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Regulation of Human Lung Mast Cell Responses by Stem Cell Factor

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ABSTRACT

Stem Cell Factor (SCF) is widely recognised as a crucial growth factor for mast cells and has been shown to mediate the development, differentiation and proliferation of mast cells from precursors. These effects of SCF are thought to be mediated by the c-KIT receptor. Whether SCF directly activates mast cells is more contentious and most of the data suggests that SCF can 'prime' mast cells for enhanced responses to IgE-dependent activation. The principal aim of the present work was to determine whether SCF activates human lung mast cells. Our studies showed that SCF was an effective direct activator of human lung mast cells. Although not as effective as anti-IgE at inducing histamine release, nonetheless, in about a third of all mast cell preparations, SCF induced substantial levels of release. Even more strikingly, SCF was as effective as anti-IgE at inducing prostaglandin D_2 (PGD_2) generation from mast cells. By contrast, SCF was relatively ineffective at inducing cytokine generation from human lung mast cells, a feature shared by other stimuli such as anti-IgE. The effects of SCF on histamine and PGD_2 release were blocked by the c-KIT inhibitors imatinib, dasatinib and nilotinib suggesting that SCF acts through the c-KIT receptor to activate mast cells. Further studies were performed to evaluate the mechanism by which SCF and anti-IgE drive PGD_2 generation from mast cells. In particular, the role of the enzyme cyclooxygenase (COX) on PGD_2 generation was evaluated. Studies utilising COX-1 and COX-2 selective inhibitors demonstrated that COX-1 was the isoform that drives PGD_2 generation from mast cells. This finding was further supported by Western blotting studies showing that COX-1 is the principal isoform expressed by mast cells. In total, these findings indicate that SCF can directly activate human lung mast cells through the c-KIT receptor to generate substantial amounts of PGD_2 and variable amounts of histamine. The data also show that COX-1 is the principal isoform involved in PGD_2 generation from human lung mast cells. Overall these studies indicate that SCF is a far more effective activator of human lung mast cells than hitherto appreciated.

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DEDICATION

This thesis is dedicated to my parents,

Khaled and Ghada

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5-HETE	5-hydroxyeicosatetraenoic
5-HPETE	5-hydroperoxyeicosatetraenoic acid
5-LO	5-lipoxygenase
5-oxo-ETE	5-oxo-6,8,11,14-eicosatetraenoic acid
Ag	Antigen
A-MuLV	Abelson murine leukemia virus
APC	Antigen presenting cell
ASM	Aggressive systemic mastocytosis
ASP	Aspirin
ATP	Adenosine triphosphate
BaP	Basophil Progenitor
bFGK-2	Basic fibroblast growth factor-2
BMCP	Basophil mast cell progenitor
BzATP	Benzyl-ATP
c-AMP	Cyclic adenosine monophosphate
Cb1	Casitas B-lineage protein
CEL	Celecoxib
CM	Cutaneous mastocytosis
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
COX	Cyclooxygenase
CTMC	Connective tissue-type mast cell
Cys-LT	Cysteinyl leukotriene
DC	Dendritic cell
DIC	Diclofenac
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FBS	Foetal bovine serum
FcεRI	High affinity IgE receptor

FDA	Food and Drug Administration
FOXO3a	Forkhead transcription factor
GAG	Glycosaminoglycan
GIST	Gastrointestinal stromal tumour
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte/macrophage progenitor
GPCR	G-protein-coupled receptor
GPI	Glucose-6-phosphate isomerase
HDC	Histidine decarboxylase
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLMC	Human lung mast cell
H-PGDS	Haematopoietic prostaglandin D_2 synthase
HSC	Haematopoietic stem cell
IBU	Ibuprofen
ICER	Inducible cAMP early repressor
IFN-1	Interferon type 1
IL	Interleukin
INDO	Indomethacin
INF-γ	Interferon gamma
ITAMs	Immunoreceptor tyrosine-base activation motifs
JAK3	Initiates Janus kinase 3
JNK	Jun amino-terminal kinase
LAT	Linker for activation of T cells
L-PGDS	Lipocalin prostaglandin D_2 synthase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MC_C	Mast cell chymase
MCP	Mast cell progenitor
MC_T	Mast cell tryptase
MC_{TC}	Mast cell tryptase and chymase

MMCS	Mucosal mast cells
MPP	Multipotent progenitor
MS	Multiple sclerosis
NAP	Naproxen
NGF	Nerve growth factor
NK	Natural killer
NLRs	NOD-like receptors
NSAIDs	Non-steroidal anti-inflammatory drugs
OXE	Oxoeicosanoid receptor
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monocytic cell
PDG2	Prostaglandin D_2
PDGRR	Platelet-derived growth factor receptor
PGHS	Prostaglandin H synthase
PI_3	Inositol-1,4,5-trisphosphate
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKC	Protein kinase C
PLC- γ1/2	Phospholipase C-γ1 and 2
PTSD	Post-traumatic stress disorder
RA	Rheumatoid arthritis
RLR	RIG-like receptor
RTK	Receptor tyrosine kinase
SCF	Stem cell factor
SHIP	Phosphatidylinositol 5' phosphatase
SLP-76	SH2-containing leukocyte-specific protein 76
SM	Systemic mastocytosis
STAT 6	Signal transducer and activator of transcription 6
TGF-β	Transforming growth factor beta
Th2	T helper cell 2

TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor α
TPO	Thrombopoietin
T_{reg}	$CD4^+CD25^+FOX3P^+$ regulatory T cell
TRPC5	Transient receptor protein channel 5
TSLP	Thymic stromal lymphopoietin
Vav	Human vaccinia virus oncogene
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor

CHAPTER ONE

1. INTRODUCTION

1.1 Overview

Mast cells are found at mucosal surfaces, in the skin and around blood vessels: sites where pathogens might first gain entry into the body. It has been suggested that the mast cell has a sentinel function, which suggests an involvement in innate immunity. The mast cell also plays an important role in adaptive immunity in the context of parasitic infestations.

Mast cells are most commonly associated with allergies. Allergens are innocuous objects that are recognised as foreign by allergic people. Allergens can activate the immune system inappropriately and the generation of IgE is strongly linked to allergic reactions. Mast cells express receptors for IgE and IgE-dependent activation of mast cells by allergens leads to mediator release. These mediators promote inflammatory responses. Dependent on the mast cells activated, particular allergic diseases develop. For example, activated lung mast cells are associated with asthma.

