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Understanding signal integration through targeted mutations of an adapter protein

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Abstract

Immunoreceptor engagement leads to the activation of multiple second messenger cascades, and integration of these pathways requires proper function of a number of adapter proteins. Although adapters possess no intrinsic enzymatic function, they nucleate the formation of multi-molecular protein complexes to support downstream signaling. Since adapters contain functionally distinct domains, intense investigation has been devoted to understanding how these regions act to integrate signals. This review describes the evolution of studies investigating one of these adapters, the SH2 domain-containing leukocyte phosphoprotein of 76 kDa. Through utilizing biochemical, genetic and imaging techniques, a model has emerged describing how this adapter regulates signals resulting in complex immune responses.

Keywords

signal transduction; immunoreceptor; adapter protein; SLP-76

Introduction

Since the discovery of the T cell receptor (TCR), investigators have sought to understand how external signals are translated to downstream events within the T cell. Stimulation of the TCR leads to the activation of Src family protein tyrosine kinases (PTKs) that initiate signaling cascades by phosphorylating immunoreceptor tyrosine-based activation motifs (ITAMs) within the TCR complex. Subsequent activation of the Syk family PTK ζ -associated protein of 70 kDa (ZAP-70) and the Tec family kinases inducible T cell kinase (ITK) and resting lymphocyte kinase (RLK) results in the phosphorylation of multiple proteins, leading eventually to the creation of an array of second messengers [1–3]. These signaling pathways must be integrated within the cell, allowing for the proper response to receptor engagement. However, as researchers first evaluated these signaling cascades, it was not obvious how this signal integration occurred.

Adapter proteins were soon identified to fill this role. Adapter proteins themselves have no intrinsic enzymatic activity but instead have intermolecular interaction domains that serve as molecular scaffolds, facilitating protein-protein and protein-lipid interactions in an

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appropriate spatial and temporal manner. Growth factor receptor-bound protein 2 (Grb2), the prototypic adapter protein, was identified in the early 1990s [4]. Grb2 was first found in *Caenorhabditis elegans* and *Drosophila melanogaster*, where it was shown to be composed of a single Src homology 2 (SH2) domain that binds phosphotyrosine residues, flanked by two Src homology 3 (SH3) domains that attach to proline-rich regions [4]. These discoveries were extended to mammalian systems, where it was shown that the Grb2 SH2 domain could inducibly bind the tyrosine phosphorylated epidermal growth factor receptor (EGFR) while bound constitutively to son of sevenless (SOS), a guanine nucleotide exchange factor for Ras. Localization of SOS to the membrane by the recruitment of Grb2 to the activated EGFR allowed for activation of Ras [4]. The identification of Grb2 and defining its role in coupling a PTK signal (the activated EGFR) to Ras signaling spurred an interest in identifying other adapter proteins as potential participants in the integration of cell signaling. One such lymphocyte protein was identified based on its inducible phosphorylation upon TCR stimulation and its ability to be “pulled out” of a cell lysate with a Grb2 fusion protein. The cDNA encoding SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) was cloned in 1995 [5] and has been shown to be present exclusively in hematopoietic lineages. Subsequent studies have revealed that SLP-76 is vital for hematopoietic cell development and function.

In the 15 years since the cloning of its cDNA, numerous laboratories have used varied approaches *in vitro* and *in vivo* to investigate the role of SLP-76 in coordinating immunoreceptor and integrin signaling in multiple cell lineages. Here, the techniques utilized to study SLP-76 and the knowledge that has been gained from these experimental approaches is reviewed.

SLP-76 is required for TCR signal transduction

Initial evidence for a possible role for SLP-76 in integrating T cell signaling came when cDNA encoding the adapter was transfected into the Jurkat human T cell line. Following TCR stimulation, overexpression of SLP-76 led to markedly enhanced induction of nuclear factor of activated T cells (NFAT) and IL-2 promoter activity [6], implicating SLP-76 in calcium-mediated signaling. Elevated SLP-76 expression also led to an increase in the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) activity and activation of the activator protein 1 (AP-1) transcription factor, suggesting a role for SLP-76 in Ras signaling [7]. While these gain-of-function studies were informative, demonstration of an absolute requirement for SLP-76 in TCR signaling required a complementary loss-of-function approach. In J14 cells, a mutant variant of Jurkat that had lost expression of SLP-76, TCR stimulation still led to PTK activation, but downstream TCR signals were lost, establishing that SLP-76 is essential for calcium and Ras signaling in T cells [8].

Given its domain structure characteristic of an adapter protein and that in Jurkat cells overexpression of SLP-76 only altered signals in the setting of TCR stimulation, it was suggested that SLP-76 function was likely regulated through modulation of the spectrum of proteins with which it interacted. Hence, an understanding of the molecular basis for SLP-76 function required an analysis of its partner proteins. Inspection of its primary structure showed that SLP-76 contains at least four recognizable protein-binding domains [3]: a sterile- α motif (SAM) domain, an amino-terminal acidic region with three conserved tyrosine residues (Y112, Y128, Y145 [numbering based on the murine sequence]) shown to be phosphorylated by ZAP-70 in activated T cells and in appropriate motifs to bind to other proteins with SH2 domains, a central proline-rich region appropriate for binding to SH3 domains, and a C-terminal SH2 domain. Initial studies, using biochemical approaches *in*

vitro (e.g. fusion protein pull downs) followed by co-immunoprecipitation studies within cells, were designed to identify the proteins that bound to each of these SLP-76 domains.

