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Cooperation of adapter molecules in proximal signaling cascades during allergic inflammation

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Summary: Activation of mast cells through their high-affinity immunoglobulin E receptor (FcεRI) plays an important role in allergic disorders. Other mast cell-activating stimuli, such as Toll-like receptor (TLR) ligands, synergize with FcεRI to enhance allergic inflammation. Thus, there is much interest in understanding how signaling occurs downstream of these receptors. One key event for FcεRI-mediated mast cell activation is the inducible formation of multimolecular proximal signaling complexes. These complexes are nucleated by adapter proteins, scaffolds that localize various signaling molecules through their multiple molecule-binding domains. Here we review recent findings in proximal signaling cascades with an emphasis on how adapter molecules cooperate with each other to generate an optimal signal in mast cells, and we discuss how signals crosstalk between FcεRI and TLRs in enhancing mast cell activation. Deciphering the molecular mechanisms leading to mast cell activation will hopefully bring new ideas for the development of novel therapeutics to control allergic diseases.

Keywords: mast cell, FcεRI, allergy, cell signaling, adapter molecules, TLR

Introduction

Mast cells are innate immune cells that are major effectors in allergic inflammation. Mast cells are derived from bone marrow-resident precursors but do not undergo full development in this compartment. Rather, they leave the bone marrow as immature precursors and reach final maturation in target tissues, including the connective tissue underlying the skin, submucosal linings of respiratory and gastrointestinal organs, and the peritoneal cavity. Due to this strategic localization at the host–environment interface, mast cells are often considered ‘sentinels of the immune system’, as they are one of the first cell types to react against external threats encountered from the environment (1, 2). Mast cells immediately respond to certain pathogens, especially parasites (3); however, parasitic infections have become exceedingly rare in developed countries. The ability of mast cells to respond quickly to environmental antigens is now often considered deleterious, as their activation leads to allergic symptomatology.

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The current epidemic of allergic responses, ranging from inconveniences to life-threatening disorders, makes a complete understanding of how mast cells respond to their triggers important not only for an appreciation of the basic biology of this cell type but also for potential clinical applicability.

The principal receptor in allergic inflammation is the high-affinity immunoglobulin E (IgE)-binding receptor (Fc ϵ RI). Expression of Fc ϵ RI is limited and is primarily found at high levels on mast cells and basophils. Fc ϵ RI binds reversibly but strongly to the Fc portion of monomeric IgE molecules (4), displaying the Fab portions for antigen recognition. Upon encounter with cognate antigen, two or more of the IgE-bound Fc ϵ RI are crosslinked, leading to the transduction of an intracellular signaling cascade that eventually results in the release of inflammatory mediators.

Mast cell activation can be divided temporally into three distinct phases. The first, occurring on the order of seconds to minutes, involves the rapid release of preformed mediators stored in granules, a process known as degranulation. Release of vasoactive substances such as histamine and serotonin stored in these granules causes increased vascular permeability, which allows the flow of inflammatory mediators and cells into the antigen-encountered site. A second slightly more delayed phase involves the formation of arachidonic acid metabolites, which in conjunction with a third protracted phase of mast cell activation involving the *de novo* production of chemokines and cytokines further recruit inflammatory cells. Cytokines produced by the mast cells can activate other innate immune cells and influence adaptive immune responses (5). These mediators cause the acute and chronic symptoms of allergic reactions such as erythema, pruritis, bronchiolar constriction, and shock. Thus, understanding the signaling mechanisms underlying mast cell activation through Fc ϵ RI is vital for developing therapeutics with the potential to ameliorate the earliest steps that lead to the symptoms of allergic responses.

Although Fc ϵ RI is most critical to mast cell function in allergy, mast cells express many other receptors relevant to allergic inflammation, including Toll-like receptors (TLRs), complement receptors, integrins, adenosine receptors, and cytokine receptors (6). Co-engagement of these receptors can influence the outcome of Fc ϵ RI activation and play an important role in modulating the mast cell response to allergens. In this review, we discuss the requirements for the formation of a proximal signaling complex for optimal mast cell activation after Fc ϵ RI engagement and the crosstalk and synergism that occur between Fc ϵ RI and TLR signaling pathways.

The Fc ϵ RI complex

Fc ϵ RI is a multimeric receptor composed of an α , β , and two disulfide-bonded γ chains (7, 8). The α chain contains an extracellular portion that binds IgE molecules (9), while the β and γ chains are involved in signal transduction through this receptor (10, 11). Fc ϵ RI α is expressed by many immune cell types including neutrophils (12), eosinophils (13), and dendritic cells (14), but the expression of Fc ϵ RI β is restricted to mast cells and basophils. Expression of the complete Fc ϵ RI complex is found only on mast cells and basophils in mice, as the Fc ϵ RI complex is not transported to the cell surface without the Fc ϵ RI β chain (15, 16). In humans, however, the trimeric form composed of one α and two γ chains can be expressed on the cell surface, albeit at much lower levels, as the extracellular portion of the human Fc ϵ RI α chain allows for some transport of the Fc ϵ RI trimer to the cell surface without Fc ϵ RI β (17). Thus, Fc ϵ RI expression is found on a wider variety of cell types in humans. However, Fc ϵ RI β not only increases Fc ϵ RI receptor density but also amplifies the Fc ϵ RI signal transduction pathway itself. Compared with transfected cells that express all components of the Fc ϵ RI complex, cells lacking Fc ϵ RI β transduce signal intensities of at least fivefold less, given similar Fc ϵ RI receptor densities (18). The nature of signals transduced in cells expressing the trimeric and tetrameric forms of Fc ϵ RI is likely to be different. The most important component of the Fc ϵ RI complex in signal transduction is Fc ϵ RI γ , as Fc ϵ RI β cannot transduce a signal alone without a functional Fc ϵ RI γ (10).

The aggregation of multiple Fc ϵ RI complexes by IgE/cognate antigen complexes results in transphosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) of the β and γ chains by Lyn, an src family protein tyrosine kinase (PTK) that is constitutively associated with the β chain (19, 20). Phosphorylation of the β chain recruits additional Lyn, while the phosphorylated γ chains serve as docking sites for spleen tyrosine kinase (Syk) (20–22). The unique ITAM structure of the β chain with an extra inserted tyrosine makes it unsuitable to bind to the src homology 2 (SH2) domains of Syk and thus explains why the β chain alone is not sufficient to initiate signal transduction (20). Localization of Syk to the Fc ϵ RI complex by the γ chain allows phosphorylation and activation of Syk by Lyn. Thus, the β chain amplifies signal transduction through Fc ϵ RI by enhancing phosphorylation of the β and γ ITAM and Syk (18). The single most important PTK in mast cell Fc ϵ RI activation appears to be Syk, as Syk-deficient mast cells fail to degranulate or release leukotrienes or cytokines upon Fc ϵ RI crosslinking (23, 24). The phosphorylation

of downstream signaling molecules such as phospholipase C γ (PLC γ) and Vav are completely absent in Syk-deficient mast cells. By contrast, the phosphorylation of Lyn, which lies upstream of Syk, is intact in Syk-deficient mast cells.

