RESEARCH ARTICLE

Protein Kinase C α and β compensate for each other to promote stem cell factor-mediated KIT phosphorylation, mast cell viability and proliferation

Lakshminarayan Reddy Teegala[1,2] | Yasmine Elshoweikh[1] |
Ravindra Gudneppanavar[1] | Sathwika Thodeti[1] | Sabita Pokhrel[1] | Erik Southard[2] |
Charles K. Thodeti[2,3] | Sailaja Paruchuri[1,2] [iD]

[1]Department of Chemistry, University of Akron, Akron, Ohio, USA

[2]Department of Physiology and Pharmacology, University of Toledo, Toledo, Ohio, USA

[3]Department of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, Ohio, USA

Correspondence
Sailaja Paruchuri, Department of Physiology and Pharmacology, Block Health Science Building, 3000 Arlington Ave, University of Toledo, Toledo, OH 43614, USA.
Email: Sailaja.Paruchuri@UToledo.edu

Funding information
HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID), Grant/Award Number: R01AI144115; HHS | NIH | National Heart, Lung, and Blood Institute (NHLBI), Grant/Award Number: R01HL148585; James Foght support

Abstract

Mast cells (MCs) develop from hematopoietic progenitors and differentiate into mature MCs that reside within connective or mucosal tissues. Though the number of MCs in tissues usually remains constant, inflammation and asthma disturb this homeostasis, leading to proliferation of MCs. Understanding the signaling events behind this proliferative response could lead to the development of novel strategies for better management of allergic diseases. MC survival, proliferation, differentiation, and migration are all maintained by a MC growth factor, stem cell factor (SCF) via its receptor, KIT. Here, we explored how protein kinase C (PKC) redundancy influences MC proliferation in bone marrow-derived MC (BMMC). We found that SCF activates PKCα and PKCβ isoforms, which in turn modulates KIT phosphorylation and internalization. Further, PKCα and PKCβ activate p38 mitogen activated protein kinase (MAPK), and this axis subsequently regulates SCF-induced MC cell proliferation. To ascertain the individual roles of PKCα and PKCβ, we knocked down either PKCα or PKCβ or both via short hairpin RNA (shRNA) and analyzed KIT phosphorylation, p38 MAPK phosphorylation, and MC viability and proliferation. To our surprise, downregulation of neither PKCα nor PKCβ affected MC viability and proliferation. In contrast, blocking both PKCα and PKCβ significantly attenuated SCF-induced cell viability and proliferation, suggesting that PKCα and PKCβ compensate for each other downstream of SCF signaling to enhance MC viability and proliferation. Our results not only suggest that PKC classical isoforms are novel therapeutic targets for SCF/MC-mediated inflammatory and allergic diseases, but they also emphasize

the importance of inhibiting both PKCα and β isoforms simultaneously to prevent MC proliferation.

1 | INTRODUCTION

Mast cells (MCs) are hematopoietic cells in the body[1,2] which initiate inflammatory responses to allergens and infectious agents.[3] MCs are mainly found around vasculature, in skin and mucosal surfaces, in the airways and intestine, and at the host–environment interphase.[4] MCs play a vital role in triggering and enhancing asthma exacerbations through the production of a myriad of inflammatory mediators.[5,6] Stem cell factor (SCF) is the principal growth factor for MCs that is secreted by fibroblasts, stromal cells, and endothelial cells.[7] It has been reported to play a critical role in the growth, differentiation, and proliferation of human and murine bone marrow MCs (BMMCs) in vitro.[8] SCF mainly relays signaling by binding to KIT, an MC surface tyrosine kinase receptor. KIT is a member of the type III subclass of receptor tyrosine kinases, comprised of an N-terminal extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic kinase domain. Activation of KIT is achieved through ligand-mediated receptor dimerization.[9] Phosphorylation of the tyrosine residues within the receptor triggers several transduction pathways which regulate cell survival, proliferation, differentiation, and migration.[7] Aberrant SCF-KIT signaling leads to the uncontrollable proliferation of MC, which in turn disrupts MC homeostasis resulting in the accumulation, proliferation, survival, and enhanced turnover rates of MCs via mitogen activated protein kinases (MAPK). MAPKs represent a family of serine/threonine kinases that includes the extracellular-regulated kinases (Erks), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs. These MAPKs integrate multiple signals from various receptors and second messengers, and they are involved in the regulation of cellular proliferation and differentiation.[10] Even though SCF/KIT axis in MC survival and proliferation via MAPK activation is well known,[11–13] there are still gaps in our knowledge regarding the role of PKCs in SCF-mediated mitogenesis. While most studies have focused on understanding the role of PKCs in antigen-stimulated MC activation,[14–18] there is still limited information on PKCs in SCF signaling.

PKC, a ubiquitous, phospholipid-dependent enzyme, is involved in signal transduction associated with cell proliferation, differentiation, and apoptosis. PKC belongs to the family of serine/threonine protein kinases, and it is activated by a number of extracellular stimuli including growth factors, adhesion, cytokines, and GPCRs.[19] At least eleven closely related PKC isozymes have been reported, each differing in their structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. They are classified as classical (α, β1, β2, γ), novel (δ, ε, η, θ), and atypical (ζ, ι, λ) isozymes depending on their requirement for the cofactors calcium, diacylglycerol (DAG), and phosphatidylserine (PS).[20–22] PKCs are widely implicated in MAPK activation in several cell types.[23,24] Several PKCs may be activated in response to an agonist, and some PKCs may positively regulate cell function while others may negatively regulate the same. The outcome observed represents a balance of both the positive and negative regulation. Further, due to the similarity between different PKC isoforms, one isoform can compensate for another resulting in redundancy. Using pharmacological and knockdown approaches, we have previously shown that two distinct PKCs regulate cysteinyl leukotriene signaling downstream of their receptor, $CysLT_1R$ in MCs, modulating their inflammatory signals.[25] Although PKCs were shown to be important in MC function, blocking individual PKCs was also shown to be dispensable for MC activity,[26] possibly due to the redundancy in PKC signaling. The redundant role of PKCs has not been studied in KIT-mediated MC activation, so our current focus is to study how redundancy in classical PKC isoforms affects MC responses. Therefore, in the present study, we investigated the role of classical and novel PKC isoforms in relaying SCF-KIT signaling and their ability to compensate for each other enhancing MC viability and proliferation.

2 | MATERIALS AND METHODS

2.1 | Animals

BMMCs were cultured from wild type C57BL/6 (WT) mice (6–8 weeks old), purchased from the Jackson laboratory and maintained at the University of Akron Research Vivarium (UARV). The animals were euthanized in accordance with standard guidelines as approved by the Animal Care and Use Committee of UA.

