Immunity
Article

Phospholipase C-β3 Regulates FcεRI-Mediated Mast Cell Activation by Recruiting the Protein Phosphatase SHP-1

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DOI 10.1016/j.immuni.2011.04.010

SUMMARY

Mast cells are major effectors in high-affinity IgE receptor (FcεRI)-dependent allergic reactions. Here we show that phospholipase C (PLC)-β3 is crucial for FcεRI-mediated mast cell activation. *Plcb3*$^{-/-}$ mice showed blunted FcεRI-dependent late-phase, but not acute, anaphylactic responses and airway inflammation. Accordingly, FcεRI stimulation of *Plcb3*$^{-/-}$ mast cells exhibited reduced cytokine production but normal degranulation. Reduced cytokine production in *Plcb3*$^{-/-}$ cells could be accounted for by increased activity of the negative regulatory Src family kinase Lyn and reduced activities of the positive regulatory protein kinases MAPKs. Mechanistically, PLC-β3 constitutively interacts with FcεRI, Lyn, and SHP-1 (protein phosphatase). SHP-1 probably recognizes its substrates Lyn and MAPKs via the recently described kinase tyrosine-based inhibitory motif, KTIM. Consistent with PLC-β3- and SHP-1-mediated repression of Lyn activity by dephosphorylation at Tyr396, FcεRI-mediated phenotypes were similar in *Plcb3*$^{-/-}$ and SHP-1 mutant mast cells. Thus, we have defined a PLC-β3- and SHP-1-mediated signaling pathway for FcεRI-mediated cytokine production.

INTRODUCTION

Mast cells are key effector cells for IgE-dependent allergic inflammatory reactions (Galli et al., 2005). Upon activation, mast cells secrete preformed proinflammatory chemical mediators (e.g., histamine), proteases, proteoglycans, and nucleotides, as well as de novo synthesized lipids (e.g., leukotrienes and prostaglandins) and polypeptides (e.g., cytokines). These substances lead to the development of allergic inflammation.

FcεRI, the high-affinity IgE receptor expressed on the mast cell surface, is a tetrameric complex consisting of an IgE-binding α subunit, a signal-modulating β subunit, and two copies of the signal-generating γ subunit (Kinet, 1999). Aggregation of IgE-bound FcεRI induced by specific multivalent antigen (Ag) results in activation of β chain-associated Lyn, a Src family kinase (SFK). Lyn phosphorylates tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic domains of the β and γ chains, which leads to the recruitment of additional Lyn to the β chain, another protein tyrosine kinase (PTK), Syk, to the γ chains, and other signaling and scaffolding molecules to the aggregated receptor complex (Gilfillan and Rivera, 2009; Gilfillan and Tkaczyk, 2006; Rivera and Gilfillan, 2006; Turner and Kinet, 1999). Another SFK, Fyn, is also known to associate with FcεRI and to play a complementary role by activating phosphatidylinositol 3-kinase (PI3K) (Parravicini et al., 2002). These PTKs phosphorylate numerous targets and activate several signaling pathways, leading to mast cell activation (Kalesnikoff and Galli, 2008).

Activation signals are strictly counterbalanced by negative signaling molecules, such as Src homology 2 (SH2) domain-containing inositol 5′-phosphatases, SHIP-1 and -2, and SH2 domain-containing protein phosphatase-1 (SHP-1). Tyrosine-phosphorylated SHIP-1 binds FcεRI β chain via a phosphorylated ITAM (Kimura et al., 1997a). SHIP-1-deficient mast cells show increased degranulation after IgE stimulation, which is correlated with a higher and more sustained intracellular calcium concentration (Gimborn et al., 2005; Huber et al., 1998). SHIP-1 also negatively regulates cytokine production through multiple pathways: in SHIP-1-deficient cells, PI3K, Akt, and protein kinase C (PKC) elevate interleukin-6 (IL-6) mRNA synthesis by enhancing the phosphorylation of the inhibitor of κBα (IκBα) and increasing NF-κB DNA binding while ERK and p38 pathways enhance IL-6 mRNA synthesis by increasing the transactivation potential of NF-κB (Kalesnikoff et al., 2002). SHIP-1 is largely phosphorylated by Lyn (Hernandez-Hansen et al., 2004; Hibbs et al., 2002; Xiao et al., 2005). Thus, Lyn negatively regulates mast cell activation in vivo and in vitro (Hernandez-Hansen et al., 2004; Kawakami et al., 2000; Odom et al., 2004), although its

positive function can be revealed under certain conditions (Poderycki et al., 2010; Tolar et al., 2004; Xiao et al., 2005). SHIP-2 is also known to inhibit degranulation and cytokine production (Leung and Bolland, 2007). On the other hand, SHP-1 is generally considered a negative regulator of mast cell activation (Ott and Cambier, 2000). However, the data on SHP-1 in mast cell activation have been inconsistent. Overexpression of SHP-1 in a rat mast cell line, RBL-2H3, results in increased cytokine production upon IgE+Ag stimulation (Xie et al., 2000). By contrast, mast cells derived from SHP-1 loss-of-expression mutant *motheaten* (*me/me*) mice show increased cytokine production, but reduced calcium mobilization and degranulation, implying that SHP-1 has both negative and positive roles (Nakata et al., 2008).

PLC is a family of enzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate, leading to the activation of PKC and the mobilization of intracellular Ca^{2+}. Molecular cloning has identified 13 mammalian PLC isozymes that are classified into six subfamilies (PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ, and PLC-η) (Suh et al., 2008). Different PLC isozymes appear to be activated by different receptors and mechanisms, for example, PLC-β by G protein-coupled receptors (GPCRs) and PLC-γ by PTK-based receptors (Rhee, 2001). In mast cells, PLC-γ2 is absolutely required for FcεRI-mediated calcium mobilization, degranulation, and cytokine production, which cannot be compensated by the concurrently expressed PLC-γ1 (Wang et al., 2000). The function of other PLC members in mast cells is largely unknown. Recently, we have reported that PLC-β3 can form the multimolecular SPS complex containing SHP-1, PLC-β3, and Stat5 and facilitate the catalytic activity of SHP-1 in hematopoietic stem cells (Xiao et al., 2009). Because SHP-1 has been shown to regulate FcεRI-mediated mast cell activation, we set out to study the function of PLC-β3 in mast cells.

RESULTS

PLC-β3 Is Required for In Vivo Mast Cell Functions

To investigate the role of PLC-β3 in in vivo mast cell functions, we induced two types of mast cell-dependent immune responses, i.e., passive cutaneous anaphylaxis (PCA) and airway inflammation, in mice. The FcεRI-dependent PCA response is promoted by histamine and serotonin released from activated mast cells in the acute phase (Inagaki et al., 1986) and in part by mast cell-derived tumor necrosis factor-α (TNF-α) in the late phase (Wershil et al., 1991). We confirmed that mast cell numbers in ear skin were comparable in 6-week-old wild-type (WT) and *Plcb3*$^{-/-}$ mice (15.92 ± 1.45 versus 18.25 ± 0.55 per high-power field; p = 0.18, n = 4 each). In PCA experiments, both WT and *Plcb3*$^{-/-}$ mice exhibited an increased vascular permeability (Figure 1A) and edema (Figure 1B) as early as 30 min after Ag challenge and there was no difference between these two genotypes, indicating that mast cell-mediated acute-phase PCA reactions do not require PLC-β3. Within 2 hr after Ag challenge, the ear thickness representing edema was reduced in *Plcb3*$^{-/-}$ mice to that similar to non-Ag-challenged control. However, ears in WT mice were still edematous, significantly more than those of *Plcb3*$^{-/-}$ mice until 6 hr later (Figure 1B; see Figure S1 available online), demonstrating that PLC-β3 is required for full late-phase reactions. To investigate the PLC-β3 function in mast cells more directly, mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were engrafted with bone marrow-derived mast cells (BMMCs) from WT or *Plcb3*$^{-/-}$ mice (see Figures 2A and 2B) and subjected to PCA experiments. Comparable numbers of the engrafted mast cells were confirmed by toluidine blue staining (WT 8.37 ± 1.06 per high-power field versus *Plcb3*$^{-/-}$ 8.68 ± 1.47). Results showed a reduced late-phase response in *Plcb3*$^{-/-}$ cell-engrafted mice compared to WT cell-engrafted mice (Figure 1C), indicating that PLC-β3 is essential for in vivo mast cell functions.