Assembly of a proximal signaling complex through adapter molecules

Following PTK activation, engagement of Fc ϵ RI results in the assembly of a multimolecular signaling complex. Once Syk is localized to the γ chains of Fc ϵ RI, it phosphorylates a number of adapter molecules that are important for full mast cell activation. Adapter molecules are proteins that have no enzymatic function. Yet, they are a vital component of signal transduction, as they colocalize key signaling molecules in both a temporal and spatial manner, thereby increasing the likelihood of molecular interactions that would otherwise occur only rarely. For example, membrane-resident adapter proteins can localize cytosolic proteins to the cell surface and thus enable the interaction of cytosolic proteins with the most proximal components of the signaling cascade. Following Fc ϵ RI activation in mast cells, one of the first adapter molecules phosphorylated by Syk is the membrane-localized adapter protein known as linker for activation of T cells (LAT). Four tyrosine residues on LAT that are inducibly phosphorylated (25–27) act as attachment sites for Grb2/son of sevenless (SOS), PLC γ , and Grb2-related adapter downstream of Shc (GADS) (27, 28). GADS is constitutively bound to another key cytosolic adapter protein, src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa (SLP-76) (29–31). SLP-76 is drawn to this multimolecular signaling complex at the plasma membrane (32), which then permits Syk to phosphorylate the SLP-76 N-terminal tyrosines (33). These SLP-76 phosphotyrosines then become binding sites for the activation of Vav, Nck, and tec family PTKs such as Bruton's tyrosine kinase (Btk) (34–36). Activation and colocalization of Vav and Nck by SLP-76 contribute to actin polymerization pathways, a process important in mast cell granule exocytosis, by activating a series of proteins including Cdc42, p21-activated kinase (PAK), and Wiskott–Aldrich syndrome protein (WASP) (37, 38). Btk is important for the activation of PLC γ (39, 40) and also mediates the activation of mitogen-activated protein kinases (MAPKs), such as s-Jun N-terminal kinase 1 (JNK1), JNK2, and, to a lesser extent, p38 (41), ultimately leading to the transcription of cytokines. Accordingly, mast cells lacking SLP-76 or LAT display significantly reduced Fc ϵ RI-mediated phosphorylation of MAPKs and PLC γ , which manifests as defects in degranulation and cytokine/chemokine production (26, 27, 33, 42, 43).

Another key function of the SLP-76/LAT molecular complex is to stabilize PLC γ at the plasma membrane. PLC γ possesses independent binding sites for both SLP-76 and LAT and therefore is cooperatively localized by these two adapters (29, 44–46). Membrane localization allows for phosphorylation and activation of PLC γ by Btk (39, 40) and/or Syk (47). Activated PLC γ converts the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol triphosphate (IP₃) and diacylglycerol (DAG), which results in a rise in intracellular Ca²⁺ (48), and activation of protein kinase C (PKC) and Ras guanyl nucleotide-releasing protein (RasGRP) (49, 50). Together, these second messengers contribute to both degranulation and cytokine production by mast cells. Mast cells express two isoforms of PLC γ , PLC γ 1 and PLC γ 2 (51). Both isoforms are phosphorylated following Fc ϵ RI crosslinking. Although PLC γ 2-deficient mice display reduced Ca²⁺ flux and degranulation (52), residual Ca²⁺ flux is still observed, which is probably mediated by PLC γ 1. Testing the contribution of PLC γ 1 to mast cell activation has been hampered by the early embryonic lethality of PLC γ 1-deficient mice (53).

Given that LAT localizes SLP-76 to the plasma membrane, it is conceivable that the requirement for LAT could be bypassed by forced localization of SLP-76 to where LAT resides. LAT is localized to plasma membrane lipid rafts, due to its transmembrane domain and two palmitoylated cysteines in its membrane-proximal cytoplasmic domain. To constitutively localize SLP-76 to lipid rafts of the plasma membrane, we generated a fusion protein between the transmembrane domains of LAT and full-length SLP-76 [membrane-targeted SLP-76 (MTS)] (46, 54). MTS was successfully targeted to the site of LAT localization in Jurkat T cells and was capable of rescuing T-cell receptor (TCR) signaling in a SLP-76-deficient Jurkat variant. Moreover, MTS rescued TCR-mediated signaling in LAT-deficient Jurkat T cells, further supporting the notion that a key role for LAT was to recruit SLP-76 to a specialized region within the cell (54). We wondered whether the MTS would function similarly in mast cells. We studied bone marrow-derived mast cells (BMMCs) obtained from mice in which the SLP-76 gene was replaced by sequence encoding the MTS (MTS knock-in mice) (55). Although MTS/MTS BMMCs developed normally and expressed equivalent levels of Fc ϵ RI and KIT compared with wildtype (WT) BMMCs (data not shown), MTS/MTS BMMCs displayed significantly defective degranulation, monocyte chemotactic protein-1 (MCP-1), and interleukin-6 (IL-6) production (Fig. 1A–C). Despite normal numbers of mast cells in the skin (Fig. 1D), MTS/MTS mice exhibited significantly reduced passive systemic anaphylaxis (PSA) responses (Fig. 1E). To

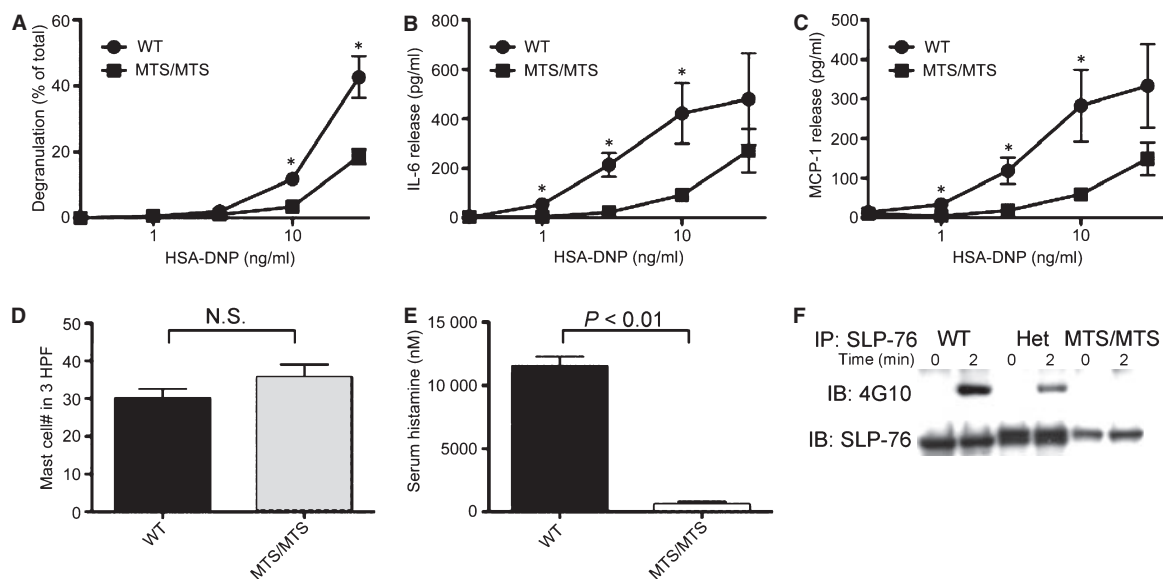


Fig. 1. BMMCs from MTS/MTS mice exhibit defective function downstream of Fc ϵ RI. BMMCs were generated from WT (circles) and MTS/MTS (squares) mice as previously described. BMMCs were sensitized with anti-DNP IgE (1 μ g/ml) and stimulated with various concentrations of HSA-DNP for 1 h (degranulation) or 24 h (IL-6/MCP-1). (A) Degranulation (β hexosaminidase activity), (B) IL-6, and (C) MCP-1 release were measured using a colorimetric enzymatic assay or using ELISA. Data are represented as mean $\pm$ SD generated from BMMCs of three individual mice per genotype. (D) Total mast cell numbers from three hpfs were counted in the ear skin of WT and MTS/MTS mice that were fixed and stained with toluidine blue. Data are represented as mean $\pm$ SD ($N = 3$ mice per genotype). (E) WT or MTS/MTS mice were injected with anti-DNP IgE (5 μ g per mouse) intravenously, followed by an intravenous injection with HSA-DNP (100 μ g per mouse) 24 h later. Ninety seconds later, the mice were bled, and serum histamine content was measured using an immunoassay. Data are presented as mean $\pm$ SD ($N = 3$ mice per genotype). (F) Cell lysates were prepared from anti-DNP IgE-sensitized HSA-DNP-stimulated BMMCs from WT, MTS heterozygous, and MTS/MTS mice and immunoprecipitated with anti-SLP-76 antibody. The proteins were resolved using SDS-PAGE and blotted for phosphotyrosine (4G10) or SLP-76. Note the slower migrating band (apparent as a double band in MTS heterozygous BMMCs) of MTS compared with WT SLP-76 due to the addition of the LAT transmembrane domain. BMMC, bone marrow-derived mast cell; hpf, high-power field; MTS, membrane-targeted SLP-76; WT, wildtype; IgE, immunoglobulin E; HSA-DNP, dinitrophenol-conjugated human serum albumin; IL-6, interleukin-6; MCP, monocyte chemoattractant protein; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LAT, linker for activation of T cells.

