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Mutation of tyrosine 145 of lymphocyte cytosolic protein 2 (*Lcp2*; SLP-76) protects mice from anaphylaxis and arthritis

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Abstract

Background—The Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76-kilodaltons (SLP-76) is an essential adaptor molecule in myeloid cells, where it regulates FcεRI-induced mast cell (MC) and FcγR- and integrin-induced neutrophil (polymorphonuclear leukocyte; PMN) functions. SLP-76 contains three N-terminal tyrosines at residues 112, 128 and 145 that together are critical for its function.

Objective—We sought to explore the relative importance of tyrosines 112, 128 and 145 of SLP-76 during MC and PMN activation.

Methods—We examined *in vitro* MC and PMN functions using cells isolated from knock-in mice harboring phenylalanine substitution mutations at tyrosines 112 and 128 (Y112/128F) or 145 (Y145F). We also examined the effects of these mutations on *in vivo* MC and PMN activation using models of anaphylaxis, dermal inflammation and serum-induced arthritis.

Results—Mutations at Y112/Y128 and Y145 both interfered with SLP-76 activity; however, Y145F had a greater impact than Y112/128F on most *in vitro* FcR-induced functions. *In vitro* functional defects were recapitulated *in vivo*, where mice expressing Y145F exhibited greater

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Key Message

Y145 of SLP-76 is critical for *in vitro* MC and PMN functions.

Mutation of SLP-76 Y145 reduces histamine release in a mouse model of passive systemic anaphylaxis and protects against dermal inflammation and serum-induced arthritis.

Y145 of SLP-76 may prove to be a novel therapeutic target in the treatment of MC- and PMN-mediated inflammatory disease.

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attenuation of MC-dependent passive systemic anaphylaxis and PMN-mediated inflammatory responses. Notably, the Y145F mutation completely protected mice against development of joint-specific inflammation in the MC and PMN-dependent K/B $\times$ N model of arthritis.

Conclusion—Our data indicate Y145 is the most critical tyrosine supporting SLP-76 function in myeloid cells. Future efforts to dissect how Y145 mediates SLP-76-dependent signaling in MC and PMN will increase our understanding of these lineages and provide insights into the treatment of allergy and inflammation.

Keywords

Mast cells; neutrophils; Fc receptors; integrins; signal transduction; SLP-76; passive systemic anaphylaxis; localized Shwartzman reaction; arthritis

Introduction

Mast cells (MC) and polymorphonuclear leukocytes (PMN; neutrophils) provide an immediate line of defense against invading pathogens. Through phagocytosis and release of inflammatory mediators, these cells kill microorganisms and amplify the ensuing immune response.^{1, 2} The aberrant activation of MC and PMN also contributes to several human diseases, including hypersensitivity disorders and asthma,³ as well as endotoxic shock and inflammatory arthritis.^{4, 5} To better understand and manipulate their roles in host defense and disease, it is important to delineate the molecules and signaling pathways that control the activation of MC and PMN.

The Fc receptors for immunoglobulin (FcR) and integrins regulate critical aspects of MC and PMN function. Much of MC activation is mediated by Fc ϵ RI, a receptor that binds with high affinity to the constant region of immunoglobulin E (IgE) molecules.⁶ Following cognate antigen exposure, antigen-specific IgE-bound Fc ϵ RI are cross-linked, leading to release of pre-formed granule contents and inflammatory mediators.⁷ PMN express other stimulatory FcR, such as Fc γ RIII, that bind IgG-opsonized particles or soluble IgG immune complexes (IC), thereby triggering PMN spreading, reactive oxygen intermediates (ROI) production and degranulation.⁸ PMN also express high levels of the β 2 integrin Mac-1 (CD18/CD11b), which binds extracellular matrix ligands or C3bi complement-opsonized particles and promotes PMN migration, ROI production and phagocytosis.^{1, 9}

Engagement of FcR and integrins results in the transduction of intracellular signals, which depend upon the recruitment of Src and Syk family tyrosine kinases to immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors/coreceptors and formation of a multimolecular signaling complex containing the adaptor molecule Src homology 2 (SH2) domain-containing leukocyte protein of 76-kilodaltons (SLP-76).^{10–14} SLP-76 is required in MC and PMN to support optimal signaling downstream of immunoreceptors and integrins, as previously demonstrated by the examination of SLP-76-deficient primary cells and mice, which exhibit marked defects in *in vitro* functional assays and *in vivo* models of allergy and PMN-dependent inflammation.^{15–19}

Although SLP-76 is critical for MC and PMN activation, the structural components supporting its function in these lineages are not fully understood. SLP-76 contains three tyrosines at residues 112, 128 and 145, which are phosphorylated following integrin and immunoreceptor engagement.^{20–22} To understand the roles of these residues in myeloid cell activation, we examined the functions of MC and PMN from knock-in mice expressing tyrosine to phenylalanine (Y $\rightarrow$ F) substitutions at positions 112 and 128 (Y112/128F) or 145 (Y145F).²³ We observe that Y112/128 and Y145 are each required for full activation of MC and PMN *in vitro*; however, Y145 plays a more critical role in controlling most Fc ϵ RI-induced MC and Fc γ R-induced PMN functions. Importantly, *in vitro* defects in Fc ϵ / γ R-induced MC and PMN

activation correlate to *in vivo* deficiencies, where mice expressing Y145F exhibit significantly reduced immediate hypersensitivity in a model of systemic anaphylaxis and no clinical or histological signs of joint inflammation in a model of antibody-mediated arthritis.