A number of proteins bind to SLP-76, either basally or inducibly following TCR engagement (Figure 1). The amino-terminal tyrosines of SLP-76 bind to three proteins known to be important for T cell activation [3]: the adapter molecule Nck (non-catalytic region of tyrosine kinase), the guanine nucleotide exchange factor Vav, and the Tec kinase ITK. Mapping studies showed that Vav [9] and Nck [10] bind cooperatively to tyrosines 112 and 128, both found in aspartic acid–tyrosine –glutamic acid– serine–proline (DYESP) motifs, while ITK binds to Y145 found within an aspartic acid– tyrosine–glutamic acid–proline-proline (DYEPP) motif [11]. Subsequent studies showed that for this binding to occur, the SLP-76 tyrosines require phosphorylation and the interactions occur through SH2 domains of the partner molecules. The proline-rich central region of SLP-76 was found to bind not to Grb2 but to its family member, Grb2-related adapter downstream of Shc (Gads) [12]. The Gads-binding site was originally defined as a 20 amino acid region, but was shown subsequently to require only an R-XX-K sequence within this larger region [13]. Initial studies failed to identify known proteins that bind to the SLP-76 SH2 domain. Therefore, this region of SLP-76 was used as probe to “pull out” potential associated molecules from lysates of activated T cells. This strategy identified a novel adapter protein, now known as adhesion and degranulation-promoting adapter protein (ADAP), which was shown also to bind to the SH2 domain of fyn [14,15]. Other SLP-76-associated proteins were then described, including phospholipase C γ 1 (PLC γ 1) [3] and Lck [16], two key effectors of TCR signaling that bound near the SLP-76 proline-rich region, and hematopoietic progenitor kinase 1 (HPK-1), a putative negative regulator of T cell activation that associates with the SLP-76 SH2 domain [17]. The negative regulatory effects of HPK1 are mediated by the recruitment of 14-3-3 proteins to a HPK-1 phosphorylated site on SLP-76 [18]. For some proteins, e.g. Gads, the rules for its association with SLP-76 are clearly defined [12,19]. For others, e.g. ITK [3,20] and PLC γ 1 [21,22], the binding to SLP-76 is more complex with multiple sites of interaction likely.

A Model for SLP-76 Function

Once the binding partners of SLP-76 were identified, investigators began to ask which if any of these interactions were critical for SLP-76 function. A structure/function analysis of SLP-76 was performed in Jurkat and J14 cells, where defined mutants of the adapter were transfected into the cell lines. Deletions of each of the protein-binding domains were constructed. Point mutations that prevented the association of phosphoproteins with the SLP-76 SH2 domain or that altered SLP-76 tyrosines to non-phosphorylatable phenylalanine were also created. In both Jurkat and J14 studies, each of these mutant SLP-76 proteins abrogated, at least partially, the effect on NFAT activation seen with wild type (WT) SLP-76, suggesting all three domains are required for optimal SLP-76 function [7,21]. It did appear, however, that there was a hierarchy of importance of these domains, as mutation of the three amino-terminal tyrosines (Y3F) had a profound effect on function, with mutation of the Gads binding site being less severe, and alteration of the SLP-76 SH2 domain being least disruptive. The importance of the three amino-terminal tyrosines led to studies where each was individually mutated, with the demonstration that Y145 seemed most critical for SLP-76 function [23,24].

A model for how SLP-76 functions to support TCR signaling (Figure 2) emerged from the results of the biochemical studies examining protein interactions and the functional analysis in Jurkat and J14 cells comparing the effectiveness of WT and the mutant variants of SLP-76. Not all of the reported SLP-76-associated proteins are incorporated into this model, as exactly when some of these interactions occur during T cell activation is not clear.

According to the model, in resting cells, SLP-76 resides in the cytosol, bound constitutively to Gads. Upon TCR engagement and activation of PTKs, the transmembrane adapter linker of activated T cells (LAT) becomes tyrosine phosphorylated, creating a docking site for Gads. Through the recruitment of Gads to phosphorylated LAT, SLP-76 is brought into this complex. PLC γ 1, which binds basally to SLP-76 and inducibly to phospho-LAT, becomes stably associated with its partner proteins and part of the complex. Because SLP-76 is also a substrate of ZAP-70, TCR stimulation results in phosphorylation of Y112, Y128, and Y145, allowing Nck, Vav, and ITK to bind these residues and positioning these proteins within the signaling complex. Finally, ADAP is recruited to the complex through its phosphorylation and association with the SLP-76 SH2 domain. ADAP recruits another set of adapter and effector molecules important for activation of integrins on the cell surface [25] (see legend to Figure 2).

This description of basal and inducible interactions provides a mechanism for key molecules critical for T cell activation to be brought to the right place in the cell so that enzymes and substrates and other effector molecules can interact appropriately. It also appears that the associations nucleated by SLP-76 and LAT are crucial for function of TCR-stimulated effectors. For example, while mutation of SLP-76 Y145 does not abrogate recruitment of ITK (ITK binds also to the proline-rich region of SLP-76 and to multiple other members of the activation complex), the interaction of SLP-76 Y145 with ITK appears critical for normal ITK function (see below) [26,27].