investigate the biochemical basis of the functional defect in these cells, the phosphorylation status of SLP-76 was examined. Although SLP-76 was phosphorylated in WT BMMCs following Fc ϵ RI-mediated stimulation, no SLP-76 phosphorylation was observed in MTS/MTS BMMCs (Fig. 1F). These results were surprising given that MTS fully rescued SLP-76-deficient Jurkat T cells. However, unlike Jurkat T cells, we recently found that MTS in MTS/MTS mice failed to colocalize with LAT, and although MTS partitioned with the membrane fraction, it was found to be mainly associated with internal membrane structures (55). Thus, proper localization of SLP-76 to LAT appears crucial for its phosphorylation and function. These results further emphasize the importance of the cooperation of adapter molecules in mediating their effector functions.

Functional domains of SLP-76 in Fc ϵ RI-mediated mast cell activation

SLP-76 is critical to Fc ϵ RI-mediated mast cell activation, as mast cells lacking SLP-76 demonstrate a near-complete failure

in degranulation and cytokine/chemokine production after Fc ϵ RI-mediated stimulation (33, 42, 43). Moreover, despite normal development and distribution of mast cells *in vivo*, SLP-76-deficient mice are unable to mount an IgE-dependent PSA response (33). SLP-76 can be divided into four functional domains: an N-terminal acidic domain, a sterile α motif (SAM) domain, a central proline-rich domain, and a C-terminal SH2 domain. All domains of SLP-76 except for the SAM domain have been shown to be important for mast cell function to varying degrees in studies using mast cells expressing mutant variants of SLP-76.

The SH2 domain of SLP-76

The SLP-76 SH2 domain appears least contributory to Fc ϵ RI-mediated mast cell function. This region of SLP-76 inducibly associates with adhesion- and degranulation-promoting adapter protein (ADAP) and the serine-threonine kinase hematopoietic progenitor kinase 1 (HPK1) (35, 56) through tyrosine phosphorylation of the SLP-76 partner molecule. Consequently, a point mutation of a key arginine residue (R448K)

that abrogates phosphotyrosine binding disrupts these interactions. Using this SLP-76 mutant, we have found consistent albeit mild defects in FcεRI-mediated degranulation and IL-6 production (43). Given that FcεRI-activated ADAP-deficient and WT mast cells are indistinguishable (43), it is possible that HPK-1 or another protein localized by the SLP-76 SH2 domain plays a role in FcεRI-mediated mast cell activation. Further studies of HPK-1-deficient mast cells are required to determine whether this is the SLP-76 partner molecule that contributes to mast cell function.

The N-terminal tyrosines of SLP-76

The SLP-76 N-terminal acidic domain contains three tyrosines located at residues 112, 128, and 145. The importance of these three tyrosines is demonstrated by the failure of SLP-76 containing three Y/F mutations (SLP-76.Y3F) to rescue FcεRI-mediated function when transfected into SLP-76-deficient mast cells (42, 43). Tyrosines 112 and 128 of SLP-76 are followed by the amino acids glutamic acid, serine, and proline (YESP motif), which are coordinately responsible for binding Vav and Nck (57–59). Tyrosine 145 of SLP-76 is part of a YEPP motif, which associates with Tec family PTKs including Btk (36, 60). As the two groups of tyrosines have distinct binding partners, we investigated the relative contribution of the individual tyrosine groups to SLP-76 function by creating knock-in (KI) mice with either SLP-76 containing Y/F mutations in Y112 and Y128 (Y112/128F KI) or in Y145 (Y145F KI) (61). BMMCs derived from both Y112/128F KI and Y145F KI mice exhibited defective FcεRI-mediated activation compared with WT mast cells. However, Y145F KI mast cells were significantly more impaired than Y112/128F KI mast cells, suggesting that Y145 plays a more dominant role in mast cell activation than Y112/128 (T. Kambayashi, unpublished observations). This result is consistent with the finding that Btk-deficient (62, 63) and Vav1-deficient (64) mast cells display severe and mild defects in FcεRI-mediated mast cell activation, respectively. Although these tyrosines were initially thought to be important for binding to these molecules, SLP-76 containing the corresponding Y/F mutations were still bound to Vav and Tec family PTKs (61). However, the activation of these effectors was diminished. It is possible that without the phosphorylated tyrosines of SLP-76, Vav and Tec family PTKs still bind to SLP-76 but not in a proper conformation, which may be critical to full activation of these signaling mediators.

Y112/128F KI and Y145F KI mast cells exhibit markedly decreased phosphorylation of PLCγ and associated Ca²⁺ flux.

This observation is consistent with the function of Btk, which is responsible for phosphorylation of several phosphorylation sites within PLCγ (39, 40). Moreover, although the mechanism is unclear, Vav1-deficient mast cells also show decreased phosphorylation of PLCγ (64). The proline-rich domain of SLP-76 associates with PLCγ (29), and similar to SLP-76.Y3F, expression of SLP-76 with a deletion of the PLCγ-binding domain also fails to rescue mast cell function in SLP-76-deficient mast cells (T. Kambayashi, unpublished observations). Thus, SLP-76 is not only important in the activation of Btk and Vav but also in bringing Btk and Vav into close proximity with PLCγ, which permits efficient phosphorylation and activation of PLCγ.

The proline-rich and SAM domains of SLP-76

Localization of SLP-76 to the plasma membrane is dependent on the GADS-binding site located in the proline-rich domain that lies slightly carboxy-terminal of the PLCγ-binding site of SLP-76 (30). The recruitment of SLP-76 to the membrane by association with LAT is a crucial step in mast cell activation. LAT indirectly localizes SLP-76 through its three tyrosines at positions 175, 195, and 235 that bind to GADS when phosphorylated (27). Thus, mutation of the three tyrosines of LAT abrogates the FcεRI-initiated SLP-76/LAT association (27). Disruption of the GADS-binding domain of SLP-76 (42, 65) has detrimental effects on the function of SLP-76. Upon FcεRI activation, SLP-76 clusters and co-localizes with LAT, Syk, and FcεRI at the cell surface (65). These clusters increase in size, contain other critical signaling molecules, and thus are thought to be actual sites of signal transduction (66). However, such SLP-76 clusters fail to appear when mast cells harboring SLP-76 with point mutations in the GADS-binding region are similarly activated. Furthermore, interference of SLP-76 binding with GADS by a dominant negative binding protein abrogates SLP-76 cluster formation at the mast cell membrane and inhibits FcεRI-associated function (65). The ability of SLP-76 to be recruited to the membrane and cluster at the mast cell surface appears required for proper functioning of SLP-76.