2.2 | Reagents

The following reagents were purchased commercially: SCF (Peprotech, NJ), Go6976 (Go) and GF109203X (GFX) (PKC inhibitors; EMD Millipore Corporation, MA), SB203580, BIRB0796 (p38 inhibitors; Tocris Bioscience, MN). All phospho-specific primary antibodies (phospho KIT Y719, cat # 3391; total KIT, cat # 3074; phospho p38 T180/Y182, cat # 4511; total p38, cat # 9212; phospho Erk T202/Y204, cat # 4370; total Erk, cat # 9102; phospho PKCβ S660, cat # 9371) are from Cell Signaling Technology (Danvers, MA), phospho PKCα S657/Y658, cat # ab23513 is from Abcam; total PKCα, cat # sc208; total PKCβ, cat # sc210 from Santa Cruz Biotechnology (Dallas, TX); GAPDH, cat # 10R-G109A from Fitzgerald (Acton, MA). PE-conjugated rat anti-mouse CD117 (KIT), and FITC-conjugated anti-mouse FcεR1 antibodies from Biolegend (San Diego CA). XTT proliferation assay kit (Trevigen, Gaithersburg, MD) and BrdU proliferation assay kit CalBiochem (EMD Millipore, La Jolla, CA).

2.3 | Bone marrow cell isolation and differentiation

Bone marrow cells (BMCs) were isolated from 6 to 8 weeks old wild type (WT) C57BL/6 mice. BMCs were re-suspended in RPMI-1640 media supplemented with L-glutamine (2 mM), Penicillin/streptomycin, Sodium pyruvate (1 mM), 10% FBS, β-mercaptoethanol (50 nM), HEPES buffer (25 mM) and were maintained at 37°C in a humidified 5% CO_2 environment. These primary cells were differentiated into BMMCs using interleukin-3 (IL-3) 30 ng/ml for 5 weeks. The maturity of BMMCs was examined by Toluidine blue staining, and >90% mature BMMCs were used for experiments. Differentiation of BMCs into BMMCs was confirmed by analyzing the surface expression of KIT and FcεR1 by flow cytometry.

2.4 | Cell activation and treatment

For phosphorylation of KIT, PKCα, PKCβ, ERK, and p38, BMMCs were stimulated with the indicated concentrations of SCF (100 ng/ml) in the absence of IL-3 for 5 min. For cell proliferation and viability assays, BMMCs were stimulated with SCF (100 ng/ml) in the absence of IL-3 for 72 h. In some experiments, cells were pretreated with Go6976 (2 μM) or GF109203X (2 μM) or SB203580 (10 μM) for 30 min in order to inhibit classical PKCs or novel PKCs or p38 MAPK respectively as described earlier,[27–29] followed by SCF treatment.

2.5 | Cell lysates and western blotting

After stimulation with the respective treatments, BMMCs (0.5×10^6) were lysed with lysis buffer (BD Bioscience, San Jose, CA) supplemented with protease and phosphatase inhibitor cocktails (Thermo fisher scientific, Waltham, MA). Immunoblotting was performed as described previously.[25] Briefly, lysates were subjected to 4%–15% SDS–PAGE and transferred to PVDF membrane. Membranes were blocked for 1 h with 5% non-fat dried milk in 1x TBS/0.1% Tween-20 at room temperature, and then subsequently incubated with respective primary phospho-antibodies diluted in 5% non-fat dried milk/1x TBS/0.1% Tween-20 (1:1000) overnight at 4°C on shaker. The next day, membranes were washed 3 times with 1x TBS/0.1% Tween-20 and then incubated with the secondary antibody (peroxidase-conjugated anti-rabbit) (1:5000) for 1 h at room temperature. Thereafter, membranes were washed again, incubated with ECL, and the bands were visualized using ProteinSimple (San Jose, CA) and quantified using Image J. Densitometric analysis was performed by normalizing the respective bands to the loading control.

2.6 | Flow cytometry

BMMCs (1×10^5) were pretreated with PKC inhibitors and stimulated with SCF as mentioned above. After stimulation, cells were washed with FACS buffer (0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS)), fixed with 4% paraformaldehyde, and then incubated with purified PE-conjugated rat anti-mouse KIT and FITC anti-mouse FcεR1 antibodies (Biolegend, San Diego, CA) for 30 min. Cells were washed with FACS buffer three times, and flow cytometric analyses were performed using BD Accuri C6 Plus Flow Cytometer.

2.7 | Short hairpin RNA (shRNA) and small interfering RNA (siRNA) knockdown

shRNA constructs targeting mouse PKCα, PKCβ were purchased from Open Biosystems. The constructs were cloned into a lentiviral vector (pLKo1, Open Biosystems, Huntsville, AL) and used to generate infectious particles with a lentiviral packaging mix (Virapower, Invitrogen, Waltham, MA), according to the manufacturer's protocol.[23] Viral stocks were titered using HIV-1 Gag p24 DuoSet® ELISA Kit (R & D Systems, Minneapolis, MN). For viral transfection, for control, and single knockdowns (PKCα or PKCβ), viral stocks of 100 transfection units (TU), and for double knockdowns, viral stocks of total 100 TU (50 TU PKCα and 50 TU PKCβ) along

with protamine sulfate (5 μg/ml) were added to 1×10^6 BMMCs suspended in RPMI medium, and the cells were incubated for 48 h at 37°C. Knockdown of PKCs was determined via western blotting. We did not observe a significant difference in PKC knockdown between 50 TU and 100 TU (not shown). For siRNA transfection, BMMCs were transfected with siGENOME SMART pool (a mix of 4 pre-made siRNA; Horizon Discovery, Lafayette, CO) of 50 nM PKCα-specific siRNA to block PKCα (Cat# D-040348-0), or non-specific siRNA (negative control; Cat# D-001206-14-05). Transfection was carried out with si-LentFect transfection reagent (Bio-Rad, Hercules, CA) according to manufacturer's instructions.

2.8 | Cell viability and proliferation

Proliferation and viability assays were performed in triplicates on cells plated at a density of (1×10^4) in each well of a 96-well plate suspended in fresh medium with no IL-3, in the presence or absence of SCF (100 ng/ml), and pretreated or not with Go6976 (2 μM) or GF109203X (2 μM).[24,25] Viability and proliferation were measured after 72 h by XTT and BrdU assay respectively, according to the manufacturer's protocol. XTT is a measure of the metabolic activity of cells. For the proliferation assay, BrdU label was added 24 h before the assay.

2.9 | Statistics

Data are expressed as mean ± SEM from at least three experiments. Significance was determined using one-way ANOVA, and comparisons between the groups were determined by Tukey's multiple comparisons test (GraphPad Prism, 7.01, La Jolla, CA, USA). *$p < .05$, **$p < .01$, ***$p < .001$.

