

Mutation of tyrosine 145 of lymphocyte cytosolic protein 2 protects mice from anaphylaxis and arthritis

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Background: Lymphocyte cytosolic protein 2, also known as Src homology 2 domain-containing leukocyte phosphoprotein of 76 kilodaltons (SLP-76), is an essential adaptor molecule in myeloid cells, where it regulates Fc ϵ RI-induced mast cell (MC) and Fc γ R- and integrin-induced neutrophil (polymorphonuclear leukocyte [PMN]) functions. SLP-76 contains 3 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 effect than Y112/128F on most *in vitro* FcR-induced functions. *In vitro* functional defects were recapitulated *in vivo*, where mice expressing Y145F exhibited greater 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 that 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 MCs and PMNs will increase our understanding of

these lineages and provide insights into the treatment of allergy and inflammation. (J Allergy Clin Immunol 2009;124:1088-98.)

Key words: Mast cells, neutrophils, Fc receptors, integrins, signal transduction, SLP-76, passive systemic anaphylaxis, localized Shwartzman reaction, arthritis

Mast cells (MCs) and neutrophils (polymorphonuclear leukocytes [PMNs]) 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 MCs and PMNs also contributes to several human diseases, including hypersensitivity disorders and asthma,³ as well as endotoxic shock and inflammatory arthritis.^{4,5} It is important to delineate the molecules and signaling pathways that control the activation of MCs and PMNs to better understand and manipulate their roles in host defense and disease.

The Fc receptors for immunoglobulin (FcRs) 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 IgE molecules.⁶ After cognate antigen exposure, antigen-specific IgE-bound Fc ϵ RI is cross-linked, leading to release of preformed granule contents and inflammatory mediators.⁷ PMNs express other stimulatory FcRs, such as Fc γ RIII, that bind IgG-opsonized particles or soluble IgG immune complexes (ICs), thereby triggering PMN spreading, reactive oxygen intermediates (ROI) production, and degranulation.⁸ PMNs 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 FcRs and integrins results in the transduction of intracellular signals, which depend on 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 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76).¹⁰⁻¹⁴ SLP-76 is required in MCs and PMNs to support optimal signaling downstream of immunoreceptors and integrins, as previously demonstrated by the examination of SLP-76-deficient primary cells and mice, that 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 3 tyrosines at residues 112, 128, and 145, which are phosphorylated after integrin and immunoreceptor engagement.²⁰⁻²² To understand the roles of these residues in myeloid cell activation, we examined the functions of MCs and PMNs from knock-in mice expressing tyrosine

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Abbreviations used

BMMC:	Bone marrow–derived mast cell
Btk:	Bruton tyrosine kinase
DB:	Double-mutant
DNP:	Dinitrophenol
FcR:	Fc receptor for immunoglobulin
GPVI:	Glycoprotein VI
HSA:	Human serum albumin
IC:	Immune complex
ITAM:	Immunoreceptor tyrosine-based activation motif
JNK:	Jun N-terminal kinase
LSR:	Localized Shwartzman reaction
LTC ₄ :	Leukotriene C ₄
MAPK:	Mitogen-activated protein kinase
MC:	Mast cell
MCP-1:	Monocyte chemotactic protein 1
PGD ₂ :	Prostaglandin D ₂
PI3K:	Phosphoinositide 3-kinase
PLCγ:	Phospholipase Cγ
polyRGD:	Fibronectin-like engineered protein polymer-plus
PSA:	Passive systemic anaphylaxis
ROI:	Reactive oxygen intermediates
SLP-76:	Src homology 2 domain–containing leukocyte phosphoprotein of 76 kDa
TCR:	T-cell receptor
Tec:	Tyrosine kinase expressed in hepatocellular carcinoma
WT:	Wild-type

to phenylalanine (Y → 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 MCs and PMNs *in vitro*; however, Y145 plays a more critical role in controlling most FcεRI-induced MC and FcγR-induced PMN functions. Importantly, *in vitro* defects in Fcε/γR-induced MC and PMN activation correlate to *in vivo* deficiencies, in which mice expressing Y145F exhibit significantly reduced immediate hypersensitivity in a model of systemic anaphylaxis and no clinical or histologic signs of joint inflammation in a model of antibody-mediated arthritis.

METHODS

Mice

Slp-76^{−/−}, *Slp-76*^{Y112/128F}, *Slp-76*^{Y145F}, and *Slp-76*^{LoxP/LoxP}*LYsM*^{Cre} mice are as previously 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 mast cells (BMMCs) were generated, as previously described,²⁵ and used when greater than 95% of cells expressed high levels of FcεRI and CD117, as determined by flow cytometry. FcεRI-induced degranulation, as measured by release of β-hexosaminidase and cytokine production, was measured, as previously described.¹⁵ Anti-dinitrophenol (DNP) IgE-sensitized (1 μg/mL for 2 hours at 37°C) BMMCs were activated with various concentrations of human serum albumin (HSA)–DNP for 30 minutes to assess prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) release. LTC₄ and PGD₂ contents were measured in cell-free supernatants by enzyme immunoassay (Cayman Chemicals, Ann Arbor, Mich), according to the manufacturer’s protocol. Passive systemic anaphylaxis (PSA) was induced in wild-type (WT) or mutant mice, as previously described.¹⁵

FcεRI-induced ROI production and Ca²⁺ flux

BMMCs were sensitized with 1 μg/mL anti-DNP IgE for 2 hours at 37°C, washed, and resuspended at 1 × 10⁷ cells/mL in PBS containing 5 μmol/L 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (Invitrogen, Carlsbad, Calif) for measuring ROIs or 1 mmol/L 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 to 30 minutes, washed, resuspended in warm Tyrode buffer, incubated at 37°C for 10 minutes, and placed on ice until stimulation. Cells were stimulated with DNP-HSA (100 ng/mL) at 37°C, and ROI production or Ca²⁺ flux was measured by using a flow cytometer (Becton Dickinson, Franklin Lakes, NJ) for 300 or 180 seconds, respectively. Baseline ROI production or intracellular Ca²⁺ levels were measured for 30 seconds before addition of DNP-HSA. Data were analyzed with FlowJo software (Tree Star, Inc, Ashland, Ore).

