Role of PTP-alpha in Integrated c-Kit and Fc-epsilon R1 Mast Cell Activation

by

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Abstract

Mast cells (MCs) play a crucial role in the induction of allergic asthma by secreting inflammatory mediators in response to allergens. In addition to MC activation via the antigen/IgE receptor FcεR1, MC tissue recruitment and responsiveness is greatly enhanced by co-stimulation with the stem cell factor (SCF). Levels of SCF are elevated in the asthmatic lung, where it stimulates the c-Kit receptor on MCs. Sufficient signaling through c-Kit and FcεR1 requires the activation of Src family kinases, which can be regulated by protein tyrosine phosphatase alpha (PTPα). Our lab has previously demonstrated that PTPα exerts positive regulatory effects on SCF-stimulated c-Kit phosphorylation and MC migration. In contrast, PTPα negatively regulates antigen-induced mast cell activation and the release of inflammatory mediators. To determine the role of PTPα in the integrated c-Kit and FcεR1 signaling that is believed to facilitate allergic inflammation, mouse bone marrow-derived WT and PTPα-KO MCs were treated with combinations of antigen and SCF, and analyzed for secretory and migratory responses as well as for the activation of key signaling proteins. However, the expected MC hyper-responsiveness due to the lack of PTPα was not observed, which may have arisen from intrinsic changes in the cultured mast cells and not from testing methodologies. Co-treatment with antigen and SCF produced synergistic degranulation and cytokine release that was similar between WT and PTPα-KO MCs. Yet, PTPα was required for the full phosphorylation of Akt and p38 after 15 min co-treatment with antigen and SCF. PTPα itself was found to be dephosphorylated at tyrosine 789, especially upon treatment with antigen. During fibronectin-aided Transwell migration towards SCF,

the addition of antigen significantly reduced the number of PTPα-KO but not WT MCs that remained attached to fibronectin. Interestingly, in the presence of fibronectin, the SCF-mediated migration of WT and PTPα-KO MCs was not significantly affected by the addition of antigen, whereas fibronectin-independent MC migration was synergistically enhanced by antigen and SCF. Taken together, despite effects on the FcεRI/c-Kit integrated activation of downstream signaling proteins, the overall SCF-enhanced mediator release and migration of mast cells were found to be independent of PTPα.

Preface

I carried out all the experiments described in this thesis, including data collection and analysis. The colony of mice used were maintained by Dr. Jing Wang, following the guidelines of the Canadian Council on Animal Care. All animal procedures were approved by the University of British Columbia Animal Care Committee (PTP Alpha Mouse Study, certificate number A09-0447-R002).

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abl	Abelson murine leukemia
ACP1	acid phosphatase 1
ADAM	A disintegrin and metalloprotease
Ag	antigen, allergen
AHR	airway hyper-responsiveness
ATP	adenosine-5'-triphosphate
BMMC	bone marrow-derived mast cell
BSA	bovine serum albumin
Btk	Bruton’s tyrosine kinase
CD	cluster of differentiation
CDC	cell division cycle
cDNA	complementary deoxyribonucleic acid
CRAC	calcium-release activated calcium
Csk	C-terminal Src kinase
DNP	2,4-dinitrophenyl
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Erk	extracellular signal regulated kinase
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FcεR1	high-affinity IgE receptor
FITC	fluorescein isothiocyanate
FN	fibronectin

FRET	fluorescence resonance energy transfer
Gab	Grb2-associated-binding protein
GATA	trans-acting T cell-specific transcription factor
GPI	glycosyl-phosphatidylinositol
Grb	growth factor receptor-bound protein
HDM	house dust mite
HP	hypersensitivity pneumonitis
HRP	horseradish peroxidase
HSA	human serum albumin
IgE	immunoglobulin E
IGF	insulin-like growth factor
IL	interleukin
IP_3	inositol triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
Jnk	c-Jun N-terminal kinases
K_v	voltage-gated potassium channel
LAT	linker for activation of T cells
LMPTP	low molecular weight protein tyrosine phosphatase
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MKP	mitogen-activated protein kinase phosphatase
mTOR	mammalian target of rapamycin
NMDA	N-methyl-D-aspartate
NTAL	non-T cell activation linker
PAMP	pathogen-associated molecular pattern

PBS	phosphate buffered saline
PE	phycoerythrin
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T) motif
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PKC	protein kinase C
PLCγ	phosphoinositide phospholipase C-gamma
PMSF	phenylmethanesulfonyl fluoride
PRL	phosphatase of regenerating liver
PTEN	phosphatase and tensin homolog
PTP	protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src family kinase
SH	Src homology domain
SHIP	SH2 domain-containing inositol 5’ phosphatase
SHP	Src homology region 2 domain-containing phosphatase
SOS	son of sevenless
STAT	signal transducer and activator of transcription
Th	T-helper type
TNFα	tumor necrosis factor-alpha
TSLP	thymic stromal lymphopoietin

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The love and inspiration of my close family in Canada has given me the strength to succeed in university and obtain higher education.

Chapter 1: Introduction

1.1 Mast Cell Biology

Mast cells specialize in the secretion of potent inflammatory mediators and facilitation of Type 2 T-helper (Th2) immunity during infection, allergies and many other immune disorders. Human mast cells originate in the bone marrow from $CD34^{+}$/c-Kit^{+} progenitors that pass through the circulatory system and home to specific tissue sites to complete their maturation (Kirshenbaum et al., 1999). Unlike the other major FcεR1-expressing (the high-affinity IgE receptor) granulocyte, the basophil, mast cells maintain expression of c-Kit receptor throughout their life, and do not stay in circulation for long (Brown et al., 2008; Karasuyama et al., 2011; Sokol et al., 2009a). Human tissue resident mast cells require stimulation by the c-Kit ligand, SCF, to survive for several months. In healthy individuals, mast cells are mainly found near mucosal and epithelial barriers with the environment where they serve as early detectors of invading pathogens. They are highly conserved through the evolution of organisms and are vital for survival since no human deficient in mast cells has been found (McNeil et al., 2007).

Mast cell development differs between tissue types in order to produce more appropriate responses. As a result, many features of mast cells exhibit considerable heterogeneity such as granule contents, receptor expression and responsiveness (Bradding 2009; Kitamura 1989). Different subtypes of mast cells have been characterized in humans and animals based on their expression of granule

proteases and tissue distribution. Human tryptase-containing mast cells are best exemplified by mucosal mast cells found in rodent lungs and intestines, whereas the chymase and tryptase subtype in humans corresponds to rodent mast cells in connective tissues such as the skin (Ekoff et al., 2007). Both subtypes also exhibit plasticity upon interactions with endothelial or epithelial cell types (Gilfillan et al., 2011). Mast cells can also fine-tune their responses towards specific pathogen types, as well as provide both pro- and anti-inflammatory immune regulation (Abraham and St. John 2010; Lu et al., 2006; Nechushtan, 2010). Due to their localization at common pathogen entry sites, mast cells are some of the first immune cells to detect bacteria, viruses, and parasites. Recently it was identified that binding of IgE antibodies to FcεR1 promotes mast cell survival in the absence of growth factors and antigen (Ekoff et al., 2007; Kashiwakura et al., 2011; Kawakami and Kitaura, 2005; Kohno et al., 2005; McNeil et al., 2007; Sly et al., 2008). Thus, the induction of allergic sensitivity that produces IgE can increase the number of Th2 effector cells as well as their sensitivity to allergens.

1.1.2 Mast Cell Effector Functions

In addition to a major role in allergic diseases (detailed in the following sections), mast cells also perform a crucial task in the body's defence against various pathogens via secretion of inflammatory mediators during early infection (Gilfillan et al., 2011; Moiseeva and Bradding, 2011). Their ability to rapidly home into barrier tissues such as the skin and lung epithelium allows them to direct specific immune responses by recruiting and activating other leukocytes. In addition to Toll-like and

other innate immune receptors, mast cells express the antigen receptor complex FcεR1. Stimulation of these receptors by microbial products, allergens, host cytokines and complement proteins contributes to the recruitment and activation of mast cells and the subsequent secretion of inflammatory molecules.

Antigen stimulation induces multiple phases of mediator release. Degranulation is the earliest event, in which bioactive compounds stored in cytoplasmic granules are released within minutes of antigen exposure. Some of the products of mast cell granule exocytosis include bioactive amines (histamine, serotonin), serine proteases (chymase and tryptase), proteoglycans (heparine, chondroitin sulfate), growth factors and enzymes (Broide et al., 2011; Brown et al., 2008). A number of positively charged proteases remain attached to negative proteoglycans, slowing their dispersal. Certain mast cell proteases help combat infections, while others promote tissue remodeling during allergic inflammation. Tryptase enzymes inhibit blood clotting and promote mucous secretion in the airways, aid in microbial elimination, and disable toxic molecules (McNeil et al., 2007). Mast cell chymases can activate matrix metalloproteases that promote tissue repair, and also recruit other leukocytes (Caughey, 2011). Histamine rapidly solubilizes from granules and induces allergic symptoms such as swelling and mucous accumulation. Following early degranulation, lipid-derived prostaglandins and leukotrienes are also released, increasing endothelial permeability and pain sensation (Abraham and St. John, 2010). Platelet activating factor produced by mast cells is an important mediator of systemic anaphylaxis (Arias et al., 2009).

Minutes to hours after the activation of mast cells by antigen, newly synthesized inflammatory cytokines and interleukins are secreted, including TNFα, IL-4, IL-5, IL-6, IL-13 and others. These cytokines mediate a variety of secondary responses, such as the recruitment of leukocytes, further induction of immune mediators, and activation of connective tissue cells. Tumor necrosis factor (TNFα) is a central pro-inflammatory mediator that promotes fever, immune cell adhesion, lymph node hypertrophy and other antimicrobial responses during infection and chronic inflammation (Babu et al., 2011; Laichalk et al., 1996; McLachlan et al., 2003). Inhibition of TNFα is being clinically tested as therapy against various severe inflammatory disorders (Tracey et al., 2008). The mast cell cytokine IL-13 has been shown to promote mucous secretion which helps eliminate parasites, but also contributes to worsening allergic reactions (Scales et al., 2007; Wang et al., 2010).

More recently, mast cells have also been implicated in the progression of various human cancers (Groot Kormelink et al., 2009; Maltby et al., 2009; Nechushtan, 2010). In mouse models of adenomatous polyposis, mast cells are recruited earlier than other leukocytes and promote colon tumor expansion (Gounaris et al., 2007). Additionally, polyp-associated mast cells can reprogram anti-inflammatory regulatory T cells into IL-17-producing pro-inflammatory cells, which lose the ability to suppress tumor growth (Colombo and Piconese, 2009). In other malignancies mast cells are recruited by various growth factors (including SCF) to promote tumor metastasis by secreting angiogenic factors and inducing tissue remodelling (Crivellato et al., 2008).

However, not all functions of mast cells facilitate cancer progression. In some forms of breast cancer and various stages of other malignancies, higher mast cell numbers can be associated with better patient outcomes (Galinsky and Nechushtan, 2008). Evidence from animal models suggests that IgE-mediated adaptive Th2 immunity can help in the elimination of cancer cells. IgE antibodies against tumor antigens can initiate lysis of tumor cells by Th2 effector cells (Jensen-Jarolim et al., 2008). In human epidemiological studies high IgE (atopy) is associated with a reduced risk of cancer mortality, while eosinophils from atopic patients also exhibit more tumoricidal functions. Taken together, an improved understanding of mast cell regulation may open new venues in the treatments of both cancer and allergic disorders. Figure 1.1 illustrates some of the key mediators of allergic Th2 immunity and mast cell responses.

1.2 Allergic Disorders

Allergic diseases are characterized by harmful inflammatory responses to normally innocuous molecules by immune mechanisms that evolved to protect against invasive pathogens. Allergic asthma affects hundreds of millions of people worldwide, placing additional financial burdens on healthcare. The prevalence of allergic disorders including asthma, food allergies and anaphylaxis has grown over the past decades, particularly among children (Finkelman 2010; Moneret-Vautrin et al., 2005; Umetsu et al., 2002). While each type of allergy may be represented by a spectrum of disorders, affected by genetic and environmental factors, they commonly involve the dysregulated activation of Th2 cell responses and symptoms caused by innate immune-effector cells (Brown et al., 2008; Moiseeva and Bradding, 2011).

Asthma symptoms are caused by chronic inflammation of the conducting airways, characterized by difficult breathing due to airway constriction and excessive mucous secretion. Seasonal plant pollen, animal dander, house dust mites, and mould allergens commonly trigger such airway hyper-responsiveness (AHR), although smoke, pollution, and exercise can also induce similar symptoms in some non-allergic individuals (Kim et al., 2010a; Zhang and Kohl, 2010). Epidemiological studies have shown that chronically elevated levels of serum IgE antibodies, broadly described as atopy, is strongly associated with the risk of developing asthma and other allergic disorders (Kim et al., 2010a). IgE molecules bind to the high affinity FcεR1 receptors of granulocytes, particularly mast cells and basophils, stimulating

them to secrete potent pro-inflammatory mediators and cytokines upon allergen (antigen, Ag) exposure. In severe reactions these Th2 immune mediators, together with activated complement proteins, may produce anaphylactic shock or even death in susceptible individuals (Matasar and Neugut, 2003; Zhang and Kohl, 2010).

The developed animal models of allergen-induced AHR have greatly improved the understanding of the cells and bioactive molecules involved in the pathophysiology of allergic asthma. According to prevailing theories, initial inhalation of an allergen induces its uptake and processing by resident lung antigen-presenting cells, such as dendritic cells (Banchereau and Steinman, 1998; Hammad et al., 2009). Antigen-loaded dendritic cells migrate to the lympth nodes, where they present the processed allergen epitopes on MHC class II molecules to naïve T cells. Other foreign molecules, such as bacterial lipopolysaccharides, can trigger the pathogen-associated molecular pattern receptors (PAMPs) on dendritic, and epithelial cells. This additional signal enhances the maturation and migration of dendritic cells, and stimulates the secretion of cytokines that determine the differentiation pathway towards specific T helper cell subtypes (Hammad et al., 2009; Sokol et al., 2008; Sokol et al., 2009a). The development of allergen-specific Th2 cells requires their stimulation with interleukin-4 (IL-4) cytokine, which can initially be provided by basophils and other cell types (Khodoun et al., 2004). These responses are crucial for the effective immunological elimination of helminth parasites *in vivo* (Pennock and Grencis, 2004; Scales et al., 2007; Yamashita et al., 1999).

The mature Th2 cells home to specific tissue sites producing large quantities of cytokines such as IL-4, IL-13, IL-5, IL-9, IL-25, which drive the inflammatory activation of effector cells. Under the influence of IL-4, B cells undergo immunoglobulin class switching to start producing IgE antibodies and memory cells. IgE molecules enhance the expression of FcεR1 receptors on mast cells and basophils, and mediate degranulation and cytokine production by these cells upon subsequent antigen exposure, causing the various allergic symptoms (Kashiwakura et al., 2011; Oka et al., 2004) (Figure 1.1). However, Th1 and $CD8^+$ cell activity has also been shown to worsen lung inflammation in severe chronic asthma as well as during viral infections (Hamzaoui et al., 2005; Meyts et al., 2006) The specific roles and functions of these immune regulators will be described below in more detail.

1.2.1 Lung Epithelium

The epithelial lining of the lungs serves as a barrier against pathogens and irritants in the air. However, in most asthmatics the epithelium and the underlying structures sustain damage from chronic allergic inflammation, which is partly due to inherent defects in the maintenance of its barrier function (Holgate, 2008). Lung epithelial cells from asthmatics allow more allergens and pollutants, such as smoke particles, to penetrate deeper into the lungs to cause damage and immune activation, than in healthy individuals. There is strong evidence that air pollution worsens asthma symptoms, and combined with the insufficient antioxidant defenses in susceptible people, may contribute to the initiation of chronic lung diseases (Nadeau et al., 2010; Rahman et al., 2006). The majority of children who develop non-atopic wheezing

from early-life allergen exposure will eventually regain healthy lung function, whereas children with atopy are much more likely to maintain airway hyper-responsiveness and develop asthma in adulthood (Illi et al., 2006). It is postulated that a combination of genetic and environmental risk factors contribute to the vulnerability of lung epithelial cells. These susceptible individuals acquire more tissue damage from early-life viral infections and pollution, which cannot be effectively repaired, resulting in a chronic lung tissue damage and inflammation (Holgate, 2008).

Furthermore, asthma patients are less resistant to many common respiratory viruses, caused directly by insufficient antiviral interferon responses and apoptosis of infected lung epithelial cells (Wark et al., 2005). Thus infection-induced damage may enhance sensitivity towards allergens and the activation of Th2 immunity. Lung epithelial cells respond to a variety of foreign molecules via innate receptors, such as Toll-like receptors, by releasing cytokines and immune cell chemoattractants (Hammad et al., 2009; Liu, 2006). These mediators will promote the development of Th2 cells and subsequent cytokine cascades to attract and activate immune effector cells. Elevated levels of stem cell factor (SCF) and IgE recruit mast cells, and prime them to release inflammatory mediators that contribute to chronic lung tissue remodelling and inflammation (Da Silva et al., 2006; Kashiwakura et al., 2011; Okayama and Kawakami, 2006). Macrophages, eosinophils, basophils, and neutrophils have also been shown to accumulate in asthmatic lungs, though the

individual contribution of each cell type towards AHR and tissue damage is still debated (Karasuyama et al., 2011; Kim et al., 2010b).

The remodeling of connective tissue is a hallmark of chronic lung inflammation, and often involves epithelial damage, fibrosis of subepithelial layers, hyperplasia of smooth muscle and goblet cells, excess mucous, and angiogenesis (Sumi and Hamid, 2007). These histological abnormalities strongly correlate with asthma severity at all ages, and may be the body's mechanism of repairing and preventing further damage to susceptible lungs. Production of epidermal growth factor facilitates repair of the lung epithelial layer, but in severe asthma it also stimulates neutrophil recruitment and inflammation (Hamilton et al., 2003). Increased numbers of mast cells accumulate in the lung with asthma progression, where they release IL-4 and other cytokines, stimulating the buildup of extracellular matrix proteins such as collagen (Plante et al., 2006). *ADAM33* (A disintegrin and metalloprotease 33) is one of the major susceptibility genes for the development of asthma, and it encodes a proteolytic enzyme expressed by subepithelial connective tissue cells. This provides more evidence that aberrant homeostasis of lung epithelial structures plays a crucial role in asthma pathogenesis.

1.2.2 Antigen Presentation and T Cells

The differentiation of Th2 cells, which mediate antiparasitic and allergic responses, requires the expression of GATA-binding protein 3 (GATA3) in antigen-presented $CD4^+$ T cells. IL-4 cytokine stimulation is sufficient to stimulate STAT6 signaling to

upregulate GATA3 in naïve T cells (Zhu et al., 2001). Activated lung dendritic cells express high levels of MHCII and the co-stimulatory molecules CD80/CD86, making them strong inducers of T cell receptor signaling during antigen presentation. However, dendritic cells alone are incapable of producing IL-4, therefore it must initially come from other types of cell (Karasuyama et al., 2011). Recently it has been postulated that basophils are important producers of IL-4 in response to allergens (Tang et al., 2010). Several animal models have demonstrated that basophils are sufficient for antigen processing, migration to lymph nodes and induction of Th2 cell differentiation in the absence of dendritic cells (Sokol et al., 2009b). Nonetheless, it remains to be seen whether human basophils are essential for sensitization to common allergens and disease development. Moreover, basophils induce weaker T cell receptor signaling than dendritic cells, which actually favours Th2 pathway differentiation (Constant et al., 1995). Interestingly, in more advanced asthma, T-helper type 1 cells (Th1) may also contribute to the severity of lung tissue damage and remodelling. The cytokine interleukin-12 is produced by dendritic cells to promote Th1 cell development, while inhibiting the Th2 pathway during initial antigen sensitization. In later phases of allergic asthma, Th1 effector cells are also recruited to the lungs, where they secrete more proinflammatory mediators (Meyts et al., 2006). Taken together, the presentation of common human allergens and the resulting allergic responses involve a complex interplay of innate and adaptive immune cells, which depending on the type of allergen, may contribute to disease progression.

1.2.3 Anaphylaxis

Anaphylaxis is a rapid, severe allergic reaction, which may result in shock or death. Close to 1% of people experience anaphylactic reactions in their lifetime, with increasing prevalence in children and young adults (Lin et al., 2008; Matasar and Neugut, 2003; Moneret-Vautrin et al., 2005). Similarly to allergic asthma, anaphylaxis is most commonly caused by allergen-induced systemic activation of highly IgE-sensitized mast cells and other FcεR1 expressing Th2 effector cells. This triggers a rapid release of inflammatory cytokines and vasoactive mediators, which contribute to swelling, reduced blood pressure and difficult breathing (Sampson et al., 2006). Anaphylactic sensitization involves similar mechanisms of Th2 cell development and antigen-specific IgE antibody production as in other allergic disorders. In susceptible individuals high levels of serum IgE increase the risk of anaphylaxis by enhancing the number and antigen sensitivity of mast cells (Kashiwakura et al., 2011; Kawakami and Kitaura, 2005). Of all food allergens in the United States, peanut products cause the most cases of severe anaphylaxis (Finkelman, 2010; Sicherer and Sampson, 2010). Recent evidence indicates that peanut antigen stimulation of mast cells via IgE/FcεR1 signaling induces IL-13 cytokine production and allergic inflammation (Wang et al., 2010). Anaphylaxis can also be triggered by allergies to drugs, immune therapy, or by direct stimulation of mast cells and basophils by temperature changes, toxins, or radiation (Simons, 2010). Hyper-proliferation disorders, such as mastocytosis, can also induce

anaphylactic reactions due to abnormally elevated numbers of immune effector cells (Jensen et al., 2008a; Orfao et al., 2007).

1.2.4 Cytokines and Complement

Various immune cytokines that normally play protective roles against pathogens have also been implicated in the development of IgE sensitization and facilitation of allergic responses. This section describes some new key molecular mediators of allergic disorders, while the following sections will focus on mast cell functions. Thymic stromal lymphopoietin (TSLP) is produced by human epithelial cells in response to microbial components, inflammation, and injury (Allakhverdi et al., 2007). TSLP facilitates the production of Th2 cytokines from various leukocytes, and is up-regulated in the lungs of asthmatics with disease progression (Ying et al., 2005). In addition to activating granulocytes such as mast cells, TSLP is also produced by them, and can serve as a feedback loop for chronic inflammation (Liu, 2006; Miyata et al., 2008). Both IL-25 and IL-33 cytokines are produced by a variety of cells in response to allergen sensitization. They mainly function to amplify Th2 cell activation and granulocyte responses, which lead to chronic airway hyper-responsiveness (Pecaric-Petkovic et al., 2009; Wang et al., 2007). Additionally, IL-33 and other Th2 cytokines enhance the development of alternatively activated macrophages, which enhance allergic AHR as well (Kurowska-Stolarska et al., 2009).

Complement proteins are an integral part of the innate immune system. Upon recognition of pathogen-associated molecular patterns (PAMPs), they activate a variety of antimicrobial pathways (Finkelman, 2010). Common allergens induce the proteolytic cascade of complement proteins, producing C3a and C5a anaphylatoxins in the lungs of asthmatics (Krug et al., 2001). Numerous genetic and animal studies have confirmed that complement proteins are crucial components of allergic disorders (Humbles et al., 2000). The recruitment and induction of inflammatory mediators by mast cells is also regulated by the complement proteins, which facilitates the pathogenesis of allergic asthma and anaphylaxis (Hogaboam et al., 1998; Metcalfe et al., 2009; Nilsson et al., 1996).

1.3 Mast Cell Activation

1.3.1 FcεR1 Signaling

Mast cells and basophils are the main Th2/IgE effector cells, and express the high affinity IgE receptor, FcεR1. This membrane receptor consists of four subunits, IgE-bound α, tetramembrane-spanning β and two disulfide-linked γ subunits (Kraft and Kinet, 2007). The β subunit is not essential, and facilitates downstream signaling via the γ-subunit. Crosslinking of FcεR1/IgE receptors by polyvalent antigens/allergens rapidly activates mast cell signaling cascades to trigger degranulation, cytokine release, and cell migration that drive allergic responses. Additionally, in the absence of antigens, IgE molecules remain strongly bound to FcεR1 and promote mast cell survival, differentiation, and activation (Kashiwakura et al., 2011; Kawakami and Kitaura, 2005; Oka et al., 2004; Sly et al., 2008). Initial antigen-induced aggregation of FcεR1 activates the receptor-proximal Src family kinase (SFK) Lyn by the protein tyrosine phosphatase, CD45 (Grochowy et al., 2009). There is evidence that formation of receptor complexes occurs within detergent-resistant glycosphingolipid-rich membrane domains, also known as lipid rafts (Field et al., 1997). However, the exact functions and temporal protein associations of lipid rafts remain to be further elucidated. In mast cells, activated Lyn phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the FcεR1 β and γ subunits to recruit the cytoplasmic tyrosine kinase Syk. Activated Lyn and Syk phosphorylate the membrane scaffolding proteins LAT and NTAL (LAT2). These scaffolds recruit other adaptor proteins to activate the PI3K, PLCγ and Ras/MAPK downstream signaling cascades, which regulate calcium signaling, gene transcription and cytoskeletal

rearrangements required for various mast cell responses (Figure 1.2). These pathways will be described in more detail in the following sections. Other Src family kinases, Fyn and Hck, also contribute to the activation of FcεR1 signaling in mast cells (Hernandez-Hansen et al., 2004; Hong et al., 2007; Parravicini et al., 2002; Sanchez-Miranda et al., 2010). Lyn also activates the negative regulator of the PI3K pathway SHIP (SH2 domain containing inositol-5-phosphatase) to inhibit various mast cell inflammatory responses (Hernandez-Hansen et al., 2004). The intensity of antigen stimulation appears to dictate the effects of Lyn kinase on mast cell activation. Weak (low-dose) antigen stimuli prevent Lyn/FcεR1 association and activate downstream signaling cascades required for degranulation. In contrast, high (supraoptimal) antigen doses promote Lyn-mediated activation of Csk (C-terminal Src kinase) and SHIP to inhibit mast cell responses (Hernandez-Hansen et al., 2004; Odom et al., 2004).

Calcium ion levels within lymphocytes play a crucial role in mediating various immune responses to specific stimuli. Different cytokines and immune receptors activate PLCγ signaling to induce an increase in intracellular calcium that triggers gene transcription, mediator release and migration of mast cells (Feske, 2007). Antigen and IgE-mediated cross-linking of the FcεR1 receptor complex rapidly activates the tyrosine kinases Lyn and Syk, which phosphorylate the membrane scaffolding protein, LAT. The recruited adaptor proteins Gads and SLP-76 allow Vav to activate LAT-bound phospholipase C gamma (PLCγ). In mast cells two isoforms of PLCγ hydrolyze lipid-bound PIP_2 into inositol triphosphate (IP_3) and diacylglycerol.