Additional support for this model of multimolecular complex formation nucleated by SLP-76 and LAT has come from elegant imaging studies of signaling complexes that form following TCR engagement. Using J14 cells, WT Jurkat cell lines, and primary cells expressing SLP-76 tagged with green fluorescence protein, it was shown using real time confocal and total internal reflection fluorescence (TIRF) imaging that SLP-76 forms microclusters along with LAT, Gads, ZAP-70, and the TCR [28–30]. Formation of these microclusters appears to be required for downstream signaling events leading to T cell activation. Examination of the behavior of SLP-76 mutants in these assays confirmed the results of the earlier biochemical studies describing the domains of the adapter critical for key intermolecular interactions.

Together, the biochemical and imaging studies performed in the Jurkat system yielded a wealth of information regarding basal and inducible interactions between SLP-76 and other proteins and provided the basis for understanding how SLP-76 may cooperate with other proteins to integrate signals downstream of the TCR. The limitation of Jurkat and other cell lines was well known of course, in that it is impossible to infer true biologic function from these model systems. Understanding the role of SLP-76 *in vivo* required extending the cell line work to murine systems, where expression of SLP-76 could be controlled genetically and the impact of altering SLP-76 on T cell function could be explored.

SLP-76 is essential for T cell development and function *in vivo*

The next phase of analysis of SLP-76 function came from the simultaneous description by two research groups of mice made deficient in SLP-76 through targeted disruption of the gene encoding the adapter protein [31,32]. In both strains of SLP-76 knock-out (KO) mice, no peripheral T cells could be found. The mice revealed a complete block in T cell development at the double negative (DN) 3 stage in the thymus as a result of the failure to transduce signals from the pre-TCR and resulting in a block in progression at the β -selection checkpoint. Thus, although developing thymocytes in the SLP-76-deficient animals undergo normal TCR gene rearrangement and receptor expression [33], the absence of SLP-76 precludes signaling by the receptor, leading to failed developmental progression. When the

DN3 block was bypassed by generating “floxed” SLP-76 mice, inducible deletion of the adapter in double positive (DP) thymocytes prevented both positive and negative selection with the developing thymocytes dying by “neglect” [34], again due to failed signaling when the mature TCR was appropriately engaged. Similarly, deletion of SLP-76 in the periphery after thymic development was complete rendered naïve T cells incapable of being activated through their TCRs [35]. Delayed deletion of SLP-76 until peripheral T cells appeared also allowed studies to be performed on mature T cells that were normal, except for SLP-76 deficiency. In addition to TCR signaling defects, these cells manifest markedly reduced integrin function, suggesting that this receptor system (that also utilizes Src and Syk family PTKs) depends on SLP-76 for integration of signaling pathways [36].

The profound T cell phenotype in the SLP-76 KO mouse provided an excellent model to examine SLP-76 structure/function *in vivo* through studies designed to ask what features of SLP-76 would be required for rescue in the T cell compartment. Experiments were performed using transgenic mice expressing WT or mutant SLP-76 under the control of a T cell-specific promoter crossed onto SLP-76-deficient mice [37–39]. WT SLP-76 expressed in this fashion completely rescued T cell development and peripheral T cell function. Consistent with the requirements of SLP-76 for TCR signaling in cell line systems, expressing the Y3F SLP-76 mutant (in which the three amino-terminal tyrosines are replaced with phenylalanines) as a transgene resulted in a poor rescue of T cell development, with the few SP thymocytes or mature T cells demonstrating severe TCR signaling defects [37]. The mutant of SLP-76 that fails to bind Gads (and hence cannot be recruited to the signaling microclusters) also showed severe signaling and functional defects, while expression of the SH2 domain mutant of SLP-76 resulted in the least severe phenotype [37]. In addition to these mutants that recapitulated the cell line studies, two other mutations of SLP-76 were expressed as transgenes in the T cell compartment, one in which the first 156 amino acids (including the three identified tyrosine phosphorylation sites) were deleted (Δ 156) and one in which the Lck-binding site (a 10 amino acid stretch near the Gads-binding site) was removed. Both of these mutations markedly abrogated SLP-76 function, with the Δ 156 mutation being even more severe than the Y3F mutant, suggesting that there are important functions beyond those served by the phosphorylatable tyrosines within the amino-terminus of SLP-76 [38] [39].