The signaling microclusters that form upon FcεRI crosslinking are thought to be nucleated predominantly by LAT. Under resting conditions, LAT is evenly distributed throughout the plasma membrane as small rafts. Upon activation, LAT is then redistributed and coalesces into larger LAT microclusters upon encounter with activated Syk (66). LAT phosphorylation by Syk is proposed to be a key event in the formation of larger LAT aggregates. The three tyrosines of LAT that bind to GADS

are also capable of binding to the SH2 domain of Grb2. Although Grb2 has only one SH2 domain, the association of the SH3 domain of Grb2 and SOS yields a Grb2–SOS–Grb2 complex containing two SH2 domains (67). Thus, the bivalency of the Grb2–SOS–Grb2 complex and the trivalency of LAT allow for the formation of the LAT microclusters. Mutation of the three LAT tyrosines or introduction of a mutant SOS fragment with only one Grb2-binding site results in diffuse rather than clustered LAT after cell activation (67). Mathematical analysis supports this model of cluster formation, suggesting that the trivalency of LAT is critical to the formation of large multimolecular LAT complexes (68).

It is possible that SLP-76 also contributes to multimolecular cluster formation. In addition to three tyrosines, the N-terminus of SLP-76 contains a SAM domain. SAM domains possess diverse functions ranging from RNA and protein binding, which also includes homotypic SAM/SAM interactions (69). The contribution of the SLP-76 SAM domain was revealed recently through a KI mouse with SLP-76 containing a deletion in the SAM domain (SLP-76.m) (70). These mice exhibit impaired T-cell development and TCR-mediated function, suggesting that the SAM domain plays an important role in the function of SLP-76 in ITAM-mediated signaling. Interestingly, although SLP-76.m was recruited to the membrane, cluster formation was disrupted in TCR-activated T cells. As SLP-76 clustering is dependent on GADS, the findings raised the possibility that the SAM domain may affect GADS binding; however, SLP-76.m associated normally with GADS. SLP-76.m KI mice also lacking GADS yielded a more severe defect than either SLP-76.m KI or GADS-deficient mice, suggesting that the SAM domain contributes to SLP-76 function independently of GADS binding.

Although the SAM domain exhibits only weak homotypic interactions in solution (69), large polymeric structures formed by SAM domain-containing proteins have been reported (71). This formation could be possible especially within a tight signaling complex at the plasma membrane, allowing polymeric clusters to form by interaction of SLP-76 SAM domains. In fact, there is evidence that SLP-76 molecules are in very close proximity within these clusters. When Y112/128F and Y145F SLP-76 alleles are coexpressed in KI mice, the defects elicited by the mutant SLP-76 molecules are completely abolished in T cells (61). This finding suggests that neighboring SLP-76 molecules are close enough within a signaling complex to allow for complementation of binding partners. However, the rescuing effect is only observed in T cells, as platelets (72), neutrophils, or mast cells (T. Kambayashi, unpublished observations) from mice

co-expressing Y112/128F and Y145F SLP-76 alleles are as defective as KI mice singly expressing either Y112/128F or Y145F. Thus, it would be interesting to see whether SLP-76.m exhibits defective function and clustering only in T cells or extends also to FcεRI-activated mast cells.

The recruitment of SLP-76 to the plasma membrane is dependent on the GADS-binding domain of SLP-76 following FcεRI crosslinking. Accordingly, GADS is crucial for optimal mast cell activation, as GADS-deficient mast cells have significantly reduced FcεRI-mediated responses and diminished SLP-76 phosphorylation (73). Although GADS is a necessary intermediary for the SLP-76/LAT interaction, the phenotype of mast cells with a disrupted SLP-76 GADS-binding domain and LAT deficiency do not phenocopy each other, suggesting that these interactions are more complex.

Redundant and non-redundant roles of LAT, NTAL, and LAX in mast cell activation

LAT-deficient mast cells display significant defects in FcεRI-mediated Ca^{2+} flux, degranulation, and cytokine/chemokine production (26, 27). Yet, mast cell function in the absence of LAT is better preserved compared with SLP-76-deficient mast cells. This finding is unexpected, as one might predict that LAT deficiency would yield an equivalent or worse phenotype than SLP-76 deficiency given that SLP-76 lies downstream of LAT. Moreover, it is puzzling that SLP-76 phosphorylation is still observed in the absence of LAT (74). This observation raises the possibility that either SLP-76 does not require membrane recruitment for its phosphorylation or that another molecule is capable of localizing SLP-76 to the plasma membrane in the absence of LAT. One likely candidate molecule that could compensate for the absence of LAT is NTAL (non-T-cell activation linker). NTAL is a membrane-bound adapter molecule containing five tyrosines that are putative binding sites for Grb2 (75), of which one is also a presumed binding site for GADS (76), thereby making it potentially suitable for recruiting SLP-76 to the plasma membrane. Initial studies with NTAL-deficient mast cells suggested that NTAL played a negative role in mast cell activation; these mast cells displayed enhanced degranulation and Ca^{2+} flux (74, 77). However, this effect was later attributed to increased phosphorylation and localization of LAT to lipid rafts. Being structurally similar to LAT, NTAL also possesses a palmitoylation site that localizes it to membrane lipid rafts (78). LAT and NTAL can compete for sites in the plasma membrane and for phosphorylation by Syk. Consistent with this notion, NTAL phosphorylation is significantly upregulated in LAT-deficient

mast cells (74, 77). Although NTAL binds to Grb2 family member proteins (75), it lacks the putative PLC γ -binding tyrosine motif (79). This PLC γ -binding tyrosine has been shown to be crucial for the function of LAT (27). Therefore, it appears that not all mast cell functions can be compensated for by NTAL in the absence of LAT. It is possible that NTAL can interact with PLC γ /SLP-76/GADS and help localize this complex to the plasma membrane, leading to the activation and phosphorylation of PLC γ . Indeed, PLC γ indirectly associates with NTAL through an unidentified cytosolic adapter (80). Subsequent studies have demonstrated a positive role for NTAL, as LAT/NTAL double-deficient mast cells exhibited less Ca²⁺ flux and degranulation than LAT-deficient mast cells (74, 77). In human mast cells, knockdown of NTAL resulted in decreased Fc ϵ RI-mediated degranulation (80). Thus, NTAL can function as a positive regulator of mast cell function, albeit with lower activity than LAT. Whether or not NTAL positively regulates mast cell function by recruiting SLP-76 to the plasma membrane remains to be determined.

Although some functions of NTAL are clearly redundant with LAT, the exact relationship between these two transmembrane adapter proteins in the regulation of mast cell signaling has not yet been completely worked out. NTAL-deficient mast cells manifest significantly upregulated phosphatidylinositol-3 kinase (PI3K) activity, suggesting that NTAL can serve to negatively regulate this second messenger cascade (77). This is in contrast to initial studies of LAT in mast cells, which demonstrated that this adapter does not impact PI3K activation or subsequent phosphorylation of Akt in mast cells (81). More recently, however, others (82) have found that Akt phosphorylation is enhanced in LAT-deficient mast cells compared with WT. In this study (82), it was reported that in addition to being a positive regulator of mast cell activation, LAT may also participate in dampening signals downstream of Fc ϵ RI by binding to SH2 domain-containing inositol phosphatase (SHIP), thereby antagonizing the effects of PI3K. In the absence of NTAL, where an increase in LAT phosphorylation is observed, the association of SHIP and LAT was further increased. Thus, the role of NTAL may be even more complex as this work suggests. NTAL may also positively affect the PI3K pathway indirectly by interfering with the phosphorylation of LAT.

