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Modulation of mast cell proliferative and inflammatory responses by Leukotriene D₄ and Stem Cell Factor signaling interactions

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Abstract

Mast cells (MCs) are important effector cells in asthma and pulmonary inflammation, and their proliferation and maturation is maintained by stem cell factor (SCF) via its receptor, c-Kit. Cysteinyl leukotrienes (cys-LTs) are potent inflammatory mediators that signal through CysLT₁R and CysLT₂R located on the MC surface, and they enhance MC inflammatory responses. However, it is not known if SCF and cys-LTs cross-talk and influence MC hyperplasia and activation in inflammation. Here, we report the concerted effort of the growth factor SCF and the inflammatory mediator LTD₄ in MC activation. Stimulation of MCs by LTD₄ in the presence of SCF enhances c-Kit-mediated proliferative responses. Similarly, SCF synergistically enhances LTD₄-induced calcium, c-fos expression and phosphorylation, as well as MIP1β generation in MCs. These findings suggest that integration of SCF and LTD₄ signals may contribute to MC hyperplasia and hyper-reactivity during airway hyper-response and inflammation.

Keywords

calcium; c-fos; c-Kit; cys-LTs; LTD₄; mast cells; MIP1β; proliferation; calcium; stem cell factor

Introduction

Mast cells (MCs) are stem cell factor (SCF)-dependent hematopoietic cells that are ubiquitously distributed throughout the body (Gurish and Boyce, 2006; Wedemeyer et al., 2000) and they initiate inflammatory responses to allergens and infectious agents (Okayama and Kawakami, 2006). MCs play an important role in asthma through the secretion of several soluble inflammatory mediators. c-Kit is a member of the type III subclass of receptor tyrosine kinases, comprising of an N-terminal extracellular ligand binding domain, a transmembrane domain and a cytoplasmic kinase domain, which is activated by ligand-mediated receptor dimerization (Mani et al., 2009). C-Kit activation by SCF is crucial for

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the survival and proliferation of human MCs and the deregulation of the c-kit/SCF axis can lead to uncontrolled proliferation as seen during systemic mastocytosis. Mastocytosis is the disturbance of the homeostatic mechanisms that control the accumulation, proliferation, survival, and turnover rates of MCs, contributing to inflammation, and remodeling. The mechanistic basis of MC hyperplasia in asthma (or in any allergic disease) is not completely understood. The importance of c-Kit signaling in MC proliferation makes it crucial to understand the basic mechanisms by which Kit regulates MC function. Although the role of c-Kit signaling is extensively studied in porcine aortic endothelial cells (Blume-Jensen et al., 1994; Blume-Jensen et al., 1993), its role in MC proliferation is still not well known.

Cysteinyl leukotrienes (cys-LTs), comprising of LTC₄, LTD₄ and LTE₄ are potent bronchoconstrictors and they play an important role in asthma and airway inflammation (Davidson et al., 1987; Drazen and Austen, 1987). They are derivatives of arachidonic acid generated by MCs, eosinophils, basophils, macrophages, and myeloid dendritic cells (Kanaoka and Boyce, 2004), and act through two main receptors, CysLT₁R and CysLT₂R (Heise et al., 2000; Lynch et al., 1999). MCs not only generate cys-LTs, but also express CysLT₁R and CysLT₂R (Mellor et al., 2003; Mellor et al., 2001). We and others have previously shown that stimulation of human cord blood-derived MCs (hMCs) with LTD₄ potently induces calcium flux and cytokine generation through CysLT₁R (Mellor et al., 2002; Paruchuri et al., 2008).

During inflammation, various mediators prime each other's responses, resulting in amplified inflammatory milieu. For example, SCF-induced prolonged activation of mast cells has been shown to play critical role in the progression of allergen-induced airway hyper responsiveness (AHR) and chronic airway hypersensitivity (Hundley et al., 2004). Also, SCF has been implicated in the induction of airway hyper-reactivity during allergy-induced pulmonary responses in mouse models (Campbell et al., 1999). Interestingly, SCF-induced airway hyper-reactivity has been shown to depend on leukotriene production (Oliveira et al., 2001). Enhanced proliferation of MCs is commonly seen at the site of inflammation and this increase in MC number correlates with the severity of AHR. Given that both SCF and leukotrienes are produced at the site of inflammation and induce AHR together with the fact that MCs express receptors for both SCF and leukotrienes and enhanced MC proliferation is seen during inflammation, we asked if c-Kit and CysLT₁R can cross-talk and influence MC proliferative and inflammatory phenotypes. Therefore, in the current study, we tested the MC proliferative and inflammatory responses in the presence of both SCF and LTD₄. We hypothesized that the growth factor SCF can boost LTD₄-mediated inflammatory signals and an inflammatory mediator, LTD₄, can increase SCF-induced, c-Kit-mediated proliferative responses. Our results suggest that LTD₄ and SCF synergistically enhance MC proliferative (c-Kit phosphorylation and proliferation) and inflammatory responses (c-fos phosphorylation, expression, and MIP1 β production).

Materials and Methods

Reagents

LTC₄, LTD₄, LTE₄, and MK571 were purchased from Cayman Chemicals, Fura-2 AM from Molecular Probes, All phospho-specific antibodies and corresponding controls from Cell

Signaling Technology, GAPDH antibody from Fitzgerald (Acton, MA), XTT proliferation assay kit was from Trevigen (Gaithersburg, MD), BrdU proliferation assay kit was from Calbiochem, MIP1 β ELISA kit was from R&D systems. All cytokines were purchased from R&D systems.

Cell culture

The LAD2 MC leukemia line (Kirshenbaum et al., 2003) was a generous gift from Dr. Arnold Kirshenbaum, NIH. LAD2 cells were cultured in stemPro-34 (Invitrogen) supplemented with 2mM L-Glutamine (Invitrogen), Pen-strep (100 IU/ml) (Invitrogen), and SCF (R&D systems) (100 ng/ml). Cell culture medium was hemi-depleted every week with fresh medium and 100 ng/ml SCF. Cells were SCF-starved overnight before stimulating with SCF in all the experiments. LAD2 cells are dependent on SCF for their proliferation and retain excellent responses to cys-LTs in inducing calcium flux, secretion of MIP1 β and other chemokines, similar to isolated hMCs. (Paruchuri et al., 2008; Paruchuri et al., 2009). The expression pattern of cys-LT receptors in these cells are also similar to hMCs with high levels of CysLT $_1$ R compared to CysLT $_2$ R (Paruchuri et al., 2008). Bone marrow derived mast cells (BMMCs) were isolated from C57BL/6 mice. Mice were euthanized according to guidelines and approval of the Institutional Animal Care and Use Committee (IACUC) of the Northeast Ohio Medical University (NEOMED). BMMCs were cultured in 80% RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-Glutamine, Pen-Strep (100UI/ml), Sodium Pyruvate (1mM), Non-Essential Amino acids, HEPES buffer (25mM) and β -Mercaptoethanol (50uM) and 20% WEHI-3 cell conditioned medium for 4–6 weeks. Maturity of BMMCs was examined by Toluidine blue staining and >90% mature BMMCs were used for experiments.

Cord blood was obtained from Cleveland Cord Blood Center and MCs were isolated as described (Mellor et al., 2002). Briefly, heparin-treated cord blood was sedimented with 4.5% dextran solution and the buffy coat was layered onto Ficoll-Hypaque and mononuclear cells (MNC) were obtained after centrifugation at the interphase. Erythrocytes were further removed from MNC by hypotonic lysis and cultured in RPMI-1640 (Gibco), 10%FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, Penicillin-Streptomycin, Gentamicin and 0.2 μ M 2-Mercaptoethanol in the presence of SCF (100ng/ml), IL-6 (50 ng/ml) and IL-10 (10 ng/ml). Non-adherent cells were transferred to fresh medium containing cytokines every week for 6–9 weeks. Maturity of hMCs was examined by Toluidine blue staining and >90% mature hMCs were used for experiments.