The IP_3 receptors on the endoplasmic reticulum open the flow of calcium ions into the cytosol, which also opens plasma membrane calcium channels (CRAC) to increase intracellular calcium and facilitate the exocytosis of mast cell granules (Kalesnikoff and Galli, 2008; Wen et al., 2002). Diacylglycerol activates protein kinase C (PKC) and the Ras/MAPK pathways, and also contributes to mast cell degranulation and mediator production.

1.3.2 C-Kit Signaling

The c-Kit receptor and its ligand, stem cell factor (SCF), mediate vital functions in the development of various cell types. The transmembrane receptor tyrosine kinase c-Kit (CD117) is expressed in hematopoietic progenitors, but also in mature intestinal and mast cells. SCF stimulation of c-Kit-expressing cells inhibits apoptosis, and promotes proliferation, migration and differentiation (Roskoski, 2005). Mutations that cause constitutive activation of c-Kit receptor signaling may give rise to a spectrum of hyper-proliferative and cancer disorders in humans. For example, the D816V c-Kit mutation in mast cells results in systemic mastocytosis and the associated risk of severe anaphylaxis (Jensen et al., 2008a; Orfao et al., 2007; Sundstrom et al., 2003). Gastrointestinal and other types of tumors are often caused by gain-of-function mutations of c-Kit as well (Bellone et al., 2001; Hirota et al., 1998). Additionally, tumors that secrete SCF can recruit mast cells, which then modify the tumor microenvironment to facilitate cancer progression (Huang et al., 2008). Expression of c-Kit and SCF proteins is also elevated in the lungs of asthmatics, where activation of c-Kit by SCF increases the number of mast cells and

their pro-inflammatory functions (Al-Muhsen et al., 2004; Da Silva et al., 2006). Various tyrosine kinase inhibitor drugs, such as Imatinib, have been used to down-regulate aberrant c-Kit signaling in cancer and mast cell-mediated inflammation (Attoub et al., 2002; Jensen et al., 2008b; Kajiguchi et al., 2008; Stahtea et al., 2007).

The c-Kit receptor comprises an extracellular SCF-binding domain, one transmembrane domain, and a split cytoplasmic tyrosine kinase domain (Roskoski, 2005). When the ligand SCF is bound by the extracellular immunoglobulin-like loops, c-Kit dimerizes and transphosphorylates its own juxtamembrane tyrosine 568/570 residues (Tyr-567/569 in mouse isoforms) (Blume-Jensen et al., 1991). This recruits the SFKs Lyn and Fyn to further phosphorylate activating tyrosine residues on c-Kit, which then serve as docking sites for Src homology 2 (SH2) domain-containing proteins. Activated c-Kit recruits and phosphorylates downstream signaling proteins that mediate various mast cell responses (Figure 1.3). Many of the components involved in FcεR1 signaling are also activated by the c-Kit receptor, including SFKs, PI3K, PLCγ and MAPKs (Orfao et al., 2007).

The PI3K pathway is ubiquitously expressed in most tissue types and controls crucial cell functions, while its dysregulation facilitates many common types of cancer (Liu et al., 2009). In mast cells PI3Ks promote growth factor-mediated survival throughout the life of the cells. SCF-mediated phosphorylation of c-Kit receptor tyrosine 721 (mouse Tyr-719) leads to the binding of class 1A PI3K p85

subunits directly and via adaptor proteins (Kim et al., 2008b). Activated receptor-proximal SFKs phosphorylate the p85 to activate its bound catalytic p110 subunit. Various heterodimers of PI3K enzyme are capable of phosphorylating phosphoinositide molecules in the plasma membrane to produce PIP_3 secondary messengers. Pleckstrin homology domain-containing signaling proteins, including PLCγ and Akt, are recruited and activated at the membrane. Akt, also known as protein kinase B, activates a multitude of downstream signaling cascades which promote mast cell degranulation, cytokine secretion, migration and proliferation (Ali et al., 2004).

C-Kit can directly phosphorylate LAT2 (NTAL) scaffolding protein, and also recruit Lyn and Syk tyrosine kinases to the receptor complex (Iwaki et al., 2005). The LAT2 adaptor protein recruits PI3Ks to activate the Akt and PLCγ pathways. Similarly to Ag/FcεR1 signaling, Fyn kinase phosphorylates the p110δ subunit of PI3K, which together with LAT2 play a crucial role in the enhancement of mast cell degranulation by SCF. The p110δ subunit is also required for antigen-triggered anaphylaxis in mice (Ali et al., 2004). The magnitude of degranulation and cytokine release is regulated via changes in calcium concentrations within mast cells. Initial FcεR1 receptor activation leads to PLCγ-mediated production of inositol 1,4,5-trisphosphate and diacylglycerol secondary messengers, which trigger the rapid release of calcium stores from the endoplasmic reticulum to induce mast cell degranulation. In the later phase of mast cell activation, the PI3K pathway activates Bruton's tyrosine kinase (Btk) which helps maintain the influx of external calcium (Iwaki et al., 2005; Tkaczyk

et al., 2003). Mast cell PI3Ks also activate the mTOR pathway and the c-Jun N-terminal kinases (Jnk) to promote synthesis of cytokines (Ishizuka et al., 1999; Kim et al., 2008a; Kim et al., 2008b). C-Kit/SCF signaling causes strong and lasting PI3K/Akt pathway activation, which leads to the synergistic enhancement of Ag/FcεR1-mediated secretory responses (Gilfillan et al., 2009).

The extracellular signal regulated kinase (Erk) and Jnk also play crucial roles in immune cell activation and the pathogenesis of asthma. Both Erk and Jnk MAPKs regulate transcription factors for T cell differentiation and the production of Th2 cytokines in allergies (Dong et al., 1998; Pelaia et al., 2005; Yamashita et al., 1999). In mast cells, the adaptor protein Grb2 (growth factor receptor-bound protein 2) binds to phosphorylated c-Kit and activates the Ras guanine nucleotide exchange factor (SOS) and downstream Erk and p38 MAPKs (Thommes et al., 1999). The c-Kit associated SFKs also activate Gab2 (Grb2-associated-binding protein 2) and the downstream Jnk pathway (Timokhina et al., 1998).

1.3.3 Mast Cell Migration

The trafficking and recruitment of mast cells is an important step in the inflammatory response of specific tissue sites. Mast cells express a variety of receptors for chemokines and extracellular matrix proteins, which mediate mast cell chemotaxis. Integrin signaling shares similar components with immune receptor and cytokine signaling pathways, and it also aids in mast cell recruitment to sites of inflammation (Abonia et al., 2006; Meininger et al., 1992; Nilsson et al., 1996; Ra et al., 1994; Tan

et al., 2003). In human asthma, the accumulation of activated mast cells facilitates chronic symptoms of the disease, including mucous obstruction. Animal studies demonstrate that the pathologic increase in the number of mast cells within antigen-challenged tissues is relatively rapid, and is unlikely to be due to the proliferation of basal resident progenitors (Ikeda et al., 2003). Very few mast cells are found within the lungs of healthy individuals. There is evidence that tissue remodeling in chronic inflammation promotes the secretion of stem cell factor (SCF), a key mast cell chemoattractant that helps recruit and activate more mast cells (Hogaboam et al., 1998). Antigen stimulation of the FcεR1 pathway also induces the production of cytokines, enhances mast cell migration, and synergizes with other chemoattractants (Ishizuka et al., 2001b; Jolly et al., 2004; Rosen and Goetzl 2005).

Numerous *in vitro* assays and animal models have been utilized to elucidate the molecular signaling mechanisms directing the recruitment of mast cells under different conditions (Kim et al., 2008a; Samayawardhena et al., 2007; Suzuki et al., 1998). Mast cell pathways involved in the production of inflammatory mediators can also regulate their migration. The MAPK p38 is involved in antigen and SCF-mediated mast cell chemotaxis (Craig and Greer, 2002; Samayawardhena et al., 2006). The Src family kinases Lyn and Fyn facilitate integrin signaling and mast cell migration through the extracellular matrix (Samayawardhena et al., 2007; Suzuki et al., 1998). The binding of mast cells to fibronectin enhances their inflammatory responses (Ra et al., 1994). Both the PI3K and Rac pathways also promote SCF- and fibronectin-mediated mast cell migration (Tan et al., 2003). Many chemotactic

stimuli converge on mast cells to regulate intracellular calcium signaling and cytoskeletal rearrangements, which are required for the directed movement of these cells (Shimizu et al., 2009; Suzuki et al., 1998; Tan et al., 2003).

1.4 Protein Tyrosine Phosphatases

Many vital cell functions such as growth, survival, migration and signaling require the phosphorylation of proteins to alter their catalytic or structural properties. In this post-translational modification specific serine, threonine, and tyrosine residues receive covalently bound phosphates on their hydroxyl groups. The energy and the phosphate for this reversible reaction are derived from ATP, and it is catalyzed by kinases, while the reverse removal of phosphates is carried out by phosphatases. A small subgroup of all protein kinases is capable of phosphorylating tyrosine residues, nonetheless their functions are crucial for human health. As described earlier, Src family protein tyrosine kinases (SFKs) such as Lyn and Fyn mediate the complex regulation of signaling by immune receptors and growth factor receptors, like FcεR1 and c-Kit, respectively. Since SFKs regulate division, migration and survival of various cell types, they can also drive the pathogenesis of human cancers (Furumoto et al., 2005; Hong et al., 2007; Kim et al., 2009; Samayawardhena et al., 2007). The Src homology domain 2 recognizes and binds phosphotyrosine domains of membrane proteins, recruiting the catalytic activity of SFKs to activate downstream signaling cascades for important cell functions. Phosphorylation of many SFK substrates is tightly regulated, and often requires timely inactivation and reversal by protein tyrosine phosphatases (PTPs). As a result, numerous PTPs

operate as tumor suppressors and immune regulators, and together with SFKs are attractive drug targets (Barr, 2010).

Close to 100 human PTPs have been identified and classified into family groups based on structural features and catalytic specificity (Alonso et al., 2004; Andersen et al., 2001; Soulsby and Bennett, 2009; Tonks, 2006). Most classes of PTPs utilize a cysteine nucleophile to catalyze the transfer of phosphates, except for the Eyes absent (EyA) protein phosphatases, which utilize a catalytic aspartic acid residue (Rayapureddi et al., 2003). Class I cysteine-based PTPs are divided into the group of classical tyrosine-specific phosphatases, and a group of dual-specificity phosphatases that also recognize serine and threonine substrates (Figure 1.4). The 38 classical tyrosine-specific PTPs comprise receptor-like, transmembrane proteins such as CD45, PTPα, PTPε, and LAR as well as the cytoplasmic nonreceptor subgroup containing PTP1B, SHP1 and SHP2, PTP-PEST and others (Akimoto et al., 2009; Alonso et al., 2004). The dual specificity phosphatases are generally subdivided into Slingshot, phosphatase of regenerating liver (PRL), CDC14, PTEN-like and myotubularin, and mitogen-activated protein kinase phosphatases (MKPs) (Patterson et al., 2009; Pulido and Hooft van Huijsduijnen, 2008).

A single small protein called low molecular weight protein tyrosine phosphatase (LMPTP) comprises class II of cysteine-based PTPs. It is encoded by the highly conserved *ACP1* human gene, which shares homology with bacterial tyrosine phosphatases. Variants of ACP1 with lower enzymatic activity are associated with

the development of atopic allergies and other diseases (Bottini et al., 2007). The class III cysteine-based PTPs (CDC25A, CDC25B and CDC25C) evolved to regulate cell cycle progression in human cells. They dephosphorylate N-terminal threonine-tyrosine motifs of cyclin-dependent kinases to induce mitosis (Aressy and Ducommun, 2008).

The PTP CD45 is expressed in various leukocytes, where it modulates immune receptor signaling (Grochowy et al., 2009; Saunders and Johnson, 2010). CD45 is required for T cell receptor signaling, and in mast cells facilitates activation via the FcεR1 receptor. CD45 can dephosphorylate tyrosine residues on Src family kinases as well as other PTPs (Maksumova et al., 2007; Ostergaard et al., 1989). The Src homology region 2 domain-containing phosphatases (SHP1 and SHP2) are also important regulators of mast cell responses in asthma and anaphylaxis (McPherson et al., 2009; Nakata et al., 2008; Zhu et al., 2010). Increased expression of PRLs is linked to metastatic progression in various human cancers (Bessette et al., 2008). MKPs are important negative regulators of MAPK signaling during cell activation, apoptosis, and migration (Pelaia et al., 2005). Taken as a whole, human PTPs are a functionally diverse group of enzymes, which regulate signal transduction during homeostasis and disease.

1.4.1 PTP-Alpha

Receptor-type protein tyrosine phosphatase alpha (PTPα) was identified in mouse brain by screening cDNA for homologs of the CD45 phosphatase domain (Kaplan et

al., 1990; Matthews et al., 1990; Sap et al., 1990). It is encoded by the *PTPRA* gene on human chromosome 20 and expressed as a heavily glycosylated transmembrane protein (Daum et al., 1994; Rao et al., 1992). More recently, the expression pattern and substrate specificity of two PTPα splice variants were characterized (Kapp et al., 2007). The smaller isoform of PTPα is ubiquitously expressed. The other isoform is larger due to the inclusion of 9 extra amino acids in the extracellular domain, and is more prominent in the brain, muscles, and fat tissue. The main enzymatic function of PTPα is to dephosphorylate the inhibitory C-terminal tyrosine residues of Src family kinases (Zheng et al., 1992). The biological effects of PTPα are diverse and include the regulation of cell cycle, tumorigenesis, neuronal differentiation, integrin and insulin receptor signaling, ion channel activity, cell adhesion and chemotaxis, and activation of T cells and mast cells (Chen et al., 2006; Chen et al., 2009; Maksumova et al., 2005; Pallen, 2003; Samayawardhena and Pallen, 2008; Wang et al., 2009).

The extracellular domain of the mature PTPα protein (both isoforms at 130 kDa) is shorter than that of most other receptor-like class I PTPs and carries N- and O-linked glycosylation, similar to the related receptor-like PTPε (Daum et al., 1994; Nakamura et al., 1996). No ligand has been identified for the extracellular domain of PTPα, yet it is required for complex formation with neuronal GPI-anchored receptor contactin in developing neurons (Zeng et al., 1999), suggesting a co-receptor function. Additionally, the extracellular domain was recently shown to mediate PTPα-induced transformation of fibroblasts and anchorage-independent growth (Tremper-

Wells et al., 2010). Similarly to other receptor-like PTPs, the cytoplasmic portion of PTPα carries two tandem phosphatase domains with different catalytic abilities. The membrane-proximal D1 domain of PTPα carries out all the main catalytic functions, while the D2 domain cannot dephosphorylate tyrosines, but is still enzymatically active with small synthetic phosphotyrosyl mimetic substrates (Lim et al., 1997; Wang and Pallen, 1991). The conservation of non-catalytic D2 domains in receptor PTPs suggests that they may function in regulating the formation of protein complexes in the absence of phosphotyrosine binding (Pallen, 2003). These membrane distal domains of PTPs have been shown to interact with adaptor proteins of NMDA receptors, calmodulin, as well as partner D1 domain within PTPα dimers (Bilwes et al., 1996; Lei et al., 2002; Liang et al., 2000).

1.4.2 Activation of Src Family Kinases

One of the earliest experiments examining the physiological functions of PTPα established it as an important regulator of oncogenic cell transformation. Overexpression of PTPα results in the dephosphorylation of the inhibitory C-terminal Tyr-527 of Src, activating the kinase and inducing cell transformation (Zheng et al., 1992). Purified PTPα can also dephosphorylate and activate Src in *in vitro* assays. PTPα expressed in embryonal carcinoma P19 cells dephosphorylated Src Tyr-527, resulting in differentiation into neuronal cells upon stimulation with retinoic acid (den Hertog et al., 1993). Other SFKs, such as Fyn, are also regulated by PTPα to induce various cell responses (Bhandari et al., 1998; Maksumova et al., 2007; Samayawardhena and Pallen, 2008; Wang et al., 2009). PTPα-mediated

dephosphorylation of the C-terminal tyrosine of Fyn opens the SH2 domain of Fyn and potently increases its kinase activity (Bhandari et al., 1998).

In the absence of cell stimulation several mechanisms maintain SFKs in an inactive state. As mentioned earlier, the loss of regulation of SFK activity promotes aberrant downstream signaling cascades, and the pathogenesis of cancer, chronic inflammation and other diseases (Hendriks et al., 2008; Kim et al., 2009). Under suppressive conditions intramolecular interactions block the Src tyrosine kinase domain from interacting with substrates. The phosphorylated C-terminal tyrosine 527 of Src is bound by its SH2 domain, while the region between the kinase and SH2 domain interacts with the SH3 domain (Bjorge et al., 2000; Xu et al., 1997). PTPα interacts with Src to bring the Tyr-527 into the phosphatase D1 domain, where it becomes dephosphorylated. The opened kinase domain of Src can phosphorylate its own Tyr-416, promoting full tyrosine kinase activation.

1.4.3 Phosphorylation and Regulation of PTPα

PTPα itself can also be a substrate of Src and other kinases. In various cell lines PTPα is phosphorylated in its C-terminal region on tyrosine 789. Src phosphorylates PTPα at Tyr-789, which is also a binding site for the SH2 domain of adaptor protein Grb2 (den Hertog et al., 1994). Phosphorylation of tyrosine 789 on PTPα does not inhibit its catalytic activity. There are conflicting reports on whether PTPα Tyr-789 phosphorylation is required for the binding of the Src-SH2 domain to induce Src Tyr-527 dephosphorylation and Src catalytic activity. In mitosis, dephosphorylation of

PTPα membrane-proximal serine residues was instead shown to play much bigger role in the binding and activation of Src. Furthermore, the non-phosphorylatable Y789F mutant of PTPα was still able to bind Src in an SH2-independent manner (Vacaru and den Hertog, 2010). In other studies phosphoTyr-789 of PTPα was shown to promote the activation of Src-induced cell transformation (Zheng et al., 2000). Little is known about the functions of PTPα Tyr-789 in immune cell regulation. During T cell receptor activation, another protein tyrosine phosphatase, CD45, directly dephosphorylates Tyr-789 of PTPα to regulate Fyn and Cbp signalling (Maksumova et al., 2007).

Similarly to various membrane tyrosine kinases and phosphatases, PTPα is capable of dimerization. In a crystal structure of the PTPα D1 domain, a membrane proximal helix-turn-helix structure inserts into the catalytic cleft of partner D1 domain, blocking the site to substrates (Bilwes et al., 1996). Such dimerization is enhanced by multiple structural interactions, and was proposed as a negative regulatory mechanism of PTPα activity. Evidence from FRET techniques shows constitutive PTPα dimerization in living cells that requires its transmembrane domain (Tertoolen et al., 2001). However, not all PTPα homodimer interactions inhibit phosphatase activity at physiological concentrations of PTPα. Oxidative stress also promotes the inhibitory dimerization of PTPα, requiring the catalytic cysteine of its D2 domain (Groen et al., 2008).

In various cell types PTPα activity and Tyr-789 phosphorylation can also be inhibited by reactive oxygen species (Hao et al., 2006). This effect is independent of the major SFKs, but inhibited by serine/threonine protein phosphatase 1. Small oxidizing molecules produced by antigen-challenged mast cells were reported to regulate the catalytic domain of PTPα and other tyrosine phosphatases (Heneberg and Draber, 2005). Taken together, PTPα is involved in a complex network of protein interactions and chemical modifications that modulate the activity of Src family kinases and adaptor proteins to regulate crucial functions of multiple cell types (Figure 1.5).

1.4.4 Biological Functions of PTPα

PTPα knockout (KO) mice have been successfully bred in our lab and others', and do not exhibit any gross anatomical abnormalities. Thus the lack of PTPα activity does not disrupt embryonic development (Ponniah et al., 1999; Su et al., 1999). Recent studies revealed effects of PTPα on important responses of specific cell types in the neural and immune systems and connective tissues. PTPα was originally identified as a tyrosine phosphatase that is highly expressed in the brain, and was later shown to regulate neural cell functions. Interestingly, work from our lab has shown that PTPα is required for the activation of Fyn tyrosine kinase signaling to induce the differentiation of oligodendrocytes and myelination (Wang et al., 2009). Activation of SFKs by PTPα plays an important role in synaptic transmission. PTPα-KO mice showed reduced phosphorylation of NMDA receptors in the brain, and defects in the associated memory and learning functions (Le et al., 2006; Lei et al., 2002; Petrone et al., 2003; Skelton et al., 2003). Activation of the m1 muscarinic

acetylcholine receptor induces PTPα to dephosphorylate tyrosine residues on the potassium ion channel $K_v1.2$ that inhibits electrochemical signal transmission. In the process PTPα becomes phosphorylated by PKC-dependent signaling (Tsai et al., 1999). Therefore, PTPα may be a key modulator of human neuromolecular processes.

SFKs are also closely involved in receptor-mediated cell migration, which is a crucial component in the recruitment of immune cells and metastasis. Various cell types can be recruited by gradients of chemoattractants with the aid of extracellular matrix proteins bound by integrin receptors. The PTPα substrates, Src and Fyn, regulate the fibronectin-induced cytoskeletal rearrangements and migration of fibroblasts (Su et al., 1999; Zeng et al., 2003). Cells lacking PTPα exhibit reduced activation of the focal adhesion kinase FAK and defective haplotaxis. PTPα was shown to regulate integrin-proximal signaling events to activate SFKs and FAK that in turn induce the phosphorylation of PTPα Tyr-789, which is required for the formation of focal adhesions, actin reassembly and cell migration. This process also depends on the catalytic activities of Src and PTPα (Chen et al., 2006). Additional research in our lab has revealed that insulin-like growth factor 1 (IGF-1) also stimulates PTPα Tyr-789 phosphorylation that promotes chemotaxis (Chen et al., 2009). In fibroblast and neuroblastoma cells IGF-1 receptor signaling activates the tyrosine kinase c-Abl to phosphorylate PTPα and induce migration (Khanna, 2011).

1.4.5 PTPα in Mast Cell Activation

As described earlier, the recruitment of mast cells into mucosal and vascular tissues is implicated in the pathogenesis of allergic asthma and several types of cancer. Stem cell factor (SCF) serves as a crucial chemoattractant and growth factor for mast cells. In the absence of antigen and IgE, PTPα positively regulates SCF-induced receptor c-Kit phosphorylation and downstream signaling events. PTPα-KO BMMCs exhibited defective migration towards SCF, spreading and polarization, compared to WT cells. PTPα was required for optimal activation of the c-Kit-associated tyrosine kinase Fyn, and downstream activation of the Rac/Jnk and MAPK pathways. This may contribute to the decreased homing of mast cells to hypodermis and submucosa in PTPα-KO mice, but increased numbers in the peritoneum (Samayawardhena and Pallen, 2008).

Furthermore, recent data from our lab demonstrated a negative regulatory role for PTPα in antigen-induced mast cell activation and secretory responses (Samayawardhena and Pallen, 2010). Degranulation and mediator release were enhanced in PTPα-KO BMMCs relative to WT cells. In the absence of PTPα, activating phosphorylation of FcεR1, Lyn and Fyn were reduced, whereas Syk, Hck, Akt and MAPK activities were increased. PTPα-KO mice sensitized with IgE and challenged with antigen displayed increased passive cutaneous anaphylaxis and serum histamine levels. The closely related receptor-like protein tyrosine phosphatase epsilon (PTPε) was also recently shown to negatively regulate FcεR1 responses (Akimoto et al., 2009). PTPε-null BMMCs treated with antigen showed

increased phosphorylation of Syk, but not Lyn tyrosine kinase, and hyperactivation of downstream MAPK and calcium signaling. Loss of PTPε activity resulted in enhanced BMMC degranulation and release of cytokines, but not of leukotrienes. Other studies demonstrate that antigen is also chemotactic to mast cells (Ishizuka et al., 2001b; Kitaura et al., 2005). Under pathologic conditions mast cells are co-stimulated with SCF and antigen, which has been shown to synergistically enhance their recruitment and secretory responses (Al-Muhsen et al., 2004; Columbo et al., 1992; Iwaki et al., 2005; Kuehn et al., 2010). However, the role of PTPα in the integrated c-Kit/FcεR1 signaling that regulates their migration and activation has not been investigated (Figure 1.6).

1.5 Hypothesis

The aberrant mast cell processes described earlier are implicated in the development of allergic diseases and cancer, and are regulated by several Src family kinases. PTPα was shown to differentially affect SFK-mediated mast cell migration to SCF and secretory responses to antigen. **I hypothesize that the balance of positive regulatory fuctions of PTPα in c-Kit signaling, and its observed negative regulation of FcεR1 signaling, combine to control the extent of Ag/SCF-induced synergistic mast cell responses.** The following experimental aims will address the specific aspects of my hypothesis utilizing mast cells derived from the bone marrow of PTPα-deficient mice.

Aim 1. Investigate the effect of PTPα on synergistic mast cell degranulation and cytokine release in response to co-treatment with antigen and SCF.

Aim 2. Determine whether PTPα also regulates antigen-mediated mast cell chemotaxis in the presence or absence of SCF.

Aim 3. Identify the crucial effector proteins of PTPα and their activation in integrated c-Kit/FcεR1 signaling.