Mast cell-dependent chronic airway inflammation was induced in mice according to the published protocol of ovalbumin (OVA) sensitization (without adjuvant) followed by intranasal OVA challenges (Williams and Galli, 2000). Inflammation characterized by the accumulation of eosinophils and goblet cell hyperplasia, hallmarks of airway inflammation, was seen. Consistent with type 2 inflammation, IL-5 and IL-13 in the lung homogenates were increased. However, these features were drastically reduced in *Plcb3*$^{-/-}$ mice, although *Plcb3*$^{-/-}$ mice had increased macrophages in lungs at baseline (Figures 1D–1F). A similar OVA sensitization and OVA challenge model shows the important role of FcεRI in airway inflammation (Taube et al., 2004). Thus, both PCA and airway inflammation data strongly suggest the important role of PLC-β3 in FcεRI-mediated in vivo mast cell activation, particularly in the late and chronic phases of allergic inflammation.

PLC-β3 Regulates FcεRI-Mediated Migration and Cytokine Production, but Not Degranulation

To study how PLC-β3 regulates the function of mast cells, we cultured bone marrow cells derived from WT and *Plcb3*$^{-/-}$ mice in the presence of IL-3. Four weeks later, more than 90% pure populations of mast cells were generated from both types of cells, as determined by surface staining of c-Kit (receptor tyrosine kinase for stem cell factor [SCF]) and FcεRI (Figure 2A). Microscopic analysis of May-Grünwald-stained mast cells revealed an indistinguishable metachromatic cell morphology (Figure 2B). These results suggest that PLC-β3 is not required for mast cell development.

Consistent with the similar acute-phase PCA reactions (Figure 1A), Ag stimulation of IgE-sensitized cells (IgE+Ag) induced similarly potent histamine release in WT and *Plcb3*$^{-/-}$ cells (Figure 2C). Moreover, IgE+Ag-induced Ca^{2+} mobilization, which is required for mast cell degranulation (Ozawa et al., 1993), was not different between WT and *Plcb3*$^{-/-}$ cells (Figure 2D).

Migration and cytokine production are cardinal features of FcεRI-induced mast cell activation (Galli et al., 2005). Therefore, we compared these responses between WT and *Plcb3*$^{-/-}$ cells. IgE-sensitized WT cells were attracted toward Ag, whereas this chemotactic response was drastically decreased in *Plcb3*$^{-/-}$ cells (Figure 3A). PLC-β3 functions downstream of GPCRs (Rhee, 2001). Therefore, we also examined chemotaxis induced by GPCR ligands, including RANTES (CCL5), MIP-1α (CCL3), and adenosine. Although, compared to antigen, all of these stimuli induced stronger migration of mast cells, there was no significant difference between WT and *Plcb3*$^{-/-}$ cells (Figure 3B and data not shown). Furthermore, SCF-induced chemotaxis

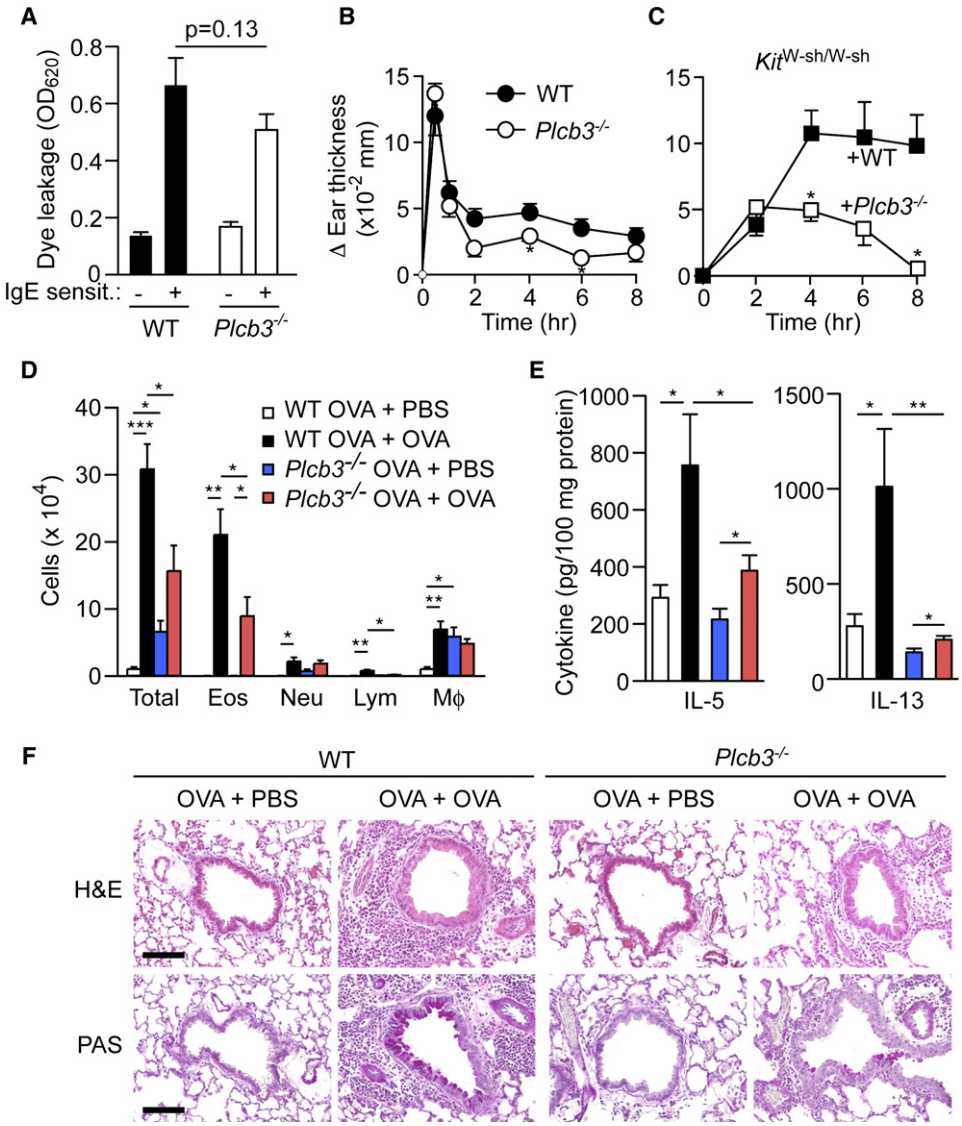

Figure 1. Reduced Late-Phase PCA Response and Mast Cell-Dependent Airway Inflammation in *Plcb3*$^{-/-}$ Mice

(A–C) PCA reactions were induced as described in the Experimental Procedures.

(A) Vascular permeability was measured by quantifying Evans blue dye that leaked into ears 30 min after antigen challenge. WT, n = 4; *Plcb3*$^{-/-}$, n = 7.

(B and C) Ear thicknesses during PCA responses were measured in WT and *Plcb3*$^{-/-}$ mice.

(C) *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were engrafted at their ears with BMMCs derived from WT or *Plcb3*$^{-/-}$ mice 6–8 weeks before PCA experiments. Results are expressed as mean ± SEM. *p < 0.05 versus WT mice. n = 5 each.

(D–F) Mast cell-dependent airway inflammation was induced as described in the Experimental Procedures.

(D) Total and differential cell counts in BALF. *p < 0.05; **p < 0.01; ***p < 0.001. Error bars represent SEM; n = 6 per group. Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes; Mϕ, macrophages or monocytes.

(E) IL-5 and IL-13 amounts in lung homogenates were measured by ELISA. n = 6 per group.

(F) Lung tissue sections were stained with H&E and PAS. Scale bars represent 200 μm. Data are representative of two independent experiments.

was not affected by PLC-β3 deficiency (Figure 3B). Collectively, these results indicate that PLC-β3 selectively and positively regulates FcεRI-mediated mast cell migration.