Calcium flux

Cells were cultured in SCF-free medium overnight. Thereafter, cells ($0.5-1 \times 10^6$ /sample) were washed and labeled with fura 2-AM for 30 minutes at 37°C. Cells were further washed and stimulated with 500 nM of LTD $_4$ and/or 100 ng/ml of SCF, and the changes in intracellular calcium were measured using excitation at 340 and 380nm in a fluorescence spectrophotometer (Hitachi F-4500) as described earlier (Paruchuri et al., 2008). In some experiments, MK571 was added 10 minutes before the addition of indicated agonists. The relative ratios of fluorescence emitted at 510 nm were recorded and displayed as a reflection of intracellular calcium concentration.

Cell activation

Cells were either stimulated with 500 nM LTD₄ and/or 100 ng/ml SCF (or with indicated concentrations) for indicated time points (phosphorylation of c-Kit for 15 min, phosphorylation and expression of c-fos for 1h, measurement of cytokines at transcript level for 2h, and protein level for 6h). The concentration of MIP1 β was measured with ELISAs according to the manufacturer's protocol (Paruchuri et al., 2008).

Cell lysates and western blotting

After stimulation with the respective agonists, MCs (0.5×10^6) were lysed with lysis buffer (BD Bioscience) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Pierce). Immunoblotting was performed as described previously (Paruchuri et al., 2002). Briefly, lysates were subjected to 4–12% SDS-PAGE and transferred to PVDF membrane. Membranes were incubated with respective primary phospho- and total antibodies diluted in $1 \times$ TBS, 5% dry milk, 0.1% Tween-20 (1:1000) overnight at 4°C on shaker, and then with secondary antibody (peroxidase-conjugated anti-rabbit or anti-mouse). Western blot was incubated with ECL, and the bands were visualized using imager (Protein Simple) and quantified using Alpha View software (Protein Simple).

Real-time Quantitative PCR

The expressions of COX-2, MIP1 β and TNF α transcripts were determined with real-time PCR performed on Light cycler 480 (Roche). Cells were cultured and treated as described above, and total RNA was isolated with an RNAeasy minikit (Qiagen) according to manufacturer's instructions. RNA concentration was determined using a Take 3 module of Epoch micro plate reader (Biotek) at 260/280 nm. 1 μ g of total RNA was used for reverse transcription using cDNA synthesis kit from Quanta Biosciences, containing MgCl₂, dNTPs, recombinant RNase inhibitor protein, qScript Reverse Transcriptase, random primers, oligo (dT) primers and stabilizers. Gene expression was assayed by quantitative real-time PCR on LightCycler® 480 II (Roche Applied Science) using LightCycler® 480 SYBR Green I Master mix, cDNA prepared as described above and COX-2, MIP1 β , TNF α and GAPDH forward and reverse Primers. Following are the forward (F) and reverse (R) primers used

| | |
|----------------|------------------------------|
| MIP1 β - | F- CCAGCCAGCTGTGGTATT |
| | R-CAGTTCAGTTCAGGTCATACA |
| TNF α - | F- CCAGGGACCTCTCTAATCA |
| | R-TCAGCTGAGGGTTGCTAC |
| COX-2- | F-CAACTCTATATTGCTGGAACATGGA |
| | R-TGGAAGCCTGTGATACTTTCTGTACT |
| GAPDH- | F-TGCACCACCAACTGCTTAGC |
| | R-GGCATGGACTGTGGTCATGAG |

The Ct values for COX-2, MIP1 β , and TNF α were calculated relative to the GAPDH levels and values were expressed as fold change over the control (Duah et al., 2013).

Cell proliferation

Cells were plated at a density of 5000 cells/well of 96 well plate, cultured in SCF-free medium overnight, and treated with increasing concentration of SCF and/or 500 nM of LTD₄. After 72 h, the proliferation was assayed either by XTT assay (Trevigen) or BrdU ELISA (Millipore) according to the manufacturer's protocol.

Data Analysis

Data is expressed as mean \pm SEM from at least three experiments except where otherwise indicated. Significance was determined using one way ANOVA and post-hoc analysis.

Results

SCF induces concentration-dependent phosphorylation of c-Kit in MCs

SCF is the major growth factor for the proliferation of MCs, and it relays its responses through the cell surface receptor, c-Kit (Galli et al., 1995; Iemura et al., 1994; Tsai et al., 1991). To confirm if c-Kit is activated in response to SCF stimulation in LAD2 cells, we first determined the effect of SCF on phosphorylation of c-Kit receptor, by treating the cells with different concentrations of SCF. Stimulation with SCF for 15 minutes led to dose-dependent phosphorylation (Fig. 1A, B) of c-Kit receptor. SCF induced phosphorylation of c-Kit receptor at doses as low as 1ng/ml with a maximum response at 100 ng/ml concentration.

C-Kit phosphorylation is synergistically activated by SCF and LTD₄

To determine if there is crosstalk between c-Kit and CysLTRs, we stimulated LAD2 cells with cys-LTs with or without SCF and analyzed c-Kit phosphorylation. We could not detect any significant phosphorylation of c-Kit by cys-LTs alone. However, LTD₄ significantly strengthened SCF-induced c-Kit phosphorylation (Fig. 2A, B, C). We also found similar synergistic activation of c-Kit by SCF and LTD₄ in BMDCs (Suppl. Fig. 1). This synergism with SCF was not observed with LTC₄ or LTE₄ treatment (Fig. 2A). Further, we found that treatment of cells with CysLT₁R antagonist, MK571 prior to stimulation with SCF and LTD₄, inhibited this synergistic effect suggesting that it is mediated through CysLT₁R (Fig. 2B, C).

LTD₄ potentiates SCF-induced MC proliferation

The fact that LTD₄ augmented c-Kit phosphorylation by SCF prompted us further to understand the significance of this potentiation. We analyzed if LTD₄ treatment together with SCF would amplify LAD2 cell proliferation by using XTT assay. Consistent with earlier results (Laidlaw et al., 2011), we found that SCF promoted proliferation of LAD2 cells in a dose-dependent manner (Fig. 3). Interestingly, a significant increment in cell proliferation was observed with combined stimulation of SCF and LTD₄ at high doses of SCF. These findings indicate that LTD₄ can enhance c-Kit responses in LAD2 cells and increase their proliferation. We also found that hMCs cultured in the presence of both SCF and LTD₄ induced around 2 fold increase in proliferation compared to SCF treatment alone

(Suppl. Fig. 2A). Taken together, these results suggest that LTD₄ in concert with SCF can modulate primary mast cell (hMC) and MC cell line (LAD2) proliferation.

SCF pre-treatment amplifies CysLT₁R function as measured by LTD₄-induced calcium flux

Above results clearly depict that combined stimulation of SCF and LTD₄ enhances c-Kit-mediated proliferative responses. Next, we asked if this cross-talk is bi-directional i.e. if combined stimulation with SCF can augment LTD₄-mediated inflammatory responses, the same way as LTD₄ can modulate SCF-induced proliferation. We have previously shown that stimulation of LAD2 cells with LTD₄ induces calcium flux, c-fos phosphorylation and expression and MIP1 β generation (Kondeti et al., 2013; Paruchuri et al., 2008). First, we asked if SCF pre-treatment influences LTD₄-mediated calcium flux. We have previously shown that LTD₄ was the most potent agonist among the cys-LTs for eliciting calcium flux and completely desensitized MC to the calcium fluxes induced by the two other cys-LTs (Paruchuri et al., 2008). We observed that SCF (100 ng/ml) induced minimal or no calcium flux by itself (Fig. 4B) or followed by priming with LTD₄ (Fig. 4A). However, stimulating cells with SCF prior to LTD₄ significantly amplified LTD₄-induced calcium flux (Fig. 4B, C). Further, this calcium flux was inhibited by prior treatment with CysLT₁R antagonist, MK571, confirming that this signal is mediated by CysLT₁R (Fig. 4C).