Stem cell factor (SCF) is intimately involved in the maturation, migration and survival of mast cells. In addition, SCF has been found to enhance mast cell responses to IgE-dependent activation. Elevated levels of SCF have been found in the lungs of people with asthma. The effects of SCF are thought to be mediated by the tyrosine kinase receptor, c-KIT. Taken together, targeting the SCF/c-KIT interaction could be a mechanism to treat allergies.

In this chapter, I will introduce the mast cell and describe the important role this cell plays in physiological and pathological responses. I will also discuss IgE and IgE-dependent activation of mast cells as this is the most common mechanism by which mast cells are activated in an allergic context. Since this

project is concerned with the responses of mast cells to SCF, background information on SCF and the mechanism by which SCF is thought to activate mast cells will also be presented.

1.2 Mast cell origins

Paul Ehrlich discovered mast cells in 1887. He was amazed by the distinctive granules of the cell and because of their appearance called Mastzellen = well-fed cells in German (Gordon, 2008). Ehrlich also noticed that connective tissue mast cells stained distinctively with an aniline dye. At the time, it was not known that the dye binds to heparin in the granules and this approach is still used to identify mast cells today (Beaven, 2009).

Histamine was first linked to anaphylaxis by Henry Dale at the turn of the 20th century (Tansey, 2003). Later studies in 1953 by Riley and West demonstrated that the mast cell was the main source of histamine (Riley, 1965). Ishizaka continued the investigation on the mechanisms by which histamine is released by mast cells. Between 1966 and 1967 the discovery was made by Ishizaka that IgE antibody was linked to this allergic reaction (Johansson, 2011). In 1989, Metzger's group successfully identified the structure of the IgE receptor (FcεRI) which is expressed at high densities on mast cells (Kulczycki et al., 1974). This discovery led to attempts to look at blocking the IgE/FcεRI interaction as a mechanism to prevent mast cell activation (Saito et al., 2013).

1.2.1 Mast cell development

Mast cells were initially thought to arise from undifferentiated mesenchymal cells (Kitamura and Ito, 2005). Human mast cells can be differentiated from

committed pluripotent haematopoietic progenitors in the bone marrow (Kirshenbaum et al., 1991). Mast cells leave the bone marrow as immature progenitors and mature at connective and mucosal tissues (Okayama and Kawakami, 2006).

Earlier studies in mice demonstrated that mast cells were derived from multipotent progenitors (MPPs) in bone marrow (Kitamura et al., 1981) but not from specific progenitors such as common myeloid progenitors (CMPs) or granulocyte/macrophage progenitors (GMPs) (Chen et al., 2005). Moreover, Okayama and Kawakami (2006) demonstrated that haematopoietic stem cells (HSCs) could differentiate into mast cells or support the production of mast cell precursors in several contexts. Other mice studies conducted by Arinobu et al. (2005) showed that a unique progenitor for mast cells and basophils called (BMCPs) could be specialised to develop basophil progenitors (BaPs) and mast cell progenitors (MCPs). Under the influence of CCAAT/enhancer-binding protein α (C/EBPα) expression, basophils develop when C/EBPα is expressed on BMCPs and mast cells develop when BMCPs lack this expression (Arinobu et al., 2005). These studies suggest that mast cells can develop by different mechanisms.

SCF is involved in mast cell development by influencing haematopoietic progenitors to be committed to the mast cell lineage. From cultured CD34+ cells studies, it has been noticed that mast cell development is regulated by growth factors such as SCF (Sawai et al., 1999). Besides SCF there are different factors which mediate mast cells development including growth factors and cytokines (*Table 1.1*) (Okayama and Kawakami, 2006).

Human mast cells can develop into different subtypes dependent on tissue influences. Mast cells exist as three different subtypes which can be identified by the content of proteases. These are tryptase-containing mast cells (MC_T) which are found in the mucosa of the small intestine and alveolar septa, chymase-containing mast cells (MC_C) which are sited in the mucosa of the colon, small intestine sub-mucosa and the mucosa and sub-mucosa of stomach. Finally, tryptase and chymase-containing mast cells (MC_{TC}) which are located in the small intestine sub-mucosa, bronchial airways' tissues and mostly in skin (Kumar and Sharma, 2010). These different subtypes of mast cells appear to be functionally heterogeneous.

Table.1.1 Effects of cytokines and growth factors on human mast cells.

Cytokine	***Effects on human mast cells***
SCF	Promotes development and proliferation
IL-3	Stimulates proliferation of bone marrow mast cells
IL-4	Enhances proliferation of intestinal mast cells
IL-5 & 9	Cofactor for proliferation
IL-6	Promotes or inhibits proliferation, anti-apoptotic
INF-γ, TGF-β & GM-CSF	Inhibits proliferation
NGF	Suppression of mast cell apoptosis, in the presence of SCF
TPO	Stimulates early stage mast cell development

[IL: Interleukin; INF-γ: Interferon gamma; TGF-β: Transforming growth factor beta; GM-CSF: Granulocyte-macrophage colony-stimulating factor; NGF: Nerve growth factor; TPO: Thrombopoietin]. Adapted from (Okayama and Kawakami, 2006).

1.2.2 Mast cell migration

MCPs leave the blood circulation and migrate into various tissues to mature and differentiate. This migration involves many biological agents such as chemokines, growth factors (SCF), integrins, and adenosine nucleotides some of which are specific for rodent mast cells (Liu et al., 2010). Chemokine receptors

such as CXCR3, CXCR2, CCR3, CXCR4 and CCR5 are expressed on human mast cells, most importantly CXCR3 which is expressed on human lung mast cells by interacting with CXCR3 ligand (CXCL10) (Brightling et al., 2005).

SCF as a growth factor facilitates development and survival of mast cells and also, plays an important role in mast cell migration (Okayama and Kawakami, 2006). SCF is involved in mast cell migration by relocating the cell to a site where it can mature or be involved in an inflammatory response. Vascular cell adhesion protein 1 (VCAM-1) and E-selectin receptors are important receptors which induce mast cell migration (Boyce et al., 2002). Mast cell migration can be induced by highly cytokinergic IgE molecules in the absence of antigen, which activates the Syk and Lyn tyrosine kinase pathways and eventually PI3 kinase γ. As a result of mast cell migration, pre-formed and lipid-derived mediators can be secreted following activation of the cell (Kitaura et al., 2005).