While much was learned about structural features of SLP-76 required for function through transgenic expression of WT or mutant variants in the T cell compartment, these experiments were limited by the fact the transgenes were not expressed under the control of endogenous elements. Hence, it was possible that altered function might represent mistiming or misexpression of the molecule. To obviate this concern, several strains of SLP-76 knock-in (KI) mice were generated. This approach also provided an opportunity to investigate the features of SLP-76 important for function in non-T cells (see below). Given the importance of the SLP-76 tyrosines for its function, the first knock-in strains reported targeted these residues with animals generated in which the tyrosines at positions 112/128 (the two tyrosines within the DYESP [Vav/nck binding] motifs) or position 145 (the tyrosine within the DYEPP [ITK binding] motif) were altered to phenylalanine [26]. These mice allowed for the evaluation of the contribution of each tyrosine unit to the development and function of T cells. Although overall thymic cellularity and subset distribution appeared grossly unaffected, detailed analysis of T cell development in these genomic KI mice demonstrated that both Y112/128 and Y145 tyrosine units were crucial for normal positive and negative selection during T cell development, with Y145F mice possessing more severe impairments in the majority of cases. These defects in selection were likely a result of poor TCR-induced phosphorylation of PLC γ 1 and ERK and diminished calcium mobilization in thymocytes of both tyrosine mutants [26]. Current work is evaluating the impact of mutation of the SLP-76 tyrosines on peripheral T cell function with early studies suggesting significant

abnormalities in effector function and development of functional memory cells in the setting of an *in vivo* immunologic challenge [40].

In addition to serving as a model for T cell development and peripheral T cell function, the genomic KI mice provided the opportunity to test in primary T cells the biochemistry of SLP-76-mediated signal transduction observed initially in Jurkat T cells. Unexpectedly, despite the proposed inducible binding of Vav to phosphorylated Y112/128 and Itk to phosphorylated Y145, these protein-protein associations remained intact in Y112/128F and Y145F thymocytes [26]. Further assessment of Vav1 and Itk subcellular localization in Y112/128F and Y145F T cells revealed no differences when compared to WT T cells (unpublished data). Closer examination of TCR-induced protein phosphorylation revealed tyrosines 112/128 and 145 were crucial for optimal phosphorylation of Vav1 and Itk, respectively [26]. Furthermore, although binding to Vav or ITK was not precluded, the SLP-76 Y112/128F mice closely phenocopy Vav-deficient animals, while the SLP-76 Y145F mice are very similar phenotypically to ITK-deficient mice [41,42]. These data suggest that although tyrosine phosphorylation of SLP-76 creates binding sites for the partner proteins identified, it is likely that a larger multimolecular complex exists that depends upon many separate intermolecular interactions. These data suggest further that SLP-76 and its association with other proteins is not only important for relocating molecules within the cell but also that the interactions mediated by SLP-76 are necessary for effector protein function. This notion was supported by the observation that TCR signaling and thymic selection is rescued in mice generated by crossing homozygous Y112/128F animals with homozygous Y145F mice [26]. Thus, expression of the two SLP-76 mutants in the same cell complements the defects of each expressed individually, suggesting the existence of large multimolecular complexes that mediate TCR signaling.

In addition to the tyrosine mutants, two other SLP-76 KI strains have been reported [43–45]. The first described the impact of deletion of the SAM domain with the finding that loss of this region of SLP-76 results in reduced cellularity in the thymus and secondary lymphoid organs, neither of which were observed in the tyrosine mutant mice [43]. Additional studies showed impaired positive and negative selection, DP to single positive (SP) transition, Erk activation, and Ca^{2+} mobilization. Interestingly, however, T cells from these mice show intact LAT and PLC- γ 1 phosphorylation, indicating that SLP-76 functions in other key signaling pathways [43]. Insights into the role of the SAM domain came from imaging studies in Jurkat T cells that revealed perturbation of SLP-76 microcluster formation in the absence of the SAM domain, implicating this region for regulation of SLP-76 localization within the cell [43]. Given the importance of the Gads-binding region of SLP-76 for its relocation following TCR activation, SAM KI mice were crossed with Gads-deficient animals. The double mutant animals exhibited a much more severe developmental block in thymopoiesis than either single mutant, suggesting non-redundant roles of these two regions of SLP-76 [43]. The precise role for the SLP-76 SAM domain in thymopoiesis and SLP-76 localization within the cell will require further studies.

The other SLP-76 KI mutant strain reported was designed specifically to alter SLP-76 localization within cells [44,45]. The rationale for creation of this mutant arose from the observation that recruitment of SLP-76 to activated LAT at the plasma membrane by Gads is a crucial step linking proximal to distal signaling events following TCR engagement [3]. Indeed, when SLP-76 cannot be localized properly at the plasma membrane, as in Gads-deficient mice, T cell development is impaired due to defective pre-TCR signaling [46]. Likewise, transgenic expression of a mutant SLP-76 protein missing the Gads-binding domain in SLP-76-deficient mice also results in defective thymopoiesis [37]. Over-expression of the Gads-binding fragment (GBF) of SLP-76 into WT bone marrow acts as dominant negative by preventing SLP-76 binding to Gads. Expression of the GBF precludes

development of mature T cells when the transduced bone marrow is transplanted into recipient mice [47]. It was therefore predicted that expression of a SLP-76 mutant that was constitutively targeted to the membrane, in particular to the region in which LAT resides, would have a gain-of-function effect on TCR signaling, obviating the need for inducible recruitment of SLP-76 to LAT.