SCF and LTD₄ treatment synergistically amplifies c-fos phosphorylation and expression

Next, we investigated if SCF has the potential to amplify other LTD₄-induced MC responses such as c-fos phosphorylation and expression. We have reported earlier that stimulation of LAD2 cells with LTD₄ led to phosphorylation and increased expression of c-fos (Kondeti et al., 2013). SCF treatment alone did not induce significant c-fos phosphorylation or expression, however, both LTD₄-induced c-fos phosphorylation and expression were synergistically enhanced in the presence of SCF (Fig. 5A, B), further suggesting the ability of SCF/c-Kit to modulate LTD₄-induced responses. We also found that treatment of hMCs with a combination of SCF and LTD₄ caused potentiation of c-fos phosphorylation and expression compared to treatment with either of the agonists (Suppl. Fig.2B) further confirming the synergistic activation in primary MCs.

Amplification of inflammatory signals by LTD₄ and SCF treatment

COX-2 activation, MIP1 β secretion and up-regulation of other inflammatory cytokine expression are major MC responses downstream of CysLT₁R signaling induced by LTD₄ (Paruchuri et al., 2008; Paruchuri et al., 2009). These responses further enhance inflammation by recruiting other immune cells. To find out if SCF can increase LTD₄-induced MC inflammatory signals, we measured LTD₄-induced inflammatory signals in the presence or absence of SCF. LTD₄ stimulation alone, but not SCF, induced the expression of COX-2, TNF- α , and MIP1 β in LAD2 cells. Interestingly, we found that LTD₄-induced expression of these inflammatory genes is significantly boosted by SCF (Fig. 6 A, B, C). Finally, consistent with our transcript data, we found that LTD₄ and SCF synergistically increased the secretion of MIP1 β protein in LAD2 cells (Fig.6 D). However, either LTD₄ or SCF treatment, or the combination of both failed to induce any degranulation (data not shown) as determined by β -hexosaminidase assay using p-NAG (p-Nitrophenyl-N-acetyl- β -D-glucosaminidine) as substrate.

Discussion

In the present study, we demonstrate that an inflammatory mediator, LTD₄, and a growth factor, SCF synergistically regulate MC function. We clearly demonstrate that LTD₄ can prime SCF-induced c-Kit phosphorylation and proliferation of MCs, and that SCF in turn can strengthen LTD₄-induced calcium flux and inflammatory responses such as c-fos phosphorylation and expression, as well as MIP1 β production, suggesting a potential cross-talk between the LTD₄ and SCF receptors, CysLT₁R and c-Kit (Fig. 7).

MCs are mediators of the early phase of allergic inflammation. MCs are released into the blood stream as progenitor cells, and are then recruited to the tissues where they undergo maturation (Gurish and Boyce, 2006; Kirshenbaum et al., 1999; Metcalfe et al., 1997). SCF is an important regulator of MC growth, differentiation, survival and chemotaxis (Grimbaldeston et al., 2006; Prussin and Metcalfe, 2006; Tkaczyk et al., 2006). SCF acts through c-Kit tyrosine kinase receptor which activates downstream signaling molecules such as PI3 Kinase and AKT to exert its effects on MCs (Ali et al., 2004; Moller et al., 2005). SCF was also shown to enhance allergen induced FC ϵ RI mediated degranulation of MCs and release of inflammatory mediators (Gilfillan and Tkaczyk, 2006; Ito et al., 2012; Jensen et al., 2007). These inflammatory mediators may further activate MCs and boost inflammation.

Cys-LTs are produced by MCs and were also shown to activate MCs. We have previously shown that LAD2 cells express both CysLT₁R and CysLT₂R and that cys-LTs (LTC₄, LTD₄ and LTE₄) induce calcium influx, c-fos expression and phosphorylation (Kondeti et al., 2013; Laidlaw et al., 2011; Paruchuri et al., 2008). Both c-Kit signaling and CysLTR signaling are shown to be mitogenic for MCs. In addition, CysLT₁R has been shown to transactivate c-Kit receptor in hMCs (Jiang et al., 2006). Interestingly, patients with systemic mastocytosis showed significantly higher urinary excretion of cys-LTs than controls (Raithel et al., 2011). Further, LTC₄ synthase knockout mice were unable to develop MC hyperplasia in the inflamed mucosal surface of the lung in a model of allergen-induced pulmonary inflammation (Kim et al., 2006). However, it is not known if these pro-inflammatory mediators act in concert with SCF. In the present study, though we did not observe any c-Kit phosphorylation or proliferation with LTD₄ alone, stimulation of MCs with SCF induced phosphorylation as well as MC proliferation. Interestingly, c-Kit phosphorylation and MC proliferation was augmented in the presence of both SCF and LTD₄ suggesting the existence of a cross-talk between their receptors. LTD₄, but not LTC₄ or LTE₄, increased c-Kit phosphorylation by SCF, indicating a specific role for LTD₄ in this cross-talk. We also showed that this potentiation of c-Kit is mediated through Cys-LT₁R. Notably, we found that stimulating MCs with SCF prior to LTD₄ enhanced LTD₄-induced calcium flux. In contrast, SCF alone did not induce any calcium flux. Furthermore, we found that c-fos phosphorylation and expression, COX-2, TNF- α and MIP1 β transcript expression, as well as MIP1 β protein secretion are amplified by combined treatment with SCF and LTD₄. Thus, our findings suggest that while LTD₄ synergistically activates SCF-induced proliferative signals, SCF in turn potentiates LTD₄-induced inflammatory signals in MCs. The concept of this bi-directional cross-talk between LTD₄ (CysLT₁R) and SCF (c-Kit) signaling is novel and may have important implications in targeting MC-mediated

inflammatory responses. Importantly, our results suggest that the inflammatory microenvironment, but not a single molecule, dictate the inflammatory phenotype of MCs. SCF was previously shown to enhance FCεRI mediated responses in MCs (Gilfillan and Tkaczyk, 2006; Ito et al., 2012; Jensen et al., 2008; Jensen et al., 2007). However, recently it was shown that while acute treatment of SCF potentiated inflammatory phenotype of MCs, prolonged treatment of SCF inhibited PGE₂ and allergen-induced MC degranulation (Gilfillan and Tkaczyk, 2006; Ito et al., 2012; Jensen et al., 2007). The underlying molecular mechanism could be alterations in the cytoskeleton by SCF-mediated regulation of src kinase, hck (Ito et al., 2012; Smrz et al., 2013).

In conclusion, our results suggest that the cross-talk between SCF and LTD₄ induces MC proliferation, gene regulation, and cytokine production. SCF signaling through c-Kit may regulate baseline maintenance of MCs. However, locally derived cys-LTs produced at the site of inflammation enhance MC numbers by cross talking with c-Kit receptor. Similarly, this c-Kit and CysLT₁R crosstalk also potentiates LTD₄ effects enhancing MC inflammatory responses leading to enhanced pathological loop (Fig. 7). Although SCF-induced MC activation in allergen mediated AHR was shown to be dependent on leukotrienes (Oliveira et al., 2001), it is not known whether cys-LTs can modulate SCF receptor. Thus, our findings that LTD₄ can enhance c-Kit-dependent proliferative response and SCF potentiate LTD₄-mediated inflammatory responses are very intriguing and may provide basis for novel therapeutic targets for asthma and allergic diseases