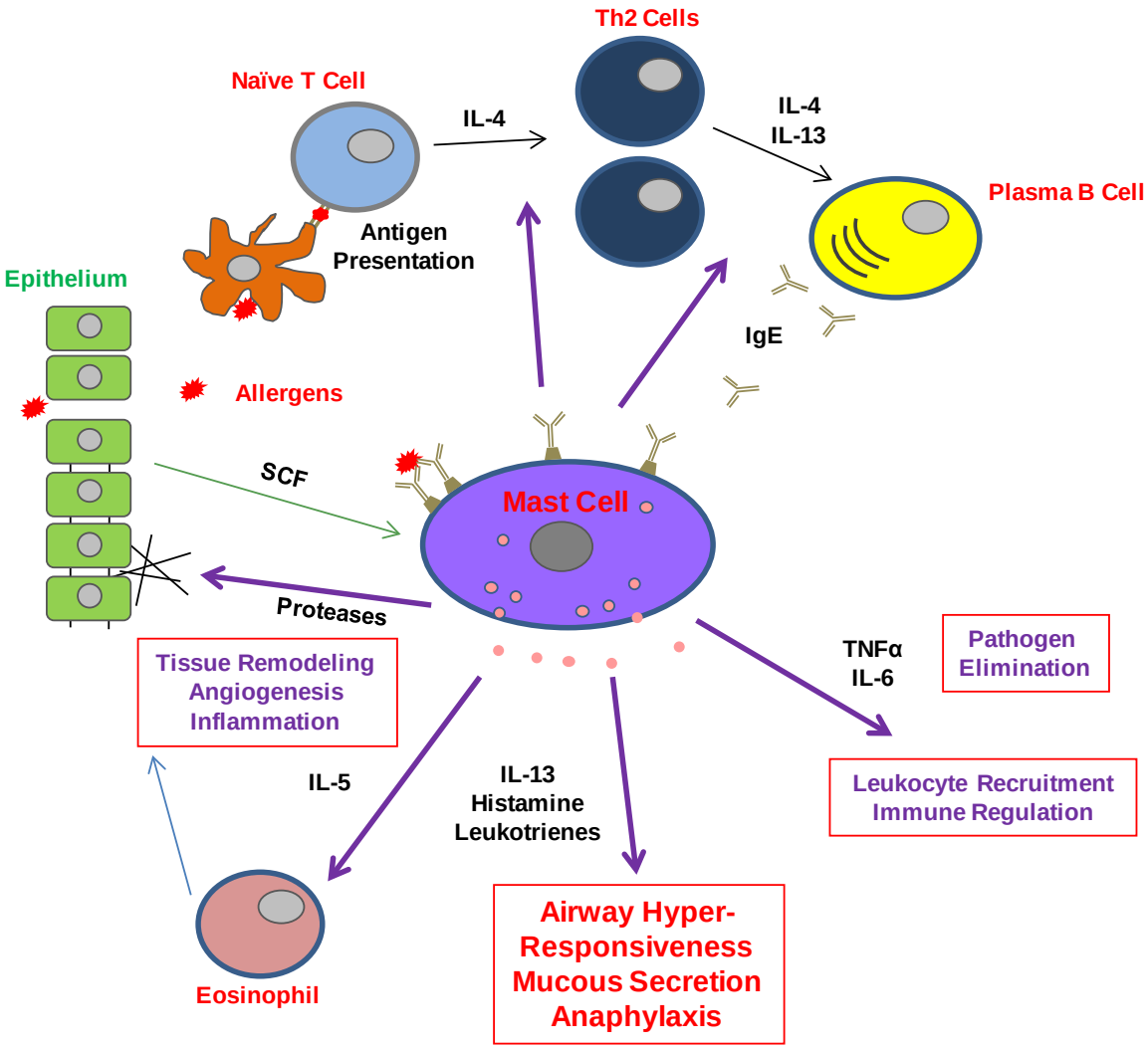


Figure 1.1. Allergens induce Th2 immunity and mast cell responses. Small protein allergens pass through the lung epithelium and are processed by antigen-presenting cells. T cells are activated via antigen presentation, develop into Th2 cells and promote IgE production by B cells. IgE binds to Fc receptors on mast cells, and becomes crosslinked by more allergen molecules, activating mast cells. Cytokines produced in inflamed connective tissues, such as SCF, recruit mast cells and other leukocytes. Allergen triggers mast cells to degranulate and secrete an array of pro-inflammatory molecules. Mast cell mediators promote various allergic symptoms, tissue remodeling, recruitment and activation of leukocytes, and defend against different types of infection and parasites.

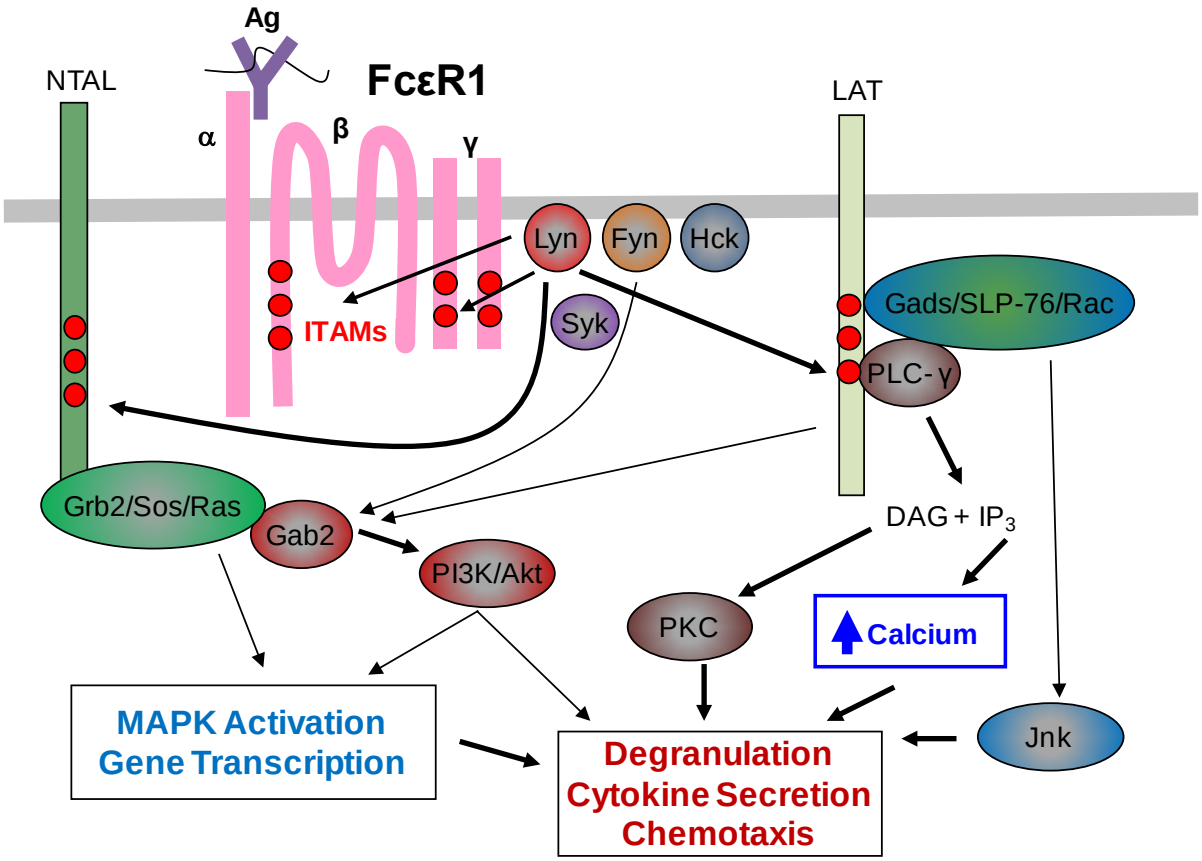


Figure 1.2. Antigen induces FcεR1 signaling cascades and multiple mast cell responses. Schematic representation of key positive regulators and signaling events. Lyn phosphorylates receptor ITAMs and recruits other tyrosine kinases, which together further phosphorylate scaffold proteins, LAT and NTAL. Adaptor proteins recruit and activate the Ras/MAPK, PI3K/Akt, PLCγ and calcium signaling cascades. Arrows indicate the direction of signaling, leading to rapid mast cell degranulation, release of cytokines, and antigen-induced chemotaxis. See list of abbreviations for full protein names.

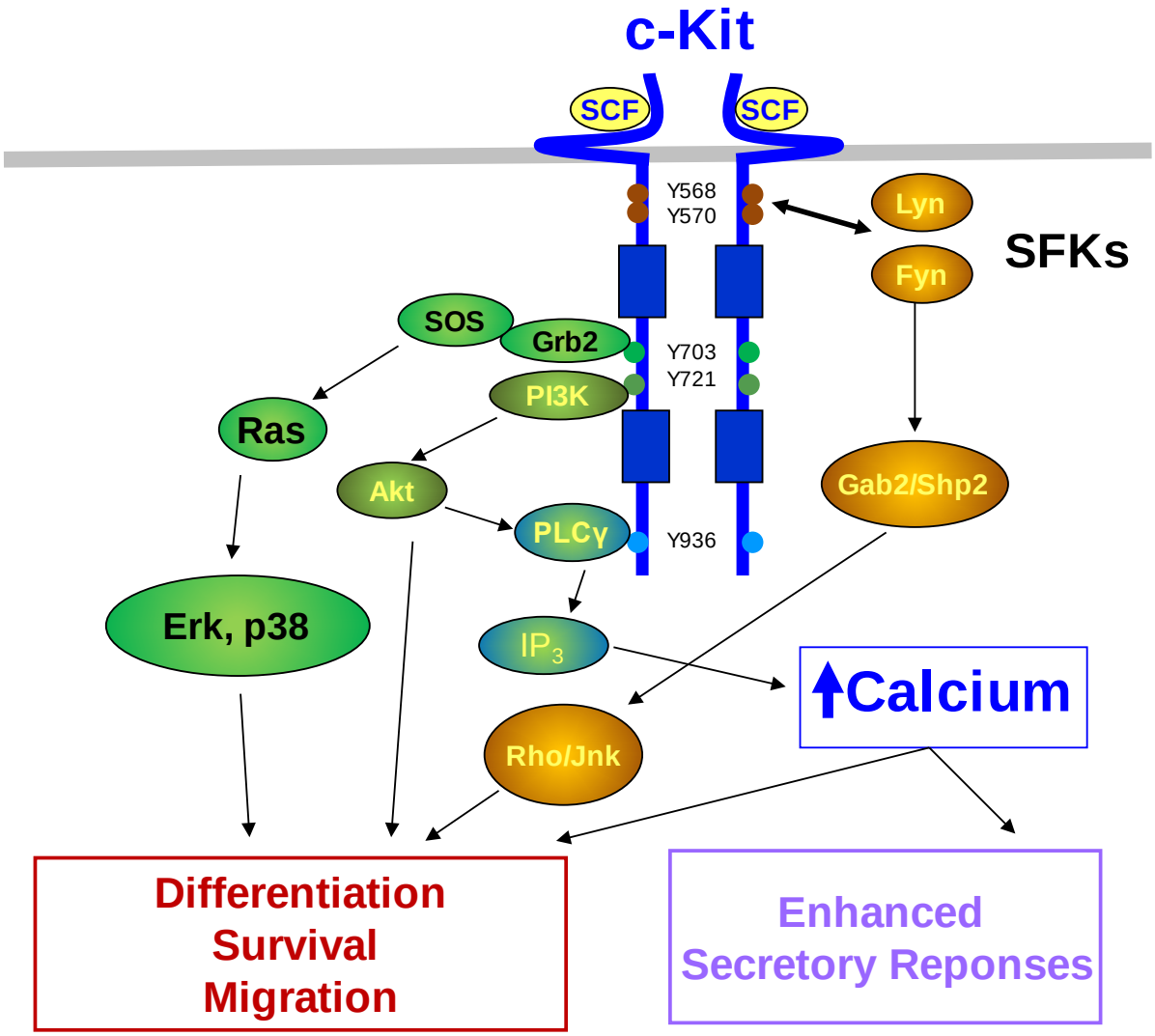


Figure 1.3. SCF induces receptor c-Kit dimerization and activation of signaling cascades. Schematic representation of key positive regulators and signaling events. C-Kit autophosphorylates several tyrosine residues, which become docking sites of adaptor proteins (human c-Kit residue numbering shown). The PI3K, MAPKs, and PLCγ pathways promote cell survival, migration, and enhancement of antigen-induced secretory responses.

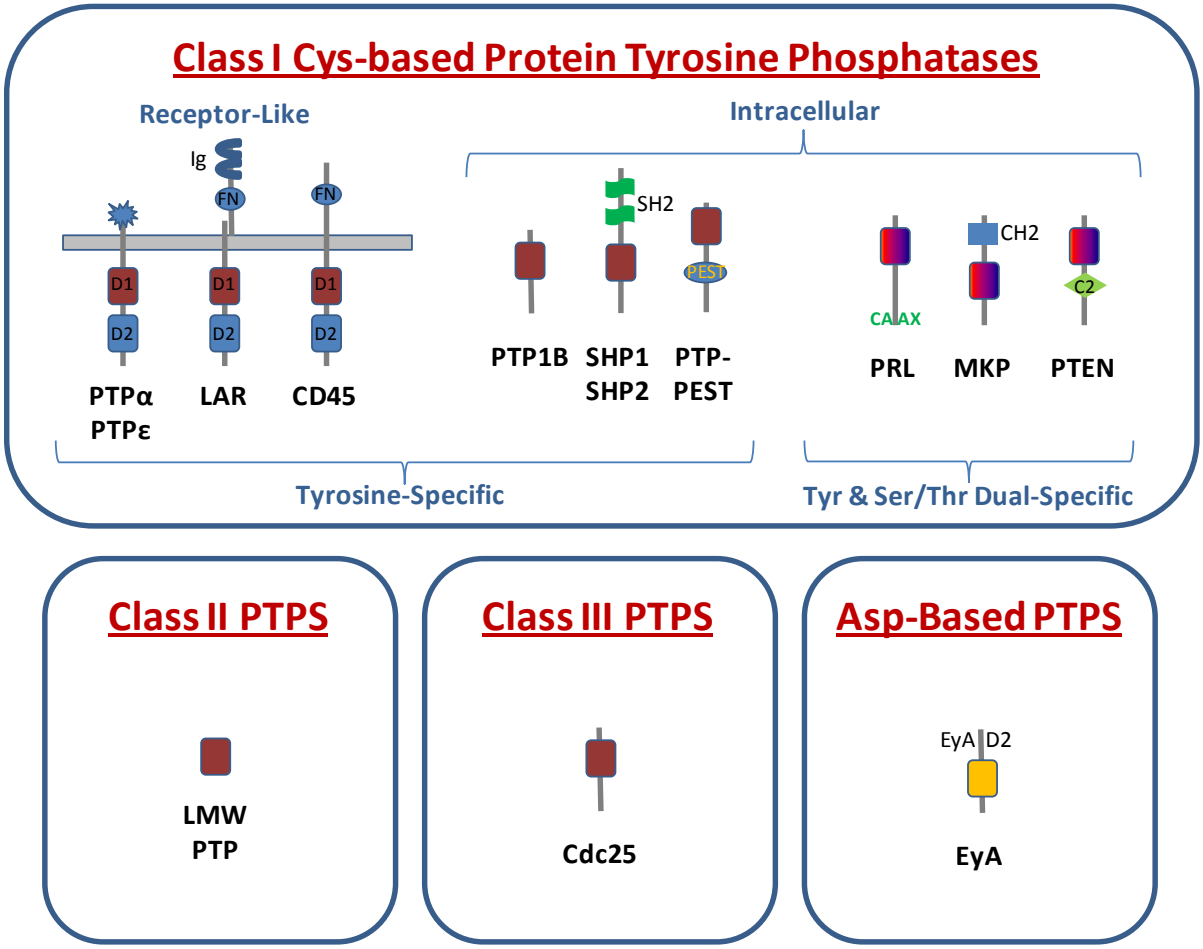


Figure 1.4. The superfamily of protein tyrosine phosphatases. Examples of proteins from the four subclasses of PTPs are represented schematically. Class I cysteine-based PTPs are subdivided into classical (receptor-like and intracellular) and dual-specificity PTPs. Others include class II Cys-based low molecular weight (LMW) PTP, class III Cys-based Cdc25 PTPs, and Asp-based EyA PTPs. Domain structures are abbreviated: D1 and D2, membrane-proximal and membrane-distal receptor PTP domains; FN, fibronectin-like; Ig, immunoglobulin-like; SH2, Src homology domain 2; PEST, rich in proline, glutamic acid, serine, and threonine; CAAX, prenylation sequence; CH2, Cdc25 homology domain 2; C2, protein kinase C conserved region 2; Eya D2, conserved domain 2 of EyA PTPs.

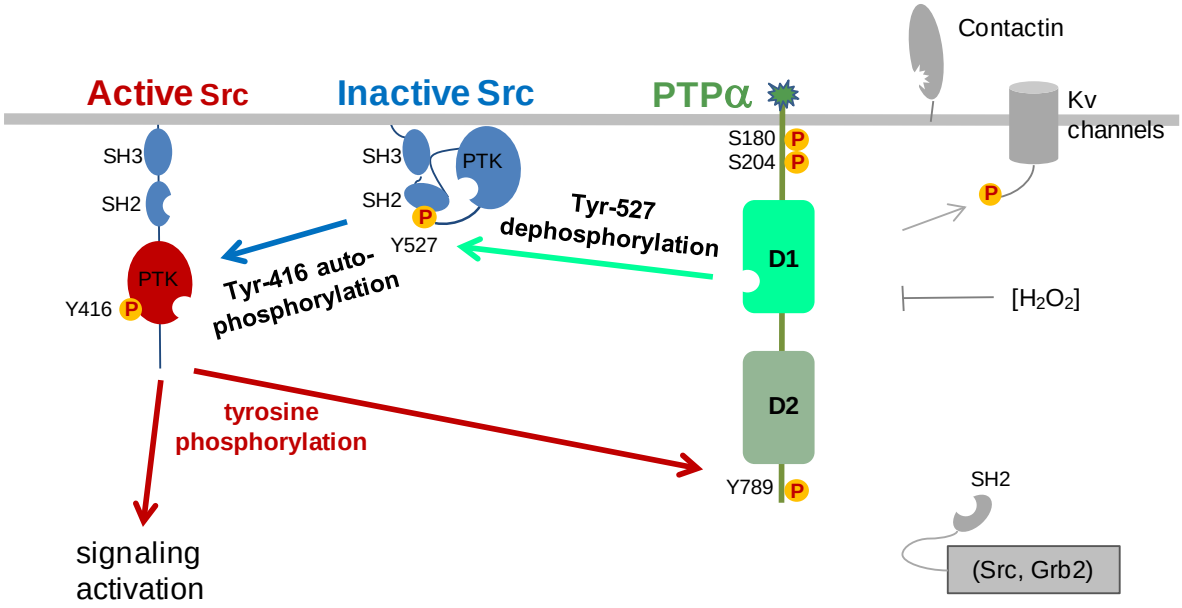


Figure 1.5. Regulation of Src family kinases (SFKs) by protein tyrosine phosphatase alpha (PTPα). SFKs such as Src remain inactive due to intramolecular interactions between the inhibitory phosphoTyr-527 and the SH2 domain, and between the SH3 domain and the region connecting the protein tyrosine kinase domain (PTK) to the SH2 domain. PTPα can dephosphorylate Tyr-527 of Src (green arrow), and interact with SH2 domain via phosphoTyr-789 of PTPα. Disruption of intramolecular interactions of Src allows its catalytic domain to autophosphorylate Tyr-416 to become fully active tyrosine kinase. SFKs mediate the activation of signaling pathways, and can promote the phosphorylation of PTPα Tyr-789 (red arrows). Various motifs of PTPα have been shown to interact (right side) with Grb2 and contactin, dephosphorylate non-SFK substrates such as potassium K_v channels, become inhibited by reactive oxygen species (e.g. H_2O_2), and become phosphorylated on regulatory serine residues.

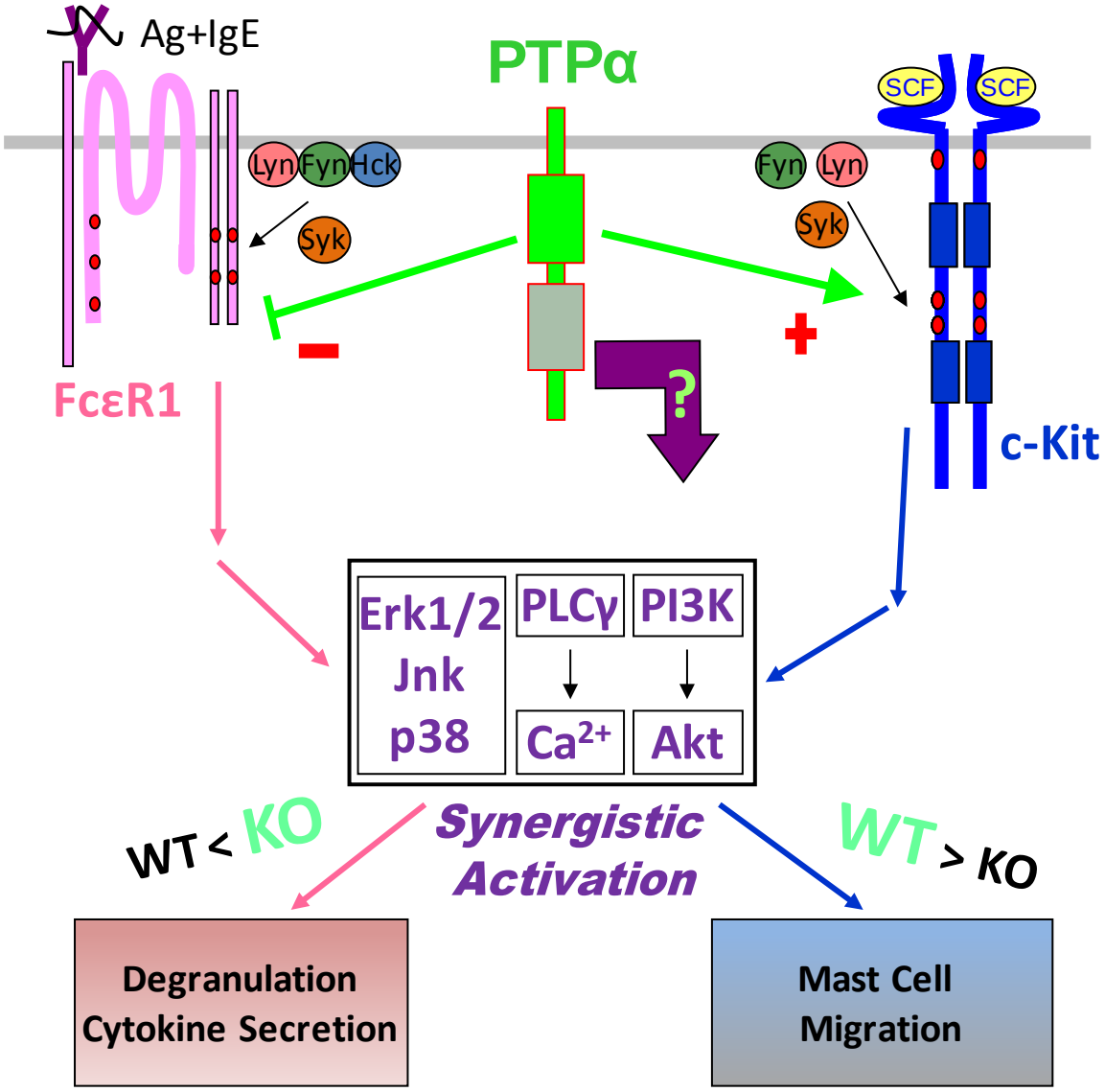


Figure 1.6. PTPα in the integrated c-Kit and FcεR1 signaling cascades. PTPα was previously shown to negatively regulate antigen-induced FcεR1 signaling, including Akt and MAPKs, down-regulating mast cell degranulation and secretion of cytokines. In the absence of IgE, PTPα promoted SCF-induced c-Kit signaling and cell migration. Co-stimulation of both c-Kit and FcεR1 receptors induces synergistic activation of PLCγ and MAPKs and enhanced migration and secretory responses. The SFKs involved in both pathways are also substrates of PTPα.

Chapter 2: Materials and Methods

2.1 Mast Cell Culture

Primary mast cells were derived from the bone marrows of PTPα$^{-/-}$ (Ponniah et al., 1999) and PTPα$^{+/+}$ C57BL/6 mice. The animals were housed in a pathogen-free environment at the CFRI Animal Care Facility. Animal care and use followed the approved guidelines of the University of British Columbia and the Canadian Council on Animal Care. At 4-8 weeks of age, sex-matched pairs of mice were killed using isoflurane gas and cervical dislocation. Their femurs were removed and flushed for bone marrow using a 25G needle and 15 ml BMMC medium consisting of Iscove's modified Dulbecco's media (Gibco), 2% of 10x concentrated WEHI-conditioned mouse IL-3 media (from UBC Biomedical Research Facility), 10% heat-inactivated fetal bovine serum (Gibco), 3 µl thioglycerol (Sigma) per 500 ml media, 1% pyruvate-sodium (Sigma), 1% non-essential amino acids (Sigma), and 1% PenStrep (Sigma). The flushed material was transferred onto 10 cm culture plates and maintained in suspension in an incubator at 37^{o}C with a humidified atmosphere containing 5% CO_2. The culture media and the plates were replaced twice a week. After 3 weeks, when no adherent cells remained, mast cell progenitors were cultured in 100 ml culture flasks at 1.0-1.5 x10^{6} cells/ml, and 80-90% of the media was replaced twice a week. After 5 weeks, BMMC purity and maturity were confirmed by FACS analysis (see section 2.9), while cell lysates were tested for expression of PTPα, c-Kit, and Akt by immunoblotting (see section 2.6). Cultures with insufficient purity of mast cells

often expressed much lower levels of Akt and c-Kit, and were discarted. BMMC cultures between 6 and 8 weeks of age were used for stimulations.

2.2 Antibodies

The antibodies used in these studies were purchased from the following companies: anti-Akt, phospho-Ser473-Akt, phospho-Erk1/2, phospho-JNK, phospho-p38, phospho-Tyr416-Src, Lyn, phospho-Tyr507-Lyn, c-Kit, and phospho-Tyr719-c-Kit (all from Cell Signaling Technology, Denvers, MA); phosphotyrosine-4G10, FcεR1β, and FcεR1γ (Upstate Biotechnology, Lake Placid, NY); phospho-Tyr567/569-c-Kit (Santa Cruz Biotechnology, Santa Cruz, CA), anti-DNP IgE and β-actin (Sigma-Aldrich, St.Louis, MO); rabbit anti-phospho-Tyr783-PLCγ1 (Invitrogen-Biosource, Camarillo, CA); rat anti-mouse CD16/32 and PE-conjugated rat anti-mouse c-Kit (Caltag Laboratories, Burlingame, CA); FITC-conjugated rat anti-mouse FcεR1-α (eBioscience, San Diego, CA), FITC rat anti-mouse-IgE (BD Pharmigen, Mississauga, ON). Anti-PTPα and phospho-Tyr789-PTPα polyclonal antibodies were described previously (Lim et al., 1998, Chen et al., 2006). Horseradish peroxidase-conjugated goat secondary antibodies against rabbit and mouse IgG were purchased from Sigma-Aldrich, St.Louis, MO. Protein-A HRP conjugate (Bio-Rad Laboratories, Hercules, CA) was used to probe some immunoprecipitation samples to minimize the appearance of denatured immunoglobulin chains.

2.3 IgE Sensitization of Cultured BMMCs

2.3.1 Old (Standard) Method of IgE Sensitization

This standard method was used as described by Samayawardhena and Pallen (2010). Mature BMMCs (obtained after 6-8 weeks of culture, as described in section 2.1) were transferred into starvation media (BMMC media lacking IL-3) at 2.0 $x10^6$ cells/ml. Mouse anti-DNP IgE (SPE-7 clone, Sigma) was added at 200 ng/ml. After 16 hr incubation the cells were washed once with starvation media and resuspended in the appropriate buffer or media and used for experimentation as described below.

2.3.2 New (Prolonged) Method of IgE Sensitization

In order to achieve strong and consistent IgE-mediated mast cell responses, particularly degranulation, the following 'New' method of IgE sensitization was utilized for all BMMC stimulation experiments unless otherwise stated. Firstly, BMMCs (2.0 $x10^6$ cells/ml) were incubated overnight in fresh BMMC media containing IL-3, and in the presence of 200 ng/ml anti-DNP IgE. After 16 hours the cells were transferred into starvation media containing 200 ng/ml anti-DNP IgE, and incubated for a further 6-8 hr. The cells were pelleted by gentle centrifugation and washed once with pre-warmed media prior to stimulation.