A similar effect on PI3K was observed with mast cells deficient in another LAT-like protein known as LAX (linker for activation of X cells). Similar to NTAL, LAX-deficient mast cells also displayed increased degranulation responses, PI3K activity, and phosphorylation of Akt (82). Although LAX is structurally similar to LAT and NTAL and contains multiple Grb2-binding sites, LAX does not contain raft-targeting palmitoylation motifs and hence is localized differently (83). LAX appears to have a positive effect on NTAL but not LAT expression, as significantly decreased NTAL expression was observed in LAX-deficient mast cells (82). Thus, the effect of LAX on PI3K might be secondary to a decreased expression of NTAL. It remains unclear how LAX affects expression of NTAL and whether LAX contributes to mast cell function in an NTAL-independent manner.

Membrane recruitment of cytosolic proteins by the transmembrane LAT family adapter proteins is a key regulatory step in signal transduction. Although many of the molecules that are involved in this process have been revealed, there is still much that is poorly understood about the complex interactions that take place between these molecules to regulate signal transduction.

Lyn as a positive and negative regulator of mast cell function

Lyn is considered the major src family PTK that phosphorylates Syk and the Fc ϵ RI β and γ chains. Lyn deficiency would be expected to phenocopy mast cells lacking Syk. An initial study demonstrated that PSA is diminished in Lyn-deficient mice (84). Phosphorylation of Syk and other downstream signaling molecules was significantly diminished in Lyn-deficient mast cells, thus supporting a positive role for Lyn in the initiation of the Fc ϵ RI-mediated signaling cascade (85). However, this view was challenged with the unexpected finding from subsequent studies showing either unchanged (84) or enhanced (86, 87) degranulation of Lyn-deficient mast cells *in vitro*. These apparently conflicting *in vitro* and *in vivo* results were attributed to the older age of mice used in the initial PSA experiments. It was reasoned that the increased endogenous serum IgE levels in older Lyn-deficient mice resulted in the competition of passively transferred IgE with endogenous IgE molecules, thus blunting the PSA response compared with WT mice (86).

The enhanced mast cell function in the absence of Lyn raises the possibility that Lyn may in fact negatively regulate Fc ϵ RI-mediated activation. The ITAM of the Fc ϵ RI β subunit not only binds to Lyn but also recruits several phosphatases including SHIP and SH2-containing phosphatase-1/2 (SHP-1/2) (21, 88). It is likely that the activation of these phosphatases is also regulated by Lyn, as phosphorylation of SHIP and SHP-1/2 (required for optimal activity of these phosphatases) is absent in Fc ϵ RI-activated Lyn-deficient mast cells (87, 89). Further dissection of this pathway revealed that activation of these phosphatases primarily occurs with strong crosslinking stimuli (89) and contributes to the commonly observed biphasic mast cell responses, where high doses of antigen results in

decreased FcεRI-mediated function. Thus, Lyn could be both a positive and negative regulator of mast cell activation, depending on the strength of the stimulus, which may explain the discrepancy in previous reports. At low antigen doses, Lyn-deficient mast cell responses may be more difficult to evoke due to the failure of Syk phosphorylation. However, at higher antigen doses, enhanced mast cell activation may be observed, owing to the lack of phosphorylation of phosphatases and perhaps compensation by other src family PTKs.

The influence of the genetic background of the mice was raised as possibly contributing to how Lyn was utilized in the cell. When mast cells from two distinct mouse strains commonly used as the genetic background for knockout mice, 129/Sv and C57BL/6, were compared in their ability to degranulate, mast cells from the 129/Sv strain responded significantly more robustly than those from the C57BL/6 mice. Moreover, Lyn possessed opposing effects in the two different strains. Lyn deficiency on the 129/Sv background showed an increase in mast cell degranulation, whereas Lyn knockout C57BL/6 mice showed decreased degranulation compared with their respective WT controls (90). Thus, Lyn predominantly acts as a negative regulator of FcεRI-mediated activation in 129/Sv but a positive regulator in C57BL/6 mice. A question still remains as to what initiates the activation of Syk in the absence of Lyn, as Syk is the central positive regulator of mast cell activation. Alternatively, it is possible that Syk-independent pathways exist downstream of FcεRI.

An alternative pathway leading from FcεRI independent of SLP-76 and LAT

A separate pathway distinct from Syk/LAT/SLP-76 in FcεRI-mediated signaling was suggested based on the residual signaling pathways that are observed in LAT-deficient mast cells. Both LAT-deficient (81, 91) and SLP-76-deficient (Fig. 2A) mast cells exhibit intact Akt phosphorylation after FcεRI crosslinking. Phosphoinositide-dependent kinase-1 (PDK-1) phosphorylates and activates Akt, a process that is dependent on colocalization of PDK-1 and Akt at the plasma membrane by interaction of their pleckstrin homology (PH) domains with phosphatidyl-3,4,5-triphosphate (PIP₃). PI3K is the enzyme responsible for converting PIP₂ to PIP₃, thereby increasing the membrane composition of PIP₃. Activation of the PI3K pathway is functionally relevant for mast cells, as PI3K inhibitors block degranulation and cytokine production (92–95). Moreover, introduction of a dominant negative form of Akt into mast cells blocks FcεRI-mediated transcription of cytokines (96).

To elucidate how PI3K was activated in mast cells, the role of Grb-associated binder 2 (Gab2), an adapter molecule that

had been shown to be involved in PI3K activation through the IL-3 receptor (97), was investigated downstream of FcεRI. Compared to WT, Gab2-deficient mast cells lacked PI3K activity and exhibited defective degranulation responses upon FcεRI crosslinking (98). The Gab2 pathway leading to PI3K activation was shown to be neither upstream nor downstream of LAT, as Gab2-deficient mast cells elicited normal LAT phosphorylation and LAT-deficient mast cells exhibited normal phosphorylation of Gab2 (81). Fyn, another src family PTK, but not Lyn was found to be involved in the phosphorylation of Gab2 (81). In fact, Lyn was shown to negatively regulate Fyn activity (86). In addition to regulating the activity of Fyn, Lyn may also decrease mRNA levels of Fyn (87). Thus, it was proposed that another reason that Lyn-deficient mast cells may be hyperfunctional was because the negative effects on Fyn were lacking in Lyn-deficient mast cells, leading to hyperactivation of the PI3K pathway. In addition to the signaling pathway mediated through Lyn/Syk/LAT/SLP-76, these results collectively argue for an entirely separate pathway mediated through Fyn/Gab2/PI3K.

Contribution of the alternative pathway in residual function of SLP-76-deficient mast cells

As mentioned in previous sections, we and others have demonstrated that SLP-76 is vital for FcεRI-mediated activation of mast cells (33, 42, 43, 65). Almost no residual function is observed in SLP-76-deficient mast cells with regard to cytokine/chemokine production and degranulation *in vitro*. However, when SLP-76-deficient mice were subjected to an IgE-dependent model of cutaneous allergy, passive cutaneous anaphylaxis (PCA), increased vascular permeability was still observed, albeit at a much lower level than WT mice (Fig. 2B). This response appears to be a *bona fide* mast cell-dependent event, as mast cell-deficient W/W^v mice demonstrate no increased permeability under these same conditions (Fig. 2B). Thus, this partial PCA response found in SLP-76-deficient mice argues for retained FcεRI-induced mast cell responses *in vivo*.