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Literature Cited

- Ali K, Bilancio A, Thomas M, Pearce W, Gilfillan AM, Tkaczyk C, Kuehn N, Gray A, Giddings J, Peskett E, Fox R, Bruce I, Walker C, Sawyer C, Okkenhaug K, Finan P, Vanhaesebroeck B. Essential role for the p110δ phosphoinositide 3-kinase in the allergic response. *Nature*. 2004; 431(7011):1007–1011. [PubMed: 15496927]
- Blume-Jensen P, Ronnstrand L, Gout I, Waterfield MD, Heldin CH. Modulation of Kit/stem cell factor receptor-induced signaling by protein kinase C. *J Biol Chem*. 1994; 269(34):21793–21802. [PubMed: 7520444]
- 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(11): 4199–4209. [PubMed: 7693453]
- Campbell E, Hogaboam C, Lincoln P, Lukacs NW. Stem cell factor-induced airway hyperreactivity in allergic and normal mice. *Am J Pathol*. 1999; 154(4):1259–1265. [PubMed: 10233863]
- Davidson AB, Lee TH, Scanlon PD, Solway J, McFadden ER Jr, Ingram RH Jr, Corey EJ, Austen KF, Drazen JM. Bronchoconstrictor effects of leukotriene E4 in normal and asthmatic subjects. *Am Rev Respir Dis*. 1987; 135(2):333–337. [PubMed: 3028218]
- Drazen JM, Austen KF. Leukotrienes and airway responses. *Am Rev Respir Dis*. 1987; 136(4):985–998. [PubMed: 2821857]

- Duah E, Adapala RK, Al-Azzam N, Kondeti V, Gombedza F, Thodeti CK, Paruchuri S. Cysteinyl leukotrienes regulate endothelial cell inflammatory and proliferative signals through CysLT(2) and CysLT(1) receptors. *Sci Rep.* 2013; 3:3274. [PubMed: 24253666]
- 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(1–3):51–53. [PubMed: 7542101]
- Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast- cell activation. *Nat Rev Immunol.* 2006; 6(3):218–230. [PubMed: 16470226]
- 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(6):751–760. [PubMed: 17011762]
- Gurish MF, Boyce JA. Mast cells: ontogeny, homing, and recruitment of a unique innate effector cell. *J Allergy Clin Immunol.* 2006; 117(6):1285–1291. [PubMed: 16750988]
- Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, Stocco R, Bellefeuille JN, Abramovitz M, Cheng R, Williams DL Jr, Zeng Z, Liu Q, Ma L, Clements MK, Coulombe N, Liu Y, Austin CP, George SR, O'Neill GP, Metters KM, Lynch KR, Evans JF. Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem.* 2000; 275(39):30531–30536. [PubMed: 10851239]
- Hundley TR, Gilfillan AM, Tkaczyk C, Andrade MV, Metcalfe DD, Beaven MA. Kit and FcεRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells. *Blood.* 2004; 104(8):2410–2417. [PubMed: 15217825]
- Iemura A, Tsai M, Ando A, Wershil BK, Galli SJ. The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *Am J Pathol.* 1994; 144(2):321–328. [PubMed: 7508684]
- Ito T, Smrz D, Jung MY, Bandara G, Desai A, Smrzova S, Kuehn HS, Beaven MA, Metcalfe DD, Gilfillan AM. Stem cell factor programs the mast cell activation phenotype. *J Immunol.* 2012; 188(11):5428–5437. [PubMed: 22529299]
- Jensen BM, Beaven MA, Iwaki S, Metcalfe DD, Gilfillan AM. Concurrent inhibition of kit- and FcεRI-mediated signaling: coordinated suppression of mast cell activation. *J Pharmacol Exp Ther.* 2008; 324(1):128–138. [PubMed: 17925481]
- 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(1):57–62. [PubMed: 17352689]
- Jiang Y, Kanaoka Y, Feng C, Nocka K, Rao S, Boyce JA. Cutting edge: Interleukin 4-dependent mast cell proliferation requires autocrine/intracrine cysteinyl leukotriene-induced signaling. *J Immunol.* 2006; 177(5):2755–2759. [PubMed: 16920908]
- Kanaoka Y, Boyce JA. Cysteinyl leukotrienes and their receptors: cellular distribution and function in immune and inflammatory responses. *J Immunol.* 2004; 173(3):1503–1510. [PubMed: 15265876]
- Kim DC, Hsu FI, Barrett NA, Friend DS, Grenningloh R, Ho IC, Al-Garawi A, Lora JM, Lam BK, Austen KF, Kanaoka Y. Cysteinyl leukotrienes regulate Th2 cell- dependent pulmonary inflammation. *J Immunol.* 2006; 176(7):4440–4448. [PubMed: 16547282]
- Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, Rao VK, Metcalfe DD. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcεRI or FcγRI. *Leuk Res.* 2003; 27(8):677–682. [PubMed: 12801524]
- Kirshenbaum AS, Goff JP, Semere T, Foster B, Scott LM, Metcalfe DD. Demonstration that human mast cells arise from a progenitor cell population that is CD34(+), c-kit(+), and expresses aminopeptidase N (CD13). *Blood.* 1999; 94(7):2333–2342. [PubMed: 10498605]
- 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(8):e71536. [PubMed: 23977066]
- Laidlaw TM, Steinke JW, Tinana AM, Feng C, Xing W, Lam BK, Paruchuri S, Boyce JA, Borish L. Characterization of a novel human mast cell line that responds to stem cell factor and expresses functional FcεRI. *J Allergy Clin Immunol.* 2011; 127(3):815–822. e811–e815. [PubMed: 21281958]

- Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateaufneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams DL Jr, Ford-Hutchinson AW, Caskey CT, Evans JF. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature*. 1999; 399(6738): 789–793. [PubMed: 10391245]
- Mani M, Venkatasubrahmanyam S, Sanyal M, Levy S, Butte A, Weinberg K, Jahn T. Wiskott-Aldrich syndrome protein is an effector of Kit signaling. *Blood*. 2009; 114(14):2900–2908. [PubMed: 19643989]
- Mellor EA, Austen KF, Boyce JA. Cysteinyl leukotrienes and uridine diphosphate induce cytokine generation by human mast cells through an interleukin 4- regulated pathway that is inhibited by leukotriene receptor antagonists. *J Exp Med*. 2002; 195(5):583–592. [PubMed: 11877481]
- Mellor EA, Frank N, Soler D, Hodge MR, Lora JM, Austen KF, Boyce JA. Expression of the type 2 receptor for cysteinyl leukotrienes (CysLT2R) by human mast cells: Functional distinction from CysLT1R. *Proc Natl Acad Sci U S A*. 2003; 100(20):11589–11593. [PubMed: 13679572]
- Mellor EA, Maekawa A, Austen KF, Boyce JA. Cysteinyl leukotriene receptor 1 is also a pyrimidineric receptor and is expressed by human mast cells. *Proc Natl Acad Sci U S A*. 2001; 98(14):7964–7969. [PubMed: 11438743]
- Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev*. 1997; 77(4):1033–1079. [PubMed: 9354811]
- Moller C, Alfredsson J, Engstrom M, Wootz H, Xiang Z, Lennartsson J, Jonsson JI, Nilsson G. Stem cell factor promotes mast cell survival via inactivation of FOXO3a-mediated transcriptional induction and MEK-regulated phosphorylation of the proapoptotic protein Bim. *Blood*. 2005; 106(4):1330–1336. [PubMed: 15855272]
- Okayama Y, Kawakami T. Development, migration, and survival of mast cells. *Immunol Res*. 2006; 34(2):97–115. [PubMed: 16760571]
- Oliveira SH, Hogaboam CM, Berlin A, Lukacs NW. SCF-induced airway hyperreactivity is dependent on leukotriene production. *Am J Physiol Lung Cell Mol Physiol*. 2001; 280(6):L1242–L1249. [PubMed: 11350804]
- Paruchuri S, Hallberg B, Juhas M, Larsson C, Sjolander A. Leukotriene D(4) activates MAPK through a Ras-independent but PKCε-dependent pathway in intestinal epithelial cells. *J Cell Sci*. 2002; 115(Pt 9):1883–1893. [PubMed: 11956320]
- Paruchuri S, Jiang Y, Feng C, Francis SA, Plutzky J, Boyce JA. Leukotriene E4 activates peroxisome proliferator-activated receptor γ and induces prostaglandin D2 generation by human mast cells. *J Biol Chem*. 2008; 283(24):16477–16487. [PubMed: 18411276]
- Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, Kanaoka Y, Conley P, Boyce JA. Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. *J Exp Med*. 2009; 206(11):2543–2555. [PubMed: 19822647]
- Prussin C, Metcalfe DD. 5. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. 2006; 117(2 Suppl Mini-Primer):S450–S456. [PubMed: 16455345]
- Raithel M, Zopf Y, Kimpel S, Naegel A, Molderings GJ, Buchwald F, Schultis HW, Kressel J, Hahn EG, Konturek P. The measurement of leukotrienes in urine as diagnostic option in systemic mastocytosis. *J Physiol Pharmacol*. 2011; 62(4):469–472. [PubMed: 22100848]
- Smrz D, Bandara G, Beaven MA, Metcalfe DD, Gilfillan AM. Prevention of F-actin assembly switches the response to SCF from chemotaxis to degranulation in human mast cells. *Eur J Immunol*. 2013; 43(7):1873–1882. [PubMed: 23616175]
- 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(3):427–450. [PubMed: 16931287]
- Tsai M, Takeishi T, Thompson H, Langley KE, Zsebo KM, Metcalfe DD, Geissler EN, Galli SJ. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc Natl Acad Sci U S A*. 1991; 88(14):6382–6386. [PubMed: 1712491]
- Wedemeyer J, Tsai M, Galli SJ. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol*. 2000; 12(6):624–631. [PubMed: 11102764]