2.4 Stimulation of BMMCs

IgE-sensitized BMMCs (described in section 2.3.2) were resuspended at 5.0 $x10^6$ cells/ml in pre-warmed 37^oC Tyrode's buffer (10 mM Hepes pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl, 1 mM MgCl, 0.1% glucose, 0.1% BSA) and equilibrated in

37^{o}C water bath for 10 min. A small volume of Tyrode's buffer, carrying antigen (DNP-HSA, Sigma) and/or recombinant murine SCF (PeproTech Inc), was mixed in to achieve the specified concentrations. Stimulation was stopped at various time points by adding an equal volume of stop solution (ice-cold PBS with 0.1 mM Na_3VO_4). Cells were pelleted by centrifugation, washed once with stop solution and solubilized in lysis buffer as described in section 2.5.

2.5 Cell Lysis and Immunoprecipitation

For direct immunoblotting of cell lysates, the cell pellet was solubilized for 20 min in ice cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1mM EDTA, 1mM Na_3VO_4, 1mM NaF, 100 µM PMSF, 10 µg/ml aprotinin and leupeptin). Cell debris was pelleted after centrifugation at 4^{o}C for 20 min and discarded. The concentrations of protein in the lysate supernatants were determined using the Bio-Rad protein assay according to the manufacturer's manual (Bio-Rad). After adding 2X SDS sample buffer, samples were boiled for 10min and resolved by SDS-PAGE.

For the immunoprecipitation of membrane proteins, 0.5% sodium deoxycholate and 0.05% SDS were added to the lysis buffer. The lysate supernatants were then pre-cleared with 20 µl Protein A/G agarose beads (Santa Cruz) for 60 min by rotation at 4^{o}C. After gentle centifugation to remove the beads, the pre-cleared samples were diluted with lysis buffer to a concentration of 200 µg protein in 500 µl lysis buffer. The appropriate immunoprecipitation antibody (2 µl) and 20 µl of Protein A/G beads were added and mixed by rotation overnight at 4^{o}C. The protein-bound beads were

collected by gentle centifugation and washed 3 times with 1 ml cold lysis buffer. The beads were resuspended in 45 µl of 2X SDS sample buffer and boiled for 5 min. The immunoprecipitation samples were then resolved by SDS-PAGE.

2.6 Immunoblotting

Polyacrylamide gels (7.5-15%, 1mm thickness) were loaded with protein samples and electrophoresed at 100 V for 90 min. The resolved proteins were transferred onto a polyvinylidene fluoride membrane for 70 min at 100 V. The membrane was blocked for 60 min with 3% BSA in PBST (PBS containing 0.1% Tween-20), before overnight incubation at 4^{o}C with primary antibodies diluted in 3% BSA/PBST. Excess primary antibody was removed by washing the membrane 3 times in PBST (10 min/wash). The membrane was incubated with HRP-conjugated secondary antibody in PBST for 60 min, and washed again 3 times with PBST. Following a 2 min incubation with the enhanced chemiluminescence reagent, the membranes were exposed to film. The films were scanned and saved as grayscale image files for densitometric quantification.

2.7 Degranulation Assay

Mature BMMCs were IgE sensitized and stimulated with antigen and/or SCF for 15 min in 37^{o}C Tyrode's buffer, and then cooled on ice for 5 min. Cells were pelleted by gentle centifugation at 4^{o}C to obtain the supernatant containing degranulation secretions. Equal volumes of Tyrode's buffer with 0.5% Triton X-100 detergent were added to solubilize the cell pellet. Aliquots (50 µL) of both the degranulation

supernatants and pellet fractions were separately loaded in duplicate onto 96-well flat-bottom plates, containing 40 µl of 4-nitrophenyl N-acetyl-β-D-glucosaminide solution (β-hexosaminidase substrate, at 3 mM in 0.1 M sodium citrate pH 4.5, Sigma). After 20 min incubation at 37^{o}C the reaction was stopped with 50 µl of sodium carbonate (0.2 M, pH 10). The β-hexosaminidase reaction product was quantified from absorbance plate readings at 405 nm, using the cell-free buffers as a blank. Degranulation in each sample was determined as the percent of absorbance in the secretion supernatant over total absorbance from the supernatant and pellet fractions.

2.8 Cytokine Secretion and ELISA

Mature BMMCs were IgE sensitized and cytokine starved as previously described in section 2.3.2. After sensitization, 1x10^{6} cells were washed once with starvation media and resuspended in 0.5 ml on 24-well plates. The media for cytokine stimulation contained 0.8% WEHI conditioned media (1/25th of the concentration of IL-3 in BMMC culture media). Each 0.5 ml of plated cell suspension, containing the specified concentrations of antigen (DNP-HSA) and/or mouse SCF, was put in a humidified cell culture incubator with 5% CO_2 at 37^{o}C for 15 hours. At the end of the incubation, the cells were pelleted by gentle centrifugation. The obtained supernatants were either immediately tested for specific cytokines by ELISA, or frozen at 80^{o}C for later analysis.

ELISA protocols were carried out according to the product manuals for detection of mouse TNF-α (R&D Systems) and IL-13 (Invitrogen). For IL-13 ELISA, samples were first mixed and incubated with an extraction buffer, but not for TNF-α detection. The supernatants were diluted with sample buffer up to 10 fold. Equal volumes of diluted samples and standards were incubated in duplicate in antibody-coated wells for 2 hr at room temperature. The wells were washed 4 times with diluted wash buffer and tapped dry. HRP-conjugated antibody was added, incubated for another 2 hr, and then washed away. A chromogenic tetramethylbenzidine solution was added to each well and incubated in the dark for 30 min. After adding a stop solution (diluted HCl) the optical densities of each sample were measured at 450 nm. Standard cytokine concentrations were plotted against their corresponding absorbances to derive the best-fit curve equation. The cytokine concentration of each experimental sample was determined using the equation of the standard curve.

2.9 FACS Analysis

To assess the purity and maturity of cultured WT and PTPα-KO BMMCs, $2x10^6$ cells cells from 5 week-old cultures were first blocked with anti-CD16/32 antibody in 100 µL PBS containing 2% serum. Cells were then washed and stained with PE-conjugated anti-c-Kit and FITC-conjugated anti-FcεR1α antibodies in the dark for 30 min. BMMCs were analyzed using a BD FACS Calibur flow cytometer. Single-stained and unstained control cells were used to adjust compensation and particle gating. The percentage of BMMCs that stained for c-Kit and FcεR1α was determined

from 20,000 counts. To assess the binding of IgE to WT and PTPα-KO BMMCs FITC-conjugated rat anti-mouse-IgE antibody was used instead of anti-FcεR1α.

2.10 Transwell Migration

2.10.1 Migration Index Determination

Migration experiments were conducted using 3.0 µm pore-sized polycarbonate membrane Transwell inserts (Corning Cat. #3415). To assess BMMC migration through an uncoated membrane, the Transwell inserts were put into 24-well plates containing 500 µl of migration media (Iscove's modified Dulbecco's media with 0.5% BSA) and the specified concentrations of antigen and SCF. IgE-sensitized WT and PTPα-KO BMMCs were washed once with migration media, and aliquotted into the centre of each Transwell insert at $4x10^5$ cells in 100 µl migration media. The plate was kept in a 37^oC humidified cell culture incubator for 3 hours. Cells that migrated into the lower chamber were pelleted by centrifugation and resuspended in a volume of 50 µl for counting. The total number of BMMCs that had migrated into the lower chamber was calculated from the average number of cells counted using a hemocytometer. The final migration index was determined as the percentage of total cells loaded that migrated into the lower chamber.

2.10.2 Adherent Cell Analysis

The ability of mast cells to migrate through fibronectin-coated Transwell inserts was also assessed. For these experiments the bottom side of Transwell membranes was coated with 20 µM fibronectin (Millipore) in PBS for 2 hr at 37^oC. The membrane

insert bottoms were rinsed with PBS and the inserts were used for migration experiments as described in section 2.10.1. In addition to determining the migration of BMMCs through the fibronectin-coated membranes to the bottom chamber, the number of BMMCs that had passed through the pores but remained attached to the fibronectin-coated membrane was also determined. After 3 hours of migration, the remaining cells inside the Transwell insert were removed by suction, and the insert washed with PBS. Adherent cells were fixed with glacial methanol for 20 min and stained with Giemsa solution (KaryoMAX Giemsa Stock, Gibco) for 1 hr. The membrane was washed in PBS, and its upper surface was gently wiped with cotton swabs to remove any cells. The membrane was excised and mounted on a glass slide with the fibronectin-coated side facing up. For each membrane mounted, five images (200x magnification) were taken using an Olympus DP72 camera on a Zeiss-Ax10 microscope. The number of adherent BMMCs in each image (covering a 0.911 mm^2 area on the membrane) was counted to determine the average cell density for each migration condition.

2.11 Statistical Data Analysis

Immunoblot protein bands were scanned and densitometrically quantified using Quantity One software (Bio-Rad). Protein phosphorylation was determined by calculating the ratio of densitometric units of phosphorylated to total protein bands. Some phosphorylation signals were normalized against total β-actin on parallel loaded gels. All bar graphs show the mean ± S.D. unless otherwise stated. Statistical *p* values were calculated using the Student's t-test.

Chapter 3: Role of PTPα in SCF-Enhanced Secretory Responses of Mast Cells

3.1 Rationale

Exposure to polyvalent antigens crosslinks FcεR1 receptors and triggers a rapid release of preformed mast cell granules, which occupy a large portion of the mast cell cytoplasm (Blank, 2011). Within minutes of this degranulation process, active pro-inflammatory mediators, including histamine, proteoglycans, serine proteases, cytokines, prostaglandins, and leukotrienes accumulate at the site. At a later phase of their activation, mast cells secrete an array of newly synthesized cytokines and growth factors, which contribute to prolonging the overall inflammatory response (Fehrenbach et al., 2009; Gilfillan et al., 2011; Kitaura et al., 2000).

Various small molecules and cytokines have been shown to strongly influence the magnitude and the type of bioactive mediators released (Gilfillan et al., 2009). The functions of the main mast cell growth factor SCF have been extensively studied. Lung tissues of asthmatic patients show higher levels of SCF in addition to serum IgE (Al-Muhsen et al., 2004; Da Silva et al., 2006). SCF can directly promote migration, growth, and survival of mast cells and enhance their pro-inflammatory responses. SCF exposure can synergistically increase *in vitro* antigen/IgE mediated mast cell degranulation and cytokine production to levels several-fold higher than with antigen alone (Fehrenbach et al., 2009; Gilfillan et al., 2009; Vosseller et al., 1997). Since previous work in our lab has shown that PTPα is a positive regulator of

SCF/c-Kit activation (Samayawardhena and Pallen, 2008) as well as a negative regulator of Ag/FcεR1 responses in BMMCs (Samayawardhena and Pallen, 2010), I investigated the role of PTPα in regulating the synergistic mediator release induced by co-stimulation of these receptors.

3.2 Mast Cell Synergistic Degranulation

To investigate the role of PTPα in SCF-enhanced early secretory responses of mast cells, antigen-triggered degranulation was first examined. Following previously established IgE sensitization protocols, mature BMMCs were incubated overnight in starvation media (lacking IL-3) with added IgE (described in Methods section 2.3.1) (Samayawardhena and Pallen, 2010). After washing off excess IgE, the cells were incubated in Tyrode's buffer and stimulated with a range of antigen (DNP-HSA) doses for 15 min. After cooling on ice, supernatant containing mast cell secretions was collected by centrifugation, and the cell pellet was lysed. The degranulation supernatants and cell pellet fractions were assayed for β-hexosaminidase enzymatic activity (a component of mast cell granules) to determine the percentage of this enzyme that was released due to antigen stimulation. However, my initial antigen-mediated degranulation experiments showed that over 90% of the total β-hexosaminidase activity still remained in the cell pellet fraction, indicating a total degranulation of less than 10% for WT BMMCs. Since the typically reported values for degranulation with 10 ng/ml antigen range from 20-40% (Bischoff and Dahinden 1992; Columbo et al., 1992; Hernandez-Hansen et al., 2004; Kuehn et al., 2008; Samayawardhena and Pallen, 2008; Vosseller et al., 1997), these initial results were

clearly abnormal. To assess whether the intrinsic degranulation ability of the BMMCs was intact, cells were also stimulated with the calcium ionophore A23187 (Sigma-Aldrich), which bypasses receptor-mediated signalling to directly induce calcium influx that triggers degranulation. In this case, 500 nM of A23187 induced over 30% degranulation, as evidenced by the decrease in β-hexosaminidase product absorbance in the pellet fraction and the corresponding increase in the secretion supernatant fraction. Thus the observed weak response to antigen was not due to defects in the inherent degranulation ability of the mast cells used.

Antigen-stimulated lysates were also immunoblotted for global protein tyrosine phosphorylation (4G10 Ab) as well as for Erk1/2 MAPK activation. In both cases there was a clear induction of protein phosphorylation in Ag-stimulated samples compared to unstimulated controls (data not shown, see Chapter 4 for signalling analysis). Therefore, FcεR1 pathway activation was being triggered by antigen to some extent. Attempts to increase IgE dosage or shorten the IL-3 starvation period had little effect, as the degranulation responses to the same Ag doses still varied between 5-15%, which is much lower than commonly reported for WT BMMCs.

Several recent reports indicate that IgE functions as more than a mere linker between antigen and the FcεR1 receptor, as it can also greatly affect the physiology of mast cells in the absence of any antigen. Prolonged exposure to monomeric IgE molecules alone promotes mast cell survival, activation, and chemotaxis (Ekoff et al., 2007; Kashiwakura et al., 2011; Kawakami and Kitaura, 2005; Sly et al., 2008),

as well as enhancement of the surface expression of FcεR1 receptor and mediator release (Hsu and MacGlashan, 1996; Lantz et al., 1997; Yamaguchi et al., 1997). This raised the possibility that perhaps the weak degranulation response to antigen was due to insufficient IgE binding and overall BMMC sensitization. To test this hypothesis I first tried sensitizing the cells in a fresh aliquot of normal growth media containing IL-3 for 16 hr with 200 ng/ml IgE (see Methods section 2.3), while maintaining the required 8 hr pre-stimulation IL-3 starvation period. Thus cells were exposed to IgE for close to 24 hours, with the last 8 hours in IL-3-free media.

This protocol resulted in a dramatic increase in Ag-mediated BMMC degranulation, particularly at the lower dose of 1.0 ng/ml of the antigen DNP-HSA. Figure 3.1A shows the effects of the 'Old' and 'New' (prolonged) sensitization methods on the degranulation of WT and PTPα-KO cells from side-by-side cultures that were stimulated with antigen at the same time. Negligible degranulation (~2%) was detected in unstimulated, control BMMCs that had been sensitized with either method. The 'New' method of prolonged sensitization more than tripled the amount of β-hexosaminidase released upon stimulation with 1 ng/ml Ag, as compared to the 'Old' method. At 100 ng/ml Ag, degranulation was only slightly increased by longer sensitization, likely due to inhibition of signaling by supraoptimal Ag concentrations (Fehrenbach et al., 2009). Furthermore, the new protocol greatly improved the overall consistency between degranulation experiments. In contrast, using the 'Old' sensitization protocol, I observed that the relatively low magnitude of WT BMMC degranulation with 10 ng/ml Ag varied between 5-15% in most experiments.

Surprisingly, PTPα-KO and WT BMMCs exhibited nearly identical degranulation responses with either sensitization protocol, which contradicts previous findings from our lab (Samayawardhena and Pallen, 2010). Earlier work by my colleague has demonstrated that BMMCs lacking PTPα produce significantly enhanced degranulation and cytokine release in response to antigen. In my hands, WT BMMCs exhibited a similar Ag degranulation dose curve to published reports, with optimal 40% degranulation at 10 ng/ml Ag and a sharp decrease with 100 ng/ml Ag (Fig.3.1A) (Bischoff and Dahinden, 1992; Columbo et al., 1992; Hernandez-Hansen et al., 2004; Kuehn et al., 2008; Vosseller et al., 1997). This suggests that the 'New' IgE sensitization protocol did not fundamentally alter how mast cells respond to antigen. Since mast cells are continuously exposed to IgE and various cytokines i*n vivo*, my experimental methods attempt to more closely simulate those conditions.

I next examined the ability of murine SCF to enhance the Ag-mediated degranulation response in WT and PTPα-KO cells with the 'New' sensitization protocol. Figure 3.1B shows that simultaneous addition of 10 or 100 ng/ml SCF and 10 ng/ml Ag to the cells synergistically enhanced the degranulation of WT BMMCs ($p<0.001$), with a maximal observed degranulation of 65%. With the supraoptimal 100 ng/ml Ag dose, additional SCF also enhanced degranulation in a dose-dependent matter, comparable to previous reports (Fehrenbach et al., 2009; Gilfillan et al., 2009; Vosseller et al., 1997). PTPα-KO BMMCs showed levels of SCF-enhanced degranulation very similar to WT cells under all the conditions tested.

Since BMMCs that lack proteins that negatively regulate FcεR1 receptor signalling, such as Lyn and SHIP, can degranulate in response to SCF alone (Hernandez-Hansen et al., 2004; Huber et al., 1998), and since PTPα is also reported to inhibit Ag/FcεR1 secretory responses (Samayawardhena and Pallen, 2010), I tested the response of PTPα-KO BMMCs to various doses of SCF alone. Figure 3.1B demonstrates that even 100 ng/ml SCF induced minimal degranulation of PTPα-KO BMMCs. Likewise, no significant degranulation was observed with 500 ng/ml SCF (data not shown). Thus unlike the situation with BMMCs lacking Lyn or SHIP inhibitory functions, the absence of PTPα does not alter c-Kit mediated signalling in a way that results in significant mast cell degranulation.

3.3 Synergistic Release of Cytokines

While the degranulation assay measures the early Ag-mediated secretory response of mast cells, prolonged stimulation promotes secretion of an array of potent pro-inflammatory cytokines that can modify immune responses. Co-stimulation of additional mast cell receptors, such as c-Kit, has been shown to synergistically boost cytokine secretion *in vitro* (Columbo et al., 1992; Fehrenbach et al., 2009; Gilfillan et al., 2009). Therefore, PTPα-KO and WT BMMCs were tested for the secretion of the cytokines TNFα and IL-13 that are linked to chronic allergic inflammation (Babu et al., 2011; Broide et al., 2011; Wang et al., 2010). BMMCs that had been IgE sensitized using both ‘New’ and ‘Old’ methods were stimulated with various combinations of Ag and SCF, and 15 hours later the media supernatants were

collected and assayed by ELISA. Figure 3.2 shows the ELISA assay results from such an experiment. Secretion of both TNFα (Fig.3.2A) and IL-13 (Fig.3.2B) was greatly enhanced by SCF co-stimulation, even in cells sensitized with the 'Old' method, while Ag or SCF alone at 100 ng/ml induced much weaker cytokine release than the combined stimuli. Optimal secretion of IL-13 and TNFα was triggered by a combination of 10 ng/ml Ag and 100 ng/ml SCF, which was also optimal for inducing the degranulation of BMMCs (Fig. 3.1B). Other combinations of Ag and SCF doses were tested for their ability to stimulate IL-6 production, yet no significant differences between WT and PTPα-KO BMMCs were observed (data not shown). Taken together, my results indicate that the absence of PTPα from BMMCs does not significantly affect antigen and/or SCF-mediated secretion of cytokines.

3.4 Analysis of Surface Binding of IgE to BMMCs

The lack of previously observed differences in Ag-induced responses between WT and PTPα-KO cells may be caused by the unequal binding of IgE to the different cell types. A lower cell surface density of IgE molecules in BMMCs lacking PTPα could compensate for their stronger FcεR1 signalling activation potential. Figure 3.3 shows a representative FACS analysis of BMMCs sensitized with the 'New' method and stained with anti-IgE and anti-c-Kit antibody probes. On average about 97% of the population of both cell types showed high levels of c-Kit and IgE staining, suggesting that WT and PTPα KO BMMCs do not differ in surface IgE binding with the new sensitization protocol.

3.5 Discussion

Mast cell secretory responses play important roles in health and disease. During the early infection of mucosal sites, mast cell-derived TNFα promotes optimal activation of antigen-presenting cells to direct proper T-cell responses (Abraham and St. John 2010; Laichalk et al., 1996; McLachlan et al., 2003). Animal studies show that IL-6 produced by mast cells is crucial for resistance to bacterial infections (Sutherland et al., 2008). Mast cell mediators, such as IL-4 and IL-13, promote mucous secretion and facilitate the elimination of helminth parasites (Scales et al., 2007). Depending on their environment and stimuli, mast cells can negatively modulate immune responses. Certain natural toxins, insect bites, and ultraviolet radiation stimulate mast cells to produce the immune-suppressive cytokine IL-10 (Kalesnikoff and Galli 2008). Interestingly, both mast cells and regulatory T-cells are required for peripheral tolerance of skin allografts (Lu et al., 2006). Since mast cell products may also promote angiogenesis and cell growth, they have recently been implicated in the regulation of cancer metastasis (Groot Kormelink et al., 2009; Maltby et al., 2009; Nechushtan, 2010).

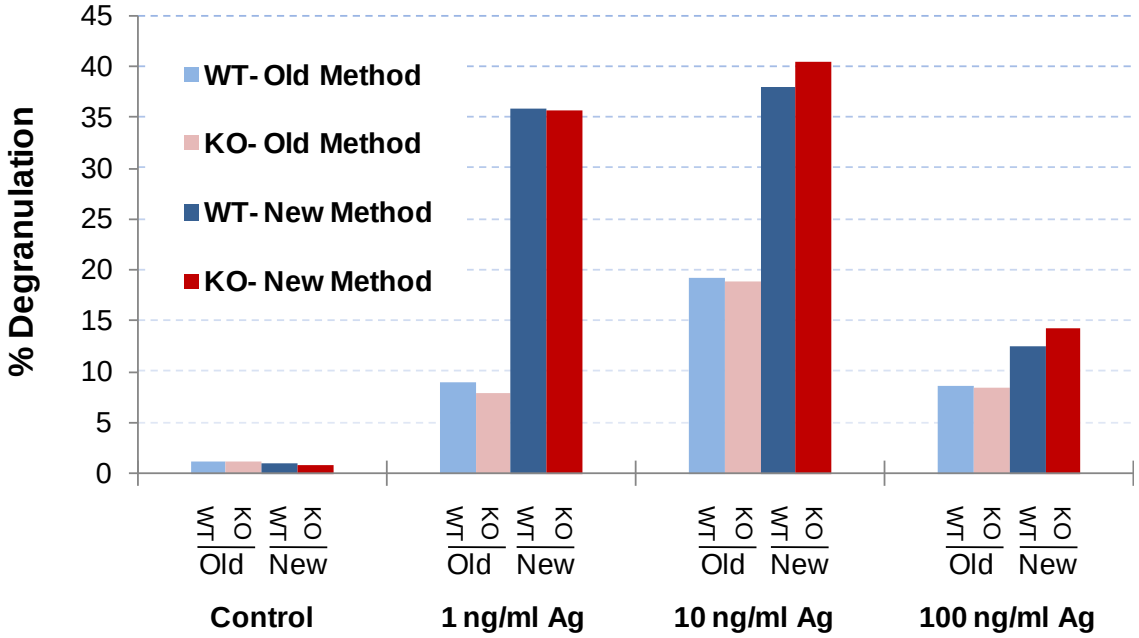
Not surprisingly, these potent mast cell-secreted mediators play important roles in autoimmune and allergic disorders. In anaphylaxis, strong activation of IgE/FcεR1 on mast cells triggers rapid secretion of vasodilators and pro-inflammatory cytokines, which can lead to life-threatening shock (Metcalfe et al., 2009; Moneret-Vautrin et al., 2005). In allergic asthma, mast cell mediators promote many of the acute and chronic symptoms of this disease (Broide et al., 2011; Da Silva et al., 2006; Holgate,

2008; Moiseeva and Bradding, 2011). Therefore, a better understanding of the mechanisms that regulate mast cell activation may reveal new insights into the pathogenesis of many immune disorders. PTPα-regulated Src family kinases are involved in immune receptor signalling pathways, in addition to cell migration (Chen et al., 2006; Chen et al., 2009; Maksumova et al., 2005; Pallen, 2003; Wang et al., 2009). Therefore, previous work in our lab has examined the role of PTPα in mast cell activation via the c-Kit and FcεR1 receptors. PTPα-KO BMMCs have been shown to have defective SCF-driven chemotaxis, while antigen/IgE stimulation resulted in elevated degranulation and cytokine production (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010).

To investigate the role of PTPα in mast cell responses in a situation that more closely approximates physiological conditions (i.e. more than one factor regulating mast cell activation), I examined the effect of PTPα in synergistic responses from the co-stimulation of c-Kit and FcεR1 receptors. Initially, my antigen stimulation experiments resulted in sub-optimal BMMC degranulation responses. Based on recent literature reports on the functions of IgE, I devised a modified BMMC sensitization protocol, which added a pre-incubation step with IgE before IL-3 starvation. This new method produced much more consistent results and a typical antigen dose-response curve for WT BMMCs. However, PTPα-KO cells displayed nearly identical extent of degranulation to WT BMMCs, with no evidence of the hyperdegranulation previously detected in our lab (Samayawardhena and Pallen, 2010). The magnitude of SCF-enhanced degranulation was also similar between

both PTPα genotypes of BMMCs. I further examined the secretion of the mast cell cytokines TNFα and IL-13 upon stimulation with various dose combinations of antigen and SCF. Once again, no significant differences were observed between WT and PTPα-KO mast cell secretory responses. To try to determine why my data differed from previous results in my lab, I used FACS analysis to measure IgE binding to PTPα-WT and KO BMMCs. Both cell populations showed comparable IgE staining intensities, suggesting equal sensitization with the 'New' protocol. I also tried to find any methodological differences between my experimental techniques and those of my previous lab co-worker that may explain our varying results. Several PTPα-KO and WT BMMCs that I had cultured were stimulated with antigen by my co-worker, but none of the phenotypic differences previously established in our lab were observed. This raises the possibility that changes in the cultured BMMCs may have instead altered their intrinsic responsiveness to antigen. Taken together, my data show that PTPα neither regulates antigen mediated secretion nor its enhancement by SCF.