Methods

Mice

Slp-76^{-/-}, *Slp-76*^{Y112/128F}, *Slp-76*^{Y145F}, *Slp-76*^{LoxP/LoxP}*LysM*^{Cre} mice are as described.^{15, 23, 24} Animal work was completed according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, as well as guidelines in place at the University of Pennsylvania.

FcεRI-induced MC activation

Bone marrow-derived MC (BMMC) were generated as described²⁵ and used when >95% of cells expressed high levels of FcεRI and CD117, as determined by flow cytometry. FcεRI-induced degranulation as measured by release of beta-hexosaminidase and cytokine production was measured as described.¹⁵ To assess prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) release, anti-DNP IgE-sensitized (1 μg/ml for 2 hours at 37°C) BMMC were activated with various concentrations of HSA-DNP for 30 minutes. LTC₄ and PGD₂ content were measured in cell-free supernatants by enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's protocol. Passive systemic anaphylaxis was induced in WT or mutant mice as described.¹⁵

FcεRI-induced ROI production and Ca²⁺ flux

BMMCs were sensitized with 1 μg/ml anti-dinitrophenol (DNP) IgE for 2 h at 37°C, washed, and resuspended at 1 × 10⁷ cells/ml in PBS containing 5 μM 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; Invitrogen, Carlsbad, CA) for measuring ROI, or 1 mM Probenecid (Sigma Aldrich, St. Louis, MO) and 3 μg/ml Indo-1 (Invitrogen) for measuring Ca²⁺ flux. Cells were protected from light, incubated at 37°C for 20–30 min, washed, resuspended in warm Tyrode's buffer, incubated at 37°C for 10 min and placed on ice until stimulation. Cells were stimulated with DNP-human serum albumin (HSA, 100 ng/ml) at 37°C, and ROI production or Ca²⁺ flux was measured using an LSR flow cytometer (Becton Dickinson, Franklin Lakes, NJ) for 300 s or 180 s, respectively. Baseline ROI or intracellular Ca²⁺ levels were measured for 30 s prior to addition of DNP-HSA. Data were analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Western blot analysis of MC lysates

BMMC were pre-sensitized with anti-DNP IgE (1 μg/ml) for 2 h, washed, rested for 2 h and stimulated with DNP-HSA (30 ng/ml) for the indicated times. Cells were then lysed, proteins resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, CA) and phosphorylation of phospholipase-C-γ (PLCγ1, PLCγ2), p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2), SLP-76 and protein kinase B (Akt/PKB) were analyzed by Western blot. All antibodies were from Cell Signaling (Danvers, MA), except for the anti-phospho-Y128 (SLP-76) antibody, which was from BD Biosciences (San Jose, CA).

FcγR and integrin-induced activation of PMN

PMN were isolated from mouse bone marrow as previously described.²⁶ PMN spreading on integrin (polyRGD) and FcγR ligands (immune complexes [IC] consisting of HSA/anti-HSA antibodies) was measured as described.¹⁵ For ROI production and degranulation, 96-well Immulon 4HBX plates (Thermo Electron Corporation, Milford, MA) were coated overnight

at 4°C with polyRGD (15 µg/ml), collagen (0.05 mg/ml, Chrono-log Corporation, Havertown, PA), anti-FcγRII/III antibody (20 µg/ml; clone 2.4G2; BD Pharmingen), an isotype control antibody (BD Pharmingen) or IC. To assess ROI production, PMN were plated on ligand-coated wells and oxidation-induced changes in ferricytochrome *c* absorbance were measured, as described.^{15, 27} To monitor degranulation, PMN were plated and lactoferrin content was measured by ELISA from supernatants of cells stimulated on coated surfaces at 37°C for 25 min.¹⁵ FcγR-induced Ca²⁺ mobilization was measured as described.²⁸ PMN were stimulated with PMA (100 nM, Sigma Aldrich) as a positive control. The localized Shwartzman reaction (LSR) was induced as described,¹⁵ with the exception that sterile 1% Evan's blue (Sigma-Aldrich) in PBS was administered i.v. 1 hr after the i.p. injection of LPS.