As SLP-76-deficient mast cells retain FcεRI-inducible Akt phosphorylation, we hypothesized that the residual function of SLP-76-deficient mast cells *in vivo* was mediated by the Fyn/Gab2/PI3K pathway. To test this hypothesis, mice deficient in both SLP-76 and Fyn (SLP-76/Fyn DKO) were generated. We first tested whether Fyn deficiency would ablate the PI3K signaling pathway in SLP-76-deficient mast cells. While the phosphorylation of Akt was preserved in SLP-76-deficient mast cells (Fig. 2A), the phosphorylation of Akt in Fyn-deficient and SLP/Fyn DKO mast cells was diminished

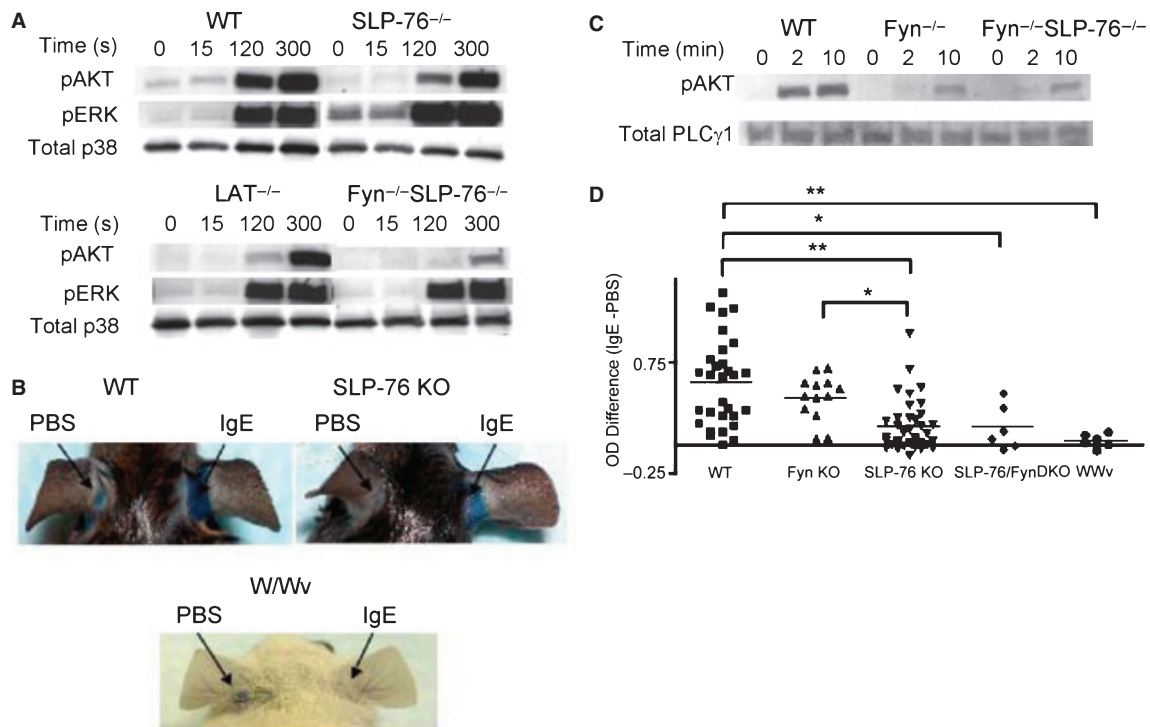


Fig. 2. Fyn is critical to Akt phosphorylation but not for residual *in vivo* function in SLP-76-deficient mast cells. (A) Cell lysates were prepared from anti-DNP IgE-sensitized HSA-DNP-stimulated BMMC from WT, SLP-76^{-/-}, LAT^{-/-}, or SLP-76^{-/-}Fyn^{-/-} mice, resolved using SDS-PAGE, and blotted for phospho-Akt (Ser473), phospho-ERK1/2 (Thr202/Tyr204), or total p38 (loading control). (B) WT, SLP-76^{-/-}, and W/Wv mice were injected with PBS (left) or anti-DNP IgE (25 ng per mouse, right) at the base of the ear. Twenty-four hours later, mice were challenged with an intravenous injection of HSA-DNP (100 μg per mouse) in 1% Evans blue dye. Images of the mice were obtained 30 min after injection. (C) Cell lysates were prepared from anti-DNP IgE-sensitized HSA-DNP-stimulated BMBCs from WT, Fyn^{-/-}, or SLP-76^{-/-}Fyn^{-/-} mice, resolved using SDS-PAGE, and blotted for phospho-Akt (Ser473) or total PLCγ1 (loading control). (D) WT, SLP-76^{-/-}, Fyn^{-/-}, SLP-76^{-/-}Fyn^{-/-} and W/Wv mice were injected with PBS (left) or anti-DNP IgE (25 ng per mouse, right) at the base of the ear. Twenty-four hours later, mice were challenged with an intravenous injection of HSA-DNP (100 μg per mouse) in 1% Evans blue dye. Thirty minutes later, dye extravasation was measured (OD610) in both ears, and the PBS-injected ear value was subtracted from the IgE-injected ear value. **P* < 0.05, ***P* < 0.01 using t-test. DNP, dinitrophenol; BMMC, bone marrow-derived mast cell; WT, wildtype; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; HSA-DNP, dinitrophenol-conjugated human serum albumin; IgE, immunoglobulin E; PLC, phospholipase C.

(Fig. 2C), confirming that the PI3K pathway was mediated by a SLP-76-independent Fyn-dependent pathway. Next, the SLP-76/Fyn DKO mice were challenged in the PCA assay. Although vascular permeability was diminished compared with WT mice, SLP-76/Fyn DKO mice were similar to SLP-76-deficient mice (Fig. 2D), and the mast cell-deficient W/Wv mice did not display any appreciable PCA response (Fig. 2D). These experiments demonstrate that the *in vivo* FcεRI-induced mast cell response in SLP-76-deficient mice is not mediated by Fyn. Thus, the proximal signaling molecules mediating the residual *in vivo* activity of mast cells in SLP-76-deficient mice remain to be determined.

The Fyn-deficient mast cells used in our experiments, however, did not display decreased degranulation *in vitro* or *in vivo* (Fig. 3A,B) as reported by others (81), a result that was most likely due to the genetic background of our mice. Similar to Lyn, the genetic background of the mice may influence

how Fyn is involved in FcεRI-mediated activation. Fyn is expressed at significantly lower levels in mast cells of mice on a C57BL/6 compared with 129/Sv background (90), suggesting that Fyn may play a more prominent role on a 129/Sv background. Indeed, when we tested Fyn-deficient mast cells from a pure 129/Sv background, the mast cells displayed significantly diminished degranulation responses (Fig. 3C). Reconstitution of Fyn as a retroviral construct into Fyn-deficient mast cells restored the ability of these mast cells to degranulate (Fig. 3D). Thus, the dependence of mast cells on different src family PTKs may relate to their genetic background. Another recent study using mice on a mixed C57BL/6 and 129/Sv background showed that Gab2 phosphorylation was dependent on the presence of not only Fyn but also Lyn and Syk (99). Syk phosphorylation appeared decreased in Fyn-deficient mast cells. Thus in addition to Lyn, Fyn may contribute to phosphorylation of Syk and subsequent