A



B

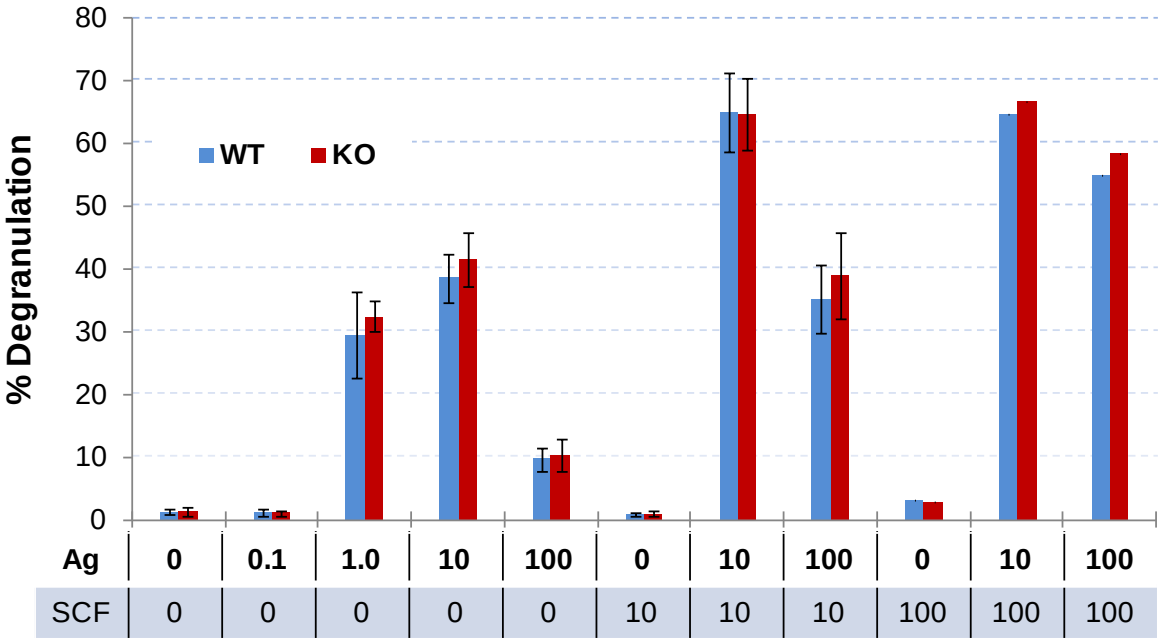
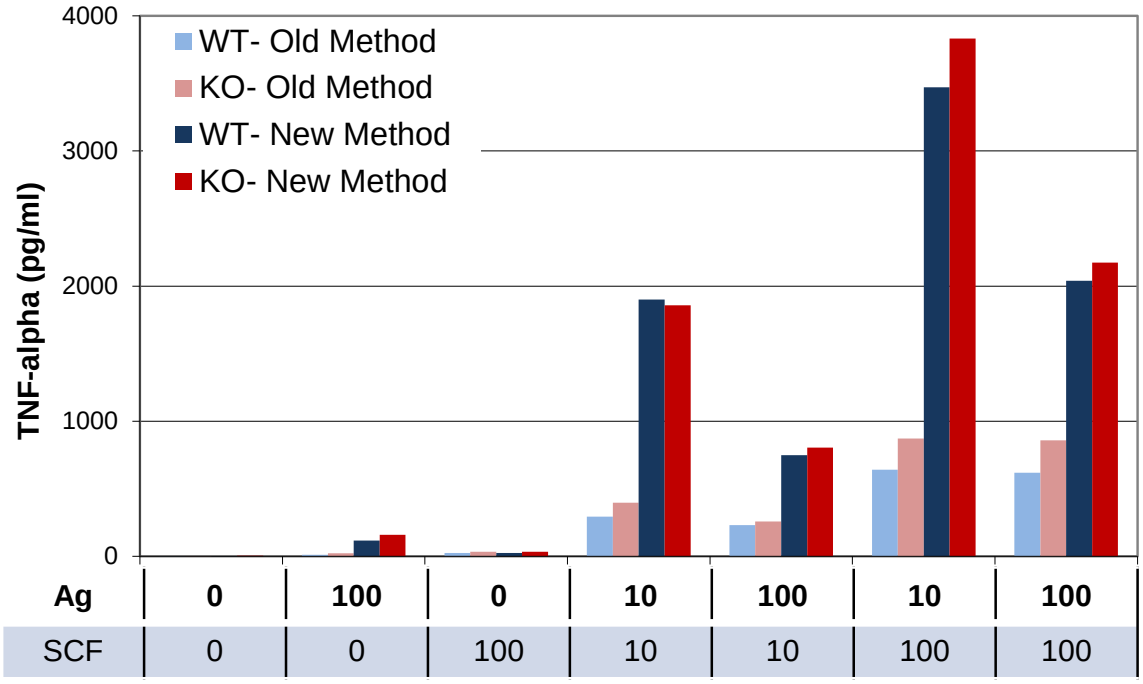


Figure 3.1 SCF enhances antigen-mediated degranulation. **(A)** Cells from the same WT and PTPα-KO BMMC cultures were sensitized using 'Old' or 'New' methods (as described in section 2.3), stimulated with the indicated doses of antigen (ng/ml DNP-HSA) for 15 min and assayed for degranulation (as described in section 2.7). Results of a single experiment are shown. **(B)** WT and KO BMMCs were sensitized with IgE (using 'New' method) and stimulated with the indicated doses of Ag or murine SCF, or the combination of both added simultaneously. The graphs show the mean ±S.D. from 4-5 independent experiments. For conditions with 100 ng/ml SCF the means of 2 independent experiments are shown.

A



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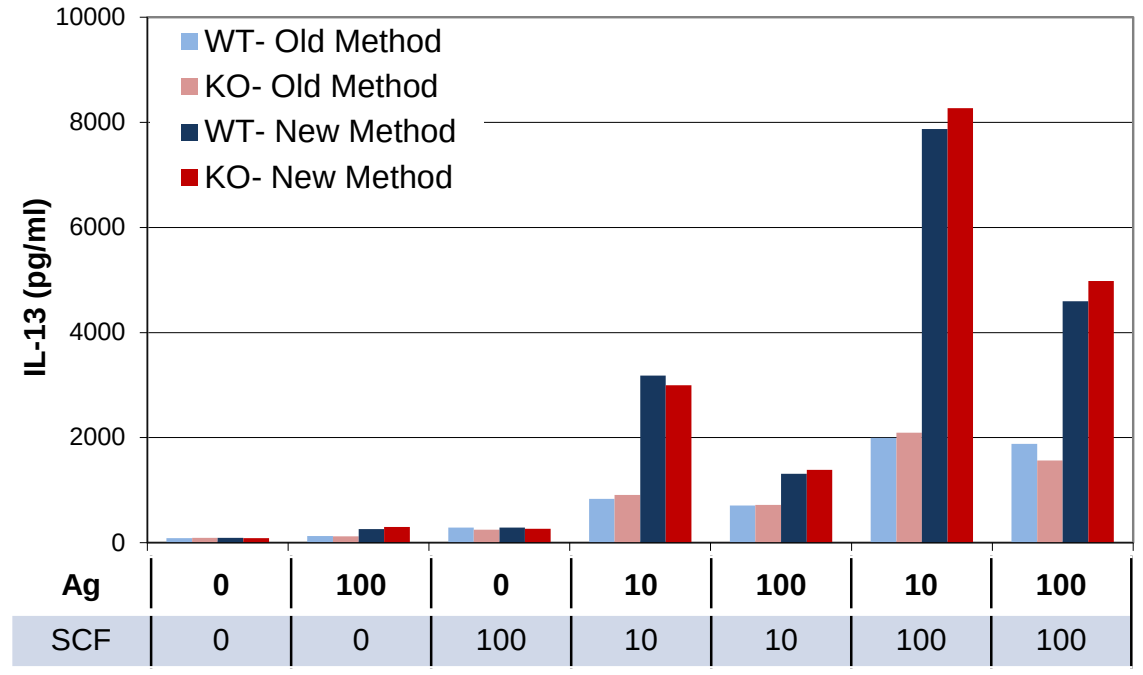


Figure 3.2 SCF-enhanced cytokine secretion. Cells from the same WT and PTPα-KO BMMC cultures were sensitized using the 'Old' or 'New' methods (as described in section 2.3). Cells were incubated in low IL-3 media with the indicated doses of antigen or murine SCF or with both simultaneously for 15 hours (as described in section 2.8). Secretion media supernatants were assayed by ELISA for **(A)** TNFα and **(B)** IL-13. The data shown are from a single overnight stimulation experiment.

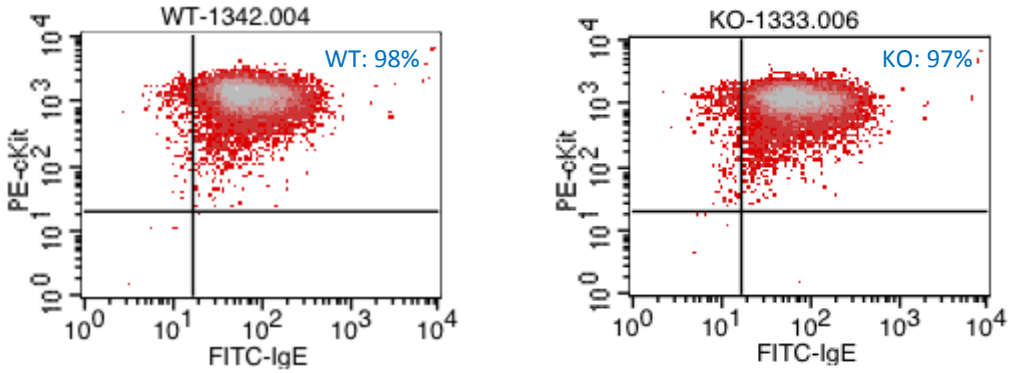


Figure 3.3 BMMC surface presentations of IgE and c-Kit after prolonged sensitization protocol. WT and PTPα-KO BMMCs were cultured for 5 weeks and were IgE sensitized using the 'New' protocol (as described in section 2.3). Cells were stained with PE-conjugated anti-c-Kit and FITC-conjugated anti-IgE antibodies and analyzed by flow cytometry. Particle size gating and signal compensation were evenly applied. Similar results were obtained from two independent experiments involving different BMMC cultures.

Chapter 4: Role of PTPα in Integrated c-Kit/FcεR1 Signaling

4.1 Rationale

Mast cells are capable of initiating and modifying immune responses that underlie various human diseases. In allergic asthma elevated levels of serum IgE are linked to the overabundance and hyperactivation of mast cells (Brown et al., 2008; Gilfillan et al., 2011; Holgate, 2008). The assortment of inflammatory mediators released by mast cells drives many allergic symptoms. Much effort has been focused on elucidating the mechanisms of antigen and IgE signaling via the receptor FcεR1 that triggers secretory responses of mast cells. Src family kinases have been shown to regulate key early events of the FcεR1 receptor activation and recruitment of scaffolding and adaptor proteins linked to downstream signaling cascades (Furumoto et al., 2005; Gilfillan and Rivera, 2009; Poderycki et al., 2010; Xiao et al., 2005). Other mast cell signaling pathways that respond to cytokines also play important roles in modulating mast cell responses. The growth factor SCF promotes the survival and development of several immune cell progenitors, as well as the tissue recruitment of mature mast cells (Bischoff and Dahinden, 1992; Fehrenbach et al., 2009; Gilfillan et al., 2009; Vosseller et al., 1997). SCF stimulates the dimerization of the receptor tyrosine kinase c-Kit, which activates the PI3K and MAPK cascades that are also involved in Ag/FcεR1 signaling. As a result, SCF increases the activation of key antigen signaling intermediates, leading to the synergistic enhancement of mast cell degranulation and cytokine release. Increased

levels of SCF in tissues promote the recruitment of mast cells, resulting in worsened allergic symptoms (Al-Muhsen et al., 2004; Da Silva et al., 2006; Moiseeva and Bradding, 2011).

Our lab has previously studied the role of PTPα in SCF-driven mast cell migration, and its regulated cell spreading and polarization (Samayawardhena and Pallen, 2008). PTPα-KO BMMCs displayed defective activation of Fyn tyrosine kinase, phosphorylation of c-Kit motifs, and reduced activation of the Rac/Jnk pathway, which promotes cell migration in response to SCF. PTPα was also shown to differentially regulate antigen-induced mast cell secretory responses. This was in part due to PTPα-mediated activation of the negative regulator Lyn and inhibition of PI3K and MAPK pathways (Samayawardhena and Pallen, 2010). Since PTPα positively regulates c-Kit, and negatively regulates FcεR1 signaling, I investigated the role of PTPα in the activation of crucial signaling proteins involved in integrated FcεR1/c-Kit signaling, which controls the synergistic release of mast cell mediators.

4.2 Global Tyrosine Phosphorylation

The newly established IgE sensitization protocol was used to assess the global protein tyrosine phosphorylation in BMMCs stimulated with combinations of antigen and SCF for various times. Treatment for 2 minutes with antigen was sufficient to activate the FcεR1 pathway, and was chosen as the earliest time point to examine mast cell signaling events. The 5 min time point was used, because it is the peak of c-Kit-mediated phosphorylation of Akt (described in section 4.4). At 15 min, the

antigen-induced global tyrosine phosphorylation and the activation of MAPKs was previously observed to diminish to near-baseline levels (Samayawardhena and Pallen, 2010). Therefore, 2 min, 5 min, and 15 min time points after stimulation were used to examine the activation of key signaling proteins. The left panel in Figure 4.1A shows the timecourse of protein tyrosine phosphorylation in WT and PTPα-KO cells that were treated with 100 ng/ml antigen (DNP-HSA) alone. WT BMMC activation peaked at 2 min and was slightly decreased by 5 min, and then declined to control levels by 15 min. Global tyrosine phosphorylation in PTPα-KO BMMCs appeared very similar to the levels in WT cells. A previous study from my lab showed that antigen-triggered global tyrosine phosphorylation peaked at 1.5 min, decreasing to minimal levels by 5 min, and appeared slightly enhanced in PTPα-KO BMMCs (Samayawardhena and Pallen, 2010). In contrast, in my experiments, Ag-mediated protein tyrosine phosphorylation remained strong by 5 min, and was not visibly affected by PTPα.

Other BMMCs from the same cultures were stimulated simultaneously with antigen and SCF. The right panel in Figure 4.1A shows that protein tyrosine phosphorylation of WT and PTPα-KO BMMCs is markedly enhanced by the addition of SCF, relative to cells treated only with antigen. At 15 min of stimulation, tyrosine phosphorylation did not appear to diminish for most proteins seen, and was especially strong for proteins of about unidentified 150 kDa (Fig.4.1A, arrow) that had little tyrosine phosphorylation with antigen alone. This SCF-enhanced activation of signaling pathways agrees well with its effect of boosting mast cell secretory responses, and

was not significantly altered in BMMCs lacking PTPα. To further elucidate the role of PTPα in c-Kit/ FcεR1 integrated signaling, specific downstream pathways were examined.

4.3 Antigen Treatment Induces the Dephosphorylation of PTPα Tyrosine 789

PTPα is an important regulator of Src family kinases (SFKs) through dephosphorylation of key tyrosine residues. PTPα can itself become a substrate of SFKs *in vivo*. Overexpression of Src in human embryonic kidney 293 cells increases the phosphorylation of PTPα at tyrosine 789 in its C-terminal region, creating a binding site for the Grb2 adaptor protein (den Hertog et al., 1994). Recent evidence suggests that PTPα Tyr-789 may regulate receptor-mediated cell migration. In fibronectin-stimulated fibroblasts PTPα becomes phosphorylated at Tyr-789, which activates SFKs and promotes migration (Chen et al., 2006). However, no reports that examined PTPα Tyr-789 phosphorylation in activated mast cells have been published.

I used a polyclonal antibody against phosphoTyr-789 of PTPα, produced in our lab (Chen et al., 2006), to probe lysates from Ag-stimulated BMMCs. Unstimulated (control) cells showed considerable phosphorylation of PTPα Tyr-789. Within 2 minutes of antigen treatment, constitutive PTPα phosphorylation decreased by close to 70%, and remained low for up to 15 min (Figure 4.1B). Stimulation with SCF alone for 5 min induced lesser (~ 25%) Tyr-789 dephosphorylation, and ~40% dephosphorylation was observed after 5 min treatment with a combination of antigen

and SCF (Figure 4.1C). No phospho-Tyr-789 signal was observed in PTPα-KO samples. These data confirm that PTPα is involved in FcεR1 signalling, though the function of PTPα phospho-Tyr-789 in mast cells is as yet unknown.

4.4 PI3K/Akt Pathway

The phosphatidylinositol 3-kinase (PI3K) pathway is central to the control of cell functions such as survival and differentiation in response to various external stimuli. In mast cells, SCF/c-Kit stimulation activates the PI3K/Akt pathway, which leads to the synergistic enhancement of Ag/FcεR1-mediated secretory responses (Gilfillan et al., 2009; Iwaki et al., 2005). To determine the role of PTPα in the induction of PI3K signaling during BMMC co-stimulation with antigen and SCF, cell lysates were immunoprobed for the activated phosphoSer-473 form of Akt at three time points. The top panel in Figure 4.2A shows Akt phosphorylation after treatment for 2 min, where the graphs on the right depict AKT phosphorylation normalized to Akt amount, averaged from several experiments. At the early 2 min time point, only SCF alone induced readily detectable Akt phosphorylation, which appeared somewhat higher in PTPα-KO BMMCs, though this difference was not statistically significant. The BMMCs challenged with 100 ng/ml antigen alone exhibited much weaker Akt phosphorylation at all time points than that induced by SCF treatment, and indeed only faint bands were visible after maximal exposure (data not shown). Figures 4.2B and 4.2C show AKT phosphorylation at 5 and 15 min time points. The timecourse of Akt phosphorylation in response to SCF alone, and to the combination of antigen

and SCF, are illustrated in Figure 4.2D. Phospho-Akt bands were strongest after 5 min of treatment with SCF.

Interestingly, the presence of antigen as a co-stimulus with SCF nearly completely abolished the early Akt phosphorylation at 2 min (Fig 4.2A). Despite this inhibition, antigen and SCF together induced increasing Akt phosphorylation between 5 and 15 min, whereas Akt phosphorylation decreased during this period of treatment with SCF alone (Fig 4.2D). A similar phenomenon has been reported by others, and shown to require the inhibitory activity of the Src family kinase Lyn to reduce Akt phosphorylation (Iwaki et al., 2005). No significant differences in Akt activation were observed between WT and PTPα-KO BMMCs after 5 min SCF stimulation. After 15 min stimulation, there was a significant reduction in phospho-Akt signal in PTPα-KO cells (Fig. 4.2C and 4.2D). SCF-stimulated Akt phosphorylation was reduced in PTPα-KO BMMCs by nearly 25% relative to WT cells, and the addition of antigen further reduced Akt phosphorylation in both cell types. Therefore, both Ag/FcεR1 signalling and the absence of PTPα appear to independently inhibit the SCF/c-Kit-induced activation of PI3K signaling to Akt.

4.5 PLCγ1 Activation

In mast cells, cytokines and immune receptors activate phospholipase C gamma 1 (PLCγ1) signalling, which promotes an increase in intracellular calcium to initiate gene transcription, mediator release, and cell migration (Fehrenbach et al., 2009; Kuehn et al., 2008; Nishida et al., 2005; Oka et al., 2004; Parravicini et al., 2002;

Sanchez-Miranda et al., 2010). While SCF stimulation alone is not capable of triggering degranulation in WT mast cells, it has been shown to boost antigen-induced secretory responses via enhanced activation of PLCγ signaling (Gilfillan et al., 2009).

My next goal was to determine for the first time the effect of PTPα in Ag- and/or SCF-induced activation of PLCγ1. Figure 4.3 shows the phosphorylation of PLCγ1 after 2, 5, and 15 min of BMMC stimulation. Using a phospho-specific antibody against Tyr-783 of PLCγ1, the phosphorylation of PLCγ1 in stimulated BMMC lysates was normalized to total β-actin (right panels). Actin was also used as a measure of total protein loading for the quantification of PLCγ1 and MAPK phosphorylation (following sections), due to less consistent protein bands seen after re-probing membranes that had been stripped of phospho-antibodies. At 2 min and 5 min time points, treatment with Ag, or Ag plus SCF, induced prominent phosphorylation of PLCγ1 (Figure 4.3A and B). The level of phosphorylation induced by SCF alone appeared similar to untreated controls. After 2 min, PLCγ1 phosphorylation in response to Ag alone or to the combination of Ag and SCF began to diminish (Figure 4.3D). Co-treatment with antigen and SCF induced much greater PLCγ1 activation than with either stimulus alone (Figure 4.3 A-C). Similar responses were observed between WT and PTPα-KO cells stimulated with SCF alone, or with Ag and SCF combined. Most notably, at all three time points PLCγ1 phosphorylation in antigen-challenged PTPα-KO BMMCs was nearly double that in WT cells. A similar synergy of antigen and SCF in the activation of PLCγ1 has previously been

reported (Iwaki et al., 2005). My data show that PTPα negatively regulates antigen-induced PLCγ1 signaling, but has no effect on its SCF-enhanced activation.

4.6 p38 Activation

The mitogen activated protein kinases (MAPKs), such as p38, mediate both secretion and migration of mast cells in response to diverse stimuli (Craig and Greer, 2002; Fehrenbach et al., 2009; Ishizuka et al., 2001a; Iwaki et al., 2005; Wong et al., 2006). It has been previously demonstrated that activation of p38 is differentially regulated by PTPα in mast cells. SCF stimulation of PTPα-KO BMMCs produced lower MAPK phosphorylation than WT cells, whereas Ag-mediated MAPK phosphorylation was enhanced in the absence of PTPα (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010). Therefore, the effects of PTPα on p38 phosphorylation in BMMCs co-stimulated with antigen and SCF was investigated. Figures 4.4A and 4.4B show that all three stimuli induce early p38 phosphorylation. Very similar levels of p38 phosphorylation were produced after 2 and 5 min stimulation with Ag, SCF, and Ag plus SCF. Surprisingly, at all the timepoints examined, Ag-induced p38 activation was not higher in PTPα-KO BMMCs compared to WT cells (Fig. 4.4A-D), in contrast to a previous finding in our lab (Samayawardhena and Pallen, 2010). Indeed, after 15 min of antigen and SCF co-stimulation, p38 activation was consistently reduced by 30% in PTPα-KO cells ($p<0.01$), and this was the only significant difference observed (Fig.4.4C). The synergistic enhancement of p38 phosphorylation after prolonged co-stimulation of WT BMMCs was previously reported (Fehrenbach et al., 2009; Iwaki et al., 2005).

My results demonstrate that PTPα positively regulates synergistic p38 activation after 15 min of Ag/SCF co-stimulation, but not with any other treatment tested.

4.7 Erk and Jnk Activation

The pro-inflammatory responses of mast cells to antigen or cytokines require sustained activation of other MAPK-mediated signaling cascades, namely involving the MAPKs Erk and Jnk (Chayama et al., 2001; Ishizuka et al., 1999; Lorentz et al., 2003; McPherson et al., 2009; Shivakrupa and Linnekin, 2005; Timokhina et al., 1998; Wong et al., 2006). PTPα has been shown to promote the SCF-induced activating phosphorylation of Erk and Jnk proteins, but to inhibit their antigen-induced activation (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010). Therefore, I investigated the role of PTPα in the phosphorylation of Erk and Jnk isoforms in BMMCs stimulated with antigen and/or SCF after 2, 5, and 15 min. Figure 4.5A shows representative blots of early (2 min, left panel) and late (15 min, right panel) Erk and Jnk phosphorylation. The phosphorylation levels after 5 min were intermediate to those at 2 min and 15 min time points (data not shown). Erk and Jnk phosphorylation after 2 min treatment with Ag, SCF, or Ag and SCF appeared similar, and was absent in unstimulated control cells (Fig.4.5, left panel; quantification data not shown). After 15 min of antigen stimulation, the phosphorylation of Erk and Jnk had diminished greatly (Fig.4.5, right panel). Co-treatment with SCF considerably enhanced the phosphorylation of Erk and Jnk after 15 min (Fig 4.5A, right panel), and in accord with previous reports of the synergistic effects of prolonged c-Kit and FcεR1 stimulation on the activation of Erk, Jnk, and

p38 MAPKs (Fehrenbach et al., 2009; Iwaki et al., 2005). However, at all time points and in all the conditions tested, the phospho-Erk and phospho-Jnk signals did not exhibit any significant differences for WT and PTPα-KO cells, as was confirmed by densitometric quantification (data not shown). Similar to p38 activation, the absence of PTPα did not have any apparent effect on antigen-induced Jnk and Erk phosphorylation, in contrast to a previous report (Samayawardhena and Pallen, 2010). Therefore, I found no evidence of PTPα regulating Erk and Jnk MAPKs with either method of mast cell stimulation examined.

4.8 c-Kit Receptor Phosphorylation

SCF/c-Kit signalling is important for the development, migration, and activation of mast cells. (Bellone et al., 2001; Da Silva et al., 2006; Gilfillan et al., 2009; Okayama and Kawakami, 2006). As shown earlier, stimulation with SCF greatly enhances mast cell secretory responses. Additionally, previous work in our lab has shown that in the absence of IgE, SCF-stimulated PTPα-KO BMMCs exhibit lower Fyn kinase activation and c-Kit phosphorylation, with decreased Rac/Jnk pathway activation, resulting in defective mast cell chemotaxis (Samayawardhena and Pallen, 2008). I further investigated whether PTPα also positively regulates SCF/c-Kit receptor phosphorylation upon Ag/ FcεR1 co-stimulation, which has been shown to modulate mast cell migration and secretory responses (Bischoff and Dahinden, 1992; Columbo et al., 1992; Ishizuka et al., 2001a; Kuehn et al., 2010; Sawada et al., 2005). IgE-sensitized WT and PTPα-KO BMMCs were stimulated with antigen, SCF, or antigen and SCF for various times. The cell lysates were immunoprobed for the

phosphorylation of key c-Kit residues, Tyr-567/569 and Tyr-719, as well as for total c-Kit protein.

Figure 4.5B shows the c-Kit phosphorylation at 2 min and 15 min time points. C-Kit phosphorylation after a 5 min treatment was also examined, and looked very similar to the response after 15 min (data not shown). No phospho-c-Kit signals were observed in untreated and antigen-challenged BMMCs after 2 min or 15 min. Treatment with SCF alone produced strong phosphorylation at both c-Kit sites, and this lasted up to 15 min. Interestingly, I observed that Ag/FcεR1 co-stimulation strongly inhibits SCF-induced c-Kit phosphorylation. After 2 min, co-treatment with antigen nearly completely abolished SCF-induced c-Kit Tyr-567/569 phosphorylation in WT and PTPα-KO BMMCs (Fig 4.2B, left panel). This inhibitory effect was seen up to 15 min after stimulation. No significant difference in c-Kit phosphorylation between WT and PTPα-KO BMMCs was observed under all the conditions tested. After 15 min of treatment with antigen and SCF, the total detectable amounts of c-Kit protein were noticeably lower than in unstimulated controls, most likely due to receptor internalization and degradation (Fig 4.2B, bottom right panel). While antigen alone did not induce c-Kit phosphorylation, it also promoted a decrease in the total c-Kit protein after 15 min stimulation, although it was not as pronounced as the reduction induced by SCF alone or in combination with antigen. Overall, IgE-sensitized PTPα-KO BMMCs showed equivalent c-Kit phosphorylation to WT cells, in response to SCF alone or in combination with antigen.