Generation of serum-induced arthritis

The K/B×N model of arthritis was induced as described²⁹ except that 150 µL of serum was injected i.p. on days 0 and 2. Footpad thickness was measured using an Absolute Digimatic caliper (Mitutoyo Corporation, Japan). A clinical score based on a 0 to 3 scale²⁹ for each ankle was generated, yielding a range of 0 to 12 per mouse. At the conclusion of the experiment, mice were euthanized, hind paws harvested, fixed in 4% paraformaldehyde in PBS for 48 h and decalcified, prior to sectioning and staining with hematoxylin and eosin.

Statistical analysis

Statistical significance was calculated using a Student's *t* test, paired *t* test or ANOVA, using Microsoft Excel or GraphPad Prism 4 software (GraphPad Software Inc., La Jolla, CA).

Results

Mutation of Y145 has a greater impact than mutation of Y112/128 on FcεRI-induced MC functions

The N-terminal tyrosines of SLP-76 can be divided into two groups based on their binding partners. Y112 and Y128 cooperatively bind the guanine nucleotide exchange factor, Vav, and the non-catalytic region of tyrosine kinase adaptor protein (Nck),^{30–34} while Y145 is implicated in the binding of tyrosine expressed in hepatocellular carcinoma (Tec) family kinases^{35, 36} such as Bruton's tyrosine kinase (Btk).³⁷ To dissect the contribution of these two groups of tyrosine units to FcεRI-induced MC functions, we generated BMMC from mice harboring Y112/128F or Y145F mutations.²³ These BMMC lacked WT SLP-76 expression but expressed mutant SLP-76 proteins comparable to the endogenous level of WT SLP-76 in control BMMC (not shown). BMMC from Y112/128F and Y145F mice developed in normal numbers and expressed normal levels of FcεRI and CD117 (c-kit, stem cell factor receptor; not shown). Upon FcεRI crosslinking, WT BMMC degranulated and produced cytokines (IL-6) and chemokines (MCP-1) robustly and in an antigen dose-dependent manner (Fig. 1A–C). In contrast, *Slp-76*^{−/−} BMMC exhibited significant reductions in each of these functions. BMMC expressing the Y112/128F and Y145F mutations also exhibited defective FcεRI-mediated functions, although not to the same extent as *Slp-76*^{−/−} cells (Fig. 1A–C). While Y145F BMMC were significantly worse than Y112/128F BMMC at secreting IL-6 (Fig. 1B), Y112/128F BMMC were more defective than Y145F BMMC in MCP-1 production (Fig. 1C). Since generation of a respiratory burst by MC has been suggested to play a role in allergic inflammation and defense against certain microorganisms,³⁸ we examined the ability of BMMC to produce reactive oxygen intermediates (ROI). Similar to IL-6 secretion, *Slp-76*^{−/−} and Y145F BMMC, and to a much lesser extent Y112/128F BMMC, exhibited defects in generating ROI in response to FcεRI crosslinking (Fig. 1D). Next, we examined the production of two key arachidonic acid metabolites, PGD₂ and LTC₄ by mutant MC (Fig. 1E). Compared to WT MC, Y145F BMMC displayed a significant reduction in the production of both mediators. In contrast, Y112/128F BMMC showed a milder yet significant decrease in LTC₄

but not PGD₂ production. Together, these data indicate that mutations affecting both tyrosine units interfere with SLP-76 function in BMMC following FcεRI crosslinking; however, Y145 plays a more critical role in certain assays, as is evident by markedly decreased IL-6, ROI and arachidonic acid metabolites production, as well as a reproducibly but not significantly reduced degranulation.

Y112/128F and Y145F BMMC exhibit altered FcεRI-induced signaling

To elucidate the biochemical pathways defective in SLP-76 mutant BMMC, we investigated the activation of key FcεRI-induced signaling pathways. Phosphorylation of PLCγ and subsequent Ca²⁺ flux are crucial SLP-76-mediated events during FcεRI-induced MC activation.^{16, 19, 39} To test the ability of BMMC to flux Ca²⁺, we stimulated cells through FcεRI and monitored intracellular Ca²⁺ levels by flow cytometry. Compared to WT BMMC, Y145F and to a lesser extent Y112/128F BMMC exhibited reduced intracellular Ca²⁺ flux (Fig. 2A). The defects in the Ca²⁺ response of the mutant cells correlated with diminished phosphorylation of PLCγ1 and PLCγ2 (Fig. 2B).

Because activation of MAPK drives the production of cytokines such as IL-6,^{40, 41} we next examined FcεRI-mediated phosphorylation of MAPK family members. Compared to WT BMMC, *Slp-76*^{-/-} BMMC displayed markedly reduced FcεRI-induced phosphorylation of p38 and JNK but preserved phosphorylation of ERK (Fig. 2B, 2C). Consistent with their diminished FcεRI-induced functions, BMMC expressing the Y145F mutation showed decreased phosphorylation of p38 and JNK, albeit to a lesser extent compared to *Slp-76*^{-/-} cells (Fig. 2B, 2C). In contrast, Y112/128F BMMC retained normal MAPK phosphorylation in response to FcεRI ligation. Therefore, the degree of reduction in FcεRI-induced IL-6 production by Y145F and Y112/128F BMMC was proportional to their ability to phosphorylate p38 and JNK.