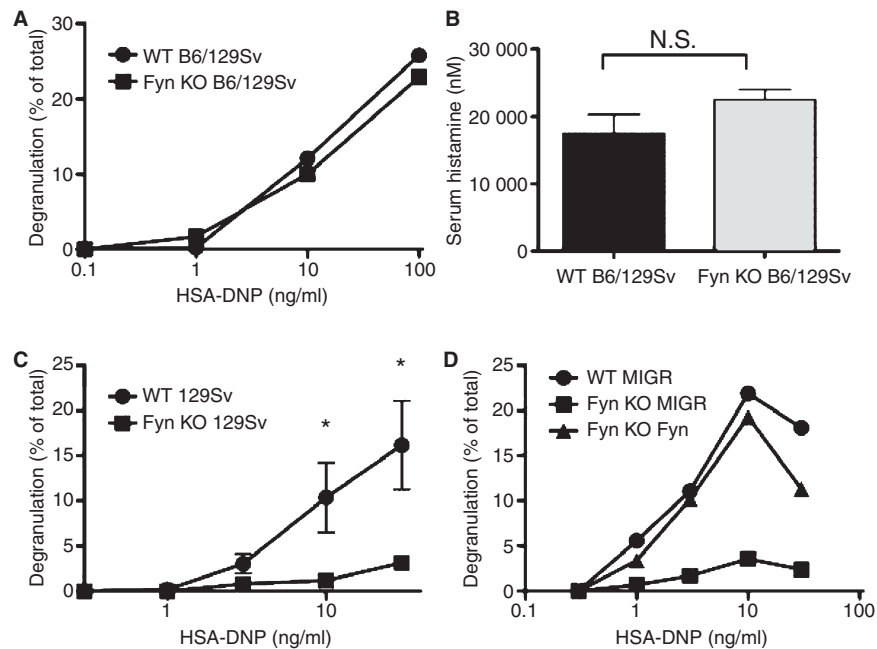


Fig. 3. Fyn deficiency impacts mast cell degranulation on a 129/Sv background but not on a mixed 129/Sv × C57BL/6 background. (A) BMMCs from WT or *Fyn*^{-/-} mice on a mixed 129/Sv × C57BL/6 background were sensitized with anti-DNP IgE (1 µg/ml) and stimulated with various concentrations of HSA-DNP for 1 h. β hexosaminidase activity as a marker of degranulation was measured using a colorimetric enzymatic assay. (B) WT and *Fyn*^{-/-} mice were injected with anti-DNP IgE (5 µg per mouse) intravenously, followed by an intravenous injection with HSA-DNP (100 µg per mouse) 24 h later. Ninety seconds later, the mice were bled, and serum histamine content was measured using an immunoassay. Data are represented as mean $\pm$ SD (N = 8 mice per genotype). (C) Degranulation was measured in BMMCs from WT or *Fyn*^{-/-} mice on a pure 129/Sv background as in (A). Data are represented as mean $\pm$ SD generated from BMMCs of three individual mice per genotype. **P* < 0.05 using t-test. (D) Degranulation of *Fyn*^{-/-} mast cells that were reconstituted with Fyn or with vector alone (MIGR) by retroviral transduction were compared with WT mast cells transduced with MIGR. BMMC, bone marrow-derived mast cell; WT, wildtype; HSA-DNP, dinitrophenol-conjugated human serum albumin.

activation of Gab2/PI3K, which is consistent with the observation that Syk-deficient mast cells show no phosphorylation of signaling molecules in both LAT/SLP-76/PLC γ and PI3K/Akt pathways (99, 100). Nevertheless, although Syk may be centrally involved in both pathways, it is clear that in certain genetic backgrounds, Fyn/Gab2/PI3K provides an alternative route that is independent of the LAT/SLP-76/PLC γ pathway.

Pathways that lead to MAPK activation in mast cells

MAPKs are a family of serine/threonine kinases, which include extracellular signal-regulated kinase (ERK), JNK, and p38. All three MAPKs are phosphorylated downstream of Fc ϵ RI and are necessary for proper mast cell function (101). Treatment of mast cells with specific MAPK inhibitors show that all three MAPKs are involved in the phosphorylation of the phospholipase A₂ (PLA₂) (102, 103), an enzyme that liberates arachidonic acid from membrane lipids. Moreover, p38 MAPK (94, 104) and JNK (105) contribute to cytokine production by mast cells. The proximal signaling pathways that lead to the activation of p38 MAPK and JNK are relatively straightforward, as disruption of either the SLP-76/LAT (27,

42) or Fyn/Gab2 (98, 106) axis leads to a reduction in phosphorylated p38 MAPK and JNK. By contrast, pathways leading to ERK phosphorylation appear more complex, as phosphorylation of ERK is intact in both SLP-76- and Fyn-deficient mast cells (43, 106) (Fig. 2A). LAT could conceivably promote ERK phosphorylation independently of SLP-76 by localizing Grb2/SOS, leading to subsequent activation of Ras GTPase and to ERK phosphorylation. In support of this notion, LAT-deficient mast cells were initially reported to exhibit decreased activity of ERK2 (26, 27). However, a more recent report demonstrated that ERK1/2 phosphorylation in LAT-deficient mast cells remains intact (74). We have also observed normal phosphorylation of ERK in LAT-deficient mast cells (Fig. 2A). As NTAL is also capable of localizing Grb2/SOS, one might argue that ERK phosphorylation occurs through NTAL in the absence of LAT. However, NTAL/LAT double deficient mast cells also exhibit normal ERK phosphorylation after Fc ϵ RI crosslinking (107), making this possibility unlikely. Aside from Grb2/SOS, activation of RasGRP by DAG/Ca²⁺ can activate ERK via Ras GTPase, linking PLC γ to the ERK pathway. However, RasGRP-deficient mast cells display defects in degranulation and cytokine production, but ERK

phosphorylation remains intact (50). PLC γ phosphorylation does not correlate with ERK phosphorylation. Thus, it seems unlikely that this pathway is a major contributor to ERK activation downstream of Fc ϵ RI and argues for another pathway independent of the SLP-76/LAT or Fyn/Gab2 pathway leading to ERK. It is clear that Syk is involved upstream of ERK phosphorylation, as Syk-deficient mast cells exhibit no functional activity (24) or phosphorylation of ERK upon Fc ϵ RI stimulation (100, 107).

Recent evidence suggests that intracellular Zn²⁺ can act as a signaling molecule that contributes to the activation of ERK (108). Upon Fc ϵ RI ligation, a Zn²⁺ wave occurs, where intracellular stores of Zn²⁺ are released into the cytoplasm in a Ca²⁺-dependent manner. Prevention of the Zn²⁺ wave by intracellular Zn²⁺ chelation leads to a decrease in ERK phosphorylation, while the addition of a Zn²⁺ ionophore leads to sustained ERK activation. The activation of ERK by Zn²⁺ may be related to its involvement in the localization of PKC β to the plasma membrane. Mast cells lacking Zn²⁺ transporter-5 (Znt5) exhibit defects in PKC β activation and membrane recruitment despite normal total levels of PKC β (109). This notion is consistent with the observation that Ras can be activated by PKC α/β (110). Upon recruitment to the plasma membrane, one of the tyrosines of PKC α/β is phosphorylated by Syk, which can localize Grb2/SOS to the membrane. Thus, it appears that PKC α/β can function as an adapter molecule aside from its role as a serine/threonine kinase. Although ERK phosphorylation is significantly decreased in PKC $\beta^{-/-}$ mast cells, further investigation is required to determine how much the adapter function of PKC contributes to ERK activation, as regulation of ERK by the kinase activity of PKC has also been described (111, 112).

Additional receptors that integrate with Fc ϵ RI to enhance mast cell responses

In addition to Fc ϵ RI, it is increasingly recognized that other receptors expressed by mast cells have an influence on mast cell effector functions (6, 113, 114). A prime example is the effect of KIT on mast cell activation. KIT is a transmembrane receptor with tyrosine kinase activity that is expressed on mast cells and is the receptor for stem cell factor (SCF), which is necessary for mast cell development and homeostasis (115). Signals mediated by KIT at the time of Fc ϵ RI crosslinking substantially enhance the magnitude of mast cell degranulation and cytokine production (116). Thus, the integration of signals transduced by mast cell receptors can result in synergistic mast cell activation and are suspected to influence mast cell responses in a physiologic setting (6). In addition to KIT,

other receptors initiate the signaling pathways that integrate with the Fc ϵ RI pathway and mediate synergic mast cell activation: the adenosine A3 receptor and the CC-chemokine receptor 1 (CCR1) (117, 118), both G-protein-coupled receptors, and TLRs (119, 120). Although much is known regarding the integration of KIT signaling and Fc ϵ RI pathways (6, 114), our knowledge regarding the integration of G-protein-coupled receptor signaling (113) and TLR signaling is more limited. Here we briefly describe emerging work regarding TLR-mediated responses in mast cells.