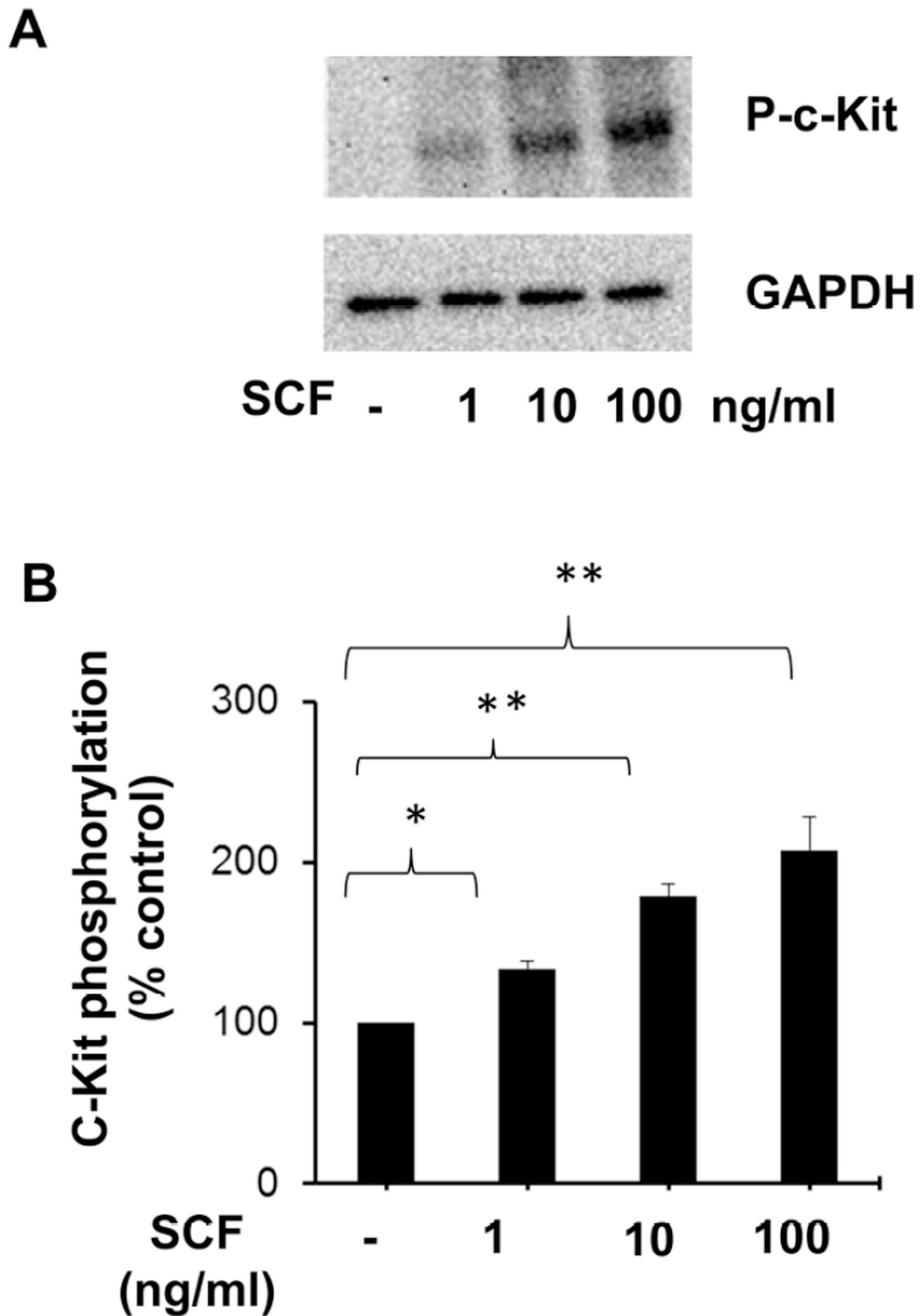


Figure 1. Dose dependent phosphorylation of c-Kit receptor by SCF

(A) LAD2 cells were stimulated with the indicated doses of SCF for 15 minutes and the c-Kit phosphorylation was assessed by western blotting using phospho-specific c-Kit antibodies. Blots were stripped and re-blotting for GAPDH to confirm equal loading. The data shown are representative of three separate experiments. (B) Densitometric analysis of data shown in A. The data represents mean \pm SEM of three separate experiments. The significance was tested using one way ANOVA and post-hoc analysis * $P < 0.05$, ** $P < 0.001$.