Y112/128F and Y145F PMN exhibit defective FcγR-dependent functions

PMN express FcγRIII, an activating FcγR that binds IC and signals in a SLP-76-dependent manner. To elucidate the role of Y112/128 and Y145 in PMN activation, we examined FcγR-induced spreading, degranulation, and ROI production. Compared to WT PMN, SLP-76-deficient, Y112/128F and Y145F PMN each exhibited significant and comparable reductions in spreading (Fig. 3A). Tyrosine mutant PMN also displayed significant decreases in IC-induced degranulation, releasing 50% less lactoferrin than WT PMN (Fig. 3B). In both of these assays, Y145F PMN were reproducibly, but not statistically, worse than Y112/128F PMN. Compared to WT PMN, PMN expressing the Y112F/128F mutation exhibited significantly reduced FcγR-dependent ROI production when plated on IC (Fig. 3C, left panel) or 2.4G2, a stimulating FcγRII/III-specific antibody (Fig. 3C, right panel). Y145F PMN had a more pronounced defect that was not statistically different than SLP-76-deficient PMN (Fig. 3C).

As in MC, Ca²⁺ mobilization is a crucial signaling event supported by SLP-76 in response to FcγR engagement.¹⁷ Therefore, we next examined whether the SLP-76 mutants could support FcγR-induced Ca²⁺ flux in PMN. The Y145F mutation, and to a lesser extent Y112/128F, impaired the ability of SLP-76 to support Ca²⁺ mobilization following FcγR cross-linking (Fig. 3D). Together, these data demonstrate that Y112/128 and Y145 are both required for optimal FcγR-induced PMN functions. However, similar to MC, Y145 plays a more prominent role during specific processes, such as ROI production and Ca²⁺ mobilization.

Y112/128 and Y145 are similarly required for integrin-induced PMN functions

Integrins can be activated in a SLP-76-independent manner by proinflammatory mediators such as tumor necrosis factor-α (TNF-α), which leads to their increased avidity and affinity for ligand.^{9, 42} Subsequently, engaged integrins transduce SLP-76-dependent signals resulting in PMN effector functions.^{9, 42} To examine whether mutations of SLP-76 impact upon integrin-

induced signaling and resulting responses, we plated PMN on surfaces coated with polyRGD, a pan-integrin ligand that directly stimulates integrins without requiring activation by proinflammatory mediators, or on the $\beta 1$ integrin ligand collagen in the presence of TNF- α . We then assessed cell spreading (Fig. 4A), degranulation (Fig. 4B) and ROI production (Fig. 4C). In contrast to WT PMN, Y112/128F and Y145F mutant PMN exhibited severely defective responses that were statistically no different from SLP-76-deficient cells. These data demonstrate that Y112/Y128 and Y145 are each key motifs within SLP-76 that mediate integrin-dependent PMN functions.

Co-expression of Y112/128F and Y145F does not support BMMC or PMN functions

Co-expression of Y112/128F and Y145F mutant proteins rescues SLP-76-dependent defects in thymocyte development²³ but not SLP-76-associated alterations in platelet function.⁴³ To further explore this discrepancy, we examined whether Y112/128F and Y145F can provide functional complementation in myeloid cells. We analyzed PMN from compound heterozygous mice harboring one *Slp-76*^{Y112/128F} and one *Slp-76*^{Y145F} allele (double-mutant mice; DB). In each assay, DB PMN exhibited altered functions that were as defective as either single mutant PMN (Fig. 3A–D, Fig. 4A–C). Similar results were obtained using BMMC, where expression of both mutations failed to rescue Fc ϵ RI-induced degranulation (Fig. 5A), Ca²⁺ flux (Fig. 5B) or biochemical signaling defects (Fig. 5C, D) to WT levels. Thus, as previously appreciated in platelets, mutant SLP-76 molecules do not function cooperatively within PMN and MC to restore FcR- or integrin-induced functions to levels greater than those imparted by a single mutation.

Y112/128F and Y145F mice exhibit altered *in vivo* MC and PMN functions

Studies examining structure/function relationships of SLP-76 in MC and PMN activation *in vivo* have not been previously described. To examine the impact of the SLP-76 tyrosine mutations on MC function *in vivo*, we subjected Y112/128F and Y145F knock-in mice to an IgE-dependent model of passive systemic anaphylaxis (PSA). Compared to WT mice, IgE-sensitized Y112/128F and Y145F mice showed significant reductions in serum histamine concentration after allergen challenge that were on average 40% and 80% less than the response of control mice, respectively (Fig. 6A). Of note, serum histamine levels in SLP-76-deficient mice are nearly undetectable after PSA challenge (data not shown). In agreement with our *in vitro* results, Y145 appears more critical than Y112/128 during Fc ϵ RI-induced MC activation *in vivo*.