1.2.3 Mast cell survival

The number of tissue mast cells is controlled by the length of survival of mature mast cells and the rate of MCPs produced (Moller et al., 2007). Apoptosis and survival is regulated by Bcl-2 gene members, members such as Bcl-2 and A1/BFL-1 which are classified as pro-survival members. Members with pro-apoptotic functions are divided into two subgroups, a multi-domain (BH1, 2 and 3) sub-group which include Bax and Bcl-GL, and another subgroup which shares 9 amino acids of BH3 domain such as Bad and Bmf (Droin and Green, 2004).

Mast cell survival is also promoted by SCF via Forkhead transcription factor (FOXO3a) inactivation and through FOXO3a Bim phosphorylation and

down-regulation (Moller et al., 2005). Mast cells activated through the IgE-dependent mechanism can promote cell survival and induce the expression of anti-apoptotic A1 protein (Moller et al., 2003). Mast cells stimulated by SCF activate PI-3 kinase and MAPK (MEK/ERK) pathways to promote cell survival and prevent Bim from inducing apoptosis (Okayama and Kawakami, 2006). Lastly, many cytokines and growth factors are involved in mast cell survival. For instance, IL-33 can enhance survival in the absence of the co-stimulator IgE in human cord blood cultured mast cells. This effect is weakened in the presence of IgE (Ho et al., 2007).

1.3 Physiological role of mast cells

1.3.1 Innate immunity

The mast cell is a fundamental cell of the immune system, and as such, expresses a wide variety of surface receptors involved in immune recognition. These receptors allow the mast cell to interact with toxins, antigens and pathogens. The detection of potentially harmful signals allows the cells to respond appropriately to pathogens and their components through the secretion of pre-formed and newly synthesized mediators (da Silva et al., 2014).

The mechanism by which mast cells recognise pathogens varies. Pathogens and their related components can directly interact with pathogen-associated molecular patterns (PAMPs) receptors that are expressed on the surface of mast cells. Also, antibody or complement-coated bacteria can bind to immunoglobulin or complement receptors. Another mechanism could potentially involve the identification of endogenous peptides following injured or infected cells (Hofmann and Abraham, 2009).

The expression of receptors such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs); a trinity of pathogen sensors and complement receptors varies between different mast cell subtypes. These receptors are required for mast cells to mediate responses associated with innate immunity. The activation of these receptors leads to the generation of inflammatory mediators which contribute to restraining the infection (Metz et al., 2008, Marshall, 2004).

The mechanism by which mast cells release their mediators through TLRs varies dependent on the ligand. For example, lipopolysaccharide (LPS) from Gram negative bacteria binds to TLR4 which leads to the production of pro-inflammatory cytokines such as IL-1β, Tumor Necrosis Factor α (TNF-α), IL-6 and IL-13 without causing degranulation in murine bone marrow-derived mast cells (Supajatura et al., 2002). However, peptidoglycans of Gram positive and Gram negative bacteria bind to TLR2 which leads to mast cell degranulation and the production of cytokines such as TNF-α. In contrast, IL-4 primed mast cell studies demonstrated that signaling through TLR2 is important for increase IL-4 production which is critical for the containment and the clearance of the pulmonary infections such as *Francisella tularensis* (Varadaradjalou et al., 2003, Rodriguez et al., 2012).

Mast cells and other immune cells such as eosinophils, natural killer (NK) cells and neutrophils are strongly involved in fighting bacterial infection and the clearance of this infection. The recruitment of these cells to the sites of infection is mainly linked to mast cells and their abilities to recognise pathogens and their components and the importance of mediators released by mast cells. All these

factors contribute to the chemoattraction of the above innate immune cells and the increase in vascular permeability (da Silva et al., 2014).

Mast cells have the capacity to produce antibacterial peptides such as cathelicidins and psidins upon degranulation that have direct bactericidal effects supporting bacterial elimination (Campagna et al., 2007, Di Nardo et al., 2003). In addition, mast cells have also been shown to be phagocytic cells, the killing mechanism is facilitated by the phagocytosis of the bacteria and then the production of reactive oxygen species (Malaviya et al., 1994).

The activation of mast cell leads to the production of mast cell extracellular traps (MCETs) which are composed of DNA, granular proteins such as cathelicidin, LL-37 and tryptase and histones which are known to have an antimicrobial function (von Kockritz-Blickwede et al., 2008). In addition, the release of mast cell proteases helps to contain the toxic effect of poisonous venoms and endogenous peptides (Akahoshi et al., 2011).

Mast cells plays an important role in viral infections. The ability of mast cells to be infected with several viruses such as human immunodeficiency virus (HIV), Influenza A virus and many others allows the cell to be activated and this activation leads to the production of several cytokines and chemokines such as IL-6, IL-1β, CCL3,4,5 and 8 (Burke et al., 2008).

Mast cells can recognise viruses and recruit $CD8^+$ T lymphocytes to the site of infection by secreting chemotactic factors such as CCL3, CCL4 and CCL5 (Bulfone-Paus and Bahri, 2015) which trigger cellular responses to these viruses by the production of interferon type 1 (IFN-1) more specifically type β. All these

functions demonstrate the important role of mast cell in immune surveillance and pathogen clearance (Orinska et al., 2005).

1.3.2 Adaptive immunity

Mast cells play a major role in the expulsion of helminth gut parasites. These effects are species dependent which can be mediated by mast cell dependent and independent mechanisms (Mukai et al., 2016). For example, in the events following infection with *Strongyloides* species, parasite antigens are identified by T helper 2 (Th2) cells which induce the production of Th2 cytokines such as IL-3 and IL-9, which in turn stimulate mast cell proliferation and activation. This activation leads to the secretion of mast cell mediators that cause physiological changes to the gut such as increases in intestinal permeability, net fluid secretion and smooth muscle contraction at the early stage of the infection. A second response can be escalated as a result of re-infection, and an immediate reaction occurs mediated by the previously synthesized IgE by parasite-specific B cells (Maizels and Holland, 1998).