Next, we measured cytokine production by mast cells stimulated by IgE+Ag. Similar to the positive role of PLC-β3 in migration, production of IL-6, TNF-α, and IL-13 from *Plcb3*$^{-/-}$ cells was substantially reduced, compared to WT cells (Figures 3C–3E). These results can explain the reduced late-phase PCA reaction in *Plcb3*$^{-/-}$ mice, because TNF-α was reported as one of the major mediators responsible for IgE-dependent late-phase PCA reactions (Wershil et al., 1991).

Increased Lyn Kinase Activity in *Plcb3*$^{-/-}$ Mast Cells

To investigate how PLC-β3 positively regulates FcεRI-induced cytokine production and chemotaxis, we compared the profiles of tyrosine phosphorylation in WT and *Plcb3*$^{-/-}$ cells. Higher

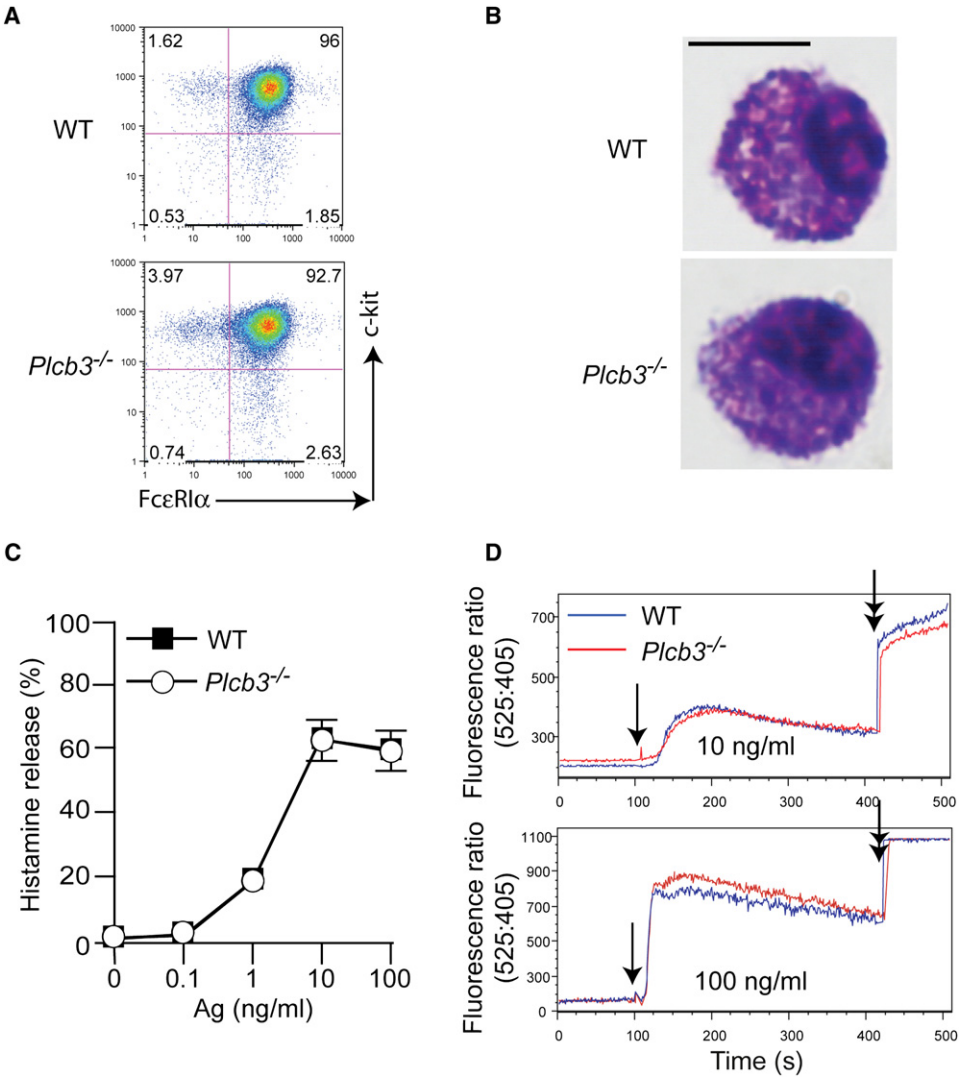

Figure 2. *Plcb3*$^{-/-}$ BMMCs Display WT Phenotypes of Degranulation and Calcium Mobilization

Bone marrow cells were harvested from WT and *Plcb3*$^{-/-}$ mice and grown in media containing IL-3 for 4–6 weeks.

(A) Surface expression of FcεRI and c-Kit on 4-week-old BMMCs derived from WT and *Plcb3*$^{-/-}$ mice was assayed by flow cytometry.

(B) May-Grünwald-stained BMMCs. Scale bar represents 20 μm.

(C) BMMCs were sensitized overnight with anti-DNP IgE, washed, and then stimulated with the indicated concentrations of DNP_{23}-HSA (Ag) for 45 min for histamine release. n = 4 each. Error bars represent SD.

(D) Ca^{2+} flux was measured with BMMCs loaded with Indo 1-AM and stimulated with the indicated concentrations of Ag (indicated by arrow), followed by the addition of 1 μg/ml ionomycin (indicated by double arrow). n = 4 each. Results shown are representative of at least three independent experiments.

tyrosine phosphorylation was observed on several proteins including those of ~53 kD and 56 kD in *Plcb3*$^{-/-}$ cells, before and after IgE+Ag stimulation (Figure 4A). Consistent with this, phosphorylation amounts of Tyr396 of $p53^{lyn}$ and $p56^{lyn}$ were dramatically increased in *Plcb3*$^{-/-}$ cells, whereas phosphorylation of Lyn-Tyr507 was only slightly increased (Figure 4B). These results indicate an increased Lyn kinase activity in *Plcb3*$^{-/-}$ cells, because Lyn activity like other SFKs is regulated positively by phosphorylation at the activation-loop tyrosine residue and negatively by phosphorylation at the C-terminal tyrosine residue (Hunter, 1995). By contrast, tyrosine phosphorylation of Fyn was not different between WT and *Plcb3*$^{-/-}$ cells (data not shown). Therefore, PLC-β3 selectively and negatively regulates Lyn kinase activity.

Consistent with the increased Lyn-Tyr396 phosphorylation in *Plcb3*$^{-/-}$ cells, tyrosine phosphorylation of downstream targets of Lyn, such as FcεRI β subunit and Syk, was increased before stimulation (Figure 4C). Previous studies demonstrate that tyrosine phosphorylation of SHIP-1, an important negative regulator in mast cells, was dependent on Lyn and FcεRI β ITAM (Furumoto et al., 2004; Hernandez-Hansen et al., 2004; Xiao et al., 2005). Consistent with the increased Lyn-Tyr396 and FcεRI β phosphorylation, tyrosine phosphorylation of SHIP-1 was upregulated in *Plcb3*$^{-/-}$ cells compared to WT cells (Figure 4D).