To test this notion, a chimeric protein in which the membrane-targeting sequences of LAT were fused to full length SLP-76 (membrane-targeted SLP-76 [MTS]) was produced [44]. Initial studies were performed in WT Jurkat cells and J14 (SLP-76 deficient) and JCam2 (LAT) Jurkat mutants. As predicted, MTS was found in lipid rafts at the membrane, the site of LAT expression. Functionally, MTS expression in Jurkat cells resulted in the predicted enhanced TCR signaling. Expression of MTS in J14 or JCam2 rescued TCR signaling suggesting that, at least in the Jurkat system, a key function of LAT is to bring SLP-76 and its associated molecules to the TCR activation complex. To extend these studies *in vivo*, the LAT-targeting sequence was knocked-in to the SLP-76 locus, creating the MTS mouse [44]. It was predicted that the MTS would also rescue TCR signaling in SLP-76- and LAT-deficient mice.

It was therefore surprising when the MTS homozygous animals were first analyzed showing very few mature T cells [44]. Unlike the SLP-76-deficient phenotype with a complete block in T cell development at the DN3 stage in the thymus, the MTS mice showed their T cell developmental arrest at the double positive (DP) stage [44]. A logical explanation for this observation was that enhanced TCR signaling due to the MTS allowed for β selection to occur at the DN stage but resulted in nearly complete negative selection, blocking developmental progression in the T cell compartment. This idea proved not to be case, as MTS did not act in a dominant fashion over WT SLP-76 and as evaluation of the signaling potential of MTS DP thymocytes revealed decreased rather than increased TCR-mediated second messenger production [44]. A likely explanation for this observation was provided by studies of the subcellular localization of the MTS. Unlike what was found in Jurkat cells, studies of the primary thymocytes revealed that MTS is membrane localized; however, it appeared to be trapped in the cytosol [44]. These data suggested that pre-TCR signaling (at the DN3 stage) has a different requirement for SLP-76 localization than does signaling by the mature TCR at the DP stage. Further, these data reveal that while the Jurkat system has been extraordinarily valuable as a model to unravel the complexities of TCR signaling, it is not always predictive of what one finds *in vivo*.

Despite the substantial defect in thymic selection, there is a population of SP thymocytes and peripheral T cells in the MTS KI mice. Assessment of these cells revealed similar signal transduction defects as observed in MTS DP thymocytes [45]. Surprisingly, however, the MTS mice consistently developed autoantibodies and significant numbers of peripheral CD4⁺ T cells poised to produce the inflammatory cytokines interferon- γ and interleukin-17 (IL-17) [45]. This phenotype arose despite an intact and functional regulatory T cell compartment. To determine if the aberrant peripheral T cell function in the MTS mice was a result solely of thymic developmental abnormalities, the MTS mice were bred to floxed SLP-76 mice. Since the WT allele is functionally dominant over the MTS allele, delaying expression of Cre allowed for the appearance of normal peripheral T cells in which the WT SLP-76 molecule could be inducibly deleted (Figure 3). CD4⁺ T cells from these mice also exhibited phenotypic and functional abnormalities, albeit not as severe as seen in the unmanipulated MTS animals [45]. Hence, it appears that expression of the MTS allele during development contributes to abnormal peripheral T cell cytokine production, but the generation of these inflammatory T cells also results partially from the expression of the abnormal SLP-76 allele after development is complete. The specific biochemical signals leading to exuberant inflammatory cytokine responses in the MTS T cells remain

unidentified and are the subject of ongoing investigation. Since the current paradigm of T helper lineage choice focuses on the dominant role of cytokines, it will be of particular interest to identify TCR signals that may bias commitment to distinct T helper lineages, whether these signals are instructive or merely support the survival of selected lineages over others.

The role of SLP-76 in non-T cell hematopoietic lineages

Although SLP-76 was originally described as regulator of TCR signaling, early studies demonstrated expression of this adapter protein in all hematopoietic lineages except for mature B cells. While there were some suggestions of a potential role for SLP-76 in non-T cell lineages (for example demonstration of its tyrosine phosphorylation in the rat basophilic leukemia mast cell line [48]), there was little concrete evidence supporting an important function of this adapter in these cell types until the development of SLP-76-deficient mice allowed for their analysis. Initial evaluation of the SLP-76 KO mice focused largely on the T cell compartment, due to the dramatic T cell phenotype that was so easily observed. In contrast, non-T cell lineages, because their development does not require the same checkpoints to test receptor function, appeared in normal numbers in the SLP-76-deficient mice. Subsequent evaluation of these cell types, however, demonstrated that similar to what was observed in T cells, SLP-76 plays an essential role in their function.

Studies of mast cells, platelets, and neutrophils from SLP-76-deficient animals revealed that signaling by receptors that use ITAMs and Src and Syk family PTKs were markedly defective. Thus, *ex vivo* stimulation of the high affinity receptor for IgE on mast cells fails to elicit the described biochemical signals downstream of initial kinase activation, resulting in defective cytokine production and a complete failure of these cells to degranulate [49]. The *in vivo* correlate is absent IgE-mediated anaphylaxis of SLP-76-deficient mice [49]. Similarly, Fc receptor stimulation on neutrophils from SLP-76-deficient animals demonstrates significantly blunted calcium and nearly absent reactive oxygen intermediate response to Fc receptor engagement [50], and platelets from these animals fail to aggregate or degranulate when stimulated through the collagen GPVI receptor [51,52], which also utilizes the common γ chain of Fc receptors. Studies in these lineages, in particular platelets and neutrophils, have also confirmed the importance of SLP-76 for signals downstream of integrins, as adherence and spreading of these cells on surfaces coated with integrin ligands is virtually absent in the setting of SLP-76 deficiency [50–52]. Finally, defects in dendritic cell and more recently natural killer cell function have been reported, although less is known about the impact of SLP-76 deficiency in these lineages [53,54].

