliferation of T cells using the thymidine incorporation assay (96) or CFSE (carboxyfluorescein succinimidyl ester) fluorochrome intracellular staining (97, 98). As my results suggest that there is a partial block in thymocyte development, especially in the DP to SP transition, I expect that there would be less proliferation in the SLP76m/m T cells compared to the SLP76m/+ T cells.

These preliminary results strongly suggest that the SAM domain in SLP-76 plays an important role in TCR-mediated signaling. Signaling events start to play an important role in the development of the thymocytes which, when impaired, led to the partial block in T cell development and in a reduced output of mature T cells. TCR-mediated signaling continues to be important in activating the mature T cells to stimulate their proliferation and effector functions, initiating an immune response. In the SLP76m/m mice, several downstream signaling pathways were affected. T cells are an integral part of our cell-mediated immunity that serves to defend our bodies from the wide range of pathogens. Therefore, characterizing the role of SAM domain in SLP-76 is important to enable us to gain a more complete understanding of T cell development and mature T cell activation. Moreover, understanding the function of the SAM domain in SLP-76 will add to our knowledge of the binding properties of this motif. This will lead us closer to identifying a unifying function of this commonly found domain.
Experimental Procedures

Mice

All mice were used in accordance with the National Institutes of Health guidelines. The experiments described above were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. Mice were housed in specific pathogen-free conditions at the Duke University Animal Care facility.

Expanding T cell population

To prepare activated T cells, lymph nodes were excised from the mice and the cells were cultured in CD3 antibody (2C11)-coated 6-well plates (3µg/ml) in the presence of murine IL-2 (10ng/ml) for 2 days. The cells were then transferred to flasks with complete RPMI 1640x growing media in the presence of IL-2 and expanded for another 4 days. Before cells were used, the activated T cells were rested in medium with IL-2 for 6 hours to return them to their inactivated states (99).

Western Blotting

Lymph node activated T cells from SLP76⁺/⁻ and SLP76⁺/⁺ mice were stained with anti-CD3-biotin (5µg/ml) and anti-CD28-biotin (2.5µg/ml) antibodies and then stimulated by crosslinking with streptavidin (40µg/ml) that binds to biotin molecule for 90 seconds. The cells were then lysed in 250µl RIPA lysis buffer on ice for 10 minutes. After centrifugation, the supernatant was then mixed with 2x SDS sample buffer for protein denaturation and as a loading buffer. Investigation of SLP-76 protein size was conducted using activated spleen cells from SLP76⁺/⁺, SLP76⁺/⁻ and SLP76⁺/⁺ mice. The cells were lysed directly in RIPA lysis buffer on ice without stimulation. The supernatant was mixed with 5x SDS sample buffer to obtain a more concentrated protein level. The samples were resolved by SDS-PAGE.
and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibodies 4G10 PY, anti-pPLCγ1, anti-PLCγ1, anti-pErk, anti-Erk2, anti-pp38 and anti-p38 (Cell signaling) (as indicated in figure 17) at 1:1000 dilution. After washing with PBST, membranes were then probed with secondary antibodies (1:5000 dilution), using either goat anti-mouse or anti-rabbit Ig conjugated with Alexa Fluor 680 (Molecular Probes) or IRDye 800 (Rockland). Membranes were then visualized with an infrared fluorescence imaging system (LI-COR Bioscience Odyssey system) (99, 100).

**FACS analysis**

Thymocytes, lymph node and spleen T cells were excised from mice and single cell suspensions were prepared according to Zhang et al. (1999). The cells were stained with the following antibodies: Texas-red - conjugated anti-CD8α, FITC-conjugated anti-CD4, phycoerythrin (PE)-conjugated anti-CD69, CD3 and CD25, APC-conjugated anti-CD44 and TCRβ and 7AAD (7-amino-actinomycin D). Standard flow cytometry was performed using a Becton Dickinson FACScan and FlowJo software. Investigation of the upregulation of CD25 and CD69 was conducted by incubating T cells either with medium alone, with anti-CD3 antibody (3µg/ml) or with PMA (40ng/ml) plus ionomycin (500ng/ml).

**Calcium Mobilization**

Thymocytes and lymph node T cells were first loaded with indo-1 in Ca2+ loading buffer (1x Hanks’ Balanced Salt Solution with 10mM HEPES and 1% FBS) for 30 min and then stained with FITC-conjugated anti-CD4, PE conjugated anti-CD8 and 7AAD (7-amino-actinomycin D). Ca2+ flux was assayed by monitoring the fluorescence emission ratio at 405/510nm with FACS. Calcium flux was initiated by addition of anti-CD3-biotin and anti-CD4-biotin or anti-CD8-biotin (1µg/ml) followed by crosslinking with strepavidin (25µg/ml) (99).
Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were either incubated with RPMI 1640 medium alone (unstimulated), with anti-CD3 and anti-CD28 antibodies, or with PMA (40ng/ml) plus ionomycin (500ng/ml). All cultures were stimulated in triplicates. Supernatents were collected at 4, 6, and 8 hours of culture and IL-2 cytokine production was assayed. Samples and IL-2 standards were incubated in a 96-well flat-bottom polyvinylchloride microtiter plate pre-coated with anti-IL-2 capture antibody (diluted 1:500) for two hours. Wells were washed with PBS and incubated with biotinylated anti-IL-2 detection antibody (diluted 1:2000) with peroxidase labeled streptavidin (diluted 1:1000) for one hour. The substrate buffer (TMB peroxidase EIA) was added and incubated until color development was sufficient before adding the stop solution (10% NaN₃) to halt the reaction. The color intensity was read by EIA reader (BioRad) (101).

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