To determine whether the SLP-76 mutations would impact PMN functions *in vivo*, we used the LSR, an integrin-dependent but MC-independent model of lipopolysaccharide (LPS)-induced dermal inflammation caused by the directed accumulation and degranulation of PMN.^{15, 44–46} Upon LSR induction, WT mice displayed significantly increased vascular permeability in LPS-injected ears compared to phosphate buffered saline (PBS)-injected ears (Fig. 6B). In contrast to WT animals, and consistent with our *in vitro* data on integrin-induced PMN activation, mice expressing Y112/128F, Y145F, or both Y112/128F and Y145F (DB) exhibited an attenuated responses that were statistically no different from mice with SLP-76-deficient PMN.

It has been observed recently that Vav and PLC γ are each essential for the induction of disease in the K/B $\times$ N model of inflammatory arthritis, a model that is dependent on the intact functions of MC and PMN as well as Fc γ R and integrins.^{5, 47–55} In this model, the passive transfer of serum from mice expressing the KRN TCR transgene and the MHC class II molecule I-A^{g7} results in the rapid and robust induction of joint-specific inflammation.^{56–58} Since the Y112/128F and Y145F mutations alter the activation of MC, PMN, Vav²³ and PLC γ , we hypothesized that both strains of mutant mice might show defects in this model. Therefore, we

administered arthritogenic serum to WT and SLP-76 mutant mice and monitored for disease by inspection for joint swelling and erythema (clinical score), measurement of footpad thickness and examination of joint histology. WT mice developed footpad swelling (Fig. 7A) and increased clinical scores (Fig. 7B) early following serum injection and continuing until joints were severely swollen and deformed (Fig. 7C). Histologic examination of WT joints showed robust leukocyte infiltration into the joint space and periarticular regions, as well as severe dilatation of the joint capsule, synovial hyperplasia and early pannus formation (Fig. 7D). Mice expressing Y112/128F exhibited a modest reduction in average footpad thickness (Fig. 7A) and overall clinical score (Fig. 7B), due to the fact that not all paws developed arthritic inflammation. However, contrary to our original hypothesis, the affected ankles from Y112/128F mice exhibited swelling and histologic features of arthritis that were indistinguishable from WT mice (Fig. 7C and D). In marked contrast, mice with SLP-76-deficient PMN, Y145F-expressing mice or DB mutant mice failed to develop clinical or histologic signs of joint inflammation (Fig. 7A–D). Thus, mutations that abrogate SLP-76 expression or interfere with the function of Y145, but not Y112/128, completely protect from the onset of antibody-mediated arthritis.

Discussion

To control allergy and inflammation resulting from aberrant MC and PMN activation, it is necessary to understand the mechanisms that govern the activation of these cell lineages. By examining knock-in mice harboring mutations in the N-terminal tyrosines of SLP-76, we demonstrate that Y145 is foremost in importance during most *in vitro* assays of FcR-induced MC and PMN function. Importantly, Y145F also has the most dramatic impact upon MC and PMN activation *in vivo*, resulting in reduced histamine release during PSA and decreased LPS-induced tissue inflammation. Furthermore, the Y145F mutation protects against leukocyte infiltration and development of joint destruction normally evident in the K/B $\times$ N model of serum-induced arthritis. These are the first data to indicate that mutation of Y145 alters MC and PMN functions, by interfering with activation of PLC γ and likely also the Tec family kinases. In conjunction with recent findings demonstrating that PLC γ itself is critical for arthritis induction in the K/B $\times$ N model,^{49, 50} our data suggest that elucidation of the Y145-dependent biochemical pathways may provide insights into how MC and PMN activation can be pharmacologically manipulated to more effectively treat patients with MC and PMN-mediated disorders such as allergy, asthma, sepsis and arthritis.

The Y145F mutation interfered with the ability of SLP-76 to activate PLC γ in MC and PMN. This result is presumably because SLP-76 signaling complexes bring PLC γ adjacent to the Tec kinases and Vav, which then are involved in phosphorylation and activation of PLC γ . Although we had anticipated that the Y145F mutation would interfere with binding of Tec kinases to SLP-76, recent data generated using primary mouse thymocytes demonstrate intact binding of Tec kinases with Y145F mutant SLP-76.²³ Nonetheless, Y145 is critical for glycoprotein VI (GPVI)-induced activation of Btk in platelets,⁴³ and T cell receptor-dependent Itk-associated functions in thymocytes. Thus, SLP-76 Y145F is required for the full activation of Tec family kinases. Our data in myeloid cells are consistent with a requirement for Y145 to fully activate Btk, since we observe reduced FcR-induced PLC γ phosphorylation in MC and diminished Ca²⁺ flux in MC and PMN. Furthermore, Y145F MC phenocopy *Btk*^{-/-} MC, which exhibit defective degranulation, cytokine production and generation of LTC₄ and PDG₂ as the result of reduced activation of PLC γ and the JNK pathway.^{59–63}