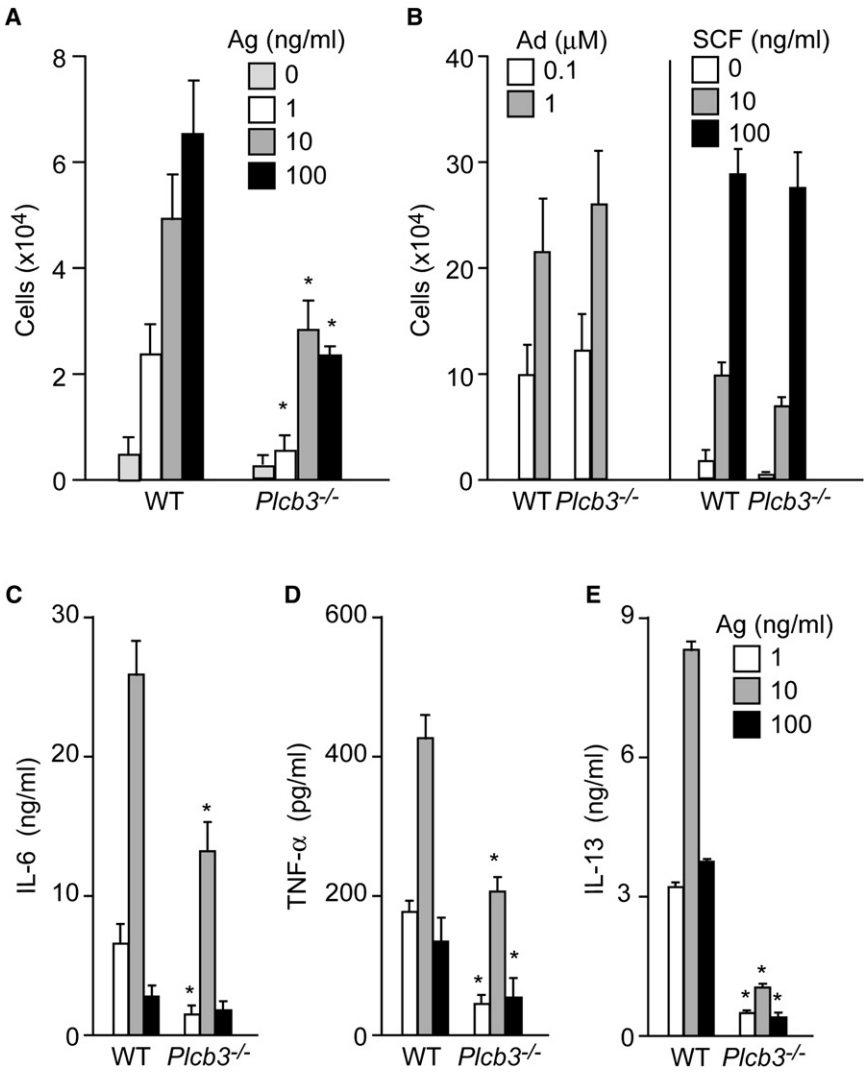

Figure 3. *Plcb3*$^{-/-}$ BMMCs Exhibit Reduced Migration and Cytokine Production

(A and B) BMMCs were sensitized overnight with anti-DNP IgE, and then migration assays were performed in transwell chambers with the indicated concentrations of DNP_{23}-HSA (Ag), adenosine (Ad), or SCF as chemoattractants. n = 5 each. Results shown are representative of three independent experiments.

(C–E) Cytokines from IgE+Ag-stimulated BMMCs were quantified by ELISA. n = 8 each. Error bars represent SD. Representative of three independent experiments.

Activation of FcεRI leads to tyrosine phosphorylation of linker of activated T cells (LAT), Bruton's tyrosine kinase (Btk), and PLC-γ2, thus triggering calcium mobilization, a crucial step for degranulation (Fluckiger et al., 1998; Saitoh et al., 2000; Wang et al., 2000). Although the proximal signaling events such as activation of Lyn and Syk were enhanced in *Plcb3*$^{-/-}$ cells, phosphorylation of LAT, Btk, and PLC-γ2 was not different from that in WT cells (Figure 5A), consistent with the comparable calcium mobilization between WT and *Plcb3*$^{-/-}$ cells (Figure 2D).

Activities of MAPKs Are Positively Regulated by PLC-β3

MAPKs are important positive regulators for FcεRI-induced mast cell activation. Therefore, we compared activities of these signaling molecules between WT and *Plcb3*$^{-/-}$ cells. Stimulation with IgE+Ag induced robust phosphorylation and thus activation of MAPKs (p38, JNK1, JNK2, ERK1, and ERK2) in WT cells (Figure 5B). By contrast, there was drastically reduced activation of the MAPKs in *Plcb3*$^{-/-}$ cells, which could account for the observed inhibition of cytokine production and chemotaxis (Figure 3). On the other hand, phosphorylation of Akt, another serine and threonine kinase involved in FcεRI-induced cytokine production (Kitaura et al., 2000), was only slightly affected by PLC-β3 deficiency. The transcription factor NF-κB is involved in production of various cytokines including IL-6 and TNF-α and regulated by its inhibitor IκBα (Hayden and Ghosh, 2004). However, IκBα degradation was comparable between WT and *Plcb3*$^{-/-}$ cells (Figure S2). Therefore, the activities of MAPKs are positively regulated by PLC-β3 in an NF-κB-independent manner.

FcεRI Stimulation Enhances the Interactions among PLC-β3, SHP-1, and Lyn

Next we investigated how PLC-β3 inhibits Lyn-Tyr396 phosphorylation. Previous studies show that Lyn kinase activity is enhanced in B cells from motheaten viable (*me*^{v}/*me*^{v}) mice that have a loss-of-function mutation in SHP-1 (Somani et al., 2001; Yang et al., 1998). We have recently shown that PLC-β3 binds to SHP-1 and enhances its phosphatase activity (Xiao et al., 2009); PLC-β3 also binds to and promotes Lyn to phosphorylate SHP-1 at Tyr536 and Tyr564, whose phosphorylation is critical for substrate recognition and enzymatic activation, respectively, in mast cells and hematopoietic stem and progenitor cells (Xiao et al., 2010). Interactions between PLC-β3, SHP-1, and Lyn are increased by growth factor stimulation in these cells. Thus, PLC-β3 in the SPS complex appears to provide a platform on which efficient enzyme-substrate interactions (i.e., SHP-1-Stat5 [signal transducer and activator of transcription 5] and Lyn-SHP-1 interactions) take place (Xiao et al., 2009, 2010). These and other (Hibbs et al., 2002; Xiao et al., 2005) observations suggest that SHP-1 might dephosphorylate Lyn-Tyr396. Consistent with this notion, Lyn, but not other SFK members, contains an evolutionarily conserved kinase tyrosine-based inhibitory motif, KTIM (Abu-Dayyeh et al., 2010), to which SHP-1 can bind to recognize its substrate (Abu-Dayyeh et al., 2008). The direct interaction between SHP-1 and Lyn was confirmed by pull-down assays with glutathione S-transferase (GST)-SHP-1 and recombinant Lyn (Figure S3), as described previously (Yoshida et al., 1999).

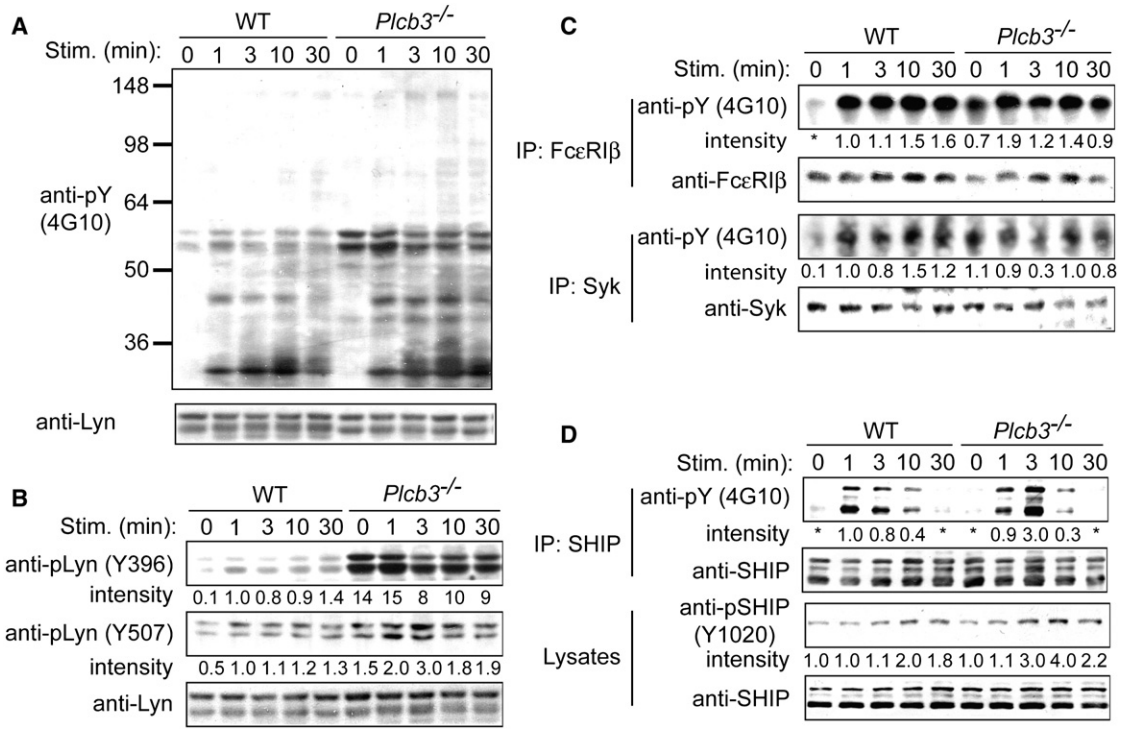

Figure 4. Lyn Activity and Tyrosine Phosphorylation of Lyn Targets Are Upregulated in *Plcb3*$^{-/-}$ Mast Cells

(A and B) BMMCs were sensitized overnight with IgE and stimulated with 100 ng/ml DNP_{23}-HSA for the indicated periods. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies.