3 | RESULTS

3.1 | Involvement of PKC isoform(s) in SCF-mediated KIT phosphorylation and internalization of BMMCs

BMCs were differentiated into BMMCs, and their maturity was assessed by staining with toluidine blue and cell surface expression of KIT and FcεR1 receptor by flow cytometry. Toluidine blue staining revealed >90% mature BMMCs at week 5 (Figure 1A). Further, FACS analysis revealed that BMMCs strongly expressed KIT and FcεR1 receptors on their surface (Figure 1B). We next analyzed the time course of SCF to activate KIT in BMMCs. We

observed rapid KIT phosphorylation (Y719) with SCF stimulation, reaching a maximum (35-fold increase) by 5 min, and then decreased over time until reaching its lowest level after 60 min (Figure 1C,D). Next, we investigated which isoform/s of PKC are involved in SCF-mediated KIT phosphorylation by employing Go6976 (Go; 0.1, 1, 2 μM), a PKC inhibitor specific for classical PKCs and GF109203X (GF109203X; 0.1, 1, 2 μM), a PKC inhibitor more specific for novel PKC isoforms.[24,25,30] The concentration of the inhibitors was chosen based on our previous experience with MCs and intestinal epithelial cells.[24,25,29,30] We pre-incubated BMMCs with these inhibitors 30 min prior to SCF treatment. Notably, we found that 1 μM and 2 μM Go6976, but not GF109203X, significantly reduced the level of KIT phosphorylation (Y719) (Figures 1E,F and S2), suggesting that only the classical PKC isoforms play a role in the SCF-mediated phosphorylation of KIT. Next, we determined the extent of KIT internalization in response to SCF stimulation. We treated cells with SCF in a time and dose-dependent manner and analyzed the KIT receptor expression at the cell surface. We found that treatment of BMMCs with SCF resulted in KIT receptor internalization (Figure S1A), as determined by a significant decrease in the percentage expression of KIT on the cell surface in a time- (Figure S1B) and dose-dependent (Figure S1C) manner with a peak internalization observed using 100 ng/ml SCF treatment for 1 h. We next analyzed the role of PKC isoforms in SCF-mediated KIT internalization. We pretreated BMMCs with Go6976 (2 μM) and GF109203X (2 μM) for 30 min before SCF treatment (100 ng/ml; 1-h) and analyzed KIT internalization. We found that pretreatment with Go6976, but not GF109203X, significantly reduced SCF-mediated KIT internalization, and it increased the percentage of KIT cell surface expression compared with the treatment of the cells with SCF alone (Figure 1G).

3.2 | Activation of classical PKC isoform(s) (PKCα and PKCβ) by SCF

Since we observed that SCF-induced KIT phosphorylation and internalization are sensitive to classical PKC isoform inhibition, we asked if SCF could activate PKCα and/or PKCβ isoforms using phospho-specific PKCα (S657/Y658) antibody and PKCβ (S660) antibodies that can detect phosphorylated α and β isoforms respectively. We observed a significant and time-dependent activation of both PKCα and PKCβ isoforms in response to SCF (Figure 2). While PKCβ phosphorylation appeared early at 5 min, peaked at 15 min and declined, PKCα phosphorylation was significantly high at 5 min, peaked at 30 min, and remained unchanged till 60 min in response to SCF (Figure 2).
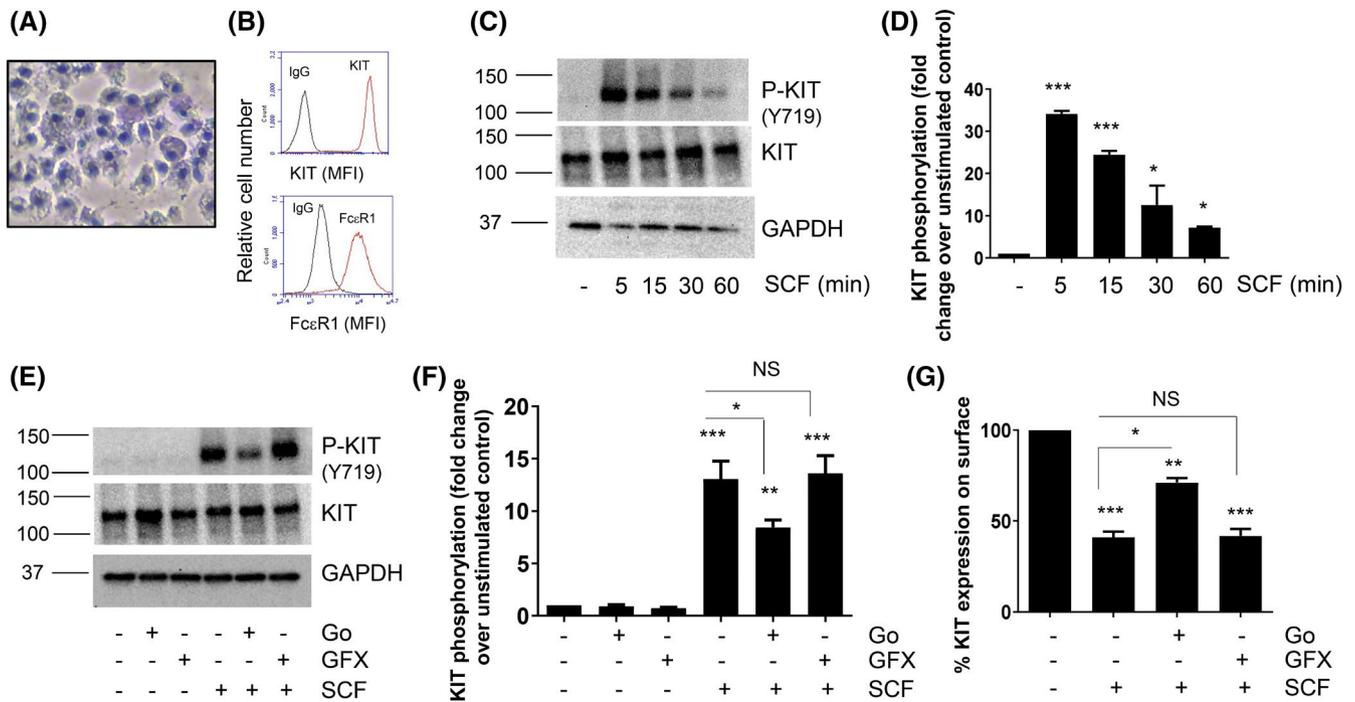
FIGURE 1 Classical PKC isoforms inhibit SCF-mediated KIT phosphorylation. BMCs were isolated and differentiated into BMMCs using interleukin-3 (IL-3) 30 ng/ml for 5 weeks. (A) Toluidine blue staining confirming maturation of BMMCs. (B) Representative flow cytometric analysis demonstrating the expression of KIT and FcεR1 in BMMCs. (C, D) Time-dependent phosphorylation of KIT receptor by SCF. BMMCs were stimulated with SCF (100 ng/ml) for the indicated time points and KIT phosphorylation was assessed by western blotting using phospho-specific KIT (Y719) antibodies. (E) Cells were pre-incubated with Go6976 (2 μM) or GF109203X (2 μM) for 30 min followed by treatment with SCF (100 ng/ml) for 5 min, and then KIT phosphorylation was assessed by western blotting using phospho-specific KIT (Y719) antibodies. Blots were either stripped and re-probed. In some instances where stripping was not complete, the same lysates were re-run and blotted for total KIT and GAPDH to confirm equal loading. (D, F) Densitometric analysis of data is shown in (C) and (E) respectively. (G) Effect of PKC inhibitors on internalization of KIT receptors in BMMCs. Cells were pretreated with PKC inhibitors Go6976 (2 μM) and GF109203X (2 μM) for 30 min, followed by SCF (100 ng/ml) for 1 h. Surface expression of KIT receptor was analyzed by flow cytometry and expressed as net mean fluorescence intensity (MFI). Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ns, not significant