Although BMMC expressing Y112/128F generally exhibited milder defects than cells expressing Y145F, MCP-1 production by Y112/128F BMMC was as defective as Y145F BMMC. The production of chemokines and cytokines have been reported have different activation thresholds.⁶⁴ For example, weak Fc ϵ RI crosslinking stimuli can elicit MCP-1

production, while an ~10 fold stronger stimulus is required to evoke IL-6 production from mast cells.⁶⁴ However, these threshold differences can not explain why the Y112/128F mutation would more selectively affect MCP-1 production, since MCP-1 would be expected to be less sensitive than IL-6 to decreases in FcεRI-induced signal strength. In addition to signal strength, the nature of the transduced signals leading to MCP-1 and IL-6 production appears different. MCP-1 production is more dependent on the Gab2-mediated signaling pathway, while IL-6 is more dependent on LAT.⁶⁴ At this time, it is unclear how these two signaling pathways intersect and whether the Y112/128F mutation has a preferential effect on the Gab2-mediated signaling pathway.

Interestingly, certain PMN functions such as spreading, demonstrate similar requirements for Y145 and Y112/128. These findings may reflect the inherent nature of PMN, which must exist harmlessly in suspension in the circulation but then execute effector mechanisms once tightly adhered to tissues. As an example of this regulatory link between cellular adhesion and signaling cascades, PMN degranulation can be triggered by PKC-dependent increases in intracellular Ca^{2+} ,^{65–69} but also requires actin reorganization to control the access granules to the plasma membrane.⁷⁰ Thus, there might be a dual requirement for guanine nucleotide exchange factor-mediated cytoskeletal rearrangements in addition to second messenger signals to support integrin- or FcγR-induced functional responses of adherent PMN. In such a scenario, mutations of Y112/128, which interfere with the phosphorylation and presumed activation of Vav, and mutation of Y145, which disrupts activation of the Tec family kinases, would be expected to similarly influence signaling and cytoskeletal reorganization. Experiments are ongoing to determine the biochemical explanation for the interdependence of Y112/128 and Y145 in cytoskeleton-mediated PMN responses.

Our studies indicate that there is a lack of cooperativity between SLP-76 molecules containing the Y112/Y128F or Y145F mutations since co-expression of both mutants in trans does not support cell signaling or function beyond the levels observed when either single mutant protein is present. This contrasts to T-cell receptor (TCR) signaling in thymocytes, where presence of Y112/Y128F and Y145F rescues developmental and signaling abnormalities,²³ but is similar to platelets where activation by the ITAM-bearing GPVI receptor requires that all phosphorylatable tyrosine units reside on the same SLP-76 molecule.⁴³ Fcε/γR and GPVI signaling depends upon the presence of the FcR γ chain⁷¹, while integrin signaling utilizes the γ chain or/and DAP12¹³, each containing one ITAM per chain. This signaling mechanism differs from that used by the TCR, where each TCR signaling complex contains 10 ITAMs within the associated CD3 subunits.²³ These observations lead us to speculate that the failure to rescue signaling and functional defects in DB mutant MC and PMN may result from the limited number of ITAMs within the Fcε/γR or integrin signaling complexes. If fewer ITAM motifs are present, the ability to deliver a successful signal might rely more heavily on individual signaling components to physically interact with and activate partner proteins. Mutations that destabilize SLP-76 dependent-protein interactions or interfere with activation of SLP-76-interacting partners would be expected to have a greater impact on downstream signaling when present in these smaller receptor complexes, where there is decreased stoichiometry between signaling molecules and fewer tertiary protein-protein interactions to facilitate the recruitment and activation of additional signaling components.

Interestingly, co-expression of Y112/128 and Y145 in PMN did lead to increased IC-stimulated superoxide generation compared to expression of the Y145 mutant alone. IC elicit a phagocytic-type response which activates multiple signaling pathways including those involving phosphoinositide 3-kinases (PI3K).^{17, 72} PI3K signaling is likely preserved in SLP-76 tyrosine mutant PMN since PI3K-dependent activation through G-protein coupled receptors is normal in SLP-76 KO PMN.^{17, 72} Thus, in cells expressing the Y112/128F mutation either alone or in combination with Y145F, IC-induced activation of PI3K could lead to generation of lipid

moieties which then activate Vav in membrane regions containing NADPH oxidase subunits, thereby coupling FcγR signaling to increases in NADPH oxidase function. While Tec kinases might also be recruited to membranous regions and support oxidase assembly, they require Y145 of SLP-76 for full activation. Thus, cells expressing Y145 alone more closely resemble SLP-76 deficient PMN in terms of their responsiveness to IC.