Western blot analysis of MC lysates

BMMCs were presensitized with anti-DNP IgE (1 μg/mL) for 2 hours, washed, rested for 2 hours, and stimulated with DNP-HSA (30 ng/mL) for the indicated times. Cells were then lysed, proteins were resolved by SDS-PAGE (Bio-Rad Laboratories, Hercules, Calif), and phosphorylation of phospholipase Cγ (PLC) 1 and PLCγ2, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK) 1/2, SLP-76, and protein kinase B (Akt/PKB) was analyzed by Western blotting. All antibodies were from Cell Signaling (Danvers, Mass), except for the anti-phospho-Y128 (SLP-76) antibody, which was from BD Biosciences (San Jose, Calif).

FcγR- and integrin-induced activation of PMNs

PMNs were isolated from murine bone marrow, as previously described.²⁶ PMN spreading on integrin (fibronectin-like engineered protein polymer-plus [polyRGD]) and FcγR ligands (ICs consisting of HSA/anti-HSA antibodies) was measured, as previously described.¹⁵ For ROI production and degranulation, 96-well Immulon 4HBX plates (Thermo Electron Corp, Milford, Mass) 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, San Jose, Calif), an isotype control antibody (BD PharMingen), or IC. To assess ROI production, PMNs were plated on ligand-coated wells, and oxidation-induced changes in ferricytochrome c absorbance were measured, as previously described.^{15,27} To monitor degranulation, PMNs were plated, and lactoferrin content was measured by ELISA from supernatants of cells stimulated on coated surfaces at 37°C for 25 minutes.¹⁵ FcγR-induced Ca²⁺ mobilization was measured, as previously described.²⁸ PMNs were stimulated with phorbol 12-myristate 13-acetate (100 nmol/L, Sigma Aldrich) as a positive control. The localized Shwartzman reaction (LSR) was induced, as previously described,¹⁵ with the exception that sterile 1% Evans blue dye (Sigma-Aldrich) in PBS was administered intravenously 1 hour after the intraperitoneal injection of LPS.

Generation of serum-induced arthritis

The K/B × N model of arthritis was induced, as previously described,²⁹ except that 150 μL of serum was injected intraperitoneally on days 0 and 2. Footpad thickness was measured with an Absolute Digimatic caliper (Mitutoyo Corporation, Kawasaki-shi, Japan). A clinical score based on a 0- to 3-point scale²⁹ for each ankle was generated, yielding a range of 0 to 12 points per mouse. At the conclusion of the experiment, mice were euthanized, and hind paws were harvested, fixed in 4% paraformaldehyde in PBS for 48 hours, and decalcified before sectioning and staining with hematoxylin and eosin.

Statistical analysis

Statistical significance was calculated by using the Student *t* test, paired *t* test, one sample *t* test, or ANOVA with Microsoft Excel or GraphPad Prism 4 software (GraphPad Software, Inc, La Jolla, Calif).