Mast cell mediators can also regulate the production of cytokines such as IL-33 and IL-25 and thymic stromal lymphopoietin (TSLP) from endothelial and epithelial cells which are important for worm removal from helminth infections and optimal Th2 responses (Hepworth et al., 2012). As well as encouraging dendritic cells (DCs) to initiate and to develop an adaptive immune response, mast cells have the ability to suppress these immune responses by releasing anti-inflammatory cytokines such as TGF-β and IL-10 (Rao and Brown, 2008).

The induction of these adaptive immune responses is promoted by DCs which are specialized antigen presenting cells (APCs) (Vermaelen et al., 2001).

More recently, mast cells have also been shown to work as antigen presenting cells by processing and presenting the antigen through major histocompatibility complex (MHC) molecules II (Stelekati et al., 2009).

Secreted mediators from mast cells regulate the migration and activation of DCs. The activation of skin mast cells through TLR7 leads to the generation of TNF and IL-1β, these mediators encourage DCs to migrate to lymph nodes and also induce cytotoxic T lymphocytes responses or directly activate T lymphocytes by generated TNF. Th2 responses can be developed by DCs as a result of the involvement of mast cell mediators such as histamine, PGD_2 and PGE_2 (McIlroy et al., 2006, Theiner et al., 2006)

Mast cells can also recruit eosinophils by the secretion of IL-5. The recruitment of eosinophils helps in the destruction of some tissue-migrating helminths. For example, in *Nippostrongylus brasiliensis* infections, eosinophils generate Th2 cytokines which promote Th2 responses and stimulate mucin secretion from effector cells such as goblet cells which trap worms and eliminate them from the gut (Maizels and Holland, 1998).

1.3.3 Immune tolerance

The concept of mast cells being involved in maintaining immune tolerance has recently been established. Mast cells and $CD4^+CD25^+FOX3P^+$ regulatory T cells (T_{reg}) are involved in peripheral tolerance and tolerance to allografts (Lu et al., 2006, de Vries et al., 2009). IL-9 secreted by activated T_{reg} recruits mast cells to allograft sites (Li fan Lu 2006). Activated mast cells can secrete cytokines with immune suppressive effects such as TGF-β and IL-10 (de Vries et al., 2009). Mast cell-derived TGF-β and IL-10 reduces inflammation, leukocyte infiltration,

necrosis, epidermal hyperplasia and ulceration which rejection of skin allografts (Grimbaldeston et al., 2007). In addition, mast cells expressing OX40L (CD252) interact with T_{reg} expressing OX40, which inhibits calcium mobilization and mast cell degranulation without interfering with cytokine generation. This process is very effective against reducing any immediate hypersensitivity responses as IgE-mediated degranulation to allografts, can interfere with this process and prevent tolerance (Frossi et al., 2011).

In addition to allograft tolerance, mast cells are involved in tolerance to self. MIP-1α recruits mast cells by interacting with the cell surface receptor, CCR1, which leads to the production of TNF-α, IL-6 and TGF-β (Davidson et al., 2007). TGF-β is crucial for T_{reg} development at the site of inflammation. Developed T_{reg} can directly supress effector T cell proliferation through the release of anti-inflammatory cytokines such as TGF-β and IL-10, which play a crucial role in self-tolerance (Zheng et al., 2004).

Mast cell mediators such as proteases, histamine and PGE_2 are also found to be involved in immune tolerance. In skin studies, skin mast cell mediators could modulate immune tolerance either by reducing antigenicity, leukocyte recruitment or mediating immune suppression (de Vries and Noelle, 2010, da Silva et al., 2014).

1.4 Pathological role of mast cells

1.4.1 Allergy

Allergy develops when mast cells or other immune cells respond to an innocuous antigen in an inappropriate fashion. Mast cells are the key effector cells which are responsible for allergic reactions mediated by IgE.

In allergic individuals, antigens that are innocuous are recognized by APCs and presented to naïve T lymphocytes that differentiate into Th2 lymphocytes (da Silva et al., 2014). As a result of this differentiation, Th2 lymphocytes generate cytokines which allows B lymphocytes to produce IgE specific for this particular antigen. Mast cells also have a direct role in this sensitization by recognising, processing and presenting these antigens to Th2 lymphocytes through MHCII (da Silva et al., 2014).

The relationship between mast cells and allergic reactions, especially asthma, has mainly focused on the acute phase reactions. The polyvalent antigen-IgE antibody interaction, via FcεRI, leads to mast cell activation and immediate secretion of pre-formed and newly-synthesized mediators, which primes immediate hypersensitivity reactions. The secretion of these mediators causes allergic symptoms such as smooth muscle contractions and increase vascular permeability among other events. In lungs, asthma is initiated because of immediate secretion of pre-formed mediators such as histamine, LTC_4 and PGD_2 that cause bronchoconstriction, respiratory mucosal oedema and mucus secretion (Hofmann and Abraham, 2009).

Mast cells are also involved in late phase allergic reactions. Immune cells such as basophils, eosinophils and T cells are recruited to the inflammation sites as a result of chemotactic factors and pro-inflammatory cytokines secreted by mast cells (Hofmann and Abraham, 2009). The continued release of mast cell mediators can promote longer term effects, such as fibrosis, tissue remodelling and persistent inflammation (chronic phase) which is observed in asthma as well as other allergic reactions (Brown et al., 2008).

1.4.2 Mastocytosis

Mastocytosis is a disorder characterized by an increase in mast cell numbers and their mediators in organs such as the gastrointestinal tract, skin, and lymph nodes (Horny et al., 2008). The clonal accumulation of mast cells at these sites is caused by constitutive activation of mutant c-KIT receptors. A number of mutations in c-KIT such as D816V, V560G, D816Y, D816F and D816H are thought to play an important role in the aetiology of the disease. These mutations sit in the activation loop domain and create a change to the enzymatic pocket of the c-KIT receptor. This change leads to ligand-independent continuous activation of the c-KIT receptor (Verstovsek, 2013).