We observe that *in vitro* MC and PMN functional deficiencies imparted by the Y112/128F and Y145F mutations correlate with impaired functions *in vivo*, as evidenced by reduced histamine release in IgE-dependent PSA and diminished inflammation in the LSR. Most striking is our finding that the Y145F mutation completely protects against serum-induced arthritis. At the earliest stages of disease, it is thought that circulating PMN are activated by soluble IC, thereby leading to their release of soluble mediators, which increase vascular permeability and allow access of IC to tissue-resident MC. The subsequent activation of MC is proposed to amplify vascular permeability, resulting in IC deposition on articular surfaces.⁷³ FcRs, particularly FcγRIII, are critical for the activation of MC and PMN and for the initiation of IC-driven inflammation.^{48, 51} Y145F mice did not display signs of joint inflammation at time of sacrifice, yet SLP-76-deficient PMN extravasate normally into inflamed tissue in other *in vivo* models,¹⁵ suggesting that the lack of arthritis in these mice may occur at the earliest time points that are dependent upon IC-driven PMN activation and MC cytokine production. Indeed, Y145F MC and PMN are defective in Fcε/γR-induced functions such as degranulation and IL-6 production. Thus without these contributions, there may no longer be sufficient changes in vascular permeability to allow full access of antibodies to articular surfaces or the proper cytokine environment to propagate subsequent inflammatory responses.

Since the Y145F mutation is germline, it is also conceivable that functional defects in other lineages, such as macrophages, dendritic cells and lymphocytes, influence disease progression.^{54, 74–78} Some of these lineages likely contribute to disease pathology after MC- and PMN-dependent activation facilitates autoantibody access to articular regions.⁷³ Despite their possible contribution to arthritis pathogenesis, our data clearly support the notion that Y145, not Y112/128, is the most critical SLP-76 tyrosine required for its function in this model of inflammatory arthritis. Future studies to delineate how Y145 mediates SLP-76 signaling will provide important insights into MC and PMN activation and may facilitate the development of new treatments for inflammatory arthritis and other allergic or autoimmune conditions.

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Abbreviations

AKT/PKB	protein kinase B
BMMC	bone marrow-derived mast cell
Btk	Bruton's tyrosine kinase
DNP	dinitrophenol
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated kinase

F	phenylalanine
FcR	Fc receptor for immunoglobulin
GPVI	glycoprotein VI
HSA	human serum albumin
IC	immune complex
IgE	immunoglobulin E
IgG	immunoglobulin G
IL-6	interleukin-6
ITAM	immunoreceptor tyrosine-based activation motif
Itk	interleukin 2-inducible T-cell kinase
JNK	Jun N-terminal kinase
Lcp2	lymphocyte cytosolic protein 2 (also known as SLP-76, SH2 domain-containing leukocyte protein of 76 kDa)
LPS	lipopolysaccharide
LSR	localized Shwartzman reaction
LTC₄	leukotriene C ₄
MAPK	mitogen-activated protein kinase
MC	mast cell
MCP-1	monocyte chemotactic protein-1
Nck	non-catalytic region of tyrosine kinase adaptor protein
p38	protein kinase of 38 kDa
PBS	phosphate buffered saline
PGD₂	prostaglandin D ₂
PLCγ	phospholipase-C γ
PI3K	phosphoinositide 3-kinase
PMN	polymorphonuclear leukocyte or neutrophil
polyRGD	fibronectin-like engineered protein polymer-plus
PSA	passive systemic anaphylaxis
ROI	reactive oxygen intermediates
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tec	tyrosine kinase expressed in hepatocellular carcinoma
TNF-α	tumor necrosis factor-alpha

WT wild type
Y tyrosine

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FIGURE 1. Y112F/128F and Y145F BMMC show defective FcεRI-mediated functions

WT, Y112/128F, Y145F and *Slp-76*^{-/-} (SLP KO) BMMC were analyzed for FcεRI-mediated (A) degranulation, (B) IL-6, or (C) MCP-1 production. Results in *top* line graphs are expressed as an average of 2 mice/genotype from a representative experiment. The *lower* plots represent the mean ± SD of at least 4 independent experiments (2 mice/genotype/experiment) normalized to WT levels stimulated with 10 ng/ml HSA-DNP. N.S. = not statistically significant. (D) WT and mutant BMMC were monitored for FcεRI-induced ROI production by flow cytometry. Results are representative of 2 independent experiments. (E) PGD₂ and LTC₄ production were measured in WT and mutant BMMC following FcεRI stimulation at the indicated antigen concentrations. Results are expressed as mean ± SD (3 mice/genotype/experiment).