4.9 FcεR1 Receptor Phosphorylation

Binding of IgE to polyvalent antigen molecules promotes FcεR1 aggregation on mast cells and the activation of receptor-proximal Src family kinases, which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the FcεR1 β and γ subunits (Gilfillan and Rivera, 2009; Sanchez-Miranda et al., 2010; Xiao et al., 2005). The tyrosine kinase Syk binds to these ITAMs and activates downstream signalling proteins, triggering mast cell secretory responses and chemotaxis. Previous work in our lab demonstrated that PTPα regulates FcεR1 signaling via key Src family kinases. Antigen-stimulated PTPα-KO BMMCs showed lower phosphorylation of FcεR1 β and γ chains, despite exhibiting higher activation of downstream MAP kinase cascades and enhanced mediator release (Samayawardhena and Pallen, 2010).

Therefore, I investigated whether PTPα also regulates antigen-induced FcεR1 activation upon SCF co-stimulation. Since receptor phosphorylation is an early signalling event, occurring soon after antigen exposure, this was probed at only the 2 min time point in IgE sensitized WT and PTPα-KO BMMCs that had been stimulated with antigen alone, antigen plus SCF, or neither (control). Antibodies against the FcεR1γ subunit were used to immunoprecipitate the receptor complex. Immunoprecipitates were first probed with anti-phosphotyrosine (4G10) antibodies, then the blots were stripped and reprobed for the FcεR1β and γ subunits. Probing for phospho-FcεR1γ produced very diffuse bands that could not be clearly compared (data not shown). However, phosphorylation of the co-immunoprecipitated FcεR1β

polypeptide was readily detected in antigen-treated WT and PTPα-KO BMMCs (Fig. 4.6A, top panel). Addition of SCF to antigen considerably enhanced the FcεR1β phosphorylation. The FcεR1β antibody did not produce clear signals that could be accurately quantified (Fig. 4.6A, bottom panel). Despite this, the phospho-FcεR1β signals appeared similar in stimulated WT and PTPα-KO BMMCs in three independent experiments. Given the observed lack of differences in the phosphorylation of FcεR1β and the downstream secretory responses, I found no evidence of PTPα-regulated FcεR1 activation in my experiments.

4.10 Lyn Activation

The Src family kinase Lyn plays a complex role in the regulation of FcεR1 signaling in mast cells (Gilfillan and Rivera, 2009). Upon antigen-mediated aggregation of FcεR1, receptor-bound Lyn phosphorylates ITAMs and the downstream adaptor proteins LAT and NTAL. However, Lyn-deficient mice display enhanced anaphylactic responses, and hyperdegranulation of their mast cells in response to antigen, or even SCF alone (Hernandez-Hansen et al., 2004; Odom et al., 2004). Lyn kinase negatively regulates mast cell activation upon strong antigen stimulation, in part by targeting C-terminal Src kinase (Csk) to the membrane, where it phosphorylates the inhibitory C-terminal tyrosine residues of Src family kinases. The functions of Csk are antagonized by PTPα, which removes these inhibitory phosphorylations (Pallen, 2003). Given its ability to regulate Src family kinases, PTPα has been previously investigated in the antigen-mediated activation of Lyn. PTPα-KO BMMCs showed reduced phosphorylation of Lyn at the activating Tyr-396 site and reduced *in vitro*

kinase activity, which corresponded with higher degranulation and cytokine production in response to antigen (Samayawardhena and Pallen, 2010). These results are in agreement with the negative regulatory functions of Lyn in response to BMMC stimulation with high doses of antigen.

To determine if Lyn was responsible for the observed lack of effect of PTPα depletion on secretory responses, I examined Lyn phosphorylation in WT and PTPα-KO BMMCs in response to antigen alone, or in combination with SCF. BMMCs were stimulated for 2 min and Lyn was immunoprecipitated from cell lysates. The immunoprecipitates were probed with anti-phosphoTyr-416-Src or anti-phosphoTyr-507-Lyn antibodies, which respectively recognize the activating Tyr-396 and the inhibitory Tyr-507 phosphorylation sites in Lyn. Total Lyn protein amount in each immunoprecipitation sample was probed after stripping off the phospho-antibodies, or from parallel blots. Typical Lyn immunoblots are shown in Figure 4.6B. In untreated WT and PTPα-KO BMMCs there was considerable Lyn phosphorylation at Tyr-396, which decreased upon stimulation with antigen, or antigen plus SCF. This was unexpected, since phosphorylation of Lyn Tyr-396 has been shown to increase with antigen stimulation (Hong et al., 2007; Poderycki et al., 2010; Samayawardhena and Pallen, 2010). This phenomenon was observed in 4 separate experiments where samples were probed with different anti-phosphoTyr-416-Src antibody dilutions. When I tried IgE sensitizing the cells using the ‘Old’ method, identical Lyn dephosphorylation was observed (data not shown). Therefore, the abnormal phosphorylation of Lyn at Tyr-396 was not the result of the ‘New’ sensitization

protocol. Lyn immunoprecipitates were also probed for inhibitory phosphorylation at Tyr-507. Antigen treatment of WT and PTPα-KO cells produced no obvious changes in Lyn Tyr-507 phosphorylation (Fig 4.6B) relative to levels in unstimulated control cells. The similarly unchanged phosphorylation of Lyn after stimulation with antigen was also reported in a study examining the role of protein tyrosine phosphatase epsilon in mast cell activation (Akimoto et al., 2009). Co-stimulation with antigen and SCF likewise did not appear to affect the phosphorylation of Lyn at Tyr-507. Taken together these data show that PTPα does not regulate Lyn activation in BMMCs stimulated with antigen alone or in combination with SCF, which is consistent with the PTPα-independent degranulation and cytokine production observed in my experiments.

4.11 Discussion

In this chapter I assessed the effect of PTPα deletion on the activation of key signaling proteins involved in integrated c-Kit/FcεR1 responses of mast cells. Antigen triggered global tyrosine phosphorylation appeared to be greatly enhanced and prolonged by the addition of SCF, as was the synergistic degranulation and cytokine release. Both WT and PTPα-KO BMMCs exhibited a similar timecourse of global tyrosine phosphorylation, in response to antigen alone, or in combination with SCF. This is in agreement with the observed lack of differences in secretory responses in the absence of PTPα.

For the first time, the phosphorylation of PTPα at tyrosine 789 was evaluated in activated mast cells. My data showed that antigen stimulation greatly reduced (by ~70%) the intrinsic phosphorylation of PTPα Tyr-789 of BMMCs over the course of 15 minutes. SCF alone produced little dephosphorylation of PTPα Tyr-789, while antigen combined with SCF induced an effect intermediate to those caused by each stimulus alone. To the best of my knowledge this is the first such result reported. Antigen treatment of mast cells produces small oxidizing molecules that regulate the activity of the catalytic domain of PTPα and other protein tyrosine phosphatases (Heneberg and Draber, 2005). This could potentially serve as a feedback loop mechanism for controlling Ag-mediated signalling through PTPα, possibly via reactive oxygen or nitrogen species produced in the process. Despite emerging findings in other cell types, the biological role of PTPα Tyr-789 in mast cells remains unknown. Overall, my findings indicate that PTPα negatively regulates antigen-induced phosphorylation of PLCγ1, while positively regulating the phosphorylation of Akt and p38 upon prolonged co-stimulation with SCF and Ag. Table 4.1 summarizes key PTPα-dependent signaling alterations.

The effect of PTPα on the activation of PI3K/Akt pathway was evaluated. After 15 minutes SCF-stimulated PTPα-KO BMMCs exhibited a significant reduction in Akt phosphorylation, which was independent of antigen. This effect of PTPα is most likely mediated via the regulation of Src family kinases associated with the c-Kit receptor. Previously, mast cell PTPα was shown to not affect PI3K signalling upon SCF stimulation. The difference observed in my experiments could be caused by the

fact that all BMMC were first sensitized with IgE. While the PI3K pathway is not required for monomeric IgE-induced mast cell survival, it does promote actin assembly in the absence of antigen (Kohno et al., 2005; Oka et al., 2004). It is plausible that prolonged IgE sensitization may alter mast cell signalling responses to SCF.

SCF-mediated phosphorylation of the upstream c-Kit receptor was also unaffected by PTPα. This differs from previous results in our lab, where PTPα was shown to positively regulate c-Kit phosphorylation in BMMCs that had not been sensitized by IgE (Samayawardhena and Pallen, 2008). Co-stimulation with antigen was shown to severely inhibit SCF-induced phosphorylation of both c-Kit receptor and downstream Akt. No reports on the effects of concurrent Ag/FcεR1 signalling on c-Kit receptor activation were found. The simultaneous activation of FcεR1 may sequester tyrosine kinases required for proper SCF-induced c-Kit phosphorylation, or it may activate negative regulators. Despite being dephosphorylated upon antigen stimulation, PTPα does not appear to be involved in the Ag-induced inhibition of c-Kit phosphorylation. Taken together, my results show that PTPα positively regulates Akt activation after prolonged SCF stimulation, but has no effect on upstream c-Kit receptor phosphorylation in sensitized mast cells.

PLCγ1 is an important activator of calcium signaling, involved in mast cell migration and secretory responses. At all 3 time points, the lack of PTPα nearly doubled the activating phosphorylation of PLCγ1 in response to antigen. However, the increased

PLCγ1 phosphorylation did not lead to the expected enhanced degranulation and cytokine release in PTPα-KO BMMCs. It is possible that other PTPα-regulated mechanisms compensate for increased PLCγ1 activity during stimulation with antigen alone. Perhaps without PTPα, defective cytoskeletal rearrangement slows down granule exocytosis to a similar rate of that in WT BMMCs. The synergistic activation of PLCγ1 by antigen and SCF co-stimulation was unaffected by the loss of PTPα, and was consistent with the observed synergistic degranulation and cytokine release. Thus, the increased phosphorylation of PLCγ1 in PTPα-KO BMMCs during treatment with antigen alone (100 ng/ml) is eliminated when SCF (100 ng/ml) is added simultaneously. This could be due to a maximal possible phosphorylation of PLCγ1 is already achieved in co-treated WT cells, given that such stimulus produces near-maximal degranulation (Fig. 3.1B). In the future it would be interesting to determine whether PLCγ1-dependent calcium signaling and cytoskeletal rearrangements are affected by PTPα.

In contrast to previous data from our lab (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010), PTPα did not show any significant effect on the activation of MAPKs (p38, Erk and Jnk) induced by stimulation with either antigen or SCF alone. After 15 min co-treatment with both antigen and SCF, the synergistic activation of p38 was reduced by 30% in BMMCs lacking PTPα whereas no alterations in Erk and Jnk activation were detected. I also found no evidence of PTPα regulating Ag-induced activation of Lyn or FcεR1, which was previously reported in our lab (Samayawardhena and Pallen, 2010). The basis for such variable

mast cell responses is unknown, but possible reasons are discussed in Chapter 6. My findings indicate that in synergistically activated mast cells, PTPα is only required for full activation of Akt and p38. Despite being dephosphorylated during stimulation, PTPα did not alter mast cell secretory responses.

Table 4.1. PTPα-dependent signaling alterations in BMMCs activated with antigen and SCF.

Signaling Molecule	Stimulus	Time	Effect of PTPα absence
pAkt	SCF Ag+SCF	15 min	~ 25% decrease
pPLCγ1	Ag	2, 5, 15 min	~ 2X increase
pp38	Ag+SCF	15 min	~ 30% decrease

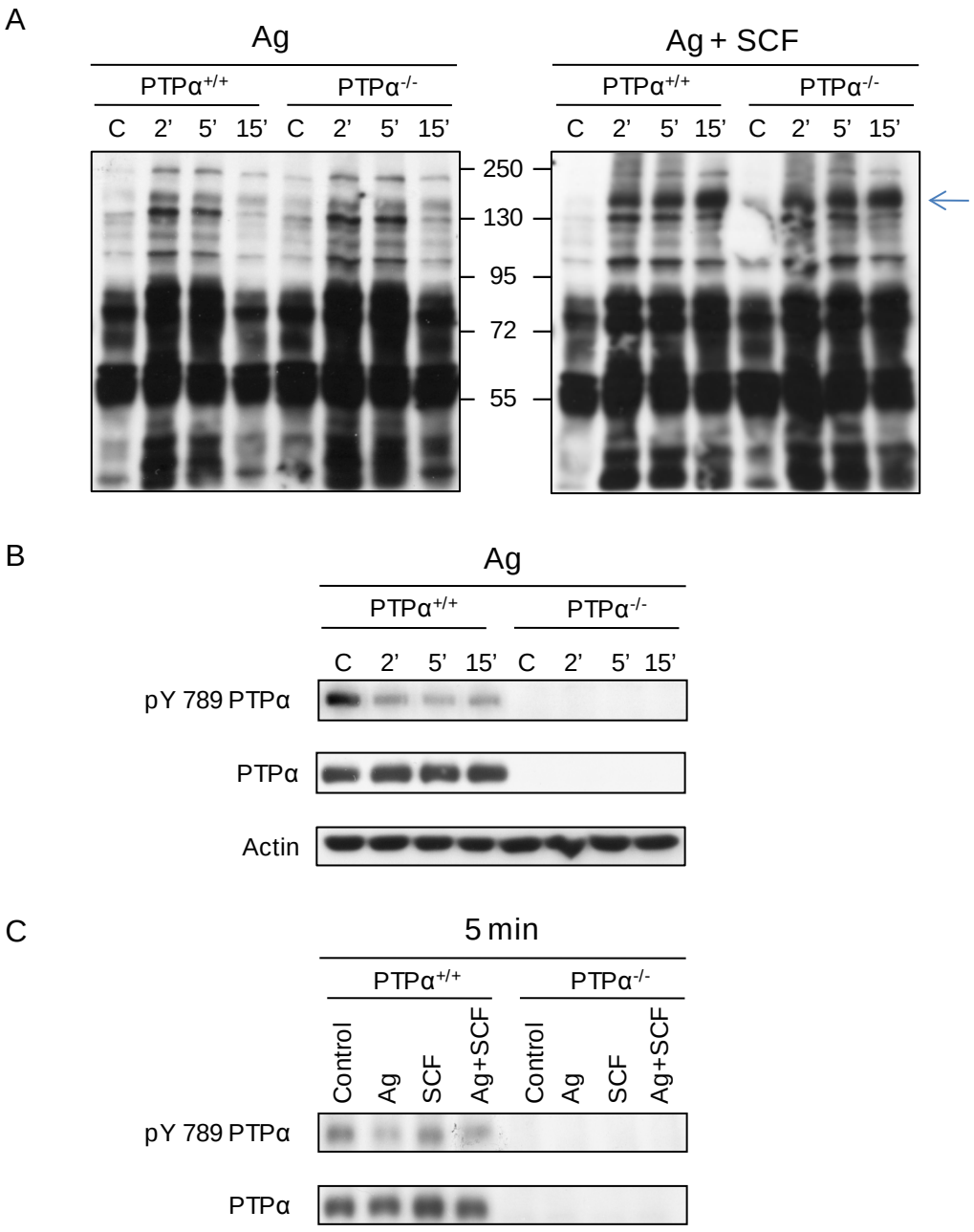


Figure 4.1. PTPα and global protein tyrosine phosphorylation. WT (PTPα$^{+/+}$) and PTPα-KO (PTPα$^{-/-}$) BMMCs were sensitized with IgE as described in Materials and Methods (section 2.3.2). The cells were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF) for the indicated times (min). **(A)** Whole cell lysates were immunoblotted with anti-phosphotyrosine (4G10) antibody. Marker sizes are represented in kDa. These blots show results from side-by-side cultures of WT and PTPα-KO cells treated at the same time. **(B)** Lysates from cells treated with antigen for 2, 5 and 15 min were immunoblotted for PTPα Tyr-789 phosphorylation. (C) WT and PTPα-KO BMMCs were treated for 5 min with antigen and/or SCF (100 ng/ml each), and probed for PTPα Tyr-789 phosphorylation.

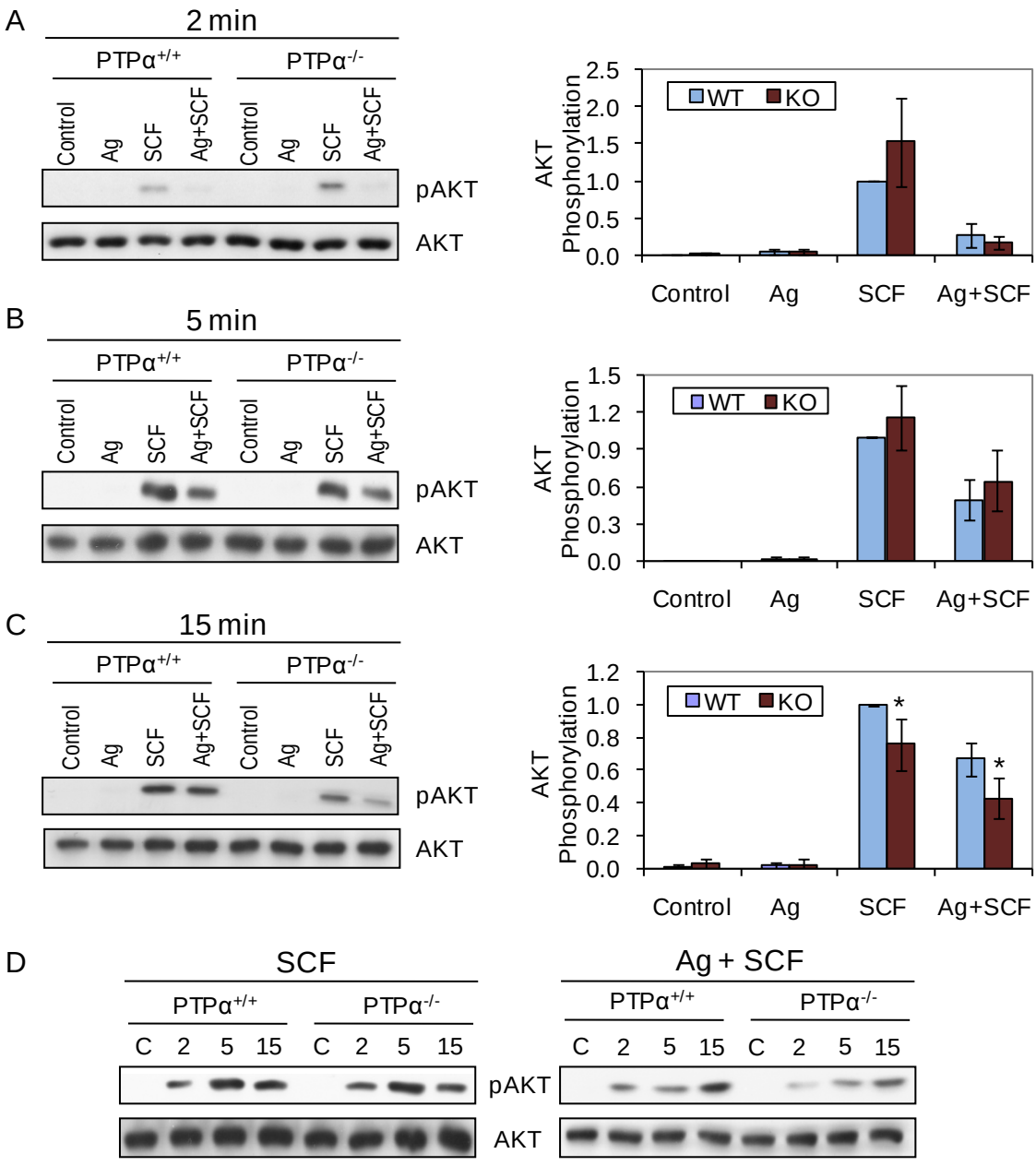


Figure 4.2. Effects of treatment with antigen and SCF on Akt activation in WT and PTPa-KO BMMCs. The sensitized BMMCs were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF). The cells were harvested at 2 min **(A)**, 5 min **(B)**, and 15 min **(C)** after treatment, and the lysates were probed for phosphoSer-473 Akt (top panels) and, on parallel blots, for Akt (bottom panels). The Akt phosphorylation per unit Akt was determined by densitometric quantification of the blots from at least 4 independent experiments, and is shown in the graphs to the right as the mean ± S.D., where Akt phosphorylation in WT BMMCs treated with SCF alone was arbitrarily taken as 1.0, and all other data are expressed relative to that. The asterisk indicates a significant difference ($p<0.05$) between WT and PTPa-KO samples for any one treatment. **(D)** The time-dependent stimulation of Akt phosphorylation was determined in response to SCF (left panels) and antigen plus SCF (right panels) as described above. The blots shown are representative of two experiments.

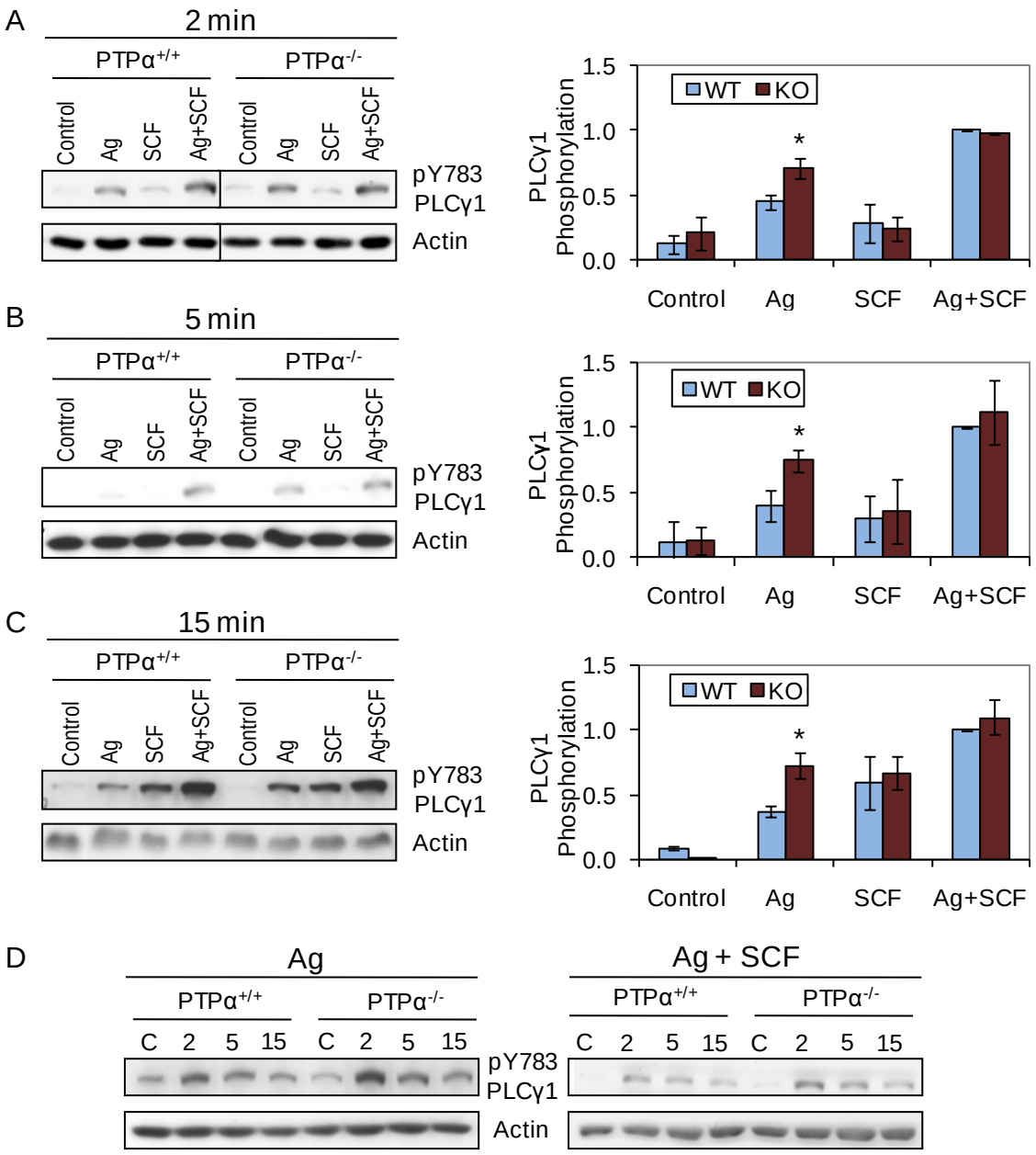


Figure 4.3. PTPα-KO BMMCs show enhanced activation of phospholipase C γ-1 upon treatment with antigen. WT (PTPα$^{+/+}$) and PTPα-KO (PTPα$^{-/-}$) cells were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF). The cells were harvested at 2 min **(A)**, 5 min **(B)**, and 15 min **(C)** after treatment, and the lysates were probed for phosphoTyr-783 PLCγ1 (top panels) and, on parallel blots, for Actin (bottom panels). The PLCγ1 phosphorylation per unit Actin was determined by densitometric quantification of the blots from at least 4 independent experiments, and is shown in the graphs to the right as the mean ± S.D., where PLCγ1 phosphorylation after each treatment was normalized to that in WT BMMCs treated with Ag plus SCF. The asterisk indicates a significant difference ($p<0.05$) between WT and PTPα-KO samples for any one treatment. **(D)** The time-dependent stimulation of PLCγ1 phosphorylation was determined in response to antigen (left panels) and antigen plus SCF (right panels) as described above. The blots shown are representative of two experiments.