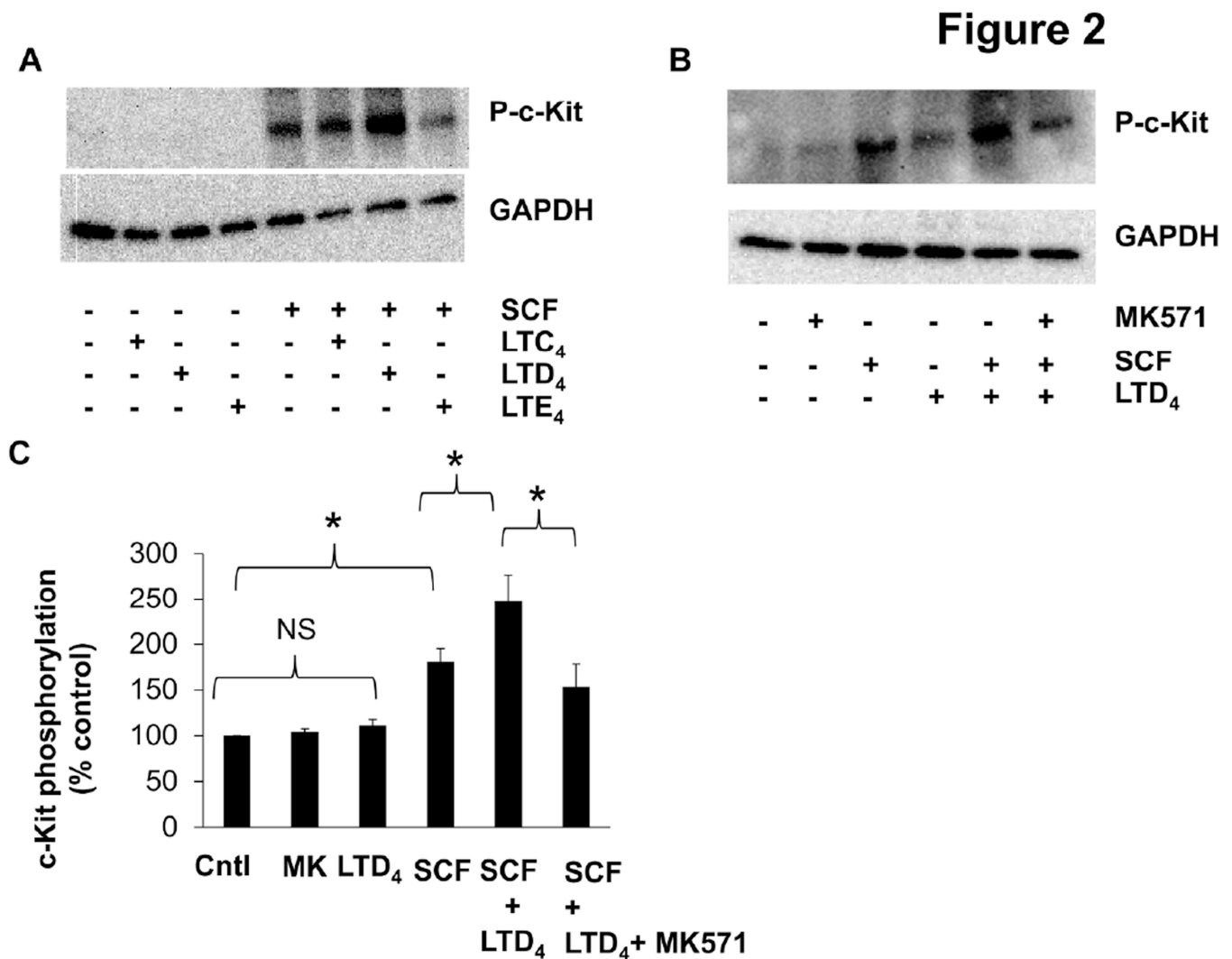


Figure 2. LTD₄ and SCF synergistically phosphorylate c-Kit

c-Kit phosphorylation was analyzed by western blotting in LAD2 cell lysates (A) stimulated with 500 nM of LTC₄, LTD₄ and LTE₄ in presence or absence of 100ng/ml SCF for 15 minutes. (B) Phospho-c-Kit levels upon stimulation with 500 nM LTD₄ and/or of 100 ng/ml SCF for 15 minutes with/without MK571 (1μM) pre-treatment (30 min). (C) Densitometric analysis of phospho-c-Kit levels upon SCF and /or LTD₄ stimulation in the presence or absence of MK571 (1μM). The data represents mean ± SEM of three separate experiments. The significance was tested using one way ANOVA and post-hoc analysis *P<0.05.

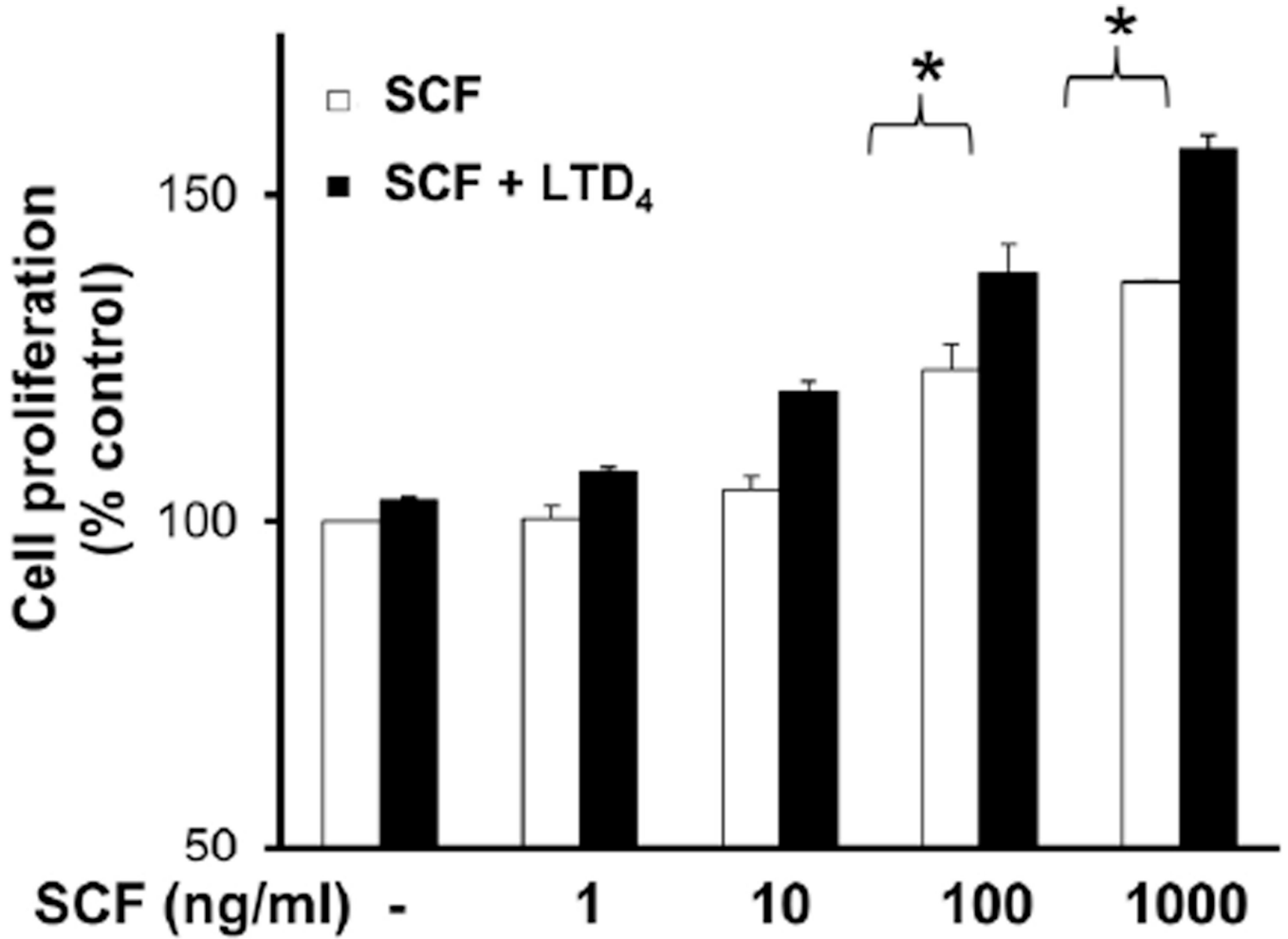


Figure 3. LTD₄ enhances SCF-mediated cell proliferation

Proliferation of LAD2 cells stimulated with 500 nM LTD₄ and/or the indicated dose of SCF was measured by XTT assay. Changes in cell proliferation were expressed as percentage of control. The data represents mean \pm SEM of three separate experiments. Data was analyzed with one way ANOVA and post-hoc analysis. *P<0.05.