Mastocytosis is characterized by different clinical symptoms such as nausea, pruritus, vascular instability, diarrhoea, vomiting and abdominal pain (Metcalfe, 2008). The disease is also categorized as two main variants: cutaneous mastocytosis (CM) and systemic mastocytosis (SM) which are based on specific inherited genetic polymorphism which then develop into somatic mutations (Metcalfe, 1991). Mastocytosis can be clinically detected by the staining characteristics of mast cell tryptase, CD117 (c-KIT receptor) and other markers using immunohistochemistry. Neoplastic mast cells also exclusively

express CD2 and CD25 which are absent under normal conditions (Escribano et al., 1998, Sotlar et al., 2004). Mastocytosis can be treated by inhibiting the action of the mediators secreted by mast cells such as histamine using H2 antihistamines or by using c-KIT inhibitors such as imatinib which inhibits the protein kinase activity of c-KIT (Metcalfe, 2008).

1.4.3 Autoimmune diseases

Autoimmune diseases occur when the immune system fails to distinguish between self and non-self molecules. Autoimmune disease can be mounted when self-reactive lymphocytes are activated by different innate immune cells such as mast cells. Mast cells have the ability to stimulate autoreactive T cells by priming and recruitment of immune cells to the inflammation sites, which can lead to tissue damage (Christy and Brown, 2007). Mast cells are involved in many autoimmune diseases especially the initiation and the progression of chronic inflammatory diseases such as type 1 diabetes, multiple sclerosis (MS) and rheumatoid arthritis (RA)(da Silva et al., 2014).

MS is a disorder affecting the central nervous system. MS is identified by degeneration in the blood-brain barrier followed by infiltration of mononuclear cells into the white matter and destruction of axon myelin (demyelination). The experimental model of MS is allergic encephalitis (EAE) (Brown et al., 2002). There is a positive correlation between the number of mast cells and the development of MS or EAE (da Silva et al., 2014). This is followed by increased mast cell activation as indicated by the presence of tryptase in the cerebrospinal fluid and an associated increase in degranulated mast cells. The progression of these diseases depends on the interaction between IgGs and the expression of Fcγ receptors (FcγIII) on mast cells (Brown et al., 2002).

RA is a disease caused by chronic inflammation in the joints. RA might be linked to the expression of glucose-6-phosphate isomerase (GPI) enzyme. According to mouse models of RA (K/B_xN), these models generate auto-antibodies to GPI and this is associated with severe inflammation (Zhang et al., 2011). The interaction between GPI and the corresponding antibody leads to the formation of immune complexes on the articular cavity surfaces. These complexes initiate a wide variety of signalling cascades including the activation of Fc receptors of mast cells and neutrophils, activation of the complement pathway and the production of the pro-inflammatory cytokines IL-1 and TNF- α (Ji et al., 2002a, Ji et al., 2002b). TNF-α in particular is important at inducing fibroblasts to secret SCF, which is one of the main factors that recruits mast cells to the site of inflammation (Benoist and Mathis, 2002).

1.5 IgE-dependent activation

1.5.1 Immunoglobulin E (IgE)

IgE (190 kDa) is similar to other immunoglobulins in that they all consist of two light and two heavy chains. However, the heavy chains contain four constant domains (Cε1- Cε4) with an extra constant domain (Cε2) compared to IgG. Circulating IgE has the ability to interact with corresponding receptors (see *Figure 1.1*). This interaction is achieved by the firm and secure attachment to the receptor via the Cε2-Cε3 regions of IgE. IgE can remain bound to the high affinity receptor (FcεRI) for about several weeks (Gould et al., 2003, Kubo et al., 2003).

The concentration of free IgE in human circulation is between 50 - 200 ng/ml which is the lowest of all immunoglobulins (10 mg/ml for IgG). The half-

life of free IgE is 2 days compared to IgG (21 days). IgE shares about 30% amino acid homology with IgG. However, IgE does not fix complement and does not promote agglutination reactions. IgE also does not exist as subclasses unlike other immunoglobulins (Waldmann 1969).

Total IgE levels increase during one's life-time but then decline with ageing. Most patients with allergic diseases or parasitic infections have increased levels of total IgE. However, allergic patients can still maintain normal total IgE levels even with high levels of IgE specific to allergens. IgE is the immunoglobulin which is involved in type I hypersensitivity reactions (Ribatti, 2016). IgE is the principal immunoglobulin that is produced by B cells and plasma cells in the presence of IL-13 and IL-4 which promote class switching from different isotypes to IgE (Lebman and Coffman, 1988, Punnonen and Devries, 1994).

The production of IgE by antigen-specific B cells requires two signals. First, the interaction between IL-4 and IL-13 with B cell surface receptors which initiates Janus kinase 3 (JAK3) and signal transducer and activator of transcription 6 (STAT 6) signal pathways (Jiang et al., 2000). Second, the interaction between CD40 ligand (CD40L) on T cells and CD40 on B cell. Both signals are required for IgE synthesis and release (Iciek et al., 1997).

The most important role of IgE is the ability to sensitize mast cells. Once sensitized to a specific antigen, the mast cell is primed to respond to that antigen upon secondary contact. Allergic reactions are caused by IgE-dependent activation of mast cells and the subsequent recruitment and infiltration of other inflammatory cells to the site of inflammation (Matthews et al., 2014).