TLRs are a major class of pattern recognition receptors that are stimulated by molecules associated with bacteria, viruses, protozoa, and fungi. These receptors are expressed on a variety of cells including professional antigen-presenting cells, phagocytes, and T cells but are especially characterized on sentinel cells such as dendritic cells. The triggering of these receptors on dendritic cells allows for infectious non-self-discrimination and initiates events important to adaptive immunity, such as costimulatory molecule expression and enhanced antigen presentation (121). TLRs are distinguished by the type of ligand they recognize: TLR2 is activated by bacterial lipopeptides, TLR4 by bacterial lipopolysaccharide (LPS), TLR3 by double-stranded RNA, TLR9 by CpG motifs in DNA, and TLR5 by the protein flagellin. These receptors have considerable homology with members of the IL-1 receptor family, each having a conserved region in their cytoplasmic tail known as the Toll/IL-1 receptor (TIR) domain (122). Except for TLR3, the core TLR/IL-1 receptor family pathway utilizes the TIR-containing adapter molecule MyD88 for recruitment and activation of the kinases IL-1 receptor-associated kinase-4 (IRAK-4) and IRAK-1. Activation of IRAK-1 results in the recruitment of tumor necrosis factor receptor-associated factor-6 (TRAF-6) into the receptor complex, which soon dissociates into the cytosol, participating in the activation of transforming growth factor- β -activated kinase-1 (TAK1), which in turn leads to the activation of both NF- κ B and MAPKs (123).

Mast cells are indeed sentinel cells and express functional TLRs, yet expression varies depending on cell maturity and origin (124–126). For example, murine fetal skin-derived mast cells, which are considered connective tissue-type mast cells, express greater levels of TLR2, TLR3, TLR4, TLR7, and TLR9 mRNA than murine BMMCs, which are considered a more immature, mucosal-type cells (125). Human mast cells cultured from peripheral blood CD34⁺ progenitor cells express protein for TLR1–7 and TLR9, whereas the human cultured mast cell (HMC) line 1 lacks TLR2 and TLR4 protein expression (124). Similarly, mast cell responses to TLR ligands also vary depending on cell maturity and origin (125–127).

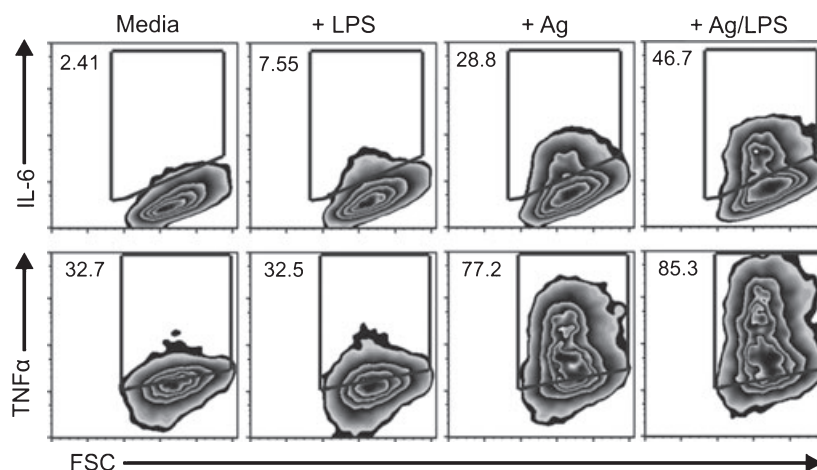


Fig. 4. FcεRI and TLR signals integrate for the enhanced production of pro-inflammatory cytokines. BMMCs sensitized with anti-DNP IgE (1 μg/ml) were cultured for 3 h in media with the indicated stimulation conditions (LPS *Salmonella minnesota* R595 100 ng/ml; Ag is DNP-HSA 100 ng/ml) in the presence of Golgi Stop (BD Pharmingen, Franklin Lakes, NJ, USA) and Golgi Plug (BD Pharmingen). Cells were harvested and processed for intracellular cytokine staining of TNF-α and IL-6. The number in the plots represents the percentage of cells expressing the indicated cytokine. TLR, Toll-like receptor; BMMC, bone marrow-derived mast cell; HSA-DNP, dinitrophenol-conjugated human serum albumin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

However, with regard to studies using murine mast cells, a model has emerged that TLR ligands directly induce mast cell secretion of pro-inflammatory cytokines and chemokines but do not induce degranulation or arachidonic acid metabolism (124–129). Perhaps most relevant to mast cell-mediated allergic inflammation in a physiologic context is the observation that FcεRI and TLR signals are integrated, resulting in enhanced production of pro-inflammatory cytokines (128) (Fig. 4). In a mouse model of airway hypersensitivity, antigen-induced eosinophilic inflammation in the lung is increased when mice are sensitized with antigen and LPS, an effect that is dependent on the expression of TLR4 by mast cells (119). However, as in these studies LPS was co-administered with antigen during the sensitization phase only, the significance of FcεRI and TLR synergism in mast cell-dependent inflammatory responses *in vivo* remains to be determined.

Our understanding of the molecular signals that mediate FcεRI and TLR signal integration is rudimentary. Consistent with the inability to activate or enhance degranulation, murine BMMCs stimulated with ligands for TLR2 (lipopeptides and peptidoglycan) or TLR4 (LPS) do not trigger calcium signals (127, 128) and have no effect on calcium signals induced by FcεRI crosslinking (128). Although both FcεRI and TLR pathways activate NF-κB, it is thought that simultaneous triggering of these pathways results in the integrated activation of MAPKs that enhance or sustain the activities of their respective downstream transcription factors. TLR2 or TLR4 stimulation of the murine MC/9 mast cell line concurrent with FcεRI crosslinking enhances and sustains the phos-

phorylation of JNK and p38 but not ERK (128). At least for p38, it appears that signals induced by TLR4 and FcεRI costimulation prolong phosphorylation much longer than is observed with FcεRI crosslinking alone, an effect that is dependent on the TLR adapter molecule MyD88 (H. Ali, unpublished observations).

As the biochemical inhibition of MAPKs does not completely abrogate the enhanced cytokine production seen with TLR and FcεRI pathway costimulation (128), it is tempting to speculate that these pathways integrate through proximal mechanisms as well. As discussed previously, in mast cells the PI3K pathway plays a major role in FcεRI-mediated cytokine production (96, 130). The PI3K pathway is also activated by TLR stimulation directly and plays a role in innate immune responses (131). TLR2 has an YXXM motif in its TIR domain that upon tyrosine phosphorylation binds the regulatory p85 subunit of PI3K (132). Furthermore, MyD88, which is utilized by all TLR except TLR3, also binds p85 and links TLR stimulation with PI3K pathway activation (133–135). Whether these proximal signals integrate and play a significant role in mast cell cytokine responses remains to be determined.

Concluding remarks

Signal transduction downstream of FcεRI in mast cells is complex and regulated by multiple signaling pathways. These pathways include activation of positive and negative feedback loops that yield a balanced signal necessary for an appropriate mast cell response. It is important that the intricate molecular

interactions that take place within the cell occur in a regulated fashion. The cooperation and synergism among adapter molecules is essential to this process, as these proteins function by nucleating appropriate molecular interactions in a temporal and spatial manner. Although our understanding of the precise molecular interactions that take place in mast cell signal transduction has deepened in recent years, much remains

poorly understood. Further research aimed at deciphering the contribution of upstream signaling molecules to the different FcεRI-mediated mast cell responses will continue to provide insights into the complex biology of these important effector cells. It is anticipated that this information will provide new ideas for the development of novel therapeutics to control allergic diseases.

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