FIGURE 2. Altered activation of intracellular signaling molecules in BMMC derived from Y112/128F and Y145F mice

(A) WT and mutant BMMC were monitored for FcεR-induced elevations in intracellular Ca^{2+} by flow cytometry. Iono, ionomycin. The curves represent the ratio of Indo-1 FL5/FL4 fluorescence, a measure of intracellular $[\text{Ca}^{2+}]$ as a function of time. (B) WT and mutant BMMC were stimulated through FcεRI and analyzed by Western blotting for phosphorylated (p) PLCγ1, PLCγ2, p38, and ERK1/2 or (C) JNK1/2. Total AKT and PLCγ1 served as loading controls. Results are representative of at least 3 independent experiments.

FIGURE 3. Mutation of SLP-76 N-terminal tyrosines diminishes Fc γ R-induced PMN activation PMN from WT, Y112/128F, Y145F, or mice expressing one Y112/128F allele and one Y145F allele (double-mutant mice, DB) were analyzed for Fc γ R-induced functions. (A) Cell spreading was assessed by microscopy after plating bone marrow PMN onto IC-coated plates for 30 minutes. (B) IC-stimulated release of lactoferrin into culture supernatant after 25 minutes was measured by ELISA and graphed as percent of WT degranulation. (C) ROI production was induced by plating PMN onto surfaces coated with IC (left) or the anti-Fc γ RII/III specific antibody 2.4G2 (right). The graphs depicted in (A), (B) and (C) are the averaged values from 4 or more experiments. (D) Fc γ R-induced Ca²⁺ mobilization was analyzed by flow cytometry. The curves represent the ratio of Indo-1 FL5/FL4 fluorescence, a measure of intracellular [Ca²⁺] as a function of time. The break in the tracings represents a temporary interruption in the flow of cells in the cytometer as ionomycin (Iono) is added to induce a maximum Ca²⁺ response. The data are a representative of >4 independent experiments performed. (*) denotes values statistically lower than WT but greater than Y145F or SLP KO PMN; P < 0.05, N.S.= not statistically significant.

FIGURE 4. The N-terminal tyrosines of SLP-76 are required for integrin-dependent PMN responses

(A) PMN were plated on polyRGD-coated wells and allowed to spread for 30 minutes. The graph depicts the average percent of cells spread, combined from 6 experiments. (B) PMN were plated on the $\beta 1$ integrin ligand collagen for 30 minutes in the presence of TNF- α . Lactoferrin release was measured by ELISA. The percent of WT degranulation was averaged from 5 experiments. (C) ROI production was induced by plating PMN onto polyRGD-coated wells and measured over time. Graph depicts the averages from 5 experiments. (*) denotes that WT PMN function was statistically greater than the function of each of the mutant strains $p < 0.05$; N.S.= no statistical difference between values measured from each mutant.

FIGURE 5. Coexpression of Y112/128 and Y145 does not rescue defective FcεRI-mediated BMMC activation

(A) WT, Y112/128F and Y145F BMMC were compared to DB BMMC by analyzing FcεRI-mediated degranulation. Results are expressed as mean $\pm$ SD (2 mice/genotype). (B) WT and mutant BMMC were monitored for FcεRI-mediated Ca²⁺ flux by flow cytometry. The curves represent the ratio of Indo-1 FL5/FL4 fluorescence, a measure of intracellular [Ca²⁺] as a function of time. (C) WT and mutant BMMC were stimulated through FcεRI and analyzed by Western blot analysis of phosphorylated PLCγ1, p38 and (D) JNK1/2. Total AKT and PLCγ1 served as loading controls. Results are representative of at least 3 independent experiments.

FIGURE 6. Mice harboring mutations in the N-terminal tyrosines of SLP-76 exhibit attenuated allergic and inflammatory responses *in vivo*

(A) Serum histamine levels were measured in WT (n = 8) and Y112/128F mice (n = 9) (left plot) or WT (n = 5) and Y145F (n = 5) mice (middle plot) undergoing PSA. Results are expressed as mean histamine concentration (nM) $\pm$ SD. In the right plot, serum histamine concentrations of Y112/128F (n = 9) and Y145F (n = 8) mice were averaged from 2 independent experiments after normalization to WT controls. (B) The LSR was induced in WT and mutant mice, and ears were assessed for vascular permeability by measurement of the extravasation of Evan's blue dye. N.S.= not statistically significant.

FIGURE 7. Mice harboring the Y145F mutation are protected from the development of serum-induced arthritis

K/B×N serum was administered to WT, Y112/128F and Y145F knock-in mice and to mice with SLP-76-deficient PMN (*Slp-76*^{LoxP/LoxP}*LysM*^{Cre}). Disease progression was monitored by footpad measurements. Average hind footpad thickness for each genotype (n = 8 paws for each mutant; n = 6 paws for WT) is graphed in (A). (B) Average clinical score for each genotype (n = 4 mice for each mutant; n = 3 mice for WT). (C) Photographs of ankle joints and a corresponding H&E stained section from these joints at day 15, pictured at higher magnification in (D), with joint capsules labeled with an arrow. (*) p < 0.05 compared to WT.