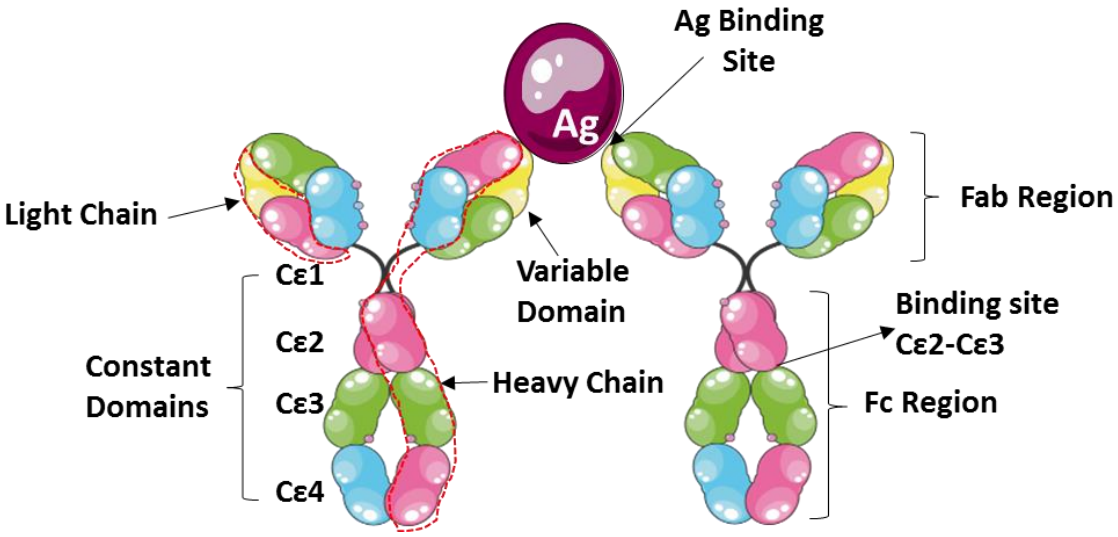


Figure 1.1 The structure of IgE. Schematic representation of the domain structure of human IgE showing two heavy and two light chains, different domains, Antigen (Ag) binding site and the receptor binding site. Diagram recreated from (Ribatti, 2016) using Servier Medical ART.

1.5.2 High affinity IgE receptor (FcεRI)

IgE can bind to two different receptors; the high affinity receptor, FcεRI, and the low affinity receptor, FcεRII (CD23). FcεRI, which is the form that is responsible for immediate hypersensitivity reactions, is highly expressed on mast cells and basophils and is composed of a tetramer (αβγγ) while another form is exists as trimer (αγγ) and is expressed on other cells such as DCs, monocytes and eosinophils (Kelly and Grayson, 2016).

The avidity and high affinity (K_d ~1 nM) of IgE for FcεRI allows this interaction to stay intact for months. FcεRI is composed of three major subunits, alpha, beta and gamma chains. The alpha chain is involved in binding to IgE, while gamma and beta chains are responsible for mediating the intracellular signals (Takahashi and Ra, 2005). The binding between IgE and FcεRI is not thought to activate mast cells, however, the crosslinking of IgE-FcεRI complexes by antigen causes mast cell activation which leads to degranulation and the release of mast cell mediators (Huber, 2013).

By contrast, FcεRII (CD23) has two isoforms CD23a and CD23b. CD23a is exclusive to B cells while CD23b is expressed on other cells. FcεRII is a type two integral membrane protein with a specific IgE binding site (C-type lectin domain) at the C-terminal end of the extracellular part of FcεRII (Weis et al., 1998). IgE binds to FcεRII with low affinity (K_d 0.1-1 μM). Interaction of IgE with FcεRII provides a negative feedback on B cells which causes the inhibition of IgE synthesis (Acharya et al., 2010, Cheng et al., 2010).

1.5.3 Signalling pathway following IgE-FcεRI cross-linking

The interaction between IgE/FcεRI and the allergen leads to receptor aggregation which initiates signalling (Kubo et al., 2003, Gould et al., 2003). This initiation requires the involvement of Src family kinase member (Lyn kinase) with the FcεRI beta subunit. The trans-phosphorylation of immunoreceptor tyrosine-base activation motifs (ITAMs) on the beta subunit and the firm interaction with Lyn SH2 domains leads to an increase in Lyn activity (Parravicini et al., 2002).

The enhancement in Lyn activity leads to the phosphorylation of ITAM gamma subunits which leads to the recruitment and the activation of the ZAP70 family member (Syk kinase). Activated Syk leads to auto-phosphorylation and interactions including those with, SH2-containing leukocyte-specific protein 76 (SLP-76), linker for activation of T cells (LAT), phospholipase C-γ1 and 2 (PLC-γ1/2). Other Src family kinase members such as Fyn may also be involved in initiating the IgE/FcεRI signalling pathway. Grb2-associated binder-link protein 2 (Gab2) is phosphorylated by Fyn which binds to phosphatidylinositol 3-kinase (PI3K) specific subunit (p85) which activates the PI3K pathway. The interaction of Gab2 and p85 leads to an acceleration in phosphatidylinositol-4,5-bisphosphate (PIP_2) conversion to phosphatidylinositol-3,4,5-triphosphate (PIP_3), the membrane PIP_3 attracts pleckstrin homology domain proteins such as Btk and PLC-γ1 and 2 (Parravicini et al., 2002, Siraganian, 2003).

Phosphorylation of PLC-γ1 and 2 accelerates the hydrolysis of PIP_2, which leads to the generation of inositol-1,4,5-trisphosphate (IP_3) and the 2nd messenger, 1,2-diacylglycerol releasing internal calcium which activates protein kinase C (PKC). The generation of an intracellular calcium signal is crucial for mast cell granules-plasma membrane fusion and degranulation (Ma and

Beaven, 2011, Nishida et al., 2005). The uptake of Ca^{2+} is regulated by the Ca^{2+} operated channel Ca^{2+} release-activated Ca^{2+} modulator 1 (CRACM1) also known as Orai1 and internal Ca^{2+} stores are controlled by the endoplasmic reticulum (ER) protein, stromal interaction molecule 1 (STIM1) which is responsible for clearing all the internal Ca^{2+} stores (Vig et al., 2008, Baba et al., 2008). STIM1 is also responsible for sending signals to CRACM1 to allow more Ca^{2+} into the cell. CRACM1 and transient receptor protein channel 5 (TRPC5) plays an important role as a gate for increasing Ca^{2+} in mast cells (Ma et al., 2008). The phosphorylation and the activation of SLP-76, Btk and LAT are crucial for signal generation and maintained Ca^{2+} influx (Siraganian, 2003).

These events leads to further activation of other enzymes and related adaptors such as Grb2, Vav, Shc and SOS which leads to the stimulation of hydrolase enzymes (GTPases) such as Ras, Rac and Rho, and to the activation of Jun amino-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK) and p38 mitogen-activated protein (MAP) kinase pathways (Siraganian, 2003). All these events lead to mast cell degranulation and the release of pre-formed mediators, the generation of newly-synthesised mediators as well as induction of transcription factors (Ma and Beaven, 2011, Garman et al., 1998) (see *Figure 1.2*).