One of the most striking phenotypes associated with SLP-76 deficiency is also one of the most intriguing. Initial evaluation of SLP-76 KO animals revealed a selective loss of mutant mice during the perinatal period. Thus, while SLP-76-null fetuses were present in Mendelian ratios, it was extremely difficult to generate adult KO mice [31,32]. A clue to the cause of the death of these animals came from inspection of the SLP-76-deficient fetuses, which appeared grossly hemorrhagic by day 12 of gestation [51,55]. Subsequent studies revealed that the few mice that survived to adulthood demonstrated significant high output cardiac failure due to a vascular communication between blood vessels and lymphatics, presumably lowering peripheral vascular resistance [55]. Re-evaluation of the SLP-76-deficient fetuses revealed that while they appeared to have gross hemorrhage, microscopic analysis showed that the blood was all intravascular, but that there was a shunt between blood vessels and lymphatics [55]. The failure to find evidence for SLP-76 in vascular endothelium and reconstitution studies transferring SLP-76-deficient bone marrow to heavily irradiated WT recipient mice recapitulating the vascular defect in developing lymphatics demonstrated that the vascular shunt was caused by an abnormality in the hematopoietic system [55]. More

recent work has identified platelets as the likely causal cells, with signaling via the Clec-2 receptor functioning in a SLP-76-dependent manner being essential for normal separation of the vasculatures [56].

Because SLP-76 is critical for integration of signal transduction in multiple cell lineages, the null animals provide an excellent platform for structure/function studies designed to compare and contrast signaling requirements in various cell types. The basic approach taken by several laboratories, which allows a single animal to be used to study multiple cell types, has been to express WT or mutant forms of SLP-76 in SLP-76-deficient hematopoietic progenitors, generally through a viral transduction approach. These progenitors are then transplanted into irradiated host animals, whose reconstituted immune system will include SLP-76-deficient cells as well as cells successfully transduced with virally encoded SLP-76 variants. Analysis of the T cell compartment in these animals is the easiest, as developing T cells arising from progenitors that were not transduced (and hence do not express SLP-76) are arrested at the DN3 stage in the thymus. Thus, all SP or peripheral T cells represent a relatively homogenous population of SLP-76 reconstituted cells. In contrast, the study of non-T cells is a bit more complex, as peripheral populations will include a mixture of SLP-76 deficient cells and cells arising from progenitors that were successfully transduced. In this case, it is necessary to include a marker for viral transduction, generally a fluorochrome tag. The spectrum of studies that can be done on these populations is restricted to experiments that can distinguish between cells positive versus those negative for the transduction marker, for example protocols making use of imaging or flow cytometry approaches. Even with these limitations, the progenitor cell transduction and immune reconstitution strategy has been highly effective for analysis of features of SLP-76 most essential for different functions in the various lineages affected by SLP-76 deficiency. These studies have also provided insights into the how key signaling pathways are controlled in different immune cell types. In addition, this approach has proved valuable for screening mutants of SLP-76 that might be of particular interest before going on to the complex but ultimately more valuable step of generating mice with SLP-76 mutations knocked into the germline.

While many of the rules for how SLP-76 functions learned from studies in T cells were corroborated in studies of other lineages, there have been a number of exceptions that underscore the complexity of how signaling pathways are integrated in lineage-specific ways. For example, although both Y112/128 and Y145 appear necessary for the generation of appropriate biochemical signals downstream of integrin and collagen receptors and for *in vitro* platelet function, only Y145 is required for normal *in vivo* thrombus formation following vessel injury [57]. Even more surprising was the observation that unlike the situation in T cells where Y112/128 and Y145 cooperate *in trans* to support TCR signaling, efforts at genetic complementation demonstrated that these three tyrosines could only act *in cis* to mediate normal platelet functions [57]. A potential explanation for this distinction is that the TCR is associated with 10 ITAMs as part of the antigen-binding molecules complexed to the CD3 chains, whereas only two ITAMs are available on Fc γ receptor dimers to support platelet immunoreceptor signaling. The increased number of TCR-associated ITAMs may allow for the formation of larger signaling complexes in T cells, each containing many SLP-76 molecules that are able to signal in a cooperative fashion. Experiments testing this notion are underway and may provide new insights into why the TCR complex evolved to contain such a large number of signaling components.

Contrasting the function of MTS in T cells and platelets has also provided insight into the importance of adapter protein localization in ITAM-associated immunoreceptor signal transduction. In contrast to the unexpected phenotype of the MTS protein in T cell development and function, MTS-expressing platelets exhibited normal (or even enhanced)

GPVI signaling when compared to platelets expressing WT SLP-76, including normal activation of biochemical intermediates and intact spreading and degranulation [44]. These data demonstrate that even in well-conserved signaling pathways, differential use of signaling machinery in diverse cell types can affect the outcome of signal transduction. Moreover, as shown by the ability of MTS cells to successfully pass β -selection but fail to signal normally as SP thymocytes or mature T cells [44], it appears that the requirements for signaling downstream of similar receptors (in this case the pre-TCR versus mature TCR) may vary within the same cell type according to developmental stage.