(C) Immunoprecipitates of FcεRI β chain and of Syk were analyzed by immunoblotting with phosphotyrosine mAb, followed by reprobing of the blots with anti-FcεRI β and anti-Syk, respectively.

(D) Analysis of SHIP-1 phosphorylation was performed as described in (A) and (C). Immunoblotting results are representative of two independent experiments.

However, it was not known whether PLC-β3 interactions with other components of the SPS complex are affected by FcεRI stimulation. Because PLC-β3 expression was low in WT BMMCs, we overexpressed PLC-β3 C-terminal (CT) domain (PLC-β3-CT), which is mapped as the SHP-1-binding site (Xiao et al., 2009). We found that Lyn was coimmunoprecipitated with SHP-1 and that the Lyn-SHP-1 interaction was enhanced by IgE+Ag stimulation (Figure 6A, lanes 1–4). This interaction was reduced in *Plcb3*$^{-/-}$ mast cells but enhanced in PLC-β3-CT-expressing *Plcb3*$^{-/-}$ cells (Figure 6A, lanes 3 and 4 versus lanes 5 and 6), indicating that PLC-β3 enhances the interaction between Lyn and SHP-1. Coimmunoprecipitations between Lyn and PLC-β3-CT (Figure 6A, lanes 3 and 4 versus lanes 5 and 6) and between SHP-1 and PLC-β3-CT (Figure 6A, lane 1 versus lane 2) were also increased by IgE+Ag stimulation. In addition to its direct interaction with SHP-1 (Xiao et al., 2009), PLC-β3-CT was shown to directly interact with Lyn in pull-down assays (Figure S3). These results show that interactions among Lyn, SHP-1, and PLC-β3 are enhanced by FcεRI stimulation. They also support the notion that the physical proximity between Lyn and SHP-1 caused by their interactions with PLC-β3-CT allows SHP-1 to dephosphorylate Lyn-Tyr396 and to repress Lyn activity.

We next examined whether PLC-β3-CT can revert the reduced cytokine production in *Plcb3*$^{-/-}$ cells. Retroviral transduction with PLC-β3-CT increased the production of IL-6 and TNF-α upon FcεRI stimulation, albeit to a lesser extent than those with WT cells (Figure 6B and data not shown). Cytokine production in *Plcb3*$^{-/-}$ cells expressing WT PLC-β3 or the catalytically inactive mutant E362G was similar to that in WT cells expressing empty vector. The results suggest that cytokine production is independent of the catalytic function of PLC-β3 but dependent on its adaptor function via the CT domain. The smaller effect of PLC-β3-CT compared to full-length PLC-β3 seems to be due to the lack of subcellular localization signals in PLC-β3-CT. Expression of PLC-β3-CT in *Plcb3*$^{-/-}$ cells reduced Lyn-Tyr396 and SHIP-1-Tyr1020 phosphorylation (Figure 6C), suggesting the importance of the PLC-β3-SHP-1-Lyn axis in regulation of cytokine production.

Physical Relationship of PLC-β3 with FcεRI and Downstream Signaling Molecules

PLC-β3 can directly interact with Lyn (Figure S3) and SHP-1 (Xiao et al., 2009), proteins previously shown to physically interact with FcεRI (Jouvin et al., 1994; Kimura et al., 1997b; Xiao et al., 2005). We next tested whether PLC-β3 can interact with FcεRI. As shown in Figure 6D, FcεRI β was constitutively coimmunoprecipitated with PLC-β3. Furthermore, three regions of PLC-β3, i.e., the N-terminal pleckstrin homology-EF hand domains, the C-terminal half of the catalytic domain Y and C2 domain, but not the CT domain, could bring down FcεRI β and γ chains in mast cell lysates (Figure S4), corroborating FcεRI's

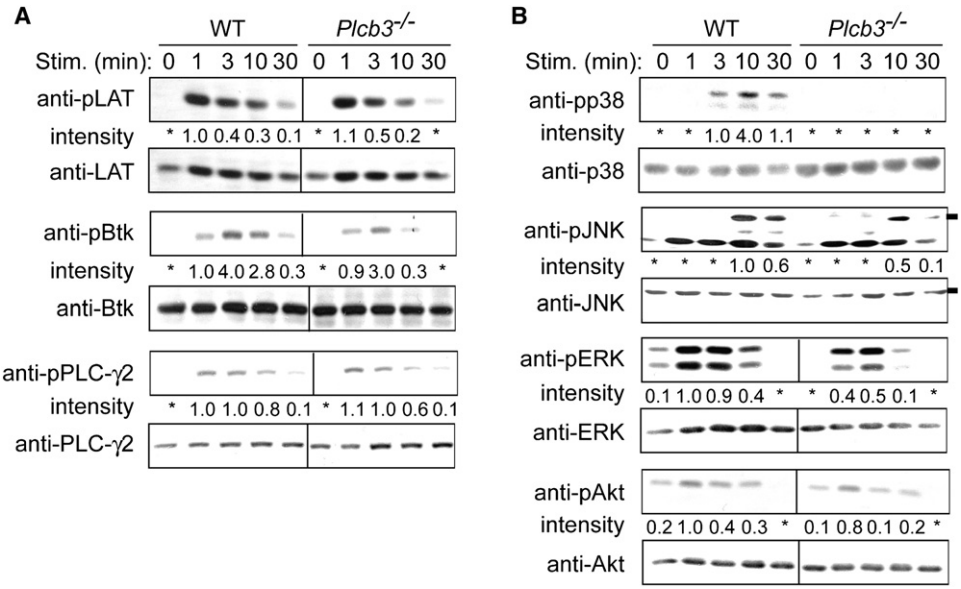

Figure 5. Phosphorylation of MAPKs Is Largely Decreased in *Plcb3*$^{-/-}$ Mast Cells

BMMCs were sensitized overnight with IgE and stimulated with 100 ng/ml DNP_{23}-HSA for the indicated periods.

(A) Phosphorylation events were analyzed by immunoblotting with antibodies to phospho-LAT (Tyr191), phospho-Btk (Tyr223), and phospho-PLC-γ2 (Tyr1217). Blots were subsequently stripped and reprobed with antibodies to LAT, Btk, and PLC-γ2.

(B) Phosphorylation of MAPKs and Akt were similarly analyzed. JNK2 is indicated by bars. The lines between lanes separate different parts of the same gel. Representative of three independent experiments.

interaction with PLC-β3. Biochemical experiments showed that PLC-β3 resides in nonlipid raft fractions (data not shown), whereas substantial proportions of FcεRI and Lyn (Field et al., 1995) and the majority of LAT molecules (Wilson et al., 2004) were present in lipid rafts.