3.3 | Role of classical PKC isoform(s) in SCF-mediated MAPK phosphorylation

Next, we examined the role of PKC isoform/s in relaying SCF signals downstream of KIT phosphorylation and internalization. Stimulation with SCF has been previously shown to induce phosphorylation of MAPK.[11–13] We asked which MAPK family of proteins are sensitive to classical PKC inhibition using Go6976, and the novel PKC inhibitor GF109203X was used as a negative control. We treated BMMCs with SCF (100 ng/ml) and analyzed phosphorylation of p38 (T180/Y182) and Erk (T202/Y204) at different time points. As reported earlier,[11–13] we observed robust phosphorylation of p38 and Erk as early as 5 min following SCF stimulation, with a decline around 60 min (Figure 3A–C). When we pretreated BMMCs with 2 μM Go6976 and GF109203X for 30 min, followed by SCF treatment for 15 min, Go6976 significantly reduced SCF-induced p38 MAPK phosphorylation, while GF109203X (0.1, 1, 2 μM) had no significant effect (Figures 3D,E and

S2): 0.1 μM Go69761 had no effect, and 1 μM Go69761 had modest effect on p38 phosphorylation (Figure S2). Notably, we found that neither Go6976 nor GF109203X had a significant effect on SCF-induced Erk phosphorylation (Figure 3D,F), although both the inhibitors alone led to significant Erk phosphorylation (Figure 3D,F).

3.4 | SCF-mediated BMMC viability and proliferation

Next, we analyzed the role of classical PKC isoforms in SCF-mediated BMMC cell viability and proliferation. BMMCs were pretreated with Go6976 (0.1, 1, 2 μM) and GF109203X (0.1, 1, 2 μM), followed by SCF treatment (100 ng/ml) for 72 h, and then measured their viability using XTT assay and proliferation using BrdU assay. SCF increased both viability and proliferation of BMMCs (Figure 4A,B), as reported earlier by other groups.[11–13] Importantly, Go6976 (1 and 2 μM) significantly inhibited SCF-mediated viability

(A)

(B)
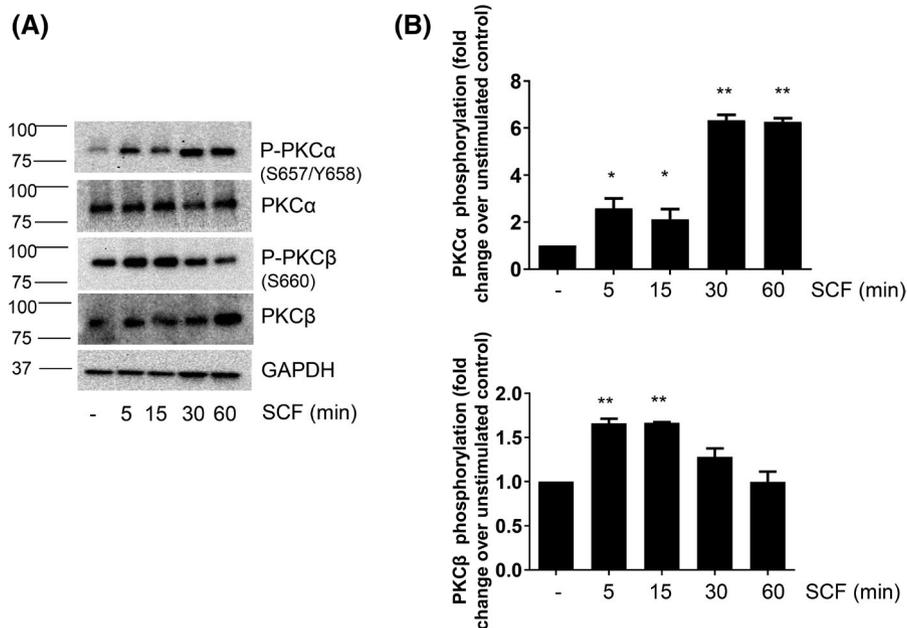
FIGURE 2 SCF stimulation induces phosphorylation of PKCα and PKCβ. BMMCs were stimulated with SCF (100 ng/ml) for the indicated time points and (A) PKCα and PKCβ phosphorylation was assessed by western blotting using phospho-specific PKCα (S657/Y658) and PKCβ (S660) antibody. Blots were stripped and re-probed for PKCα, PKCβ, and GAPDH. (B) Densitometric analysis of data is shown in (A). Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. *$p < .05$, **$p ≤ .01$

and proliferation, while GF109203X at any concentration had no effect on the same (Figure 4A,B). In agreement with our earlier results, BMMC viability was partially sensitive to p38 inhibition by SB203580 (10 μM, but not 0.1 μM and 1 μM) (Figure S3A), and also, importantly, the selective p38 MAPK inhibitor BIRB0796 (Figure S3B).[31]

3.5 | Effect of PKCα, PKCβ, or both knockdowns on SCF-induced signaling events

To analyze if SCF-mediated viability and proliferation signals require PKCα and/or PKCβ, we knocked down PKCα, or PKCβ, or both PKCα and PKCβ by shRNA and then analyzed SCF-induced downstream signaling events. Knocking down PKCα and both PKCα and PKCβ resulted in significant down regulation of PKCα expression. Similarly, knocking down PKCβ and both PKCα and PKCβ resulted in significant down regulation of PKCβ expression (Figure 5A,B). Surprisingly, neither PKCα nor PKCβ knockdown led to any reduction in SCF-induced KIT phosphorylation or p38 phosphorylation (Figure 5C–E), or viability and proliferation (Figure 6A,B). In contrast, knockdown of both PKCα and PKCβ led to a significant reduction in both KIT and p38 phosphorylation (Figure 5C–E) as well as viability and proliferation by SCF (Figure 6A,B). In support, the downregulation of PKCα

using PKCα siRNA did not affect SCF-induced viability or proliferation (Figure S4).

4 | DISCUSSION

In the present study, we demonstrated that SCF regulates MC proliferation and viability through classical PKC isoforms PKCα and PKCβ, but not through the novel PKC isoforms. Further, we demonstrated that PKCα and PKCβ can compensate for each other's function in phosphorylating KIT and p38 MAPK, and in regulating MC proliferation and viability in response to SCF.