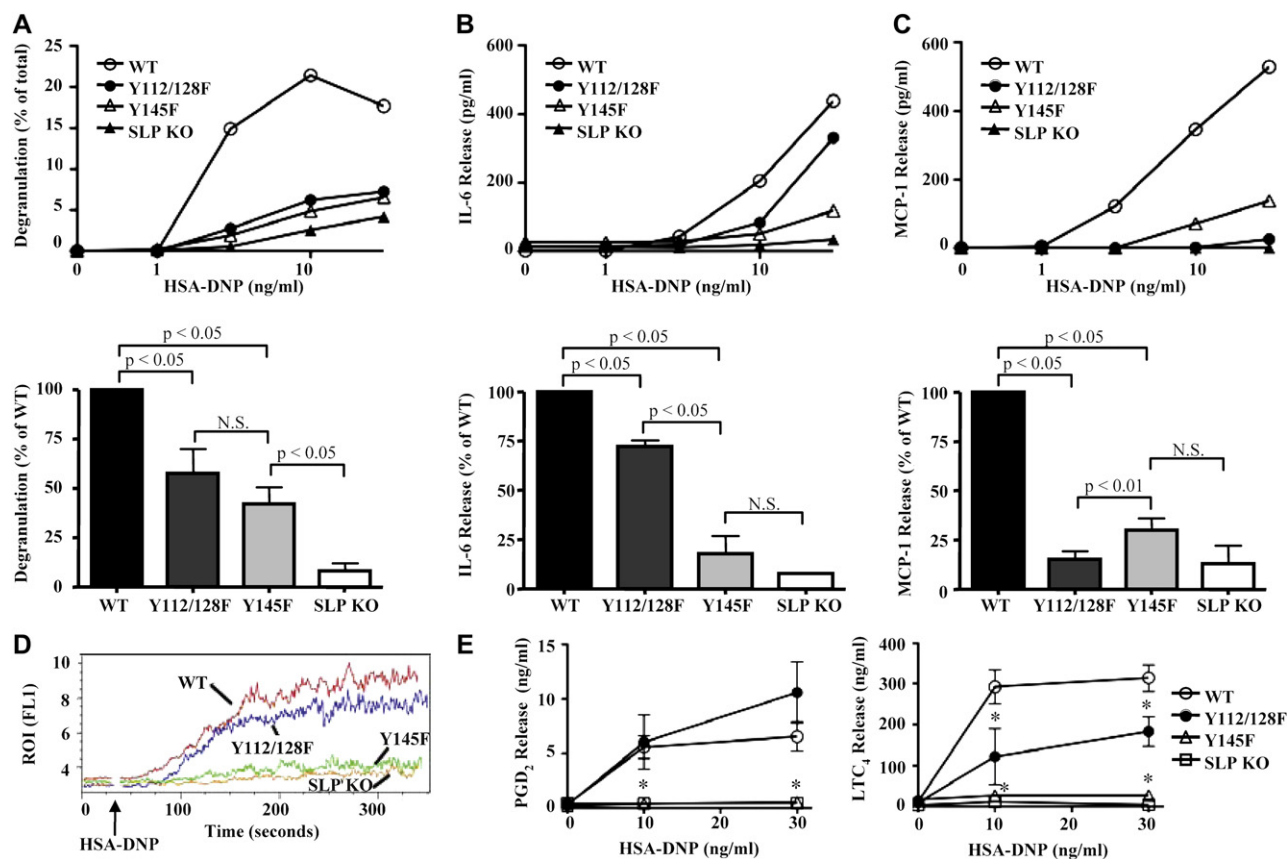


FIG 1. Y112F/128F and Y145F BMMCs show defective FcεRI-mediated functions. WT, Y112/128F, Y145F, and *Slp-76*^{-/-} (SLP KO) BMMCs were analyzed for FcεRI-mediated degranulation (A) and IL-6 (B) or MCP-1 (C) production. Results in top graphs are expressed as an average of 2 mice per genotype from a representative experiment. The lower plots represent the means ± SDs of at least 4 independent experiments (2 mice per genotype per experiment) normalized to WT levels stimulated with 10 ng/mL HSA-DNP. N.S., Not statistically significant. D, WT and mutant BMMCs were monitored for FcεRI-induced ROI production by means of flow cytometry. Results are representative of 2 independent experiments. E, PGD₂ and LTC₄ production were measured in WT and mutant BMMCs after FcεRI stimulation at the indicated antigen concentrations. Results are expressed as means ± SDs (3 mice per genotype per experiment). KO, Knockout.

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 2 groups based on their binding partners. Y112 and Y128 cooperatively bind the guanine nucleotide exchange factor Vav and the noncatalytic region of tyrosine kinase adaptor protein,³⁰⁻³⁴ whereas Y145 is implicated in the binding of tyrosine expressed in hepatocellular carcinoma (Tec) family kinases,^{35,36} such as Bruton tyrosine kinase (Btk).³⁷ To dissect the contribution of these 2 groups of tyrosine units to FcεRI-induced MC functions, we generated BMMCs from mice harboring Y112/128F or Y145F mutations.²³ These BMMCs lacked WT SLP-76 expression but expressed mutant SLP-76 proteins comparable to the endogenous level of WT SLP-76 in control BMMCs (not shown). BMMCs 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). On FcεRI cross-linking, WT BMMCs degranulated and produced cytokines (IL-6) and chemokines (monocyte chemoattractant protein

1 [MCP-1]) robustly and in an antigen dose-dependent manner (Fig 1, A-C). In contrast, *Slp-76*^{-/-} BMMCs exhibited significant reductions in each of these functions. BMMCs expressing the Y112/128F and Y145F mutations also exhibited defective FcεRI-mediated functions, although not to the same extent as *Slp-76*^{-/-} cells (Fig 1, A-C). Although Y145F BMMCs were significantly worse than Y112/128F BMMCs at secreting IL-6 (Fig 1, B), Y112/128F BMMCs were more defective than Y145F BMMCs in MCP-1 production (Fig 1, C). Because generation of a respiratory burst by MCs has been suggested to play a role in allergic inflammation and defense against certain microorganisms,³⁸ we examined the ability of BMMCs to produce ROI. Similar to IL-6 secretion, *Slp-76*^{-/-} and Y145F BMMCs and, to a much lesser extent, Y112/128F BMMCs exhibited defects in generating ROI in response to FcεRI cross-linking (Fig 1, D). Next we examined the production of 2 key arachidonic acid metabolites, PGD₂ and LTC₄, by mutant MCs (Fig 1, E). Compared with WT MCs, Y145F BMMCs displayed a significant reduction in the production of both mediators. In contrast, Y112/128F BMMCs showed a milder yet significant decrease in LTC₄ but not PGD₂ production. Together, these data indicate