Interestingly, tyrosine phosphorylation amounts of LAT, SHIP-1, and other downstream signaling molecules were not high in unstimulated *Plcb3*$^{-/-}$ mast cells, unlike Lyn, FcεRI β, and Syk (Figures 4 and 5). Pull-down experiments showed that no parts of PLC-β3 bound to LAT or SHIP-1 in lysates of mast cells (Figure S4). Thus, it is unlikely that PLC-β3 can interact with these molecules constitutively. This was corroborated by the absence of coimmunoprecipitation of PLC-β3 with LAT or SHIP-1 (Figure 6D). FcεRI β chain did not coimmunoprecipitate with either LAT or SHIP-1 under the conditions used (Figure 6D). The ability of PLC-β3 to physically interact with FcεRI and Lyn without FcεRI stimulation may be related to constitutive tyrosine phosphorylation of FcεRI β, Lyn, and Syk in *Plcb3*$^{-/-}$ cells, whereas the absence of PLC-β3 interactions could explain the absence of constitutive tyrosine phosphorylation of LAT, SHIP-1, and other downstream signaling molecules.

me^{v}/*me*^{v} and *Plcb3*$^{-/-}$ Mast Cells Exhibit Similar FcεRI-Dependent Phenotypes

If SHP-1 suppresses Lyn kinase activity in a PLC-β3-dependent manner, one would expect similar phenotypes in *me*^{v}/*me*^{v} and *Plcb3*$^{-/-}$ mast cells. Indeed, IgE+Ag-stimulated *me*^{v}/*me*^{v} cells produced significantly lower amounts of IL-6 and TNF-α than WT cells (Figure 7A and data not shown). Consistent with the notion that Lyn is a SHP-1 substrate, Lyn-Tyr396 phosphorylation was increased in resting and IgE+Ag-stimulated *me*^{v}/*me*^{v} cells (Figure 7B), whose aberrant SHP-1 proteins have only 10%–20% activity of WT enzyme (Kozlowski et al., 1993). Phosphorylation of SHIP-1, another Lyn target, was also enhanced (Figure 7B). Moreover, phosphorylation of MAPKs was reduced in *me*^{v}/*me*^{v} cells, similar to that in *Plcb3*$^{-/-}$ cells (Figure 7C).

To further investigate the positive regulatory roles of PLC-β3 and SHP-1 in cytokine production, we retrovirally transduced WT mast cells with PLC-β3-CT and SHP-1. We confirmed that PLC-β3-CT and SHP-1 were expressed by 3- to 5-fold more over the respective endogenous proteins in the transduced cells (data not shown). Consistently, PLC-β3-CT- or SHP-1-overexpressing cells produced higher amounts of IL-6 and TNF-α upon stimulation with IgE+Ag (Figure 7D and data not shown). Interestingly, this positive effect of SHP-1 is dependent on PLC-β3, as shown by the fact that SHP-1 overexpression failed to increase cytokine production in *Plcb3*$^{-/-}$ mast cells (Figure 7D). On the other hand, the positive effect of PLC-β3-CT is dependent on SHP-1, as shown by the fact that PLC-β3-CT overexpression did not increase cytokine production in *me*^{v}/*me*^{v} cells (Figure 7D). These results collectively demonstrated that SHP-1 and PLC-β3 behave similarly for FcεRI-mediated mast cell activation.

Suppression of SHIP-1 Phosphorylation and Activity by PLC-β3

Tyrosine phosphorylation of SHIP-1 is Lyn dependent, indicating that SHIP-1 is the downstream target of Lyn and might be responsible at least in part for the negative regulatory role of Lyn in mast cell activation (Hernandez-Hansen et al., 2004; Phee et al., 2000; Xiao et al., 2005). On the other hand, SHIP-1 tyrosine phosphorylation was increased in *Plcb3*$^{-/-}$ (Figure 4D) and *me*^{v}/*me*^{v} (Figure 7B) mast cells, compared to WT cells.

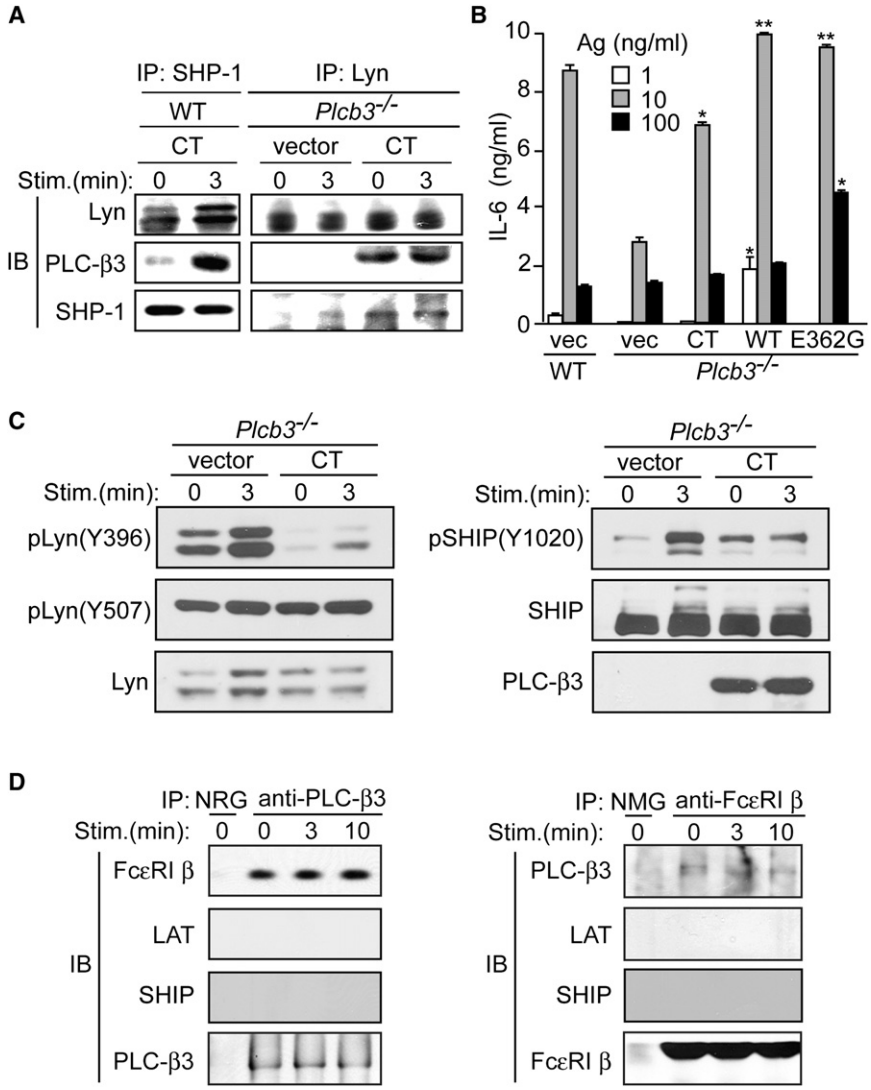

Figure 6. PLC-β3 Physically Interacts with FcεRI, Lyn, and SHP-1 and Regulates Cytokine Production

(A) WT and *Plcb3*$^{-/-}$ BMMCs were retrovirally transduced with PLC-β3-CT or empty vector and stimulated with DNP_{23}-HSA for the indicated periods. Immunoprecipitates (IP) of SHP-1 or Lyn were analyzed by immunoblotting (IB) with the indicated antibodies.

(B) WT or *Plcb3*$^{-/-}$ BMMCs transduced with the indicated retroviral vectors were sensitized overnight with IgE and stimulated with DNP_{23}-HSA for 16 hr. IL-6 production was measured. Representative of two independent experiments. *$p < 0.05$; **$p < 0.01$ versus vector control. Error bars represent SEM.

(C) *Plcb3*$^{-/-}$ BMMCs transduced with PLC-β3-CT or empty vector were stimulated with DNP_{23}-HSA for the indicated periods. Immunoblotting was performed for the indicated proteins. The lot of anti-pLyn (Y507) antibody used here was different from that used for Figure 4B and detected $p56^{lyn}$ preferentially.

(D) BMMCs were stimulated with DNP_{23}-HSA for the indicated periods. Immunoprecipitates with anti-PLC-β3 or anti-FcεRI β were analyzed by immunoblotting for the indicated proteins. NRG, normal rabbit IgG; NMG, normal mouse IgG.

these results are consistent with the notion that PLC-β3 and SHP-1 positively regulate mast cell activation by suppressing Lyn and SHIP-1 activities.