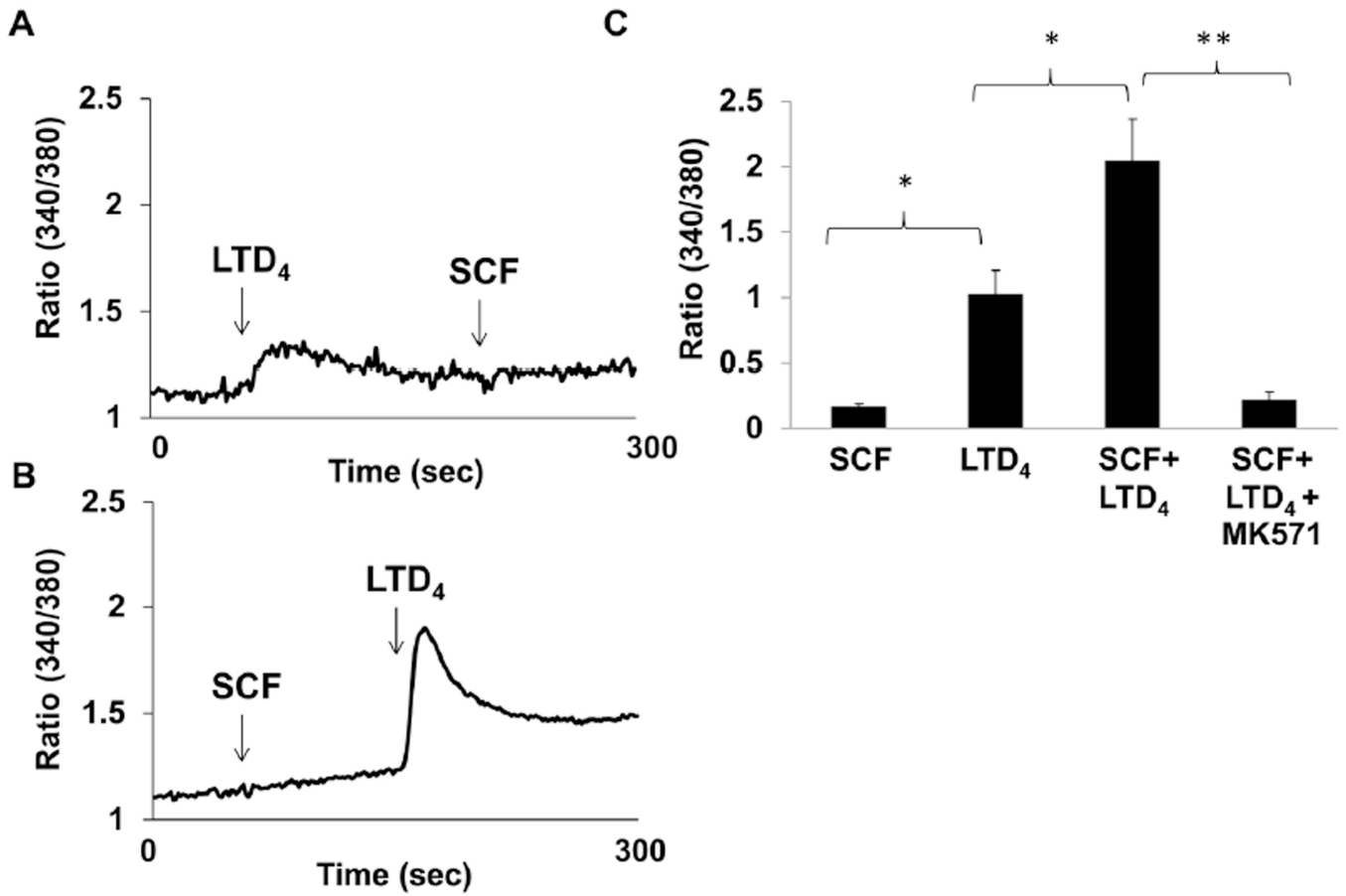


Figure 4. SCF primes LTD₄-induced calcium flux

(A, B) LAD2 cells were loaded with Fura-2AM and stimulated with 500 nM LTD₄ and 100ng/ml SCF at indicated times (arrows) and changes in intracellular calcium concentration were measured. (C) Quantitative analysis of the three experiments performed. The data represents mean \pm SEM of three separate experiments. Data was analyzed with one way ANOVA and post-hoc analysis. * $P < 0.05$, ** $P < 0.001$.

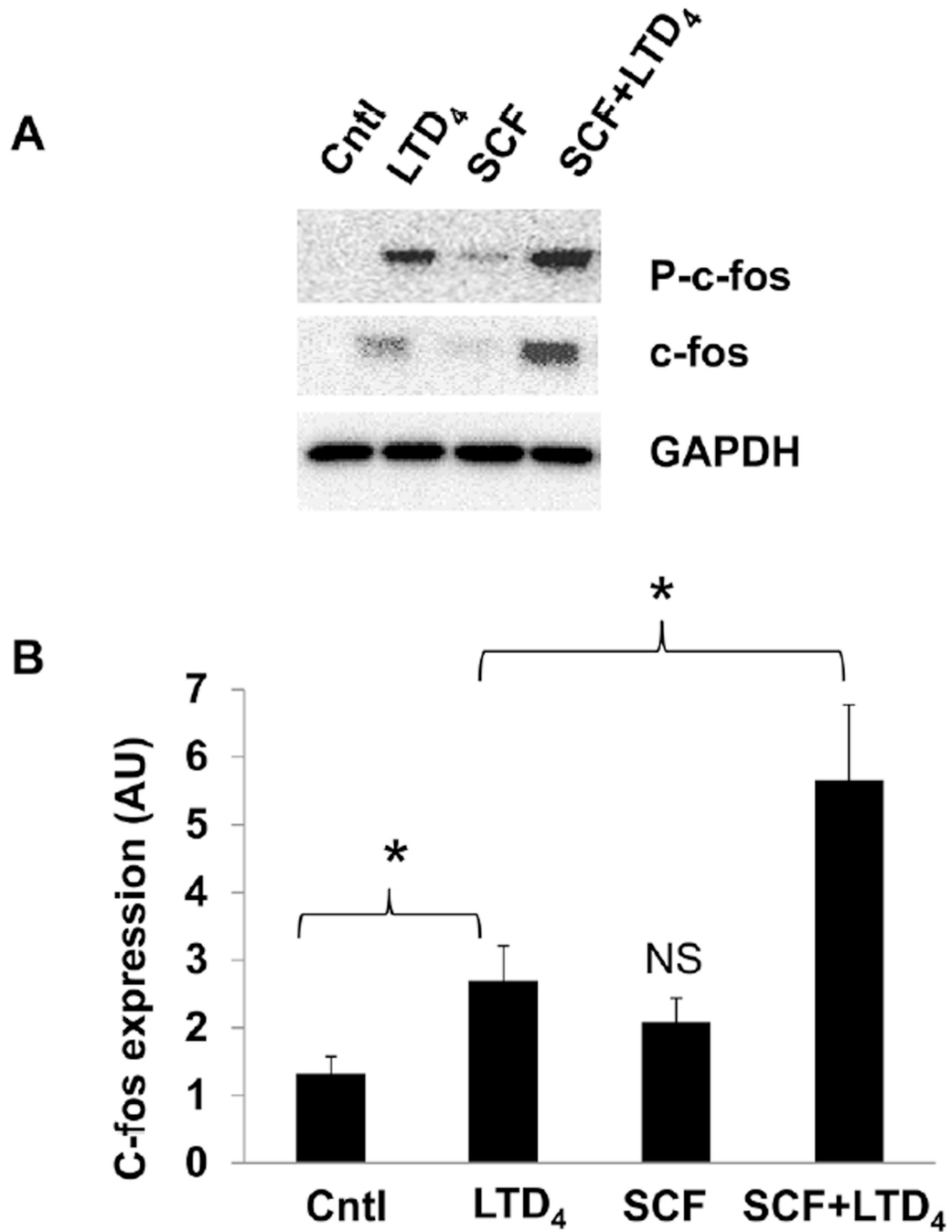


Figure 5. SCF potentiates LTD₄-induced c-fos phosphorylation and expression

LAD2 cells were treated with 500 nM LTD₄ and/or 100 ng/ml of SCF for 1 h and the phospho- and total c-fos levels were evaluated by western blotting. (B) Densitometric analysis of c-fos expression shown in (A). The data represents mean \pm SEM of three separate experiments. Data was analyzed with one way ANOVA and post-hoc analysis. $P < 0.05$.