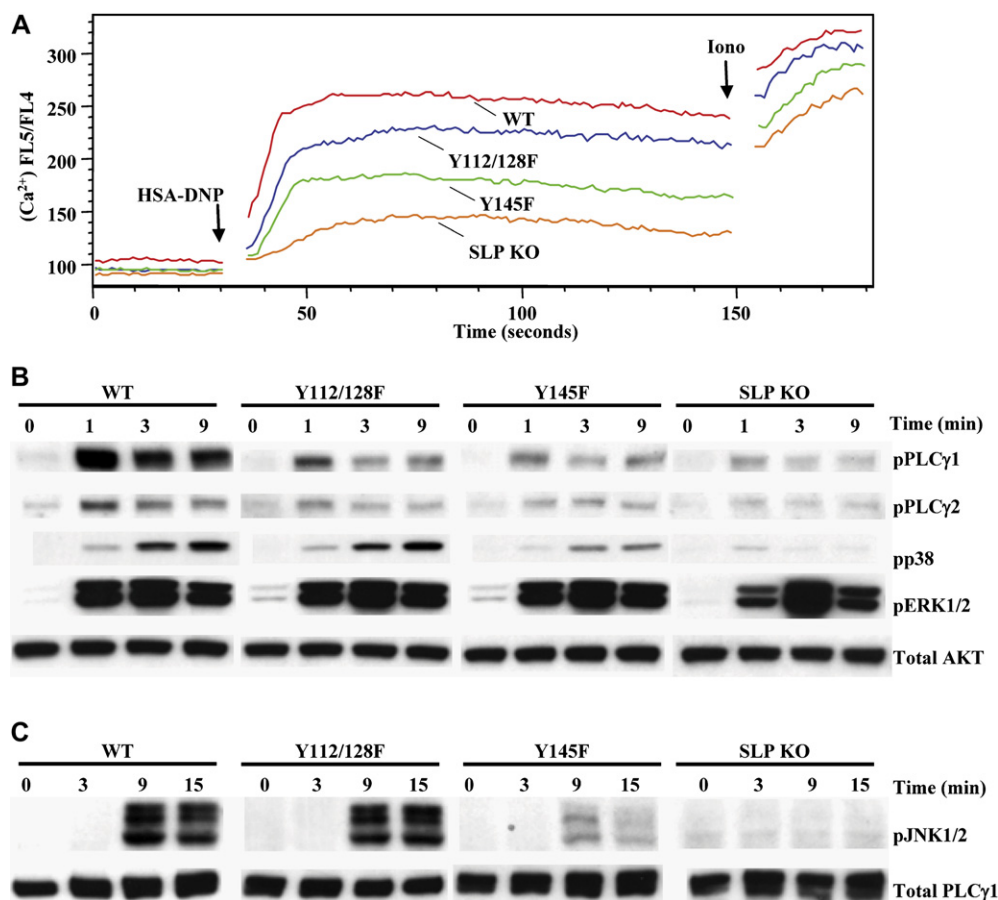


FIG 2. Altered activation of intracellular signaling molecules in BMMCs derived from Y112/128F and Y145F mice. **A**, WT and mutant BMMCs were monitored for FcεRI-induced increases in intracellular Ca^{2+} levels by flow cytometry. *Iono*, ionomycin. The curves represent the ratio of Indo-1 FL5/FL4 fluorescence, a measure of intracellular Ca^{2+} as a function of time. **B** and **C**, WT and mutant BMMCs were stimulated through FcεRI and analyzed by Western blotting for phosphorylated (p) PLCγ1, PLCγ2, p38, and extracellular signal-regulated kinase 1/2 (ERK1/2; all Fig 2, B) or JNK1/2 (Fig 2, C). Total protein kinase B (Akt/PKB) and PLCγ1 served as loading controls. Results are representative of at least 3 independent experiments. KO, Knockout.

that mutations affecting both tyrosine units interfere with SLP-76 function in BMMCs after FcεRI cross-linking; however, Y145 plays a more critical role in certain assays, as is evident by markedly decreased IL-6, ROI, and arachidonic acid metabolite production, as well as a reproducibly but not significantly reduced degranulation.

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

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

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 with WT BMMCs, *Slp-76*^{-/-} BMMCs displayed markedly reduced FcεRI-induced phosphorylation of p38 and JNK but preserved phosphorylation of ERK1/2 (Fig 2, B and C). Consistent with their diminished FcεRI-induced functions, BMMCs expressing the Y145F mutation showed decreased phosphorylation of p38 and JNK, albeit to a lesser extent compared with that seen in *Slp-76*^{-/-} cells (Fig 2, B and C). In contrast, Y112/128F BMMCs 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 BMMCs was proportional to their ability to phosphorylate p38 and JNK.

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

PMNs express FcγRIII, an activating FcγR that binds ICs and signals in an 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.