DISCUSSION

PLC-β3 deficiency did not affect FcεRI-mediated degranulation. This is consistent with the normal calcium mobilization and Fyn activity in *Plcb3*$^{-/-}$ BMMCs, implying that neither the calcium-dependent nor the calcium-independent but rather Fyn-, Gab2-, and RhoA-dependent steps for degranulation (Nishida et al., 2005) are regulated by PLC-β3. Therefore, we noticed no significant differences in acute-phase PCA response between WT and *Plcb3*$^{-/-}$ mice. By contrast, we observed reduced cytokine production in *Plcb3*$^{-/-}$ BMMCs. In line with this, *Plcb3*$^{-/-}$ mice had less severe late-phase PCA and airway inflammation. Thus, selective regulation by PLC-β3 of mast cell-mediated late-phase PCA and airway inflammation, but not acute-phase PCA, is similar to that in mice deficient in B cell lymphoma 10 (Klemm et al., 2006), mucosa-associated lymphoid tissue 1 (Klemm et al., 2006), and Zn transporter 5 (Nishida et al., 2009). NF-κB activity that is reduced in the latter three mice selectively regulates cytokine production. However, IκBα degradation was not affected in *Plcb3*$^{-/-}$ BMMCs, indicating that a different mechanism(s) is responsible for the uncoupled regulation of cytokine production versus degranulation in *Plcb3*$^{-/-}$ mice.

We showed that PLC-β3 regulates many signaling molecules downstream of FcεRI. This is striking, because PLC-β3 is a

Tyrosine phosphorylation of SHIP-1 is a prerequisite for SHIP-1 localization to the plasma membrane and thus its function (Edmunds et al., 1999; Phee et al., 2000). Therefore, we expected to restore SHIP-1 activity in Lyn-deficient cells by using a rat CD2-SHIP-1 fusion protein that is constitutively located to the plasma membrane and functions as a constitutively active form (Freeburn et al., 2002). Indeed, overexpression of CD2-SHIP-1 in *Lyn*$^{-/-}$ and *Lyn*$^{-/-}$;*Plcb3*$^{-/-}$ cells strongly inhibited IL-6 and TNF-α production (Figure 7E and data not shown). Silenced SHIP-1 by lentiviral expression of short-hairpin RNA increased cytokine production in *Plcb3*$^{-/-}$ mast cells (Figures S5A and S5B). SHIP-1 can recruit Dok-1 and Dok-2 adaptor proteins that, upon tyrosine phosphorylation by various PTKs including Lyn (Liang et al., 2002), bind RasGAP (a potent inhibitor of Ras) and leads to inhibition of the Ras-ERK pathway (Abramson et al., 2003; Mashima et al., 2009). Phosphorylation of Dok-2 at Tyr351 was higher in *Plcb3*$^{-/-}$ than WT mast cells (Figure S5C), suggesting that the signaling through this pathway contributes to the reduced ERK activation in *Plcb3*$^{-/-}$ mast cells. Collectively,

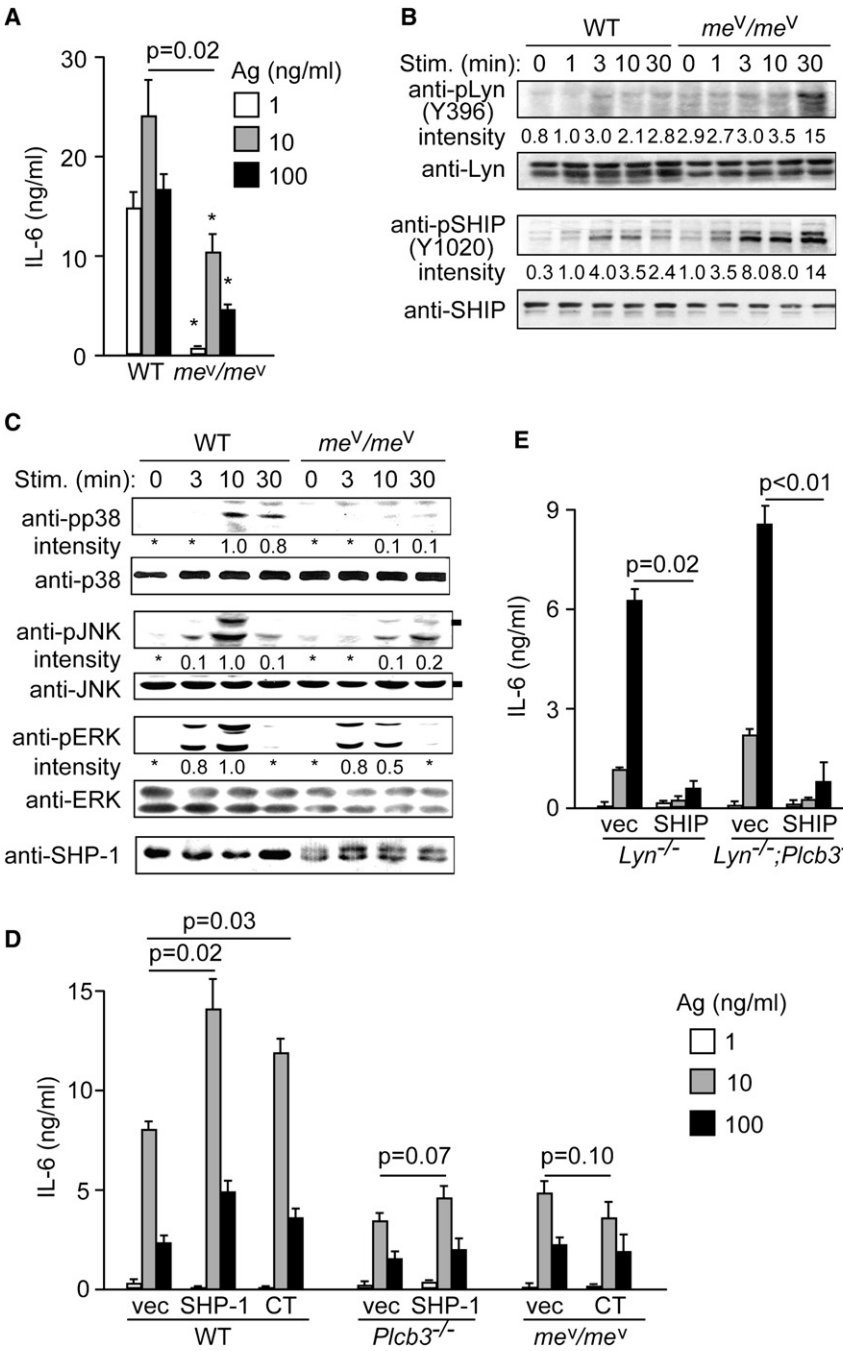

Figure 7. SHP-1 Inhibits Lyn Activation and Enhances Cytokine Production in a PLC-β3-Dependent Manner

(A) IL-6 production by me^v/me^v BMMCs was measured as in Figure 3C. n = 4 each.

(B and C) Lysates derived from WT and me^v/me^v BMMCs were analyzed by immunoblotting for phosphorylation of Lyn, SHIP-1, and MAPKs. JNK2 is indicated by bars.

(D) WT, $Plcb3^{-/-}$, and me^v/me^v BMMCs were retrovirally transduced with SHP-1 or PLC-β3-CT and stimulated with DNP_{23}-HSA for 16 hr. IL-6 production was measured. Results are representative of two independent experiments.

(E) $Lyn^{-/-}$ or $Lyn^{-/-}$;$Plcb3^{-/-}$ BMMCs were retrovirally transduced with rCD2-SHIP1, and IL-6 production was measured after IgE+Ag stimulation. Data are representative of two independent experiments. Error bars represent SEM.

target downstream of GPCRs and is activated to various extents by the Gα subunits of the Gq class and βγ subunits of the $G\alpha_{i/o}$ family of G proteins (Rhee, 2001). It should be emphasized that the function of PLC-β3 in FcεRI signaling is mediated by its adaptor but not enzymatic function. Here we found that overexpression of the noncatalytic PLC-β3-CT region was sufficient to increase cytokine production in WT BMMCs. These data and our previous study showing that PLC-β3-CT can bind SHP-1 (Xiao et al., 2009) indicate that the regulatory role of PLC-β3 in FcεRI-mediated mast cell activation does not require its catalytic activity but its adaptor function that facilitates SHP-1 interaction with its substrates. The phenocopy between $Plcb3^{-/-}$ and me^v/me^v BMMCs further supports the notion that SHP-1 and PLC-β3 function along the same signaling pathway(s). Interestingly, PLC-β3-CT domain shares a very low homology with other PLC-β isoforms. Consistent with this, other PLC-β members do not interact with SHP-1, and FcεRI-mediated mast cell activation was not affected by PLC-β2 deficiency (data not shown). Thus, the role of PLC-β3 in FcεRI-mediated mast cell activation is unique and nonredundant among PLC-β isoforms.