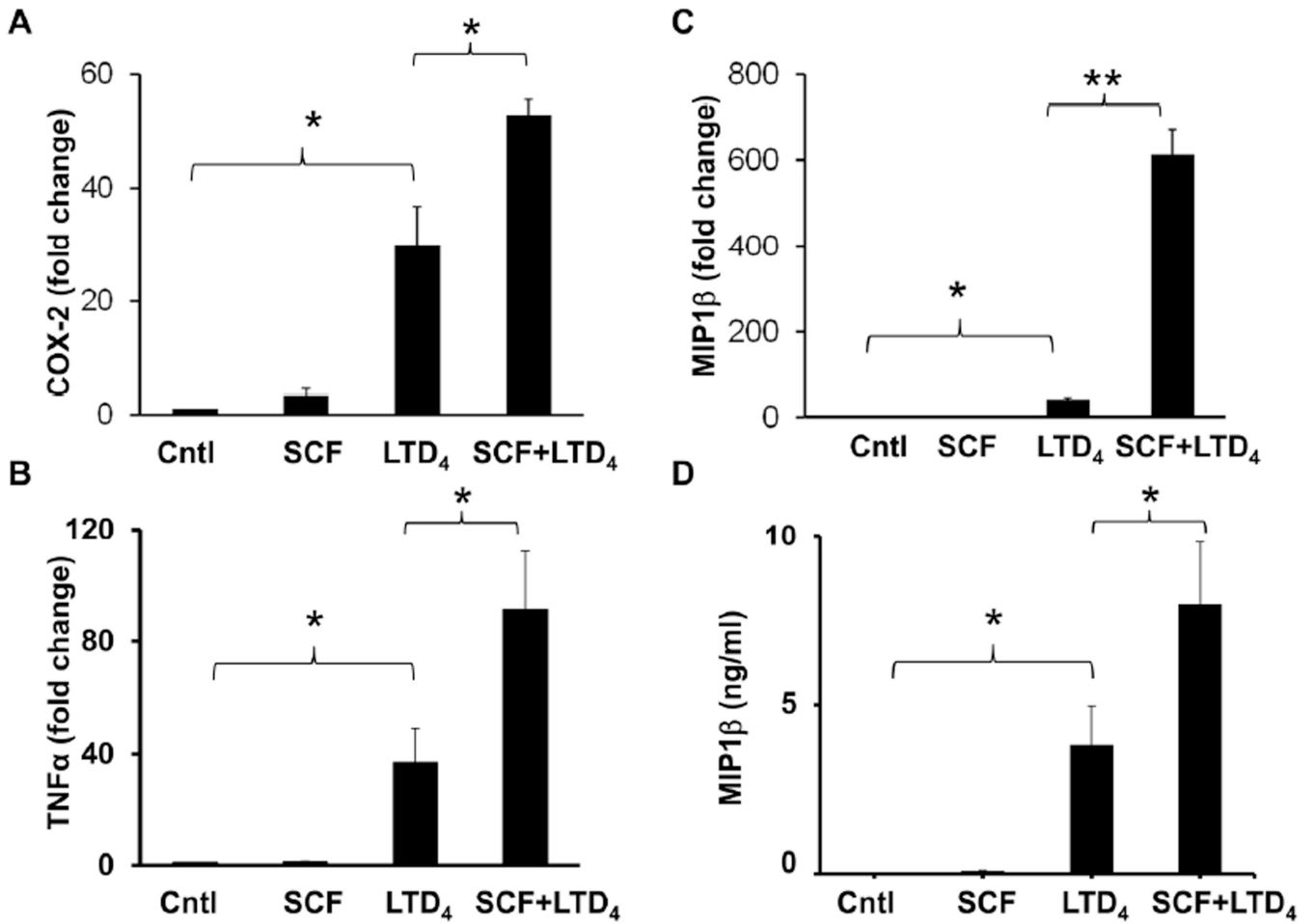


Figure 6. SCF augments LTD₄-induced inflammatory gene repertoire

LAD2 cells were treated with 500 nM LTD₄ and/or 100ng/ml of SCF for 2 h, followed by mRNA extraction and cDNA synthesis. Transcript levels of COX-2 (A), TNFα (B), and MIP1β (C) were analyzed in these cDNAs using respective real time primers and were analyzed compared to GAPDH. The graph represents fold change in the level of transcripts compared to controls from three separate experiments. (D) LAD2 cells were treated with 500 nM LTD₄ in the presence or absence of 100ng/ml SCF for 6h. Culture medium was collected and analyzed for secreted MIP1β protein by ELISA. The data shown represents mean ± SEM of three separate experiments. Data was analyzed with one way ANOVA and post-hoc analysis. *P<0.05, **P<0.001.

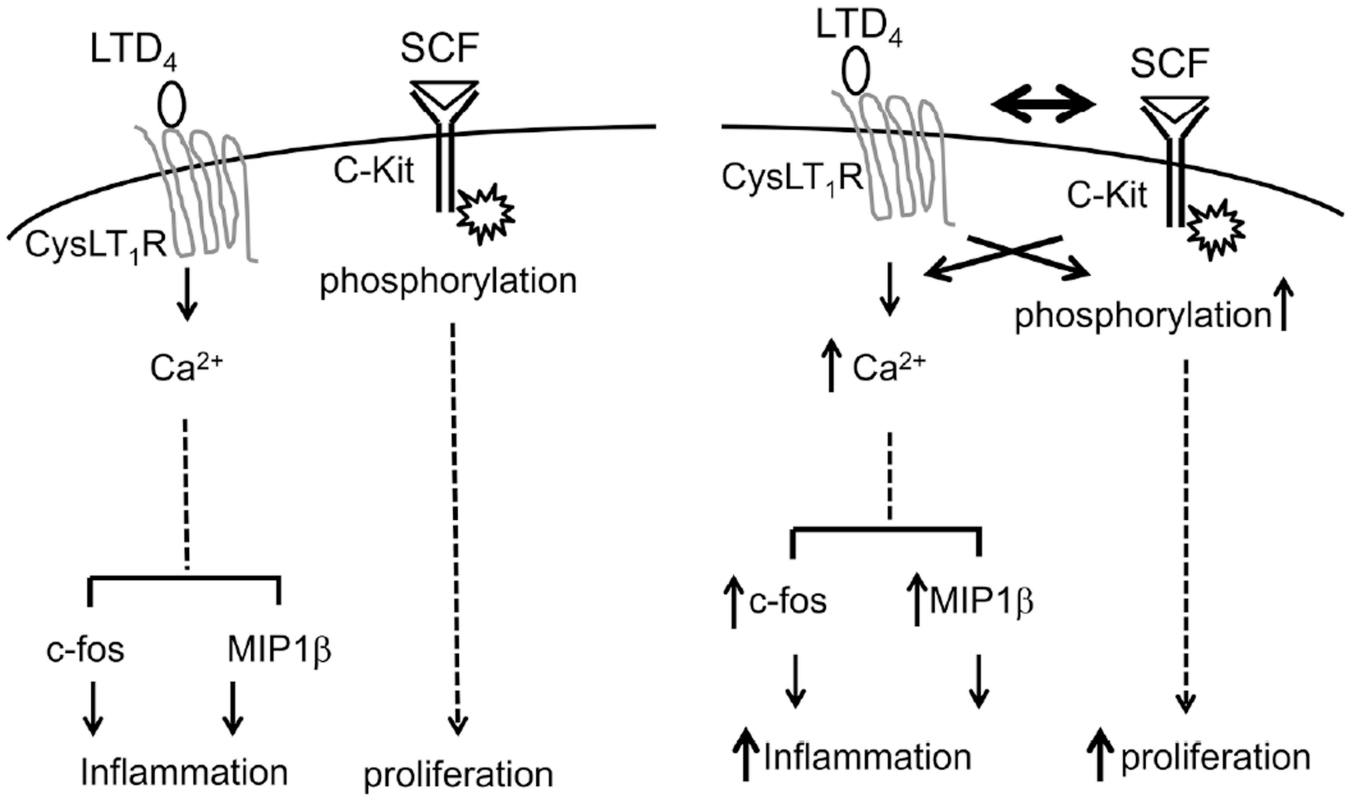


Figure 7. Schematic showing the possible cross-talk between LTD₄ and SCF in mast cells LTD₄ alone induces calcium influx via CysLT₁R leading to activation of c-fos and generation of inflammatory chemokine, MIP1β in MCs, while SCF alone enhances their proliferation through the activation of C-kit. However, simultaneous stimulation of MCs with both LTD₄ and SCF synergistically enhance each other's responses via cross-talk between associated signaling leading to augmented inflammatory and proliferatory responses.