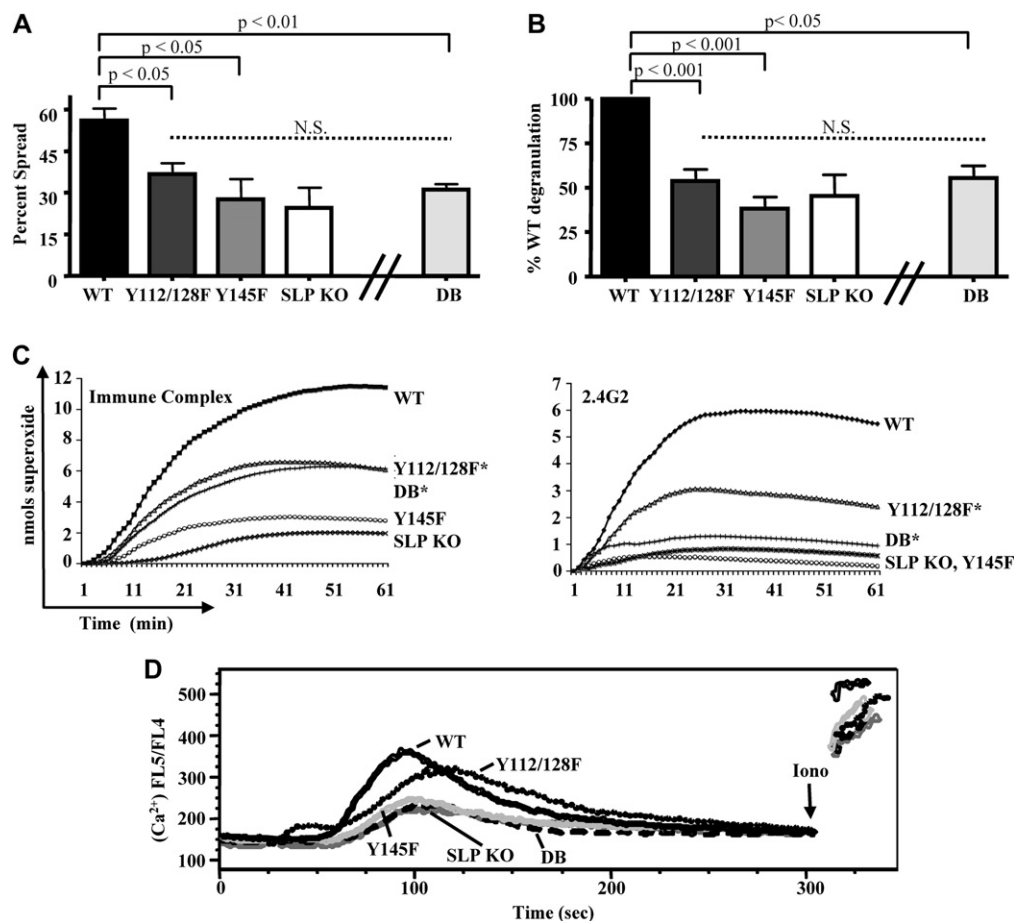


FIG 3. Mutation of SLP-76 N-terminal tyrosines diminishes Fc γ R-induced PMN activation. PMNs from WT, Y112/128F, or Y145F mice or from double-mutant (DB) mice expressing 1 Y112/128F allele and 1 Y145F allele, were analyzed for Fc γ R-induced functions. **A**, Cell spreading was assessed by microscopy after plating bone marrow PMNs 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 a percentage of WT degranulation. **C**, ROI production was induced by plating PMNs onto surfaces coated with ICs (*left*) or the anti-Fc γ R/III-specific antibody 2.4G2 (*right*). The graphs depicted in Fig 3, 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*) was added to induce a maximum Ca²⁺ response. The data are representative of more than 4 independent experiments performed. *Values statistically lower than seen in WT PMNs but greater than seen in Y145F or SLP KO PMNs ($P < .05$). N.S., Not statistically significant; KO, knockout.

Compared with WT PMNs, SLP-76-deficient Y112/128F and Y145F PMNs each exhibited significant and comparable reductions in spreading (Fig 3, A). Tyrosine mutant PMNs also displayed significant decreases in IC-induced degranulation, releasing 50% less lactoferrin than WT PMNs (Fig 3, B). In both of these assays, Y145F PMNs were reproducibly but not statistically worse than Y112/128F PMNs. Compared with WT PMNs, PMNs expressing the Y112F/128F mutation exhibited significantly reduced Fc γ R-dependent ROI production when plated on ICs (Fig 3, C, left panel) or 2.4G2, a stimulating Fc γ R/III-specific antibody (Fig 3, C, right panel). Y145F PMNs had a more pronounced defect that was not statistically different than that seen in SLP-76-deficient PMNs (Fig 3, C).

As in MCs, Ca²⁺ mobilization is a crucial signaling event supported by SLP-76 in response to Fc γ R engagement.¹⁷ Therefore we next examined whether the SLP-76 mutants could support

Fc γ R-induced Ca²⁺ flux in PMNs. The Y145F mutation and, to a lesser extent, Y112/128F impaired the ability of SLP-76 to support Ca²⁺ mobilization after Fc γ R cross-linking (Fig 3, D). Together, these data demonstrate that Y112/128 and Y145 are both required for optimal Fc γ R-induced PMN functions. However, similar to MCs, 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 an 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}

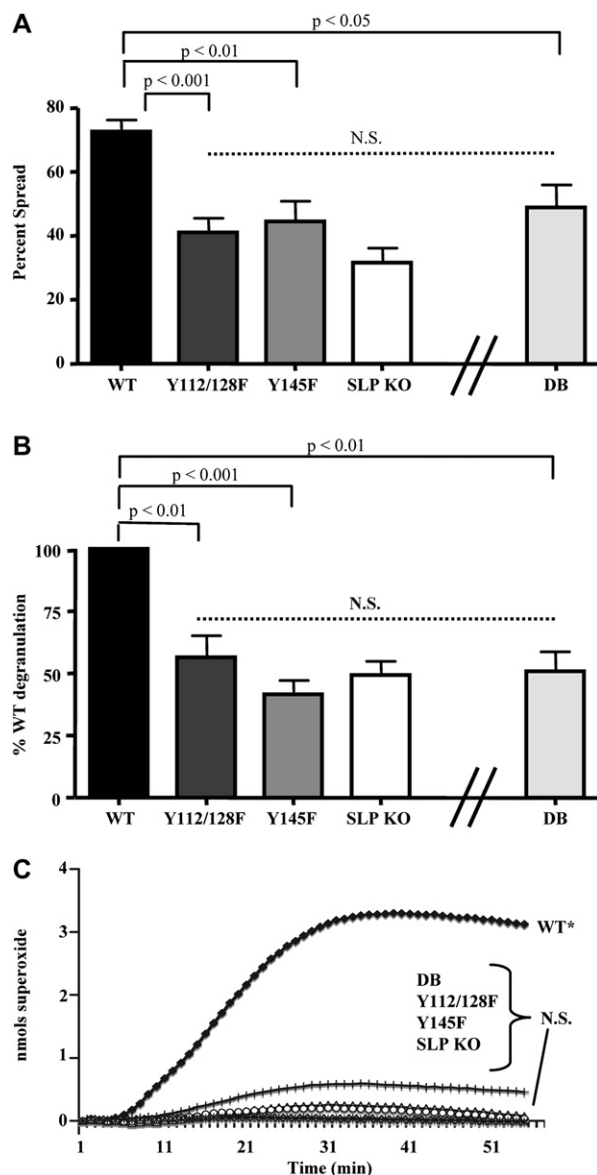


FIG 4. The N-terminal tyrosines of SLP-76 are required for integrin-dependent PMN responses. **A**, PMNs were plated on polyRGD-coated wells and allowed to spread for 30 minutes. The graph depicts the average percentage of cells spread combined from 6 experiments. **B**, PMNs were plated on the β_1 -integrin ligand collagen for 30 minutes in the presence of TNF- α . Lactoferrin release was measured by ELISA. The percentage of WT degranulation was averaged from 5 experiments. **C**, ROI production was induced by plating PMNs onto polyRGD-coated wells and measured over time. The graph depicts the averages from 5 experiments. *WT PMN function was statistically greater than the function of each of the mutant strains ($P < .05$). N.S., No statistical difference between values measured from each mutant; KO, knockout.

To examine whether mutations of SLP-76 affect integrin-induced signaling and resulting responses, we plated PMNs on surfaces coated with polyRGD, a pan-integrin ligand that directly stimulates integrins without requiring activation by proinflammatory mediators, or on the β_1 -integrin ligand collagen in the presence of TNF- α . We then assessed cell spreading (Fig 4, A), degranulation (Fig 4, B), and ROI production (Fig 4, C). In contrast to WT PMNs, Y112/128F and Y145F mutant PMNs exhibited severely defective responses that were statistically no different from those

of 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.