The functions of SHP-1 in mast cells are complex, which might be in part due to the presence of numerous substrates of SHP-1. A recent study has identified a number of protein kinases with KTIM ((I, V, L or S)-x-Y-x-x-(L or V)) that are potential SHP-1-recognition sites (Abu-Dayyeh et al., 2010). Interestingly, Lyn is the only SFK member containing a KTIM, accounting for the increased activation-loop tyrosine phosphorylation of Lyn, but not Fyn, in $Plcb3^{-/-}$ and me^v/me^v BMMCs. Lyn has been shown to suppress mast cell activation largely via FcεRI β chain and SHIP-1 (Furumoto et al., 2004; Hernandez-Hansen et al., 2004; Xiao et al., 2005). Tyrosine phosphorylation of FcεRI β chain was increased only at a resting state in $Plcb3^{-/-}$ cells, compared to WT cells. SHIP-1 phosphorylation was also increased in $Plcb3^{-/-}$ and me^v/me^v BMMCs over a >10 min period after FcεRI stimulation. Constitutive active SHIP-1 suppressed cytokine production in $Lyn^{-/-}$ and $Lyn^{-/-}$; $Plcb3^{-/-}$ cells and silencing of SHIP-1 expression enhanced cytokine production in $Plcb3^{-/-}$ cells, placing SHIP-1 downstream of Lyn and

PLC-β3. Therefore, in our model, PLC-β3 and SHP-1 positively regulate cytokine production by inhibiting Lyn and SHIP-1 activation; Lyn can interact directly with SHP-1, and the Lyn-SHP-1 interaction is increased in a PLC-β3-CT-dependent manner. Thus, it is likely that PLC-β3 docks SHP-1 and Lyn to facilitate the phosphatase activity of SHP-1 toward Lyn. This is just like the ability of PLC-β3-CT to augment SHP-1's activity to dephosphorylate Stat5 (Xiao et al., 2009). Enhanced Lyn activation in both *Plcb3*$^{-/-}$ and *me*^{v}/*me*^{v} cells could lead to reduced phosphorylation of MAPKs via the SHIP-1-Dok-RasGAP (Mashima et al., 2009) and other pathways.

In addition to their positive regulatory functions, we found negative regulatory activities of PLC-β3 and SHP-1 in the absence of Lyn (data not shown). Thus, amounts of expression and catalytic activity of Lyn and SHP-1 may be important in changing the balance between the positive and negative regulation. Slight changes in the genetic background or cellular environment might affect the outcomes of FcεRI stimulation (Yamashita et al., 2007). We have recently demonstrated that a slight change in Lyn kinase activity shifts its positive regulatory role to a negative one (Poderycki et al., 2010). Therefore, a study on these regulatory pathways might shed insights into the inconsistent phenotypes reported among motheaten (*me*/*me*), *me*^{v}/*me*^{v}, and SHP-1-overexpressing cells. Indeed, Xie et al. (2000) have shown that overexpression of WT SHP-1 in RBL-2H3 cells results in increased FcεRI-dependent cytokine production without causing changes in degranulation. By contrast, Nakata et al. (2008) have demonstrated increased cytokine production and reduced degranulation in *me*/*me* BMMCs. Thus, further analysis is warranted to clarify the roles of catalytic activity versus adaptor function of SHP-1. Another interesting issue is how PLC-β3 deficiency differentially affects basal tyrosine phosphorylation of the proximal signaling molecules (FcεRI β, Lyn, Syk) versus more distal signaling molecules (LAT, Btk, PLC-γ2, MAPKs, Akt). The ability of FcεRI β to constitutively coimmunoprecipitate with Lyn (Eiseman and Bolen, 1992) and PLC-β3 but not LAT is consistent with immunoelectron microscopy data showing that resting FcεRI colocalizes with Lyn (Wilson et al., 2000) and LAT occurs in small clusters separate from the receptor (Wilson et al., 2004). Thus, it will be interesting to study the mechanism by which the proximal versus distal signaling molecules are demarcated. In summary, this study uncovered unexpected roles of PLC-β3 and SHP-1 in mast cell activation and provided a new potential therapeutic target for the treatment of allergic diseases.

EXPERIMENTAL PROCEDURES

Cell Culture and FcεRI Stimulation

Bone marrow cells from WT and mutant mice were cultured in IL-3-containing medium for 4–6 weeks to generate >95% pure (c-Kit^{+}FcεRI^{+}) BMMCs. For stimulation of BMMCs with IgE+Ag, cells were sensitized for 16 hr with 0.5 μg/ml H1 DNP-ε-206 IgE and washed twice with Tyrode's buffer or media before stimulation with the indicated concentrations of Ag (DNP_{23}-HSA).

Retroviral Transduction

pMig or pMX-puro plasmids harboring SHP-1, PLC-β3 and its mutant, or constitutively active rat CD2-SHIP phosphatase fusion cDNAs were transfected into Plat-E packaging cells to generate recombinant retroviruses. Bone marrow cells in culture medium containing IL-3 and SCF were infected with retrovirus-containing supernatants; then GFP^{+} cells were sorted by FACSAria or puromycin-resistant cells were selected.

Measurement of Histamine, Cytokines, and Migration

Histamine released from BMMCs was measured as previously described (Kitaura et al., 2003). Cytokines were measured by ELISA. Chemotaxis of BMMCs was performed in fibronectin-coated Transwell chambers: 1×10^6 cells were loaded into the upper chamber and migration into the lower chamber containing the indicated concentration of chemoattractant was measured after 6 hr.

Immunoblotting and Immunoprecipitation

Cells were lysed in 1% NP-40-containing lysis buffer (20 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors. Lysates or immunoprecipitates were analyzed by SDS-PAGE and transferred to PVDF membranes. Binding of primary and horseradish peroxidase-conjugated secondary antibody and detection with enhanced chemiluminescence reagents (PerkinElmer) were performed.

PCA

6-week-old mice were sensitized by administration of 500 ng anti-DNP IgE or PBS (10 μl) to each ear. 24 hr later, mice were challenged intravenously with 100 μg DNP_{23}-HSA in 100 μl PBS or 1% Evans blue dye. Alternatively, 10 μl of 0.3% dinitrofluorobenzene in acetone and olive oil (4:1) was painted on both sides of ears. When Evans blue was injected, it was extracted from the ears 30 min after antigen injection. Ear thickness was periodically measured when the dye was not injected. The Animal Care and Use Committee of the La Jolla Institute for Allergy and Immunology approved all mouse experiments.

Airway Inflammation

6-week-old mice were intraperitoneally sensitized with OVA (10 μg/100 μl) at days 0, 7, 14, 21, 28, and 35. At days 40, 43, and 46, mice were intranasally challenged with OVA (20 μg/20 μl) or PBS. 24 hr after the last challenge, mice were sacrificed and bronchoalveolar fluids (BALF) were collected. Lung tissues were fixed with 10% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

Statistical Analysis

Statistical significance was determined with an unpaired two-tailed Student's t test. Results are expressed as mean ± SD unless otherwise stated; $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.immuni.2011.04.010.

ACKNOWLEDGMENTS

We are grateful to C.A. Lowell for providing us with *lyn*$^{-/-}$ mice. We thank S.G. Ward for providing the rat CD2-SHIP-1 construct and A. Rao and S. Trifari for helping with the lentiviral transduction experiments. This study was supported in part by grants from the National Institutes of Health AI50209 (T.K.) and the Diabetes and Immune Disease National Research Institute (W.X.). This is Publication 1286 from the La Jolla Institute for Allergy and Immunology.

Received: July 6, 2010
Revised: January 18, 2011
Accepted: April 8, 2011
Published online: June 16, 2011

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