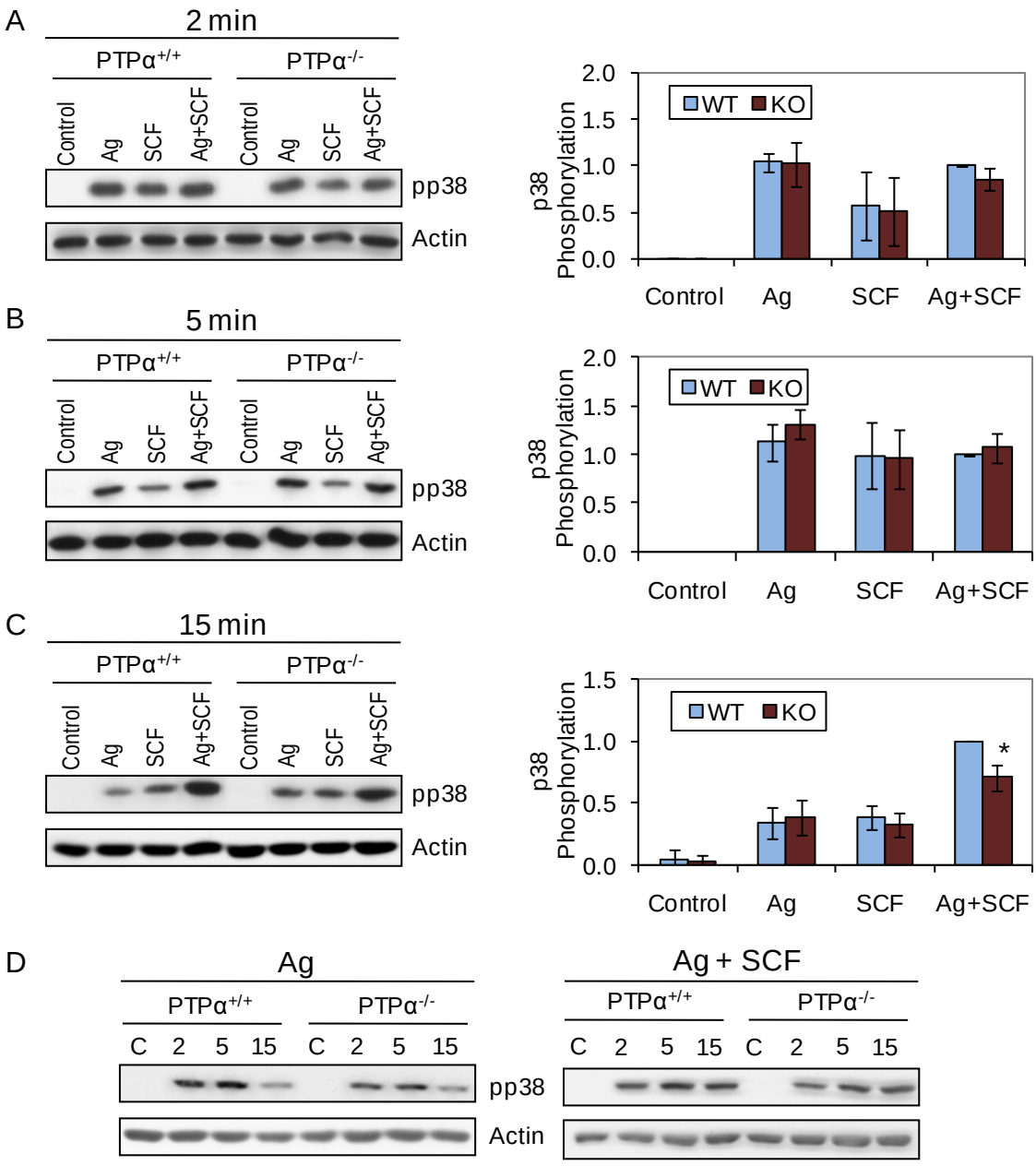
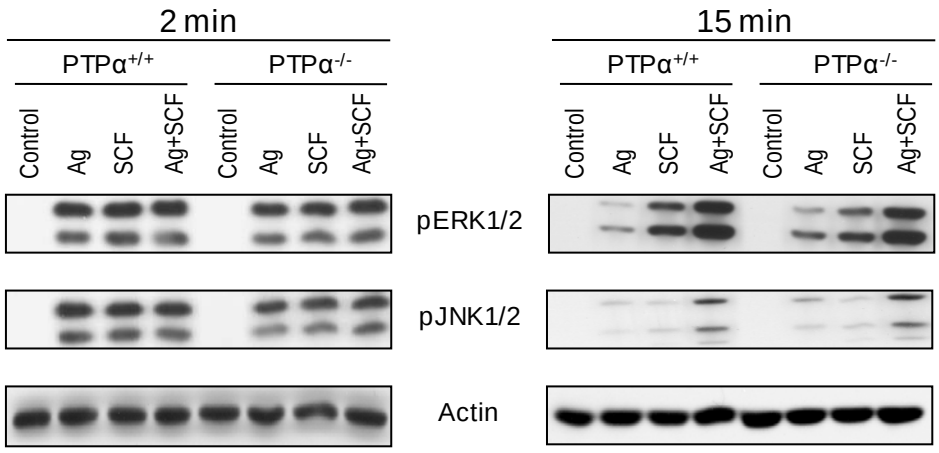


Figure 4.4. Antigen and/or SCF-induced phosphorylation of p38. WT (PTPα$^{+/+}$) and PTPα-KO (PTPα$^{-/-}$) cells were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF). The cells were harvested at 2 min **(A)**, 5 min **(B)**, and 15 min **(C)** after treatment, and the lysates were probed for phospho-p38 (pp38, top panels) and, on parallel blots, for Actin (bottom panels). The p38 phosphorylation per unit Actin was determined by densitometric quantification of the blots from at least 3 independent experiments, and is shown in the graphs to the right as the mean ± S.D., where p38 phosphorylation after each treatment was normalized to that in WT BMMCs treated with Ag plus SCF. The asterisk indicates a significant difference ($p<0.05$) between WT and PTPa-KO samples for any one treatment. **(D)** The time-dependent stimulation of p38 phosphorylation was determined in response to antigen (left panels) and antigen plus SCF (right panels) as described above. The blots shown are representative of two experiments.

A



B

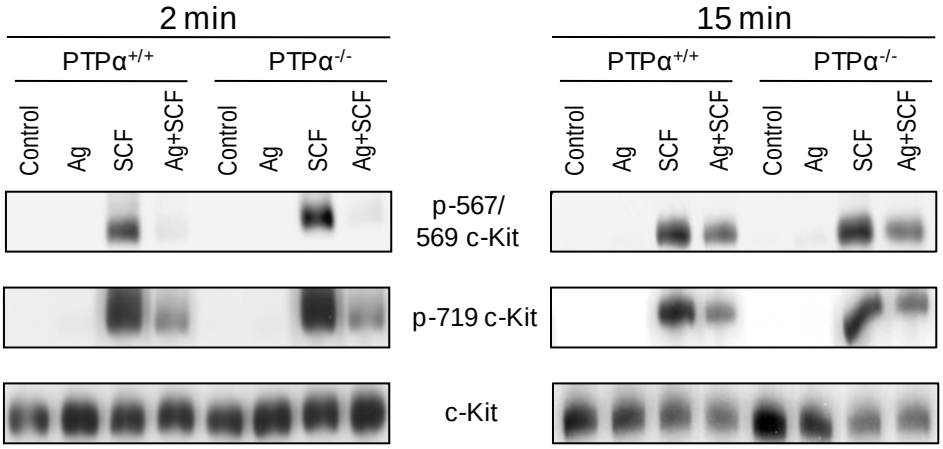
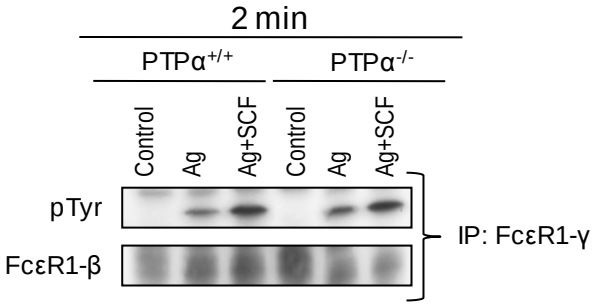


Figure 4.5. PTPα does not significantly affect the phosphorylation of ERK, JNK, and c-Kit upon treatment with antigen and/or SCF. WT ($PTP\alpha^{+/+}$) and PTPα-KO ($PTP\alpha^{-/-}$) cells were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF). The cells were harvested at 2 min and 15 min after treatment, and the lysates were probed on parallel blots with the indicated antibodies. **(A)** BMMCs lysates were immunoprobed for phosphoErk, phosphoJnk and actin. **(B)** BMMC lysates were immunoprobed for phosphoTyr-567/569-c-Kit, phosphoTyr-719-c-Kit, and total c-Kit. For each timepoint, the sets of parallel blots shown **(A,B)** are representative of four independent experiments.

A



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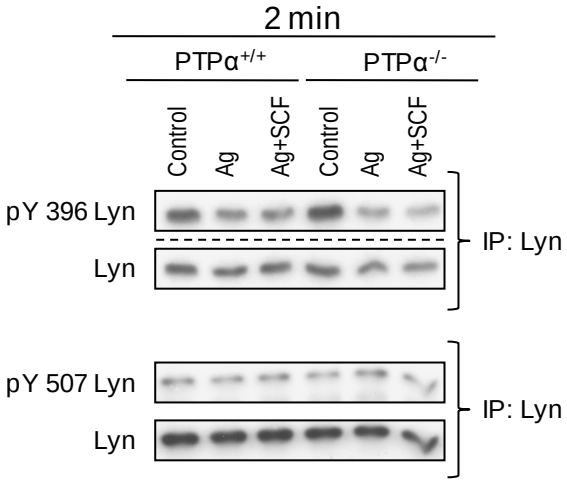


Figure 4.6. PTPα does not significantly alter the phosphorylation of Lyn and FcεR1-β upon treatment with antigen and SCF. WT (PTPα$^{+/+}$) and PTPα-KO (PTPα$^{-/-}$) cells were untreated (Control, C), or stimulated with 100 ng/ml antigen (Ag, DNP-HSA), 100 ng/ml SCF, or co-stimulated by simultaneous addition of antigen and SCF (Ag + SCF). The cells were harvested after 2 min of treatment, and the lysates were used to immunoprecipitate (IP) the indicated proteins. **(A)** FcεR1-γ antibody was used to precipitate the FcεR1 compex from BMMC lysates. The immunoprecipitates were probed for tyrosine phosphorylation of the FcεR1-β protein (using 4G10 antibody). The membrane was stripped and reprobed for total FcεR1-β protein. **(B)** Lyn immunoprecipitates from WT and PTPα-KO BMMCs were probed with phosphoTyr-416-Src or with Lyn antibodies on parallel blots (top panels). Lyn immunoprecipitates were also probed for phosphoTyr-507-Lyn, then stripped and reprobed for total Lyn protein (bottom panels). The blots shown **(A, B)** are representative of at least two independent experiments.

Chapter 5: Role of PTPα in Mast Cell Migration Towards Antigen and SCF

5.1 Rationale

A mixture of monomeric IgE, antigen, prostaglandins, complement proteins, cytokines and other mast cell attractants contribute to the migration of mast cells *in vivo* that facilitates chronic allergic inflammation (Kitaura et al., 2005; Kuehn et al., 2010; Nilsson et al., 1996). Our lab has previously shown that PTPα is required for SCF-mediated Fyn and Rac/Jnk pathway activation, which facilitate the migration of mast cells. PTPα-KO BMMCs exhibited defective spreading, polarization, and chemotaxis towards an SCF gradient. The tissue distribution of mast cells was also abnormal in PTPα-KO mice (Samayawardhena and Pallen, 2008), suggesting that PTPα regulates *in vivo* recruitment of mast cells. Several reports have shown that SCF-driven migration can be enhanced by concurrent stimulation of mast cells with antigen or fibronectin (Kuehn et al., 2010; Tan et al., 2003). The SFKs Lyn and Fyn are substrates of PTPα, and are involved in antigen signaling and migration of mast cells (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010; Suzuki et al., 1998). Therefore, chemotaxis driven by co-stimulation of c-Kit and FcεR1 signaling, as is likely to occur during allergic lung infiltration by mast cells, could also be regulated by PTPα.

5.2 BMMC Migration in the Presence of Fibronectin

Given the PTPα-dependent changes I observed in signaling pathways are involved in mast cell migration upon stimulation with antigen and/or SCF, I compared the migratory abilities of WT and PTPα-KO BMMCs towards these chemoattractants to determine whether this is also affected by PTPα. Equal numbers of IgE-sensitized WT and PTPα-KO BMMCs were cytokine starved and loaded into Transwell migration inserts that had been precoated with fibronectin on the bottom surface of the porous membranes (Methods, section 2.10). The insert was placed in a well containing serum-free media with either 10 ng/ml antigen (DNP-HSA) or 25 ng/ml SCF, or a combination of both. After a 3 hour incubation at 37^{o}C, cells that had migrated into the medium in the bottom well were collected and counted to determine the number of BMMCs that migrated. These data were used to calculate the migration index, using the formula:

Migration Index =[# of migrated cells]÷[$4x10^5$ total cells loaded]x100.

Figure 5.1 shows the migration indices of WT and PTPα-KO BMMCs exposed to different chemotactic stimuli. In the absence of stimulus virtually no cells migrated through the membrane (data not shown). Both WT and PTPα-KO cells migrated towards 10 ng/ml antigen in the bottom well with a migration index of about 1.0. The biggest index of migration (~5) was induced by 25 ng/ml SCF. On average, migration towards SCF alone was slightly lower for PTPα-KO than WT BMMCs, but this difference was not statistically significant (p=0.28). The absence of PTPα was previously shown to reduce SCF-driven migration of BMMCs nearly threefold in the

absence of IgE (Samayawardhena and Pallen, 2008). In my experiments all cells were IgE sensitized, and the migrations were carried out for 3 hours instead of 1.5 hours. In the absence of antigen, IgE alone can induce mast cell activation and migration (Kashiwakura et al., 2011; Kitaura et al., 2005), and may possibly affect signaling events that minimize the role of PTPα in SCF-driven chemotaxis. The addition of antigen appeared to reduce SCF-mediated migration to a similar extent for WT and PTPα-KO BMMCs, but this reduction did not reach statistical significance (WT: $p=0.15$, KO: $p=0.23$). A migration index of about 3.5% was achieved by WT and similarly PTPα-KO cells migrated towards the combination of antigen and SCF.

5.3 Adherent Cell Analysis

The Transwell membranes themselves were also analyzed for the number of cells that passed through the pores, but remained attached to the fibronectin-coated underside of the membranes. After a 3 hr migration period, the adherent cells were fixed and stained with Giemsa reagent, and then the top surface of the membrane was swabbed to remove cells that had not migrated through the pores. The membranes were cut out, mounted on slides, and visualized on a microscope. Figure 5.2A shows the representative staining of WT and PTPα-KO BMMCs attached to the membrane underside after migration towards 25 ng/ml SCF and 10 ng/ml antigen. For each migration condition, the number of adherent cells was counted in five different fields of the membrane to determine the mean density of adherent cells. Figure 5.2B shows the average number of migrating adherent cells per view field. Equivalent numbers of migrated adherent WT and PTPα-KO cells

were detected in response to antigen, in accord with the equivalent number of cells that moved into the media in the lower chamber (Fig. 5.1). Of all the stimuli tested, 25 ng/ml SCF alone attracted the largest number of mast cells to the underside of the fibronectin-coated membrane. More PTPα-KO than WT SCF-driven BMMCs were found on the membrane, though the difference was not statistically significant ($p=0.09$). Interestingly, PTPα appeared to regulate the co-stimulatory effect of antigen on the migration of adherent mast cells. While the addition of antigen to SCF did not significantly alter the number of WT adherent cells, the migration of SCF-driven PTPα-KO adherent mast cells was reduced by over 40% in the presence of antigen ($p=0.01$). As a result, the numbers of WT and PTPα-KO BMMCs attached to the membrane upon co-stimulation with antigen and SCF were very similar to the number of adherent WT cells attracted to SCF alone (Fig. 5.2B, third column).

Since the numbers of cells that migrated into the media in the lower well as well as those that still remained attached to the membrane were counted, it was possible to determine the total number of WT and PTPα-KO BMMCs that passed through the porous membrane under various conditions. Figure 5.3 shows the total numbers of migrated cells. As was seen with each subpopulation of cells (adherent and detached), 10 ng/ml antigen induced equivalent total numbers of WT and PTPα-KO BMMCs to pass through the membrane (Fig. 5.3). The total number of WT cells that migrated towards 25 ng/ml SCF alone was about five times higher than with antigen alone. The addition of antigen and the loss of PTPα appeared to independently lower the average number of SCF-driven BMMCs by about 30% and 15%,

respectively (right four columns, Fig. 5.3). However, these total cell populations were not significantly different under these conditions ($p>0.10$). The ratios of adherent to detached cells appears to be mostly independent of the overall number of migrated cells. An average of about 1 in every 5 cells that passed through the membrane remained attached to fibronectin.

5.4 Fibronectin-Independent Mast Cell Migration

It was recently reported that a 10 ng/ml dose of SCF can synergize with antigen (10 ng/ml) to enhance mast cell migration in the absence of fibronectin binding (Kuehn et al., 2010). This effect involves the same PI3K/Btk/PLCγ pathway that enhances calcium signaling and mediator release from mast cells (Iwaki et al., 2005). In the migration experiments shown earlier, a higher dose of SCF (25 ng/ml) was used. When the lower dose (10 ng/ml) of SCF was also tested with fibronectin-coated inserts, only about 2% of WT and PTPα-KO BMMCs migrated into the media in the lower well (n=2), and about 1.5% migrated with the addition of 10 ng/ml antigen to both cell types. Thus even the suboptimal dose of SCF did not synergize with antigen during fibronectin-aided migration. I also tested whether fibronectin-independent mast cell migration towards SCF and/or antigen (both at 10 ng/ml) was affected by PTPα. As shown in Fig. 5.4 the assessment of migration using uncoated Transwell inserts revealed no differences between WT and PTPα-KO BMMCs stimulated with 10 ng/ml Ag, 10 ng/ml SCF, or the combination of both stimuli. Interestingly, antigen and SCF co-stimulation synergistically enhanced the migration of WT and PTPα-KO cells, in agreement with the previously reported enhancement

(Kuehn et al., 2010). However, this was in contrast to my findings from fibronectin-aided migration, where added antigen appeared to reduce SCF-driven chemotaxis (Fig. 5.1), and was not a result of using a higher dose of SCF (25 ng/ml). This suggests that fibronectin stimulation may determine whether antigen enhances or inhibits SCF-driven mast cell chemotaxis.

5.5 Discussion

In this chapter, I investigated the role of PTPα in mast cell chemotaxis in response to the combined stimuli of Ag and SCF. In agreement with other studies, antigen alone was capable of stimulating the migration of BMMCs through a porous membrane with or without fibronectin coating (Ishizuka et al., 2001b; Kitaura et al., 2005; Kuehn et al., 2010). My results demonstrate that this process is independent of PTPα expression. Previously, I showed that Ag-stimulated activation of PLCγ1 is higher in PTPα-KO BMMCs. PLCγ1 regulates calcium signaling to promote mast cell migration and mediator release, however the increased PLCγ1 activation in PTPα-KO BMMC was obviously insufficient to enhance migration towards antigen. Much higher magnitude of mast cell chemotaxis was induced by 25 ng/ml SCF, and in response to this stimulus PTPα-KO BMMCs were slightly less efficient than WT-BMMCs migrating into the bottom chamber. However, the number of PTPα-KO cells that passed through the membrane and remained attached to the fibronectin-coated underside was somewhat higher than for WT cells. Although not statistically significant, this trend suggests that PTPα may affect mast cell binding to fibronectin

(via cytoskeletal rearrangements or integrin activation) more than it affects the overall chemotaxis to SCF.

PTPα did not significantly affect the total number of cells that passed through the membrane pores, since after 3 hours the majority (~80%) of migrated cells had detached into the media in the lower well. SCF-mediated enhancement of mast cell activation and migration depend on PI3K signaling, whereas adhesion to fibronectin was shown to depend more on protein kinase C activation (Ra et al., 1994; Tan et al., 2003). In my experiments, SCF-induced PI3K activation, as reflected by Akt phosphorylation, was significantly reduced in IgE-sensitized PTPα-KO BMMCs (Fig. 4.2C). However, the migration experiments demonstrate that this reduced Akt activation was not sufficient to significantly affect the migration of PTPα-KO BMMCs. There was noticeable variability in the overall migratory responses of WT and PTPα-KO BMMCs to SCF between experiments, which contributed to the standard deviation for the numbers of migrated cells. In most experiments fewer PTPα-KO than WT cells had migrated towards SCF irregardless of the magnitude of the overall migration. My data suggest that statistically significant differences might be detected by testing BMMC culture batches of similar age, performing migration experiments in triplicate, and using cell counting methods that are more accurate for low cell concentrations.

The addition of antigen to SCF appeared to inhibit migration in the presence of fibronectin, but it synergistically enhanced fibronectin-independent chemotaxis

towards the same concentration of SCF. The higher number of SCF-driven PTPα-KO BMMCs bound to fibronectin was significantly reduced by antigen co-stimulation, while WT cells were unaffected by the added antigen (Fig. 5.2B). Thus, the loss of PTPα may facilitate the reduction in binding to fibronectin brought by antigen co-stimulation, which in WT cells is inhibited by PTPα signaling. On the other hand, the lack of PTPα may just be increasing the affinity of mast cells to fibronectin during SCF-driven chemotaxis, while the co-induction of FcεR1 signaling may be ablating this effect and restoring affinity to fibronectin to the level of WT cells migrating to SCF alone (Fig. 5.2B). Elucidation of these mechanisms will require analysis of the activation of key signaling proteins in adherent mast cells that are stimulated with antigen and SCF.

Previously, I showed that PTPα positively regulates the activation of p38 and Akt in BMMCs co-stimulated with antigen and SCF. Although both p38- and PI3K/Akt-mediated signaling can regulate chemotaxis (Craig and Greer, 2002; Ishizuka et al., 2001b; Kuehn et al., 2010; Samayawardhena et al., 2006; Tan et al., 2003), the presence or absence of PTPα did not significantly effect the total number of IgE-sensitized BMMCs that migrated towards antigen and SCF. As already mentioned, our lab has previously demonstrated a crucial regulatory role of PTPα in integrin signaling and migration of various cell types (Chen et al., 2006; Chen et al., 2009; Samayawardhena and Pallen, 2008; Wang et al., 2009). It would be interesting to also examine whether PTPα affects outside-in integrin signaling, i.e. how much integrin binding influences mast cell secretory responses to antigen and/or SCF.

Based on my experiments in *ex-vivo* settings, PTPα does not appear to be a major regulator of mast cell recruitment by antigen and SCF.

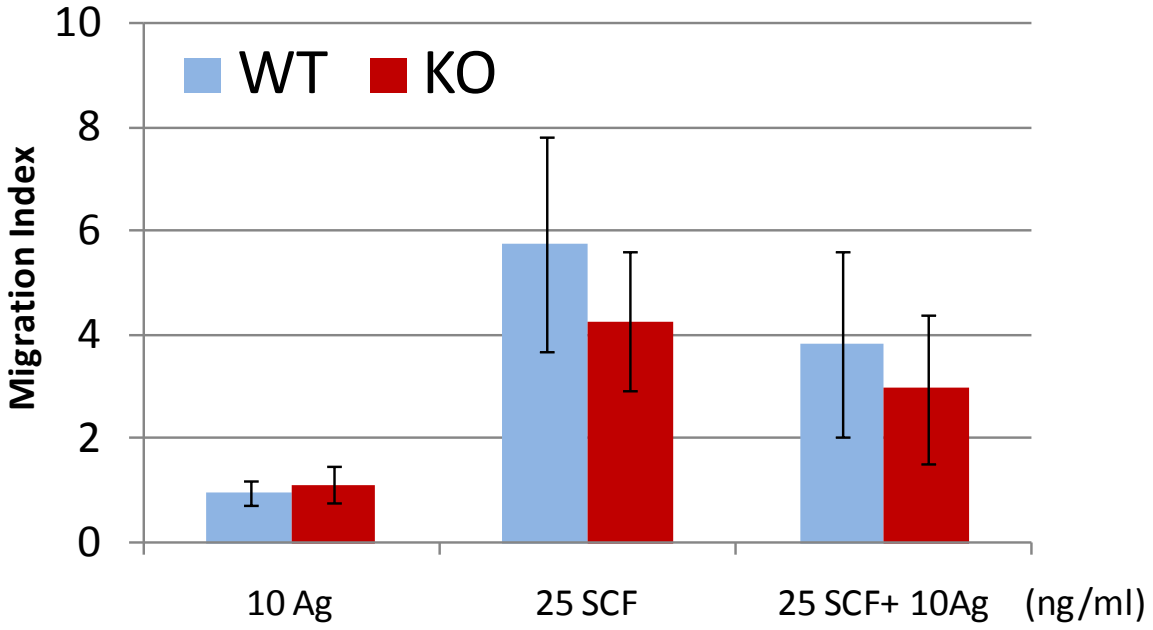
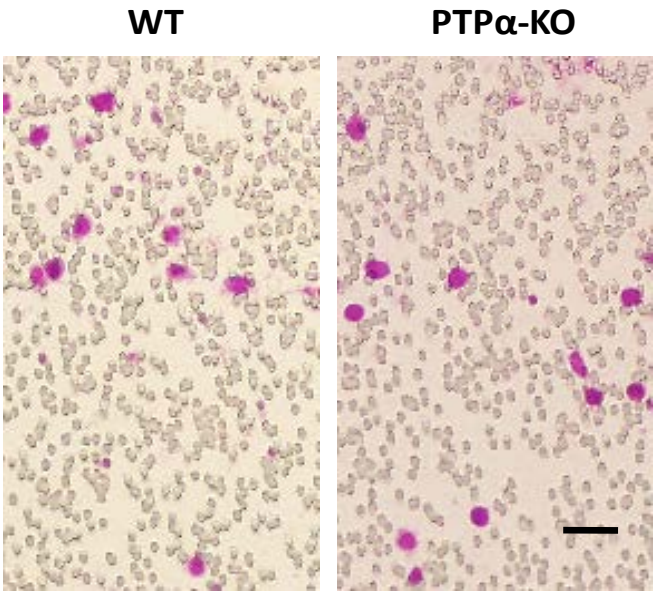


Figure 5.1. Antigen and SCF-induced migration of detached BMMCs. WT and PTPα-KO BMMCs were IgE sensitized using the 'New' method (section 2.3.2) and placed in Transwell inserts which had been precoated with fibronectin (as described in section 2.10). The bottom chamber contained the indicated doses of antigen and/or SCF (ng/ml). After 3 hours of incubation, cells that had appeared in the media in the bottom chamber media were counted. The migration index represents the percentage of cells that passed through the membrane and detached, expressed as mean ± S.D. from 3-4 independent experiments.

A



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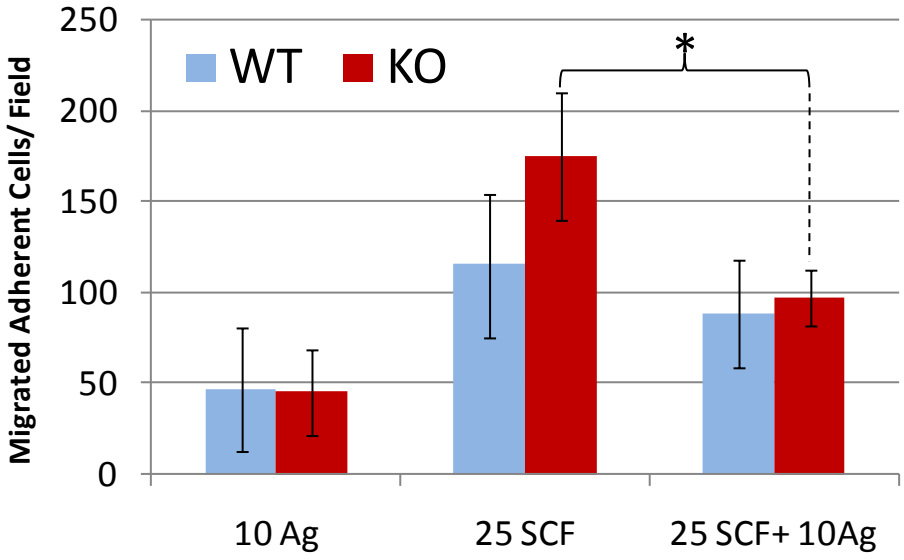

Figure 5.2. The number of fibronectin-bound, migrating cells after chemotaxis. WT and PTPα-KO BMMCs were IgE sensitized using the 'New' method (section 2.3.2) and placed in Transwell inserts which had been precoated with fibronectin (as described in section 2.10). The bottom chamber contained the indicated doses of antigen and/or SCF (ng/ml). After 3 hours of incubation, adherent cells were fixed, stained with Giemsa and photographed (200x magnification). **(A)** Representative photographs of migrated BMMCs (towards 25 ng/ml SCF plus 10 ng/ml Ag) on the fibronectin-coated bottom surface of the Transwell membrane (scale bar=20 μm). **(B)** The bar graphs represent the number of mast cells that passed through the membrane and remained attached to the bottom surface. Cells were counted from five 0.9 mm^2 fields on each membrane, and the mean ± S.D. adherent migrated cells from 3-4 independent experiments are shown. Only PTPα-KO (p=0.01), but not WT cells (p=0.32), showed significant reduction in the number of migrated adherent cells due to the addition of antigen to SCF.