Coexpression of Y112/128F and Y145F does not support BMNC or PMN functions

Coexpression 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 PMNs from compound heterozygous mice harboring 1 *Slp-76*^{Y112/128F} and 1 *Slp-76*^{Y145F} allele (double-mutant [DB] mice). In each assay DB PMNs exhibited altered functions that were as defective as either single-mutant PMNs (Fig 3 and Fig 4). Similar results were obtained with BMNCs, in which expression of both mutations failed to rescue Fc ϵ RI-induced degranulation (Fig 5, A), Ca^{2+} flux (Fig 5, B), or biochemical signaling defects (Fig 5, C and D) to WT levels. Thus, as previously appreciated in platelets, mutant SLP-76 molecules do not function cooperatively within PMNs and MCs 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 effect 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 PSA. Compared with WT mice, IgE-sensitized Y112/128F and Y145F mice showed significant reductions in serum histamine concentrations after allergen challenge that were, on average, 40% and 80% less than the responses of control mice, respectively (Fig 6, A). 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 affect PMN functions *in vivo*, we used the localized Shwartzman reaction (LSR), an integrin-dependent but MC-independent model of LPS-induced dermal inflammation caused by the directed accumulation and degranulation of PMNs.^{15,44-46} On LSR induction, WT mice displayed significantly increased vascular permeability in LPS-injected ears compared to that seen in PBS-injected ears (Fig 6, B). 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 mice) exhibited attenuated responses that were statistically no different from those of mice with SLP-76-deficient PMNs.

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 MCs and PMNs, as well as Fc γ R and integrins.^{5,47-55} In this model the passive transfer of serum from mice expressing the KRN T-cell receptor (TCR) transgene and the MHC class II molecule I-A^{g7} results in the rapid and robust induction of joint-specific inflammation.⁵⁶⁻⁵⁸ Because the Y112/128F and

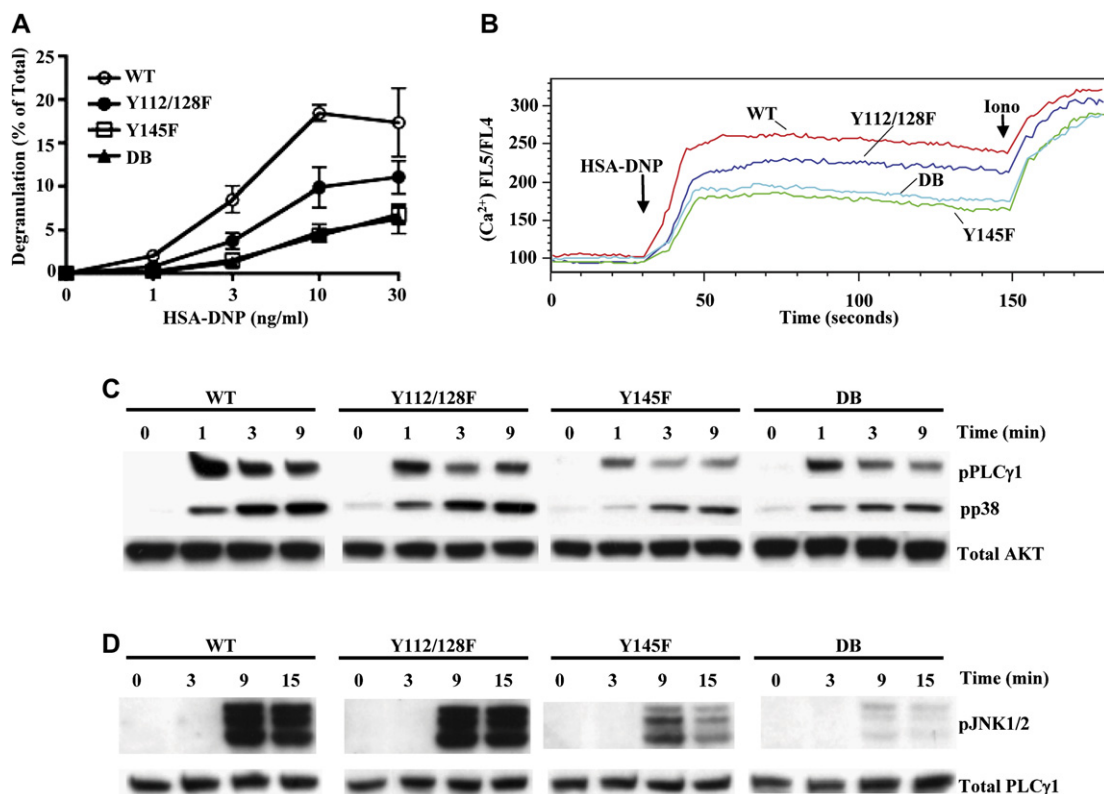


FIG 5. Coexpression of Y112/128 and Y145 does not rescue defective FcεRI-mediated BMMC activation. **A**, WT, Y112/128F, and Y145F BMMCs were compared with DB BMMCs by analyzing FcεRI-mediated degranulation. Results are expressed as means $\pm$ SDs (2 mice per genotype). **B**, WT and mutant BMMCs were monitored for FcεRI-mediated Ca^{2+} flux by flow cytometry. The curves represent the ratio of Indo-1 FL5/FL4 fluorescence, a measure of intracellular Ca^{2+} as a function of time. **C** and **D**, WT and mutant BMMCs were stimulated through FcεRI and analyzed by using Western blot analysis of phosphorylated PLCγ1 and p38 (**Fig 5, C**) and JNK1/2 (**Fig 5, D**). Total Protein kinase B (Akt/PKB) and PLCγ1 served as loading controls. Results are representative of at least 3 independent experiments.