Experiments performed with neutrophils and mast cells from Y112/128F and Y145F KI mice implicated the importance of the amino-terminal tyrosines of SLP-76 in the function of these hematopoietic lineages as well. Similar to the graded responses observed in T cells and platelets, in which the Y145F mutant typically possessed more severe impairments than the Y112/128F mutant, this functional hierarchy emerged consistently in myeloid lineage cells [58]. A prominent example of this was demonstrated in the K/B $\times$ N model of arthritis, an *in vivo* system whose pathology depends strongly on the actions of both mast cells and neutrophil [59]. Whereas WT mice developed fulminant joint disease, Y112/128F and Y145F KI mice received increasing degrees of protection from arthritis and joint inflammation [58]. In fact, Y145 is so critical to SLP-76-dependent myeloid cell function that the Y145F mice resembled SLP-76-deficient mice, in that they displayed no pathology upon arthritis induction. As with platelets and unlike the scenario in T cells, Y112/128F and Y145F did not complement genetically to restore either mast cell or neutrophil functions *in vitro* or *in vivo* [58]. Similar to platelets, mast cells and neutrophils depend on SLP-76 for signaling from ITAM-associated proteins that contain limited numbers of this signaling motif, suggesting further that the TCR, with its 10 ITAMs may be the outlier in how its signaling machinery is organized.

Tools for next generation of studies

Over the past 15 years, using a combination of biochemical, imaging, and genetic tools in cell lines and murine models, a great deal has been learned about the role played by SLP-76 in immunoreceptor and integrin signaling. Starting with the identification of this adapter protein as a substrate of the TCR stimulated PTKs, a series of investigations have taken a step wise approach discovering its importance first as an integrator of TCR signals, then more broadly of receptor signaling in multiple immune cell lineages. The initial studies in Jurkat cells suggested that SLP-76 might be an important positive mediator of TCR signals. The development of SLP-76 deficient mice with their complete absence of T cells beyond the DN3 stage in the thymus provided clear evidence of how essential a single adapter protein could be in coordinating signaling events. Subsequent studies, using advanced imaging approaches and novel genetically modified animals provided the data for a model of how SLP-76 modulates signaling events by nucleating a multimolecular complex including other adapter molecules and key effectors of signaling pathways. While the details of how SLP-76 functions is particular to the cell types in which it is expressed, many of the approaches employed by investigators studying this adapter and much of what has been learned from probing its biology has been generalizable to other receptor systems, both in immune and non-immune cell lineages. However, despite the wealth of knowledge gained from these studies, the complete mechanism by which SLP-76 (and other similar adapter protein) functions and the precise role these proteins play in regulating responses *in vivo* remain incompletely understood.

For future studies of SLP-76 to be more informative, it will be necessary to develop more powerful biochemical, imaging, and genetic tools. The ability to study signaling molecules in single cells is essential for the next generation of experiments as most gene transduction

approaches in primary cells are not 100% efficient. Hence, investigators typically must study mixed populations of cells. Assessing individual cells with a particular SLP-76 mutant within that mixed population is now possible using flow cytometric approaches to evaluate signaling events. Further development of these techniques will greatly facilitate assessing features of SLP-76 (or other key regulators of signal transduction) that are required for discrete signal transduction pathways. Combining these approaches with advances in imaging will make it possible to visualize the multiple interactions mediated by SLP-76 simultaneously in real time in living cells to determine which of these intermolecular associations are essential for particular biochemical consequences following receptor engagement.

A major issue that limits our ability to draw rigorous conclusions regarding the *in vivo* importance of SLP-76 in peripheral T cell responses using the “standard” genetic tools most easily available currently is the inability to separate developmental effects of signal disruption from effects in mature lineages. This is particularly problematic since precisely coordinated signals are required for normal β selection at the DN3 stage and for normal positive and negative selection at the DP stage of development. Thus, in experiments where mutant variants of SLP-76 are expressed either as KI alleles or as T cell specific transgenes, it is impossible to determine if effects seen in peripheral T cell populations are due to aberrant thymic selection or due instead to a primary defect in mature T cell function because of the alteration of SLP-76. Similarly, it is not always clear if an *in vivo* abnormality in the T cell compartment is cell intrinsic or due instead to the altered environment associated with global loss of SLP-76 or expression of a SLP-76 mutant in all tissues of the animal. To address these complexities, experimental approaches are required allowing for precise temporal and lineage specific control of WT versus mutant SLP-76 molecules.