SCF is an important regulator of MC growth, differentiation, survival, and chemotaxis.[32–34] When SCF binds to its receptor KIT, there is a dimerization of receptors, followed by cross-phosphorylation, that leads to the activation of its intrinsic kinase activity. This in turn recruits adaptor molecules and initiates cascades of signaling pathways, including phospholipase C, protein kinase C, MAPK, PI3K, and the Jak-Stat. Further, KIT has been shown to be phosphorylated by PKC in response to stimulation with SCF in vivo.[11] Although the SCF/KIT axis in MC survival and proliferation via activation of MAPKs is well known,[11–13] there is a further need to define the role of upstream signaling molecules, such as PKCs in KIT signaling. In fact, most studies in literature are focused on understanding the role of PKCs in antigen-stimulated
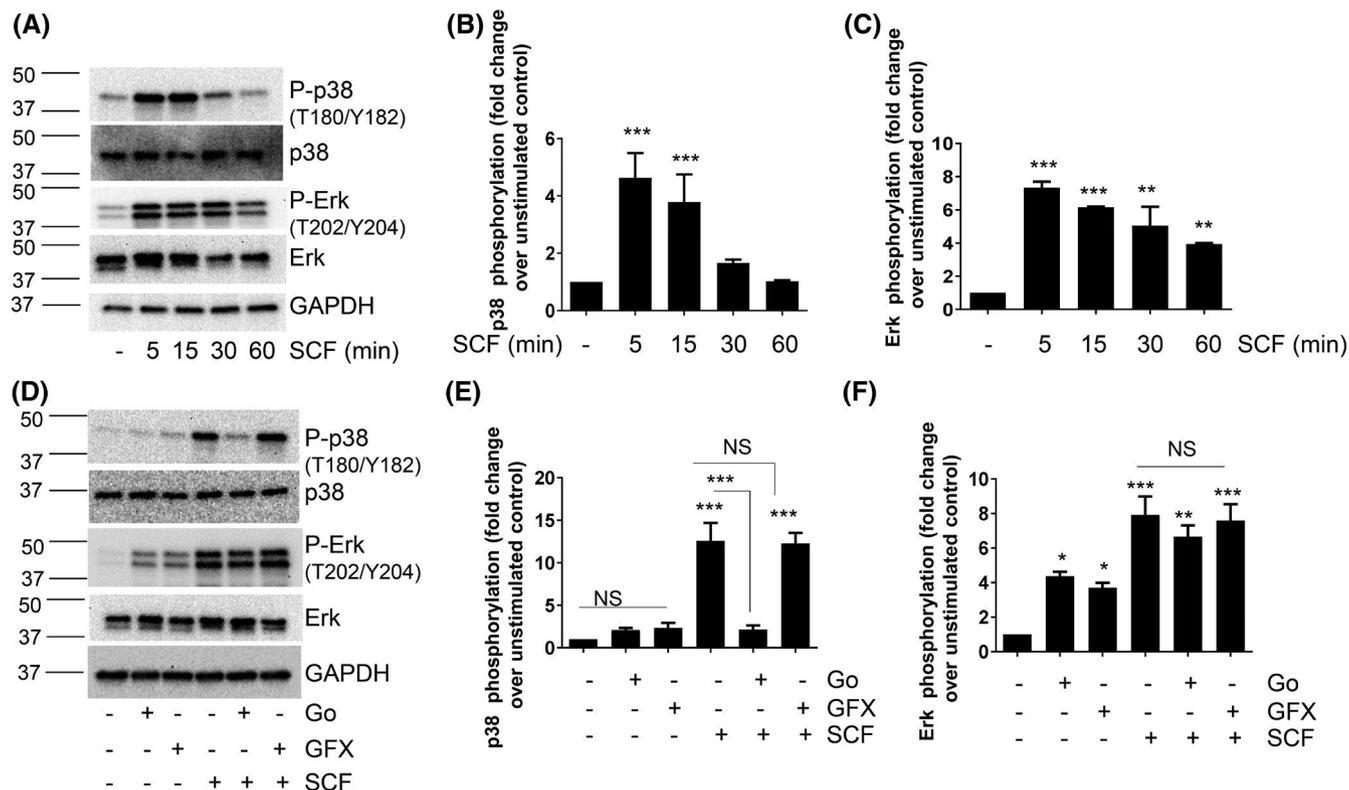
FIGURE 3 SCF promotes phosphorylation of p38 MAPK in a PKC-dependent manner via KIT. BMMCs were stimulated with SCF (100 ng/ml) for the indicated time points and (A) p38 and Erk phosphorylation were assessed by western blotting using phospho-specific p38 (T180/Y182) and Erk (T202/Y204) antibodies. (B, C) Densitometric analysis of p38 and Erk respectively as seen in (A). Blots were stripped and re-probed for total p38, Erk, and GAPDH. (D) p38 and Erk phosphorylation were assessed by western blotting using phospho-specific p38 and Erk antibodies. BMMCs were pretreated with Go6976 (2 µM) or GF109203X (2 µM) for 30 min followed by treatment with SCF (100 ng/ml) for 5 min. (E, F) Densitometric analysis of p38 and Erk respectively as seen in (D). Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. *$p \leq .05$, **$p \leq .01$, ***$p \leq .001$, ns, not significant
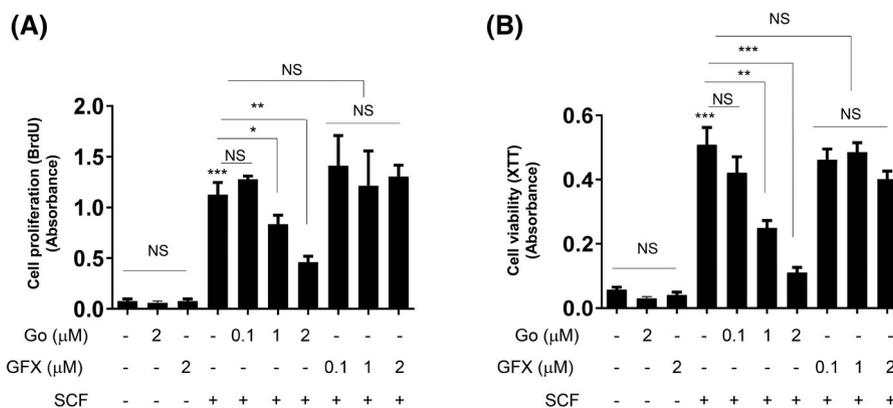
FIGURE 4 PKC inhibitors attenuate SCF-mediated BMMC viability and proliferation. BMMCs were plated in triplicate at a density of (1×10^4) in each well of a 96-well plate suspended in fresh medium without IL-3 and in the presence or absence of SCF (100 ng/ml), pretreated or not with Go6976 (0.1, 1, 2 µM) or GF109203X (0.1, 1, 2 µM). (A) Cell proliferation was measured after 72 h by BrdU ELISA. BrdU label was added 24 h before the assay. (B) Cell viability was analyzed by XTT assay. Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. **$p \leq .01$, ***$p \leq .001$, ns, not significant