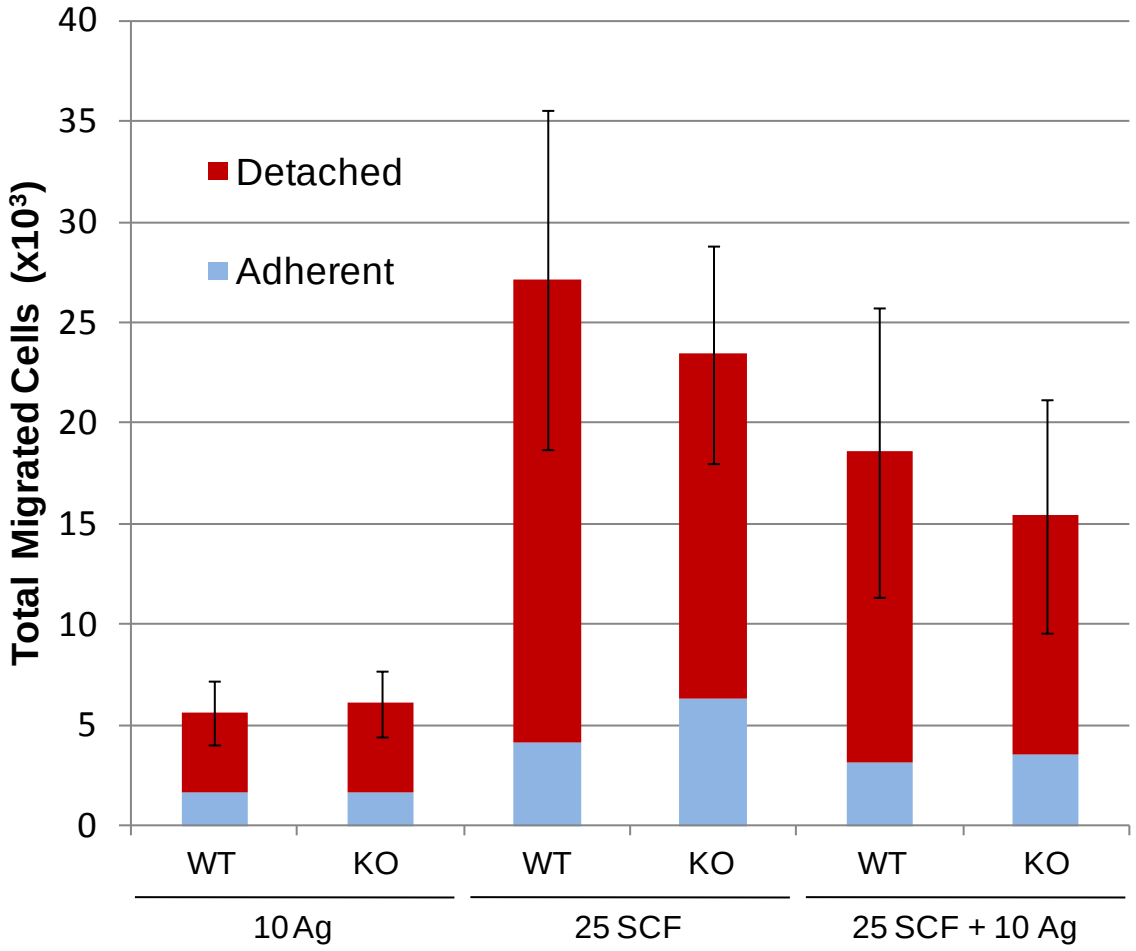

Figure 5.3. Total migrating mast cells after fibronectin-aided chemotaxis. Bars represent the sum of the number of mast cells attached to the entire Transwell membrane bottom surface and the total number of cells detached into the bottom well media. Data show the mean sum of both cell populations ± S.D. (derived from the addition of subpopulation variances) from 3-4 independent experiments. The total number of adherent cells was determined by extrapolating the mean density of counted adherent cells (Fig. 5.2B) to the whole membrane surface (33mm^2). The total number of detached cells was determined from the concentration of cells in the bottom wells; also equal to the migration index (from Fig. 5.1) times the total number of cells loaded (4x10^5). The total numbers of WT and PTPα-KO migrated cells in the right four columns (SCF ± Ag) were not significantly different ($p>0.10$).

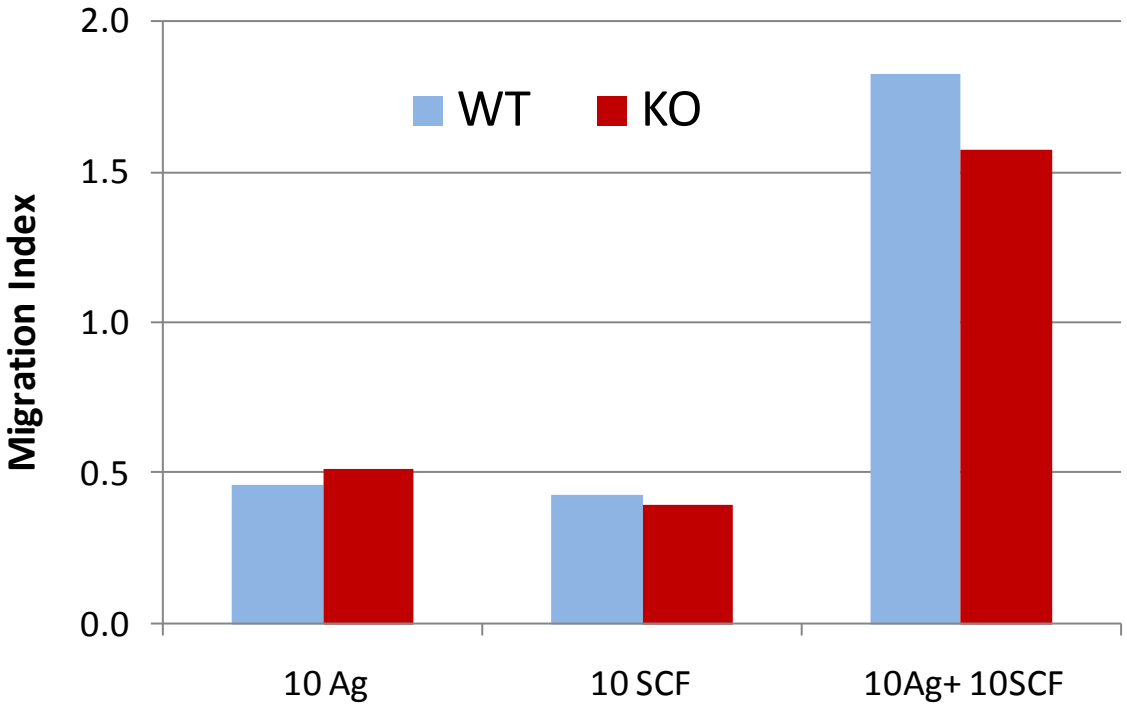

Figure 5.4. PTPα does not affect fibronectin-independent chemotaxis. WT and PTPα-KO BMMCs were IgE sensitized using the 'New' method (section 2.3.2) and placed in uncoated (no fibronectin) Transwell inserts (as described in section 2.10). The inserts were placed in wells containing the indicated doses of antigen and/or SCF (ng/ml). After 3 hours of incubation the cells that had migrated into the medium in the bottom well were counted. The migration index represents the percentage of cells that passed through the membrane. The results shown are from one experiment and similar findings were made in a second independent experiment.

Chapter 6: General Discussion and Future Directions

This investigation was based on previous work from our lab that demonstrated that PTPα regulates important mast cell responses, including SCF-driven chemotaxis and antigen-mediated secretion of bioactive molecules (Samayawardhena and Pallen, 2008; Samayawardhena and Pallen, 2010). The combination of these stimuli is implicated in the exacerbation of allergic asthma symptoms. SCF levels are elevated in the lung epithelia of asthmatics (Al-Muhsen et al., 2004), where it is believed to enhance the sensitivity of mast cells to allergen and result in a greater release of inflammatory mediators that cause allergic airway hyperresponsiveness (Campbell et al., 1999). As described earlier, treatment with IgE and/or antigen, SCF, or other mast cell activators also promotes the migration of these cells. In addition to inflamed lung tissues, certain types of cancer cells also produce SCF and other chemoattractants to recruit mast cells and modify the tumor tissue microenvironment (Colombo and Piconese, 2009; Gounaris et al., 2007).

6.1 Antigen-Mediated Responses

In order to determine how PTPα affects the integration of these stimuli to regulate mast cell effector functions, a baseline of activation with antigen alone needed to be established in WT BMMCs. However, in most of my experiments the magnitudes of antigen-induced degranulation and production of TNFα and IL-13 cytokines were far below the typically expected values. To resolve this issue I modified the IgE sensitization protocol to allow binding of IgE to mast cells before withdrawal of the

growth factor IL-3. Given that mast cells *in vivo* are continuously exposed to circulating IgE molecules, and since my protocol produces equal surface presentation of IgE on both WT and PTPα-KO BMMCs, I believe that the 'New' protocol is an effective *in vitro* replication of mast cell sensitization. The magnitudes of degranulation in response to doses of antigen of WT BMMCs sensitized with the new protocol were similar to typical values reported. Unexpectedly however, the degranulation responses of PTPα-KO BMMCs to antigen were virtually identical to WT cells. This contrasts with earlier data from our lab, where the lack of PTPα was shown to significantly enhance antigen-induced degranulation and cytokine release (Samayawardhena and Pallen, 2010).

In my other experiments, WT and PTPα-KO cells were treated with antigen for 2, 5, and 15 minutes and assessed for the activation of key signaling proteins. Antigen stimulation induced the phosphorylation of the three MAPKs (p38, Erk, Jnk) to similar extents in WT and PTPα-KO cells, in contrast to the previously shown hyperphosphorylation of MAPKs in the absence of PTPα (Samayawardhena and Pallen, 2010). Interestingly, I found that antigen-induced phosphorylation of PLCγ1, a key activator of calcium signaling that we had not previously examined, was enhanced nearly two-fold in BMMCs lacking PTPα. Therefore, the lack of enhanced secretory responses that I observed in PTPα-KO BMMCs contrasts with PLCγ1 hyperactivation, but correlates with the PTPα-independent activation of MAPKs. I also examined the antigen-stimulated phosphorylation of the IgE/Ag receptor FcεR1 and that of its key effector, the Src family kinase Lyn. I found no differences in the

phosphorylation of FcεR1 and Lyn between WT and PTPα-KO BMMCs, again in contrast to previous findings in our lab, where Lyn and FcεR1 phosphorylation were reduced in antigen-treated PTPα-KO cells (Samayawardhena and Pallen, 2010).

I could not find any significant deviations in my experiments from the methodologies previously used by my colleague to conduct experiments that revealed a hyperresponsiveness of antigen-treated PTPα-KO mast cells. To find any abnormalities in the critical features of mast cell activation in my experiments, I examined the phosphorylation of Lyn kinase, the binding of IgE to the surface of mast cells, and compared the secretion of cytokines with the ‘Old’ and ‘New’ sensitization methods, but found no differences between WT and PTPα-KO cells. Other uncontrolled factors such as possible changes in the composition of purchased serum and IL-3-conditioned media used for the culturing of mast cells may have contibuted to the overall reduced maginitude of secretory responses when using the ‘Old’ method of IgE sensitization and to the lack of previously observed PTPα-dependent effects.

PTPα undergoes phosphorylation on a tyrosine residue (Tyr-789) in its C-terminal tail in response to integrin and growth factor receptor stimulation (Chen et al., 2006; Chen et al., 2009), but the regulation of PTPα Tyr-789 in mast cells has not been previously investigated. Treatment with antigen produced rapid and strong dephosphorylation of Tyr-789 that lasted at least 15 min, while SCF treatment also induced dephosphorylation, but to a lesser extent. Although phosphoTyr-789 of

PTPα is known to regulate chemotaxis of other cell types (Chen et al., 2009) as well as the activation of T cell receptor signaling (Maksumova et al., 2007), its role in modulating PTPα function in mast cells is still unknown. Furthermore, I examined the antigen-driven chemotaxis of mast cells with or without the aid of fibronectin. In all cases, PTPα-KO BMMCs migrated just as effectively as WT cells.

Since I found antigen-mediated responses of mast cells to be broadly unaffected by the expression of PTPα, this raises the possibility that the observed dephosphorylation of PTPα Tyr-789 may reflect the inhibition of its effector functions during mast cell activation. In T cells the phosphorylation of Tyr-789 enhances PTPα-mediated dephosphorylation of several regulatory tyrosine residues on Fyn kinase, reducing overall Fyn catalytic activity. The PTP CD45 dephosphorylates PTPα at Tyr-789, thus inhibiting some effects of PTPα in T cells (Maksumova et al., 2007). CD45 is also expressed in mast cells, where it promotes secretory responses (Grochowy et al., 2009), and thus may be the major enzyme that dephosphorylates PTPα upon antigen treatment. It is plausible that the negative regulatory functions of PTPα in FcεR1 signaling are inhibited by CD45, most likely via dephosphorylation of PTPα Tyr-789. If this is correct, then the phosphorylation of PTPα Tyr-789 should be elevated in CD45-null mast cells, as was seen in T cells, whereas the Y789F substitution in PTPα should minimize the reduction in secretory responses upon the loss of CD45.

The dephosphorylation of PTPα may also be a side-effect of mast cell products. Antigen-challenged mast cells produce reactive oxygen species that inhibit the catalytic domains of various PTPs, while in other cell types oxidation inhibits the phosphorylation of PTPα at Tyr-789 (Hao et al., 2006; Heneberg and Draber, 2005). Therefore, the induction of degranulation may feed back to modify the activation of PTPα and other phosphatases via reactive oxygen species or intermediates.

To determine why the elevated activation of PLCγ1 in antigen-treated PTPα-KO BMMCs did not produce greater degranulation, it would be interesting in the future to examine the timecourse of calcium signaling. If calcium signaling is also enhanced in antigen-treated PTPα-KO cells, then processes downstream of PLCγ1 signaling such as cytoskeletal rearrangement may be preventing an increase in degranulation. Additionally, the effect of PTPα on the activation of Fyn kinase should be determined, as Fyn has crucial positive regulatory functions in the activation of calcium signaling during degranulation, and also promotes chemotaxis (Samayawardhena et al., 2006; Sanchez-Miranda et al., 2010). In previously examined antigen-stimulated PTPα-KO BMMCs, Fyn activation was reduced and contrasted with hyperdegranulation due to the lack of PTPα (Samayawardhena and Pallen, 2010). If Fyn activation was found to be increased in my stimulated mast cells, this could explain the elevated phosphorylation of PLCγ1.

6.2 SCF-Mediated Responses of IgE-Sensitized BMMCs

WT and PTPα-KO BMMCs were stimulated with SCF alone parallel to antigen-treated cells. Analysis of signaling proteins showed no effect of PTPα on the phosphorylation of the SCF receptor c-Kit, downstream MAPKs, or PLCγ1 at any timepoint. In contrast, Akt phosphorylation was reduced in PTPα-KO BMMCs by about 25% after 15 min treatment with SCF. In all of my experiments IgE-sensitized BMMCs were used, while in previous work in our lab SCF-treated cells were not pre-exposed to IgE (Samayawardhena and Pallen, 2008), which may explain their varied responses to SCF.

The migration of mast cells towards SCF with the aid of fibronectin was also examined. The total number of PTPα-KO cells that passed through the membrane pores was fairly similar to WT BMMCs. A slightly higher fraction of PTPα-KO cells appeared to remain attached to the fibronectin-coated membrane instead of detaching into the chemotactic media, but this difference was not statistically significant. The lack of previously reported (Samayawardhena and Pallen, 2008) defects in c-Kit signaling activation and migration due to the loss of PTPα may be a result of sensitization with IgE. To investigate whether this is indeed a factor in my distinct findings, IgE-free mast cells would need to be tested again for responses to SCF.

6.3 Synergistic Mast Cell Activation by Antigen and SCF

The phosphorylation of key proteins in the integrated c-Kit/FcεR1 signaling events was investigated. Interestingly, the addition of the other ligand had opposing effects on the phosphorylation of the receptor for each cognate ligand. The antigen-induced phosphorylation of the FcεR1β subunit was noticeably enhanced by adding SCF, and in contrast c-Kit receptor phosphorylation was nearly abolished by the addition of antigen to SCF in WT and PTPα-KO BMMCs. This raises the possibililty that activation of FcεR1 signaling concurrently with c-Kit sequesters tyrosine kinases away from the c-Kit receptor complex to inhibit its downstream signaling, while increasing their tyrosine kinase activity near the FcεR1 receptor complex. To determine if co-stimulation with antigen reduces the association of tyrosine kinases with c-Kit and increases their association with FcεR1, the ligand-stimulated receptors could each be immunoprecipitated to evaluate the changes in their associations with Lyn, Fyn, and the cytoplasmic Syk due to the co-activation of the other receptor.

Synergistic Ag/SCF-induced degranulation and cytokine secretion were not significantly affected by the loss of PTPα. Additionally, PTPα-KO BMMCs exhibited identical c-Kit and FcεR1 phosphorylation to that in WT cells upon co-stimulation with antigen and SCF. Upon prolonged (15 min) co-treatment, the phosphorylation of PLCγ1, Erk, Jnk, and p38 was higher than with either stimulant alone. However, only the phosphorylation of p38 and Akt was reduced in PTPα-KO cells after 15 min co-treatment with antigen and SCF, suggesting that these signaling changes alone are not sufficient to significantly affect the resultant secretory responses of mast cells.

The role of PTPα in the antigen-induced modulation of mast cell migration was also examined in the contex of SCF and extracellular matrix proteins that facilitate mast cell recruitment *in vivo*. SCF-driven chemotaxis of WT BMMCs in the presence of fibronectin was mostly unaffected by the addition of antigen. On the other hand, the number of fibronectin-bound PTPα-KO BMMCs was reduced by about 40% due to the addition of antigen. After 3 hours, the majority of migrated cells had already detached from fibronectin, leaving a small fraction of cells that are still bound to the membrane. Thus, the overall number of migrated cells was not significantly affected by PTPα, suggesting that PTPα may have bigger effects on cytoskeletal interactions with extracellular matrix proteins than on the chemotactic drive of mast cells. In the future, methods to examine cytoskeletal rearrangement, such as staining with phalloidin or anti-Rac antibodies, could be used to determine the role of PTPα in the regulation of mast cell adhesion to fibronectin or other extracellular matrix proteins in the contex of IgE and SCF.

Interestingly, in the absence of fibronectin, antigen and SCF synergistically stimulated mast cell migration, as was also recently reported by another group (Kuehn et al., 2010). In contrast, during fibronectin-aided chemotaxis in my experiments, the same doses of antigen and SCF did not have additive effects on migration.

In summary, my *ex vivo* experiments including immunoprobing the activation of key signaling proteins, degranulation and cytokine assays show that PTPα is not a major regulator of the co-stimulatory activation of mast cells via c-Kit and FcεR1, nor of the induced synergistic secretory responses. The role of dephosphorylation of PTPα Tyr-789 in mast cell activation still remains unknown. Additionally, the absence of PTPα appears to promote antigen-induced detachment of mast cells from fibronectin during SCF-driven chemotaxis. Despite the positive regulation of Akt and p38 by PTPα upon co-stimulation with antigen and SCF, the overall magnitude of migration appeared to be independent of PTPα. My overall results suggest that at least under the *ex vivo* experimental conditions that I employed, PTPα is not a crucial regulator of mast cell responses to antigen and SCF. A summary of my key findings is illustrated in Figure 6.1.

6.4 Future Directions:

The Role of PTPα in a Murine Model of Chronic Lung Inflammation

Many Src family kinases are substrates of PTPα, and are important modulators of immune cell activation and migration. In naïve thymocytes PTPα regulates the activation of Fyn kinase and thymocyte proliferation (Maksumova et al., 2005). Our lab has previously demonstrated that the loss of PTPα alters the distribution of mast cells in the mouse (Samayawardhena and Pallen, 2008), and the hyperresponsiveness of PTPα-null mice to IgE-mediated systemic anaphylaxis supports a regulatory role of PTPα in mast cell recruitment and activation (Samayawardhena and Pallen, 2010). The complex interplay of cytokines,

extracellular matrix proteins, and other bioactive molecules can drastically influence immune cell responses *in vivo*. The loss of PTPα could influence the overall allergic inflammation via the recruitment and activation of multiple cell types in ways that may not be apparent through *ex vivo* studies alone. In order to determine the role of PTPα in the pathogenesis of allergies, it must be examined in an *in vivo* model of the disease. Recent preliminary data suggest that mice lacking PTPα have altered T cell subtype-specific cytokine responses to various lung allergens. Therefore, the susceptibility of PTPα-deficient mice to develop Th2 cell differentiation and antigen-induced lung inflammation will also be examined, which models the pathogenesis of human asthma. We are collaborating with Dr. Kelly McNagny (and Matthew Gold, a graduate student in the McNagny laboratory), who has extensive experience in studying mouse models of allergic inflammation (Blanchet et al., 2007; Blanchet et al., 2011).

To investigate allergic Th2 cell responses, cohorts of WT and PTPα-KO mice will be sensitized intranasally to the house dust mite (HDM) allergen. Lung tissues will be stained and scored for histological traits of inflammation. Isolated lymphoid and lung tissues will also be re-stimulated with antigen and assayed for cytokines. Other mouse cohorts will also be tested for the development of Th1/Th17 type Hypersensitivity Pneumonitis (HP). Both asthma and HP are initiated by alternative T cell subtypes, the differentiation of which may be regulated by PTPα via cytokine and/or T cell receptor signaling. To test this hypothesis, Th1, Th2, Th17, Treg and

neutral T cells will be stimulated to produce cytokines, which will be assayed by ELISA and FACS analysis.

If these experiments show that PTPα significantly affects allergic inflammatory responses *in vivo*, then future transplantation of WT and PTPα-null BMMCs into mast cell-deficient mice will elucidate the role of PTPα in mast cell responses, and the efficacy of therapeutic targeting of PTPα. These experiments will improve our understanding of the processes that regulate adaptive immunity and facilitate human allergic diseases.

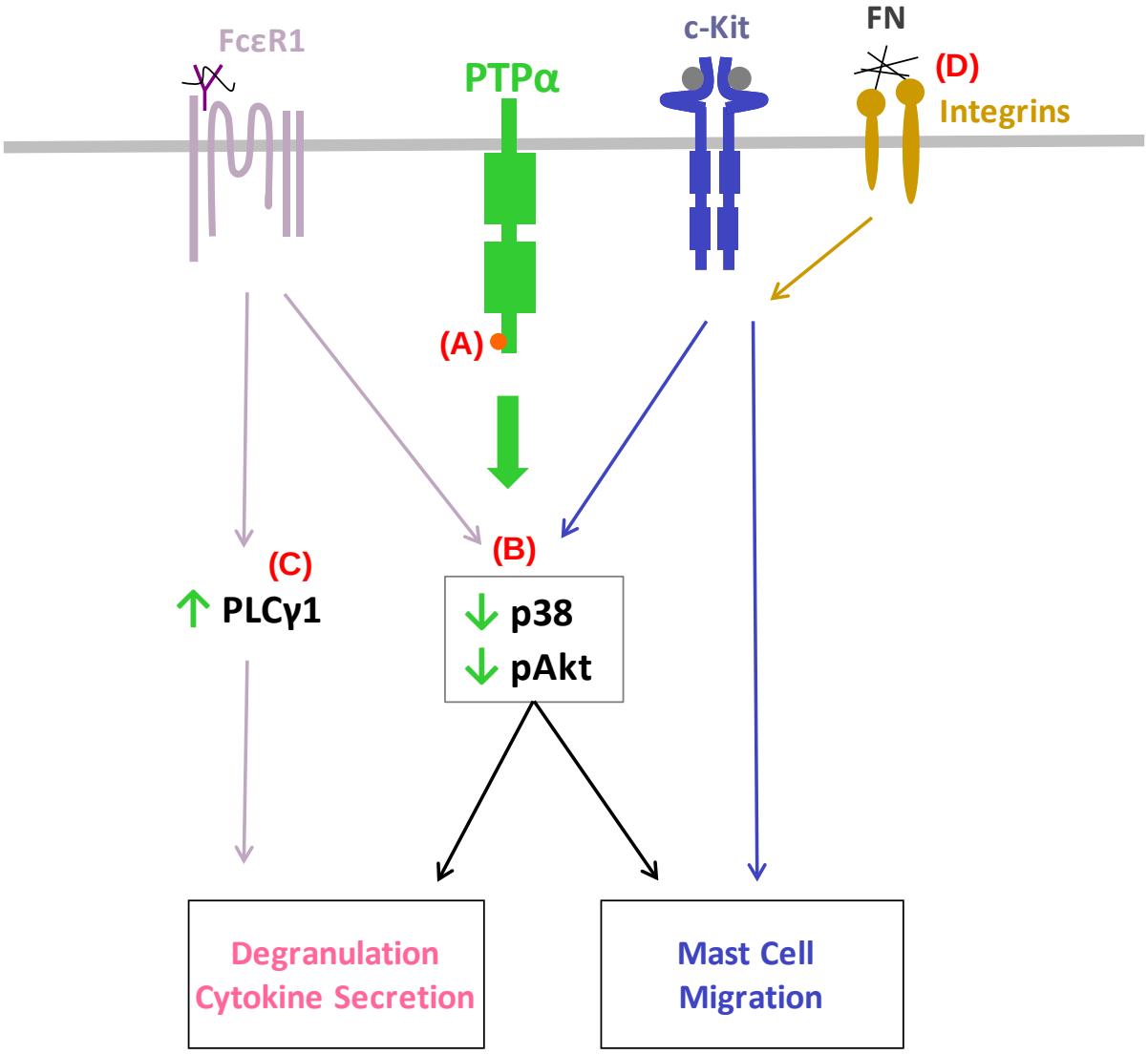

Figure 6.1 Proposed PTPα-dependent processes during mast cell activation. (A) PTPα becomes dephosphorylated at tyrosine 789 upon cell stimulation, especially with antigen. **(B)** PTPα promotes the activation of p38 and Akt upon prolonged stimulation with antigen and SCF. **(C)** Antigen-induced phosphorylation of PLCγ1 is enhanced in PTPα-null mast cells. **(D)** In the absence of PTPα antigen promotes cell detachment from fibronectin (FN) during SCF-driven migration.

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