The first generation of such approaches is now being employed. These experiments make use of floxed SLP-76 mice crossed with mice in which the SLP-76 gene has been replaced by a mutant knock-in allele. Progeny of these crosses are heterozygous for the mutant allele and heterozygous for a WT SLP-76 gene that can be excised at the discretion of the investigator [45]. The strategy is to allow these mice to develop a normal mature immune system, then excise the WT SLP-76 gene selectively in the lineage of interest (for instance, a particular T cell subset). For these studies to be informative, however, a number of criteria must be met. First, the mutant SLP-76 allele must be recessive when expressed with the floxed WT protein to avoid having expression of the mutant alter normal thymocyte development. Then, it is necessary to employ a Cre expression system that allows for complete deletion with lineage specificity. Finally, it is critical to begin experiments only after the WT SLP-76 protein, which had been translated before gene deletion occurs, is fully metabolized. While reagents are being developed to meet these criteria, absolute control over deletion of the WT floxed SLP-76 allele still represents a limitation for these studies. As tools develop for controlled gene deletion, it is anticipated that these same reagents will become available for rigorous control of gene expression. Hence, it should be possible soon to ask questions about what happens to cells that had developed in the setting of a SLP-76 mutant knock-in, when these cells now express WT SLP-76. It is possible, for example, that these cells may have selected a TCR with higher affinity for self, thus allowing for positive selection to occur in the presence of reduced signaling. Examining the impact of restoring TCR signaling capability with WT SLP-76 in these cells may provide new information not only about SLP-76, but also more generally about the impact of TCR signal strength on T cell development and TCR selection.

Thus, it is anticipated that as new tools become available, their application to studies of how SLP-76 regulates signaling events, both in T cells and in other immune cells, will provide new insights into the biology of this adapter protein. It is also anticipated that lessons

learned from these studies will be applicable to understanding of how other adapter proteins function, and more generally to how signaling networks are integrated in biological systems. The combination of new molecular, cell biologic and genetic approaches will soon open new areas of investigation that will enable investigators to modulate adapter protein function *in vivo* in ways that will hopefully teach us much about the basic biology of immune cell activation and suggest new ways that interventions can be designed for therapeutic advances.

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Figure 1. SLP-76 binding partners

SLP-76 contains four protein-binding domains: a SAM domain, an N-terminal acidic region with three conserved tyrosine residues (Y112, Y128, Y145), a central proline-rich region, and a C-terminal SH2 domain. The SH2 domains of Vav and Nck bind cooperatively to phosphorylated Y112 and Y128, both found in DYESP motifs, while the SH2 domain of ITK binds to phosphorylated Y145 found within a DYEPP motif. The proline-rich central region of SLP-76 associates constitutively with the SH3 domains of PLC γ 1, lck, and Gads and inducibly to 14-3-3 proteins at phosphorylated S (serine) 376. The SH2 domain of SLP-76 inducibly binds to either ADAP as well or HPK-1, both of which compete for SLP-76 binding. Note that some of these SLP-76 binding proteins have multiple sites of interaction with SLP-76 and several bind both directly to SLP-76 and indirectly through associations with other proteins.

Figure 2. A model for regulation of TCR signaling by SLP-76

In resting T cells, SLP-76 resides in the cytosol, bound constitutively to Gads. Upon TCR engagement and activation of *lck* and ZAP-70, LAT becomes tyrosine phosphorylated, creating a docking site for Gads. Gads is recruited to phosphorylated LAT, bringing SLP-76 to the activation complex. PLC γ 1, which binds basally to SLP-76 and inducibly to phospho-LAT, becomes stably associated with its partner proteins in this complex, leading to release of calcium and activation of NFAT and the production of diacylglycerol (DAG) resulting in Ras and PKC dependent signals. Because SLP-76 is also a substrate of ZAP-70, TCR stimulation results in phosphorylation of Y112, Y128, and Y145, allowing Nck, Vav, and ITK to bind these residues and positioning these proteins within the signaling complex. Finally, ADAP is recruited to the SLP-76 SH2 domain. ADAP is constitutively bound to 55-kDa src kinase-associated phosphoprotein (SKAP-55), which in turn is bound to Rap1-GTP interacting adapter molecule (RIAM). RIAM can recruit active Rap1 to the membrane, leading (by an unknown mechanism) to activation of integrins, including LFA-1, on the T cell surface (inside-out signaling).

Figure 3. Bypassing the effects of MTS in T cell development to study the function of peripheral T cells expressing the MTS protein

A. Schematic of the strategy for conditional deletion of SLP-76 that can be tracked on a single cell level. Conditional deletion is achieved through a combination of four genetic loci. One SLP-76 allele is comprised of a SLP-76 gene with LoxP sites flanking its third exon (which when deleted results in a SLP-76 null phenotype). The second SLP-76 allele is WT (to generate SLP-76 positive control cells), floxed SLP-76 (to generate cells with no residual SLP-76), or a knock-in mutation of SLP-76 (in this case the MTS). The third locus encodes a yellow fluorescent protein (YFP) reporter preceded by a floxed stop codon under the Rosa26 promoter and the fourth locus controls deletion using an estrogen responsive Cre recombinase under the human ubiquitin C promoter. **B.** Peripheral T cells, bearing the MTS/floxed SLP-76 or the control WT SLP-76/floxed SLP-76 genotype, are allowed to develop in the thymus with WT SLP-76 protein, which is functionally dominant over the MTS protein. In the periphery, T cells are generated in which WT SLP-76 protein is expressed prior to genetic manipulation. Tamoxifen treatment of mice with T cells bearing one floxed SLP-76 allele and an MTS allele results in the generation of T cells expressing only the MTS protein and the YFP reporter, which allows for single cell tracking and/or manipulation. T cells from control mice (with one floxed SLP-76 allele and one WT SLP-76 allele) express WT SLP-76 protein and the YFP reporter after tamoxifen treatment.