MC activation. PKCβ deficiency in MCs has been demonstrated to inhibit degranulation and interleukin-6 production in response to IgE-Ag.[16] Interestingly, PKCδ was initially suggested to mediate MC pro-secretory function,[17] but was later identified as a negative regulator of MC degranulation in response to antigen stimulation.[15]
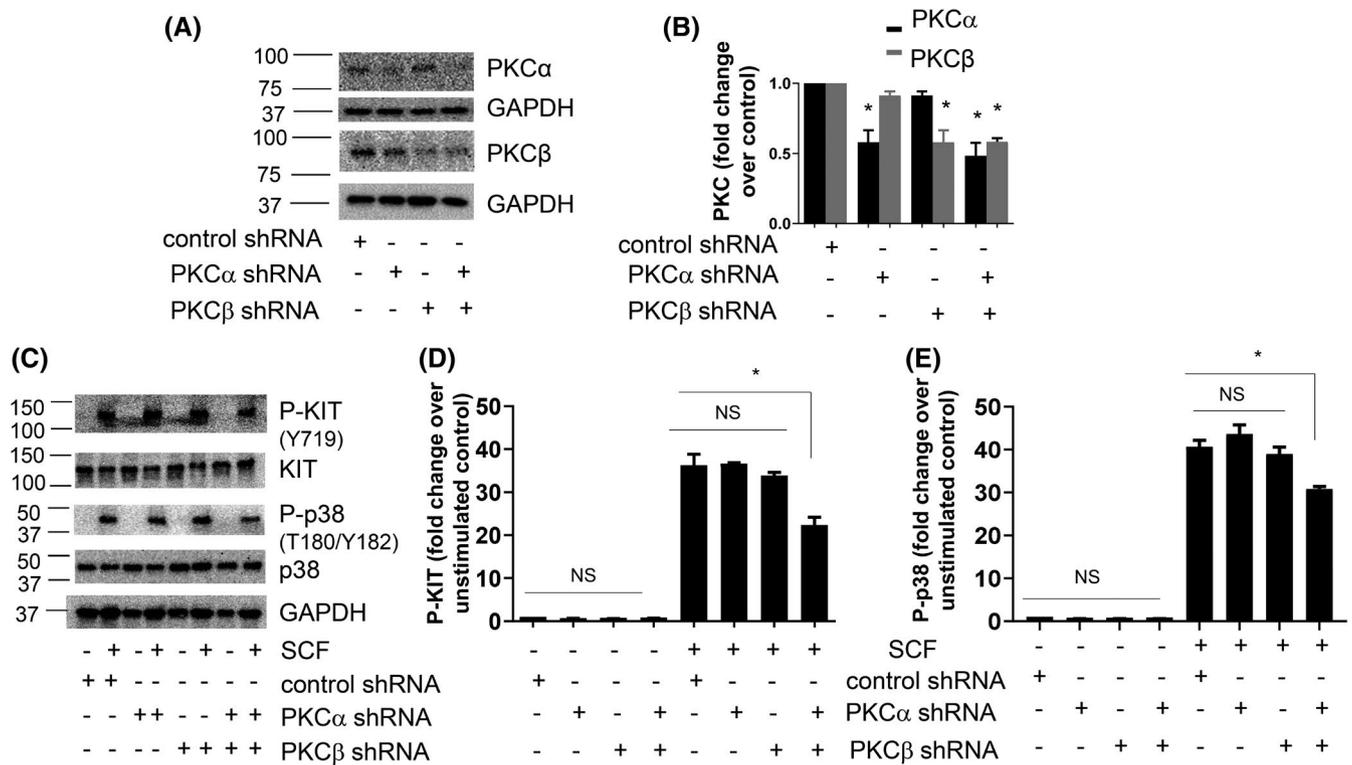
FIGURE 5 Effect of shRNA-knockdown of PKCα and PKCβ or both on SCF-mediated activation of KIT and p38 MAPK in BMMCs. BMMCs were treated with lenti-viral particles directed against PKCα or PKCβ or both PKCα and PKCβ, or empty vector for 48 h. (A) The extent of downregulation was monitored using antibodies for PKCα and PKCβ. PKCα and PKCβ expression were assessed by western blotting. Blots were stripped and re-blotted for GAPDH to confirm equal loading. (B) Densitometric analysis of data is shown in (A). (C) Phosphorylation of KIT (Y719) and p38 (T180/Y182) in BMMCs treated with SCF (100 ng/ml) for 5 min after lentiviral knockdown, assessed by western blotting. Blots were stripped and re-reprobed for total KIT, p38, and GAPDH. (D, E) Densitometric analysis of data is shown in (C). Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. *$p \leq .05$, ns, not significant
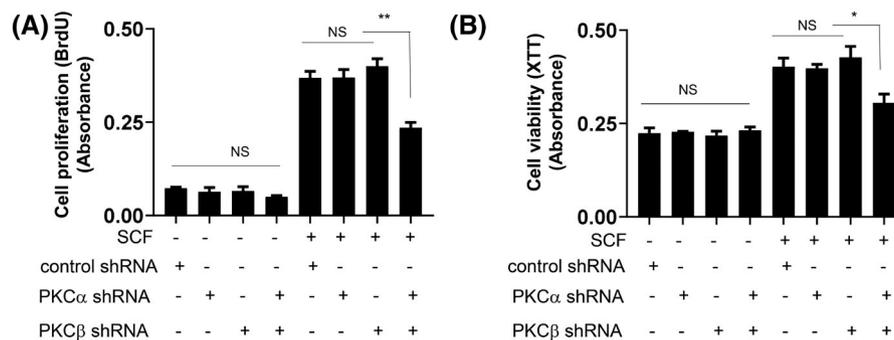
FIGURE 6 Effect of shRNA-knockdown of PKCα and PKCβ or both on SCF-mediated viability and proliferation in BMMCs. BMMCs were treated with lenti-viral particles directed against PKCα, PKCβ, both PKCα and PKCβ, or empty vector (control) for 48 h. (A) Proliferation was measured using BrdU ELISA and (B) viability was assessed by XTT assay. Data are represented as mean ± SEM of three separate experiments. The significance was tested using one-way ANOVA and post hoc analysis. *$p \leq .05$, **$p \leq .01$, ns, not significant

Importantly, although the role of PKCs in MC degranulation and cytokine release is well documented, their role in regulating SCF-mediated MC survival and proliferation is not well understood. PKCs have been suggested to negatively regulate KIT phosphorylation and cell proliferation in porcine aortic endothelial cells stably transfected with human SCF.[11] However, in the current study, we found

that classical PKCs positively regulate SCF-mediated KIT phosphorylation and internalization in BMMCs. It is possible that the differences observed could be due to differences in signaling intermediates and second messengers between the two cell types. Since pharmacological inhibition of classical PKC isoforms attenuated SCF-induced phosphorylation, we analyzed the activation of PKCα and

PKCβ in response to SCF using phospho-specific antibodies, and then observed the activation of both the isoforms. Next, we focused on the SCF-induced signaling downstream of PKC activation. Stimulation with SCF has previously been shown to induce phosphorylation of ERK, p38, and AKT in mature human MCs,[12] murine MCs,[13] and porcine aortic endothelial cells stably transfected with human *KIT*.[11] In accordance to earlier results, we observed the time-dependent activation of p38 MAPK and Erk in response to SCF. PKCα, βI, and βII isoforms were shown to be activated in response to FcεR1 stimulation, and they all play a role in the activation of JNK, MEKK2, and ERK5 in MCs.[35] We observed that, while SCF-mediated p38 phosphorylation is sensitive to inhibition by classical PKC inhibitors, SCF-mediated Erk phosphorylation had no effect, suggesting that the SCF/PKCα/β axis activates p38 MAPK, but not Erk. Further, we found that SCF-mediated viability is partially sensitive to p38 MAPK inhibition. Based on this data, it is possible that SCF may enhance viability via signaling intermediates other than p38 MAPK. Unexpectedly, we observed that when treated alone, both Go6976 and GF109203X led to significant Erk phosphorylation, suggesting that inhibition of PKCs in BMMCs may activate other intracellular pathways that may potentiate Erk phosphorylation.