Y145F mutations alter the activation of MCs, PMNs, Vav,²³ and PLCγ, 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 using inspection for joint swelling and erythema (clinical score), measurement of footpad thickness, and examination of joint histology. WT mice had footpad swelling (**Fig 7, A**) and increased clinical scores (**Fig 7, B**) early after serum injection that continued until joints were severely swollen and deformed (**Fig 7, C**). 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 7, D**). Mice expressing Y112/128F exhibited a modest reduction in average footpad thickness (**Fig 7, A**) and overall clinical score (**Fig 7, B**) because not all paws showed 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 that seen in WT mice (**Fig 7, C** and **D**). In marked contrast, mice with SLP-76-deficient PMNs, Y145F-expressing mice, or DB mutant mice did not manifest clinical or histologic signs of joint inflammation (**Fig 7**). 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

It is necessary to understand the mechanisms that govern the activation of these MCs and PMNs to control allergy and inflammation resulting from their aberrant activation. 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 PLCγ and likely also the Tec family kinases. In conjunction with recent findings demonstrating that PLCγ 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 might 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 PLCγ in MCs and PMNs. This result is presumably

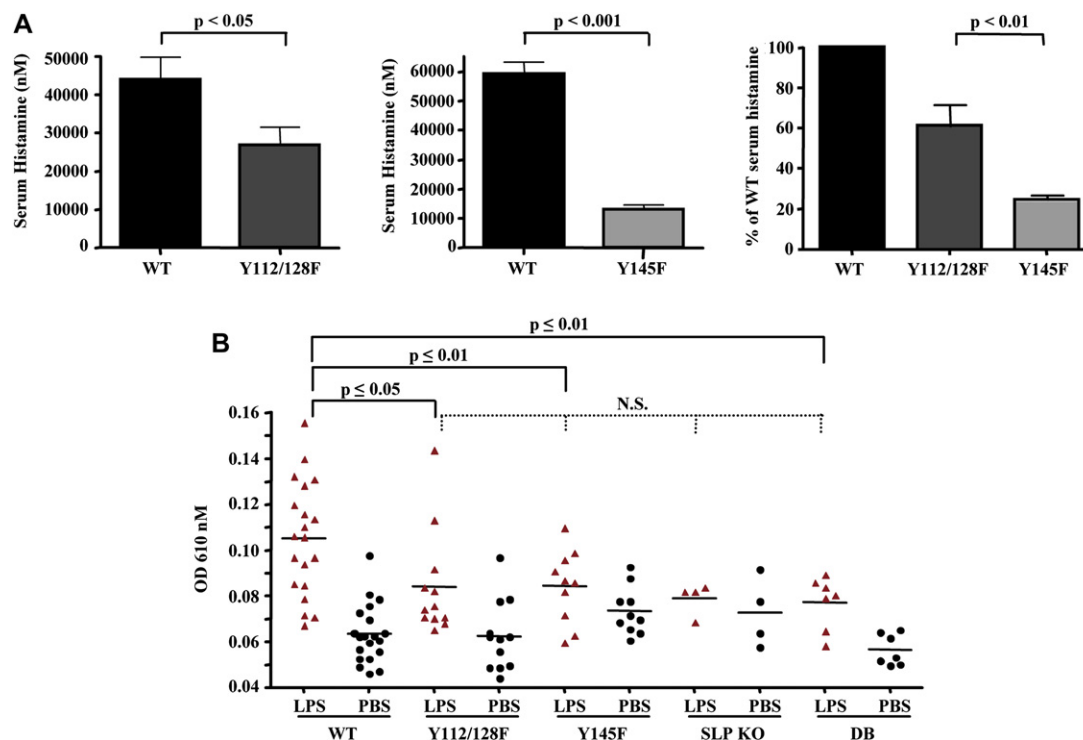


FIG 6. Mice harboring mutations in the N-terminal tyrosines of SLP-76 exhibit attenuated allergic and inflammatory responses *in vivo*. **A**, Serum histamine levels were measured in WT ($n = 8$) and Y112/128F ($n = 9$) mice (left plot) or WT ($n = 5$) and Y145F ($n = 5$) mice (middle plot) undergoing PSA. Results are expressed as mean histamine concentrations (in nanomoles per liter) $\pm$ SDs. 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 means of measurement of the extravasation of Evans blue dye. N.S., Not statistically significant; KO, knockout.

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 with primary murine 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 platelets⁴³ and T cell receptor-dependent Itk (IL-2-inducible T cell kinase)-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 because we observe reduced FcR-induced PLC γ phosphorylation in MCs and diminished Ca²⁺ flux in MCs and PMNs. Furthermore, Y145F MC phenocopy Btk^{-/-} MCs, 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 BMMCs expressing Y112/128F generally exhibited milder defects than cells expressing Y145F, MCP-1 production by Y112/128F BMMCs was as defective as that seen on Y145F BMMCs. The production of chemokines and cytokines has been reported to have different activation thresholds.⁶⁴ For example, weak Fc ϵ RI cross-linking stimuli can elicit MCP-1 production, whereas an approximately 10-fold stronger stimulus is required to evoke IL-6 production from MCs.⁶⁴ However, these threshold differences cannot explain why the Y112/128F mutation would

more selectively affect MCP-1 production because MCP-1 would be expected to be less sensitive than IL-6 to decreases in Fc ϵ RI-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 (GRB2-associated binding protein 2)-mediated signaling pathway, whereas IL-6 is more dependent on Linker of activated T cells (LAT).⁶⁴ At this time, it is unclear how these 2 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 might reflect the inherent nature of PMNs, 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 protein kinase C-dependent increases in intracellular Ca²⁺ levels⁶⁵⁻⁶⁹ but also requires actin reorganization to control the access of 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 Fc γ R-induced functional responses of adherent PMNs. In such a scenario, mutations of Y112/128, which interfere with the phosphorylation and presumed activation of Vav, and mutation of Y145, which disrupts