Mouse studies suggest the involvement of phosphatidylinositol 5' phosphatase (SHIP) as a gatekeeper of IgE-mediated responses. This function has also been demonstrated in human basophils, the function of SHIP in human mast cells has yet to be demonstrated (MacGlashan, 2007, Gibbs et al., 2006). Hck is another Src family kinase member which has a negative influence on

other Src family kinase members (Lyn and Fyn) and so may play a role in attenuating the extent of activation (Hong et al., 2007).

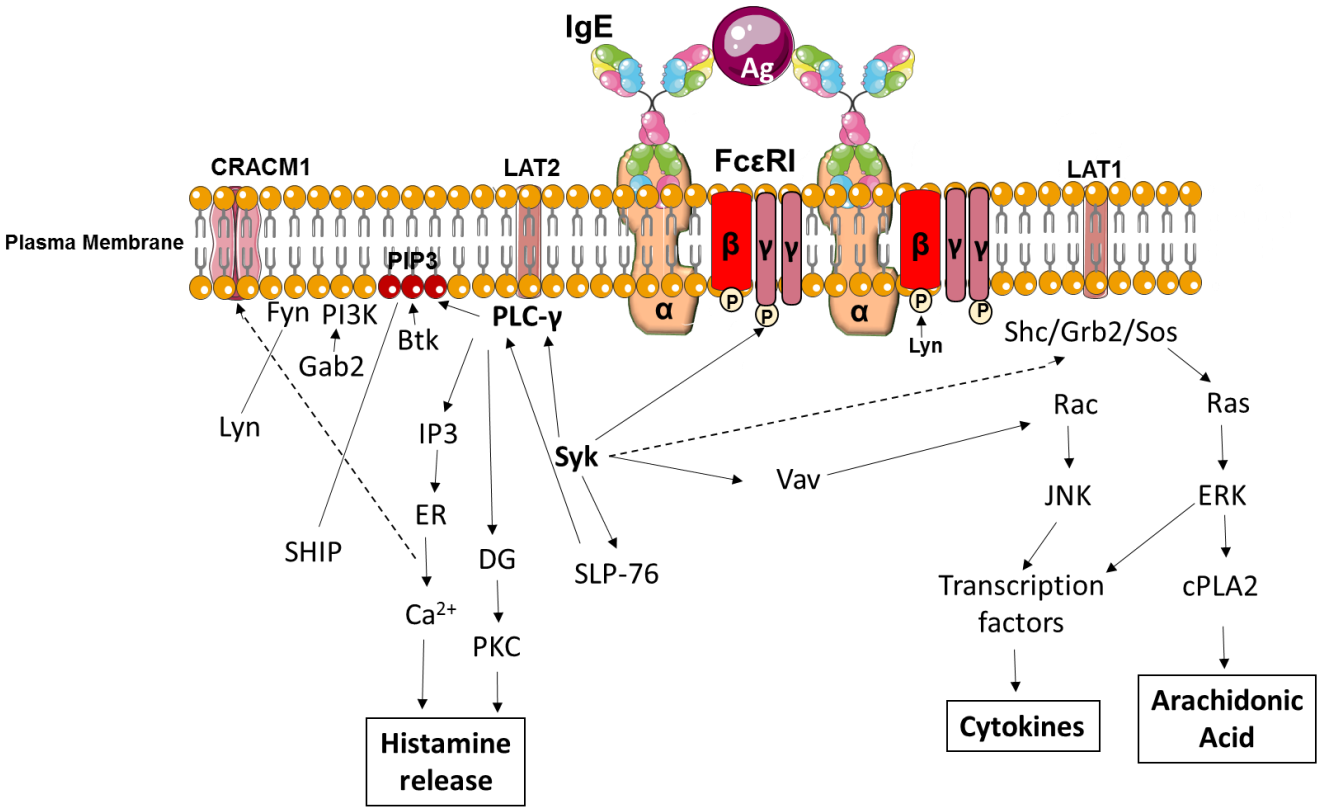


Figure 1.2 IgE-FcεRI signalling pathway. The pathways shown simplify the events involved in the initiation of this cascade. The interaction between Ag and the IgE bound to the α chain of the FcεRI leads to receptor aggregation and the phosphorylation of β and γ domains of the receptor by lyn. The recruitment and the activation of Syk leads to the phosphorylation of other related proteins, enzymes and adaptors. All these contribute to the release of histamine and arachidonic acid and the production of cytokines. Diagram recreated from (Siraganian, 2003, MacGlashan, 2008) using Servier Medical ART.

1.6 Stem cell factor dependent activation

1.6.1 SCF

As has already been discussed (see *Section 1.2*), SCF is a critical growth factor for mast cells. SCF is also called mast cell growth factor and steel factor (Reber et al., 2006). SCF is involved in mast cell survival, hyperplasia, adherence and chemotaxis (Da Silva et al., 2006b). SCF is known to be involved in cell proliferation and differentiation of immature $CD34^+$ and their progenitors as well as other cells (Huang et al., 1990, Rottem et al., 1994). SCF is also involved in the function and development of other cells such as germ cells and melanin producing cells although it is primarily associated with mast cells (Sette et al., 2000, Yoshida et al., 2001). SCF mediates its effects through the c-KIT receptor.

The human SCF gene is located on chromosome 12q22-q24 and encoded at the steel locus (Anderson et al., 1991). SCF is expressed and produced by different cells such as bronchial epithelial cells, human lung mast cells, pulmonary fibroblasts and lung smooth muscle cells (Reber et al., 2006). SCF is expressed as two isoforms; isoform one is a 165 aa soluble form (sSCF) generated from a 248 aa glycoprotein, isoform two is a membrane form (mSCF) generated from a 220 aa glycoprotein (Da Silva et al., 2006b, Ashman, 1999a). Both isoforms are expressed at the cell membrane, but the latter form remains in a membrane form unless secondary cleavage forms the soluble form (Majumdar et al., 1994).