Using pharmacological inhibitor (Go6976) that blocks classical PKCs, we observed that SCF-mediated viability and proliferation are sensitive to classical PKCs. Overexpression of PKCα has been shown to enhance proliferation in many cell types.[36] Further, classical PKC isoforms have been implicated in differential MC responses such as the degranulation and release of pro-inflammatory mediators like cytokines, prostaglandins, and leukotrienes.[16,35] PKC inhibitors have broad range of action, and the IC50 for Go6976 was determined to be in nM range for classical PKCs in cell free conditions. Notably, at the higher doses used in the current study, these inhibitors can potentially inhibit other kinases apart from PKCs.[14] Therefore, we complimented our experiments employing pharmacological inhibitors with shRNA-mediated PKC knockdown experiments as well. Although pharmacological inhibitors that block classical PKCs inhibited SCF/KIT-mediated viability and proliferation, to our surprise, the knockdown of either PKCα or PKCβ by shRNA did not inhibit SCF-mediated signaling events or BMMC proliferation. However, knockdown of both PKCα and PKCβ attenuated SCF-induced KIT phosphorylation, p38 phosphorylation, and BMMC viability and proliferation, suggesting that a redundancy exists in the mediation of SCF-induced MC survival and proliferation via classical PKCs (Figure 7). We speculate that PKCα and PKCβ compensate for each other when one of the isoforms is blocked, shifting the
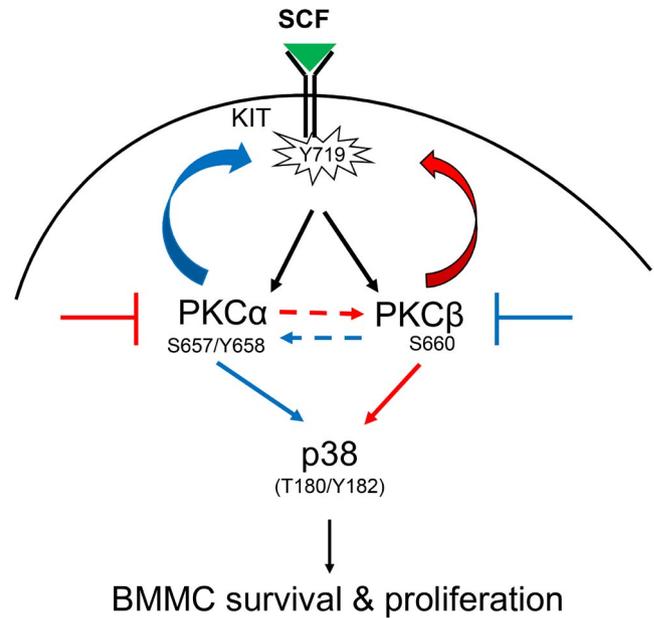
FIGURE 7 Schematic suggesting that PKCα and PKCβ compensate for each other to promote SCF-mediated KIT signaling. Both PKCα and PKCβ are activated in response to SCF via KIT, which in turn enhance SCF and p38 MAPK phosphorylation, promoting BMMC survival and proliferation. Blocking PKCα shunts the signaling through PKCβ, whereas blocking PKCβ shunts the signaling through PKCα validating the redundant nature of the PKC isoforms. We propose that targeting both PKCα and PKCβ is required for the inhibition of SCF-induced BMMC survival and proliferation

signal to the other, and that both the isoforms need to be simultaneously inhibited to hinder SCF-induced viability and proliferation. PKCε was previously shown to be activated in response to antigen stimulation in BMMCs, and it negatively regulates calcium mobilization and/or MAPK activation.[37] In contrast, PKCε was shown to positively affect transcription of Fos/Jun transcription factors,[18] indicating a positive effect of PKCε on the activation of MAPK pathways. Notably, PKCε deficiency had no specific effect on SCF or antigen-induced signal transduction in BMMCs.[26] In response to the antigen cross-linking of IgE bound to FcεRI, pro-inflammatory cytokine release was shown to be controlled in part by a partnership between one conventional and one novel PKC isoform, with PKCα and PKCθ acting as positive regulators and PKCβ and PKCε acting as negative regulators of IL-6 and TNF-α secretion.[38] Further, PKCα^{−/−} BMMCs were reported to exhibit elevated expression of PKCε in comparison with wild-type BMMCs, suggesting that the downregulation of a positive modulator (PKCα) may be accompanied by the upregulation of a negative modulator (PKCε), further suppressing the cytokine release.[38] These findings suggest that different PKCs have divergent, sometimes opposite roles in MC function. Additionally, as shown in the present study, absence of

one classical PKC isoform may be compensated by the recruitment of other classical PKC isotypes. However, based on the literature and availability of relatively less specific PKC inhibitors, we cannot rule out the possibility that novel PKCs may play a role in SCF signaling when both PKCα and PKCβ are inhibited.[38]

SCF is an essential growth factor for the maintenance of mature MCs in the body, enhancing MC proliferation and suppressing MC apoptosis.[39] SCF has been shown to induce MC hyperplasia after subcutaneous administration and, more importantly, SCF expression is increased in the airways of asthmatic patients.[40] Enhanced MC numbers were observed in the airway tissues of asthmatics, with increased levels of MC-derived mediators observed in the bronchoalveolar lavage (BAL) fluid of asthmatic patients.[41] Since MC numbers are increased at sites of inflammation in allergic diseases, exploring SCF-mediated MC viability and proliferation pathways and also investigating the pharmacological interference of key molecules in the pathway could be a promising strategy in controlling the activation of MCs in allergic diseases. In the present study, we demonstrated that inhibition of both PKC α and β simultaneously, but not individually, attenuates SCF-induced KIT activation, signaling, and KIT-mediated viability and proliferation, validating that PKCα and PKCβ compensate for each other in mediating SCF-induced activation of MCs. Based on our results, we propose that PKCα and PKCβ isoforms or p38 MAPK could be novel therapeutic targets for SCF-induced MC viability and proliferation in asthma and other allergic diseases.

ACKNOWLEDGMENTS

DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Lakshminarayan Reddy Teegala, Yasmine Elshoweikh, Ravindra Gudneppanavar, Sathwika Thodeti, Sabita Pokhrel, and Erik Southard performed experiments, analyzed the data, and edited the manuscript; Charles K. Thodeti designed some of the experiments and edited the manuscript; and Sailaja Paruchuri designed experiments, performed research, analyzed and interpreted data, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data to support the findings of the present study are available from the corresponding author upon request.