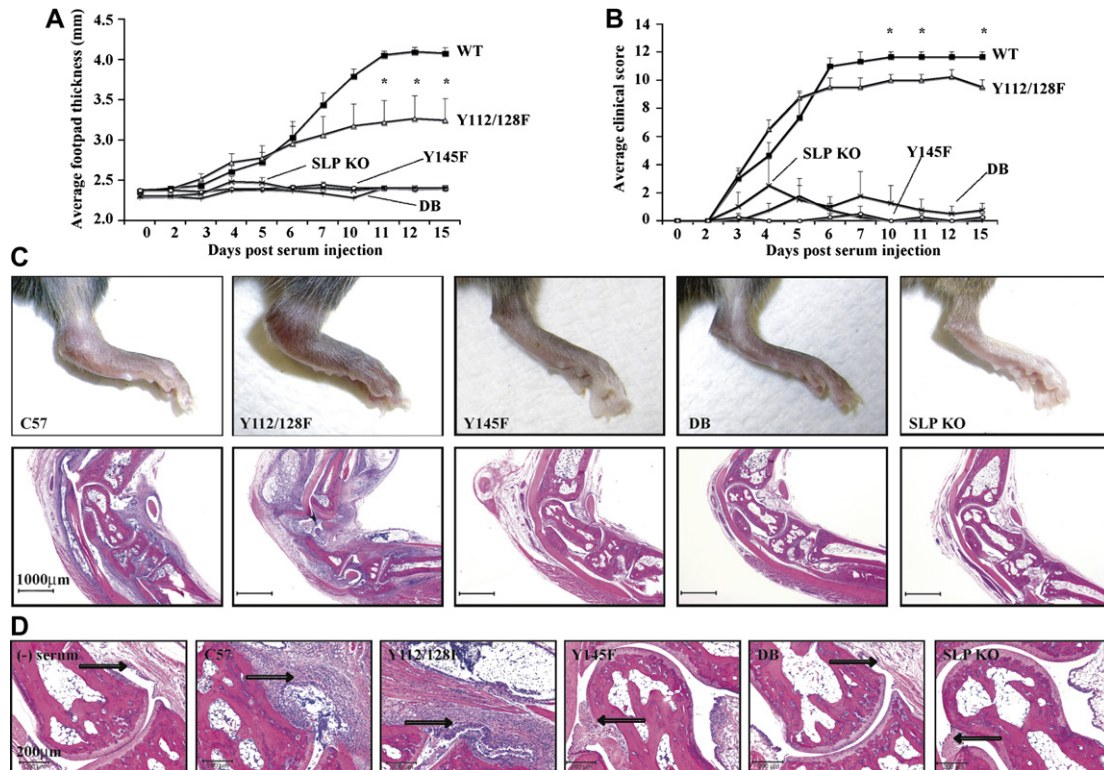


FIG 7. Mice harboring the Y145F mutation are protected from the development of serum-induced arthritis. K/B $\times$ N serum was administered to WT, Y112/128F, and Y145F knock-in mice and to mice with SLP-76-deficient PMNs (*Slp-76^{LoxP/LoxP}LysM^{Cre}*). Disease progression was monitored based on footpad measurements. Average hind footpad thickness for each genotype ($n = 8$ paws for each mutant and $n = 6$ paws for WT) is graphed in **A**. **B**, Average clinical score for each genotype ($n = 4$ mice for each mutant and $n = 3$ mice for WT). **C**, Photographs of ankle joints and a corresponding hematoxylin and eosin–stained section from these joints at day 15 (**D**, higher magnification), with joint capsules labeled with an arrow. * $P < .05$ compared with WT mice. KO, Knockout.

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 because coexpression 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 TCR signaling in thymocytes, in which the presence of Y112/Y128F and Y145F rescues developmental and signaling abnormalities,²³ but is similar to platelets, in which activation by the ITAM-bearing GPVI receptor requires that all phosphorylatable tyrosine units reside on the same SLP-76 molecule.⁴³ Fc ϵ / γ R and GPVI signaling depends on the presence of the FcR γ chain,⁷¹ whereas integrin signaling uses the γ chain, DNAX-activating protein of molecular mass 12 kilodaltons (DAP12),¹³ or both, each containing 1 ITAM per chain. This signaling mechanism differs from that used by the TCR; 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 MCs and PMNs might result from the limited number of ITAMs within Fc ϵ / γ R 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 effect 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, coexpression of Y112/128 and Y145 in PMNs did lead to increased IC-stimulated superoxide generation compared with expression of the Y145 mutant alone. ICs elicit a phagocytic-type response that activates multiple signaling pathways, including those involving phosphoinositide 3-kinases (PI3Ks).^{17,72} PI3K signaling is likely preserved in SLP-76 tyrosine mutant PMNs because PI3K-dependent activation through G protein–coupled receptors is normal in SLP-76 knockout PMNs.^{17,72} Thus in cells expressing the Y112/128F mutations 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 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits, thereby coupling Fc γ R signaling to increases in NADPH oxidase function. Although 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 PMNs in terms of their responsiveness to ICs.

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 PMNs are activated by soluble ICs, thereby leading to their release of soluble mediators, which increase vascular permeability and allow access of ICs to tissue-resident MCs. The subsequent activation of MCs is proposed to amplify vascular permeability, resulting in IC deposition on articular surfaces.⁷³ FcRs, particularly FcγRIII, are critical for the activation of MCs and PMNs and for the initiation of IC-driven inflammation.^{48,51} Y145F mice did not display signs of joint inflammation at the time of death, yet SLP-76-deficient PMNs extravasate normally into inflamed tissue in other *in vivo* models,¹⁵ suggesting that the lack of arthritis in these mice might occur at the earliest time points that are dependent on IC-driven PMN activation and MC cytokine production. Indeed, Y145F MCs and PMNs are defective in Fcε/γR-induced functions, such as degranulation and IL-6 production. Thus without these contributions, there might 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.

Because 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, but 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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Key messages

- Y145 of SLP-76 is critical for *in vitro* MC and PMN functions.
- Mutation of SLP-76 Y145 reduces histamine release in a murine model of PSA 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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