ORCID

Sailaja Paruchuri https://orcid.org/0000-0003-2377-4327

REFERENCES

1. Gurish MF, Boyce JA. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. *J Allergy Clin Immunol*. 2006;117:1285-1291.
2. Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol*. 2000;12:624-631.
3. Okayama Y, Kawakami T. Development, migration, and survival of mast cells. *Immunol Res*. 2006;34:97-115.
4. Metz M, Grimbaldeston MA, Nakae S, Piliponsky AM, Tsai M, Galli SJ. Mast cells in the promotion and limitation of chronic inflammation. *Immunol Rev*. 2007;217:304-328.
5. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;23:749-786.
6. Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev*. 1997;77:1033-1079.
7. Galli SJ, Tsai M, Wershil BK, Tam SY, Costa JJ. Regulation of mouse and human mast cell development, survival and function by stem cell factor, the ligand for the c-kit receptor. *Int Arch Allergy Immunol*. 1995;107:51-53.
8. Lennartsson J, Ronnstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev*. 2012;92:1619-1649.
9. Mani M, Venkatasubrahmanyam S, Sanyal M, et al. Wiskott-Aldrich syndrome protein is an effector of Kit signaling. *Blood*. 2009;114:2900-2908.
10. Garrington TP, Johnson GL. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol*. 1999;11:211-218.
11. Blume-Jensen P, Siegbahn A, Stabel S, Heldin CH, Ronnstrand L. Increased Kit/SCF receptor induced mitogenicity but abolished cell motility after inhibition of protein kinase C. *EMBO J*. 1993;12:4199-4209.
12. Feuser K, Feilhauer K, Staib L, Bischoff SC, Lorentz A. Akt cross-links IL-4 priming, stem cell factor signaling, and IgE-dependent activation in mature human mast cells. *Mol Immunol*. 2011;48:546-552.
13. Sundstrom M, Alfredsson J, Olsson N, Nilsson G. Stem cell factor-induced migration of mast cells requires p38 mitogen-activated protein kinase activity. *Exp Cell Res*. 2001;267:144-151.
14. Fehrenbach K, Lessmann E, Zorn CN, et al. Steel factor enhances supraoptimal antigen-induced IL-6 production from mast cells via activation of protein kinase C-beta. *J Immunol*. 2009;182:7897-7905.
15. Leitges M, Gimborn K, Elis W, et al. Protein kinase C-delta is a negative regulator of antigen-induced mast cell degranulation. *Mol Cell Biol*. 2002;22:3970-3980.
16. Nechushtan H, Leitges M, Cohen C, Kay G, Razin E. Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase Cbeta. *Blood*. 2000;95:1752-1757.

17. Ozawa K, Szallasi Z, Kazanietz MG, et al. Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca2+ and purified isozymes in washed permeabilized cells. *J Biol Chem.* 1993;268:1749-1756.

18. Razin E, Szallasi Z, Kazanietz MG, Blumberg PM, Rivera J. Protein kinases C-beta and C-epsilon link the mast cell high-affinity receptor for IgE to the expression of c-fos and c-jun. *Proc Natl Acad Sci U S A.* 1994;91:7722-7726.

19. Jaken S, Parker PJ. Protein kinase C binding partners. *BioEssays.* 2000;22:245-254.

20. Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem J.* 1998;332(Pt 2):281-292.

21. Newton AC. Regulation of protein kinase C. *Curr Opin Cell Biol.* 1997;9:161-167.

22. Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J.* 1995;9:484-496.

23. Paruchuri S, Jiang Y, Feng C, Francis SA, Plutzky J, Boyce JA. Leukotriene E4 activates peroxisome proliferator-activated receptor gamma and induces prostaglandin D2 generation by human mast cells. *J Biol Chem.* 2008;283:16477-16487.

24. Paruchuri S, Sjolander A. Leukotriene D4 mediates survival and proliferation via separate but parallel pathways in the human intestinal epithelial cell line Int 407. *J Biol Chem.* 2003;278:45577-45585.

25. Kondeti V, Duah E, Al-Azzam N, Thodeti CK, Boyce JA, Paruchuri S. Differential regulation of cysteinyl leukotriene receptor signaling by protein kinase C in human mast cells. *PLoS One.* 2013;8:e71536.

26. Lessmann E, Leitges M, Huber M. A redundant role for PKC-epsilon in mast cell signaling and effector function. *Int Immunol.* 2006;18:767-773.

27. Gombedza F, Kondeti V, Al-Azzam N, et al. Mechanosensitive transient receptor potential vanilloid 4 regulates Dermatophagoides farinae-induced airway remodeling via 2 distinct pathways modulating matrix synthesis and degradation. *FASEB J.* 2017;31:1556-1570.

28. Paruchuri S, Broom O, Dib K, Sjolander A. The pro-inflammatory mediator leukotriene D4 induces phosphatidy-linositol 3-kinase and Rac-dependent migration of intestinal epithelial cells. *J Biol Chem.* 2005;280:13538-13544.

29. Paruchuri S, Hallberg B, Juhas M, Larsson C, Sjolander A. Leukotriene D(4) activates MAPK through a Ras-independent but PKCepsilon-dependent pathway in intestinal epithelial cells. *J Cell Sci.* 2002;115:1883-1893.

30. Zeidman R, Pettersson L, Sailaja PR, et al. Novel and classical protein kinase C isoforms have different functions in proliferation, survival and differentiation of neuroblastoma cells. *Int J Cancer.* 1999;81:494-501.

31. Bain J, Plater L, Elliott M, et al. The selectivity of protein kinase inhibitors: a further update. *Biochem J.* 2007;408:297-315.

32. Grimbaldeston MA, Metz M, Yu M, Tsai M, Galli SJ. Effector and potential immunoregulatory roles of mast cells in IgE-associated acquired immune responses. *Curr Opin Immunol.* 2006;18:751-760.

33. Prussin C, Metcalfe DD. 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol.* 2006;117:S450-456.

34. Tkaczyk C, Jensen BM, Iwaki S, Gilfillan AM. Adaptive and innate immune reactions regulating mast cell activation: from receptor-mediated signaling to responses. *Immunol Allergy Clin North Am.* 2006;26:427-450.

35. Li G, Lucas JJ, Gelfand EW. Protein kinase C alpha, betaI, and betaII isozymes regulate cytokine production in mast cells through MEKK2/ERK5-dependent and -independent pathways. *Cell Immunol.* 2005;238:10-18.

36. Reyland ME. Protein kinase C isoforms: multi-functional regulators of cell life and death. *Front Biosci.* 2009;14:2386-2399.

37. Chang EY, Szallasi Z, Acs P, et al. Functional effects of overexpression of protein kinase C-alpha, -beta, -delta, -epsilon, and -eta in the mast cell line RBL-2H3. *J Immunol.* 1997;159:2624-2632.

38. Bell KS, Al-Riyami L, Lumb FE, et al. The role of individual protein kinase C isoforms in mouse mast cell function and their targeting by the immunomodulatory parasitic worm product, ES-62. *Immunol Lett.* 2015;168:31-40.

39. Jensen BM, Metcalfe DD, Gilfillan AM. Targeting kit activation: a potential therapeutic approach in the treatment of allergic inflammation. *Inflamm Allergy Drug Targets.* 2007;6:57-62.

40. Da Silva CA, Reber L, Frossard N. Stem cell factor expression, mast cells and inflammation in asthma. *Fundam Clin Pharmacol.* 2006;20:21-39.

41. Hart PH. Regulation of the inflammatory response in asthma by mast cell products. *Immunol Cell Biol.* 2001;79:149-153.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.