SCF is known to be involved in enhancing histamine release as well as leukotriene C_4 generation in response to IgE activating mechanisms without inducing mediator release itself (Bischoff and Dahinden, 1992). Our data shows

that in some mast cell preparations, SCF might actually be involved in inducing mediator release as well as enhancing IgE dependent activation (Lewis et al., 2013)

1.6.2 c-KIT receptor (CD117)

The c-KIT receptor (CD117) is a receptor tyrosine kinase (RTK), a member of the type III receptor tyrosine kinase family (Edling and Hallberg, 2007). The human c-KIT gene is located on chromosome 4q11-q12 and encoded at the white (W) locus. c-KIT receptor is a 145kDa glycoprotein expressed by haematopoietic cells, haematopoietic progenitor and precursor cells and most importantly mast cells (Broudy, 1997). CD117 can also be expressed by B and T lymphocyte progenitor cells (Palacios and Nishikawa, 1992) as well as other non-haematopoietic cells.

c-KIT receptor is present in two isoforms, a soluble form that is most likely to be responsible for SCF activity and a membrane bound form (Broudy et al., 1994). CD117 expresses structurally distinct domains: the extracellular domain involves five Ig-like motifs; the 2nd and 3rd motifs create the ligand binding pocket and the 4th motif is the site for dimerization (SCF binding to two c-KIT receptors together to form a dimer), the intracellular domain composes of a juxtamembrane domain, distal and proximal kinase domain with kinase insert domain between them. Other domains associated with the c-KIT receptor are the N-terminal signal sequence, the activation loop and C-terminal tail (see *Figure 1.3*) (Broudy, 1997, Edling and Hallberg, 2007, Da Silva et al., 2006b).

CD117 is involved in several biological activities such as differentiation, survival, proliferation and activation of mast cells and other haematopoietic cells (Edling and Hallberg, 2007). Activation of c-KIT by SCF has been linked to many inflammatory diseases such as rheumatoid arthritis, skin inflammation, asthma and allergic rhinitis (Reber et al., 2006). The SCF/c-KIT complex triggers receptor dimerization and phosphorylation, which leads to the activation of several signal transduction pathways (Heldin, 1995).

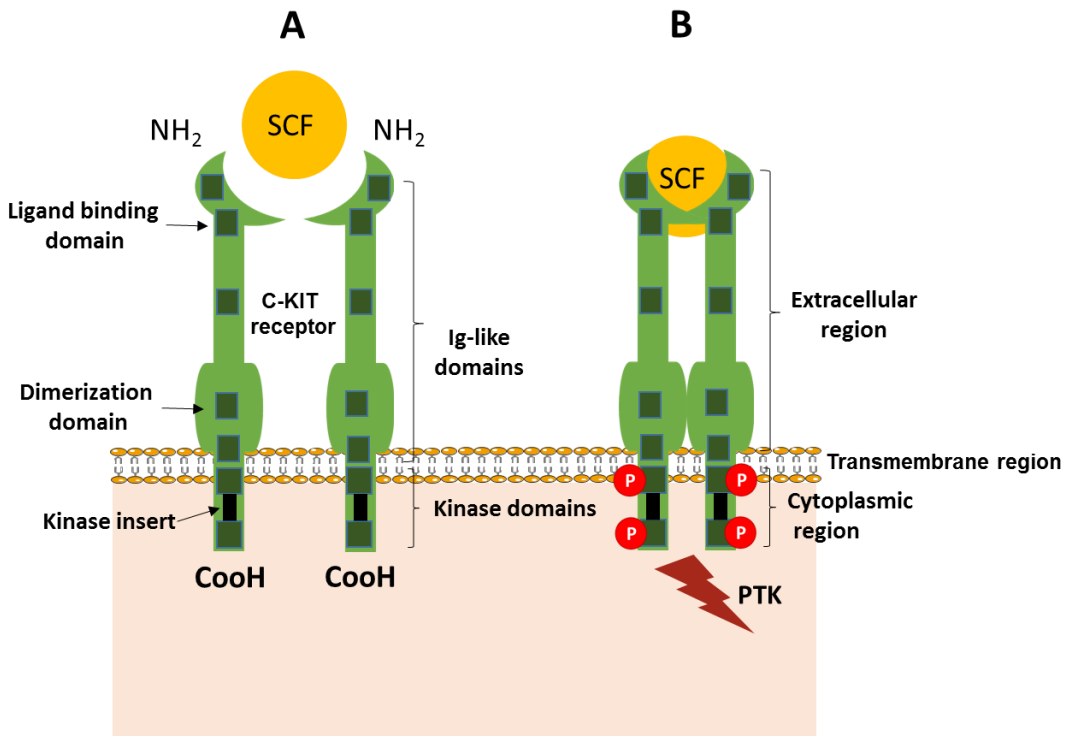


Figure 1.3 Structure of c-KIT receptor. (A) demonstrates the different domains of the c-KIT receptor. (B) illustrates the interaction between SCF and c-KIT which leads to receptor dimerization and auto-phosphorylation which leads to protein tyrosine kinase activity. Diagram recreated from (Reber et al., 2006) using Servier Medical ART

1.6.3 c-KIT signalling

The dimerization of c-KIT receptor by c-KIT ligand induces the activation of intrinsic tyrosine kinase activity (see *Figure 1.4*). This leads to the phosphorylation of specific tyrosine residues of the cytoplasmic tail of c-KIT. Tyrosines Y^{568} and Y^{570} in the juxtamembrane domain, Y^{823} and Y^{900} in the C-catalytic domain, Y^{703}, Y^{721}, Y^{730} and Y^{947} in the linker region of the insert domain and Y^{936} in the C-terminus domain may all play a role in intracellular signalling (Gilfillan and Rivera, 2009, Ronnstrand, 2004).

The phosphorylation of the receptor offers docking sites for signalling proteins or adaptor proteins with SH2 domains (Da Silva et al., 2006b). Prominent interactions include the recruitment and activation of Src signalling pathway family members Src, Lyn, Fyn and Tec at the phosphorylated tyrosine residues Y^{568} and Y^{570} (Da Silva et al., 2006b). Src activation ultimately leads to the activation of the mitogen activated protein (MAP) kinase pathway (Reber et al., 2006).

Adaptor protein growth factor receptor-bound protein 2 (Grb2) is recruited to interact with sos (son of sevenless proteins) at the phosphorylated residues Y^{703} and Y^{936} of the c-KIT receptor (Reber et al., 2006). This interaction leads to the activation of the MAP kinase signalling pathway by the activation of G-proteins Ras and Raf 1. This leads to the activation of p38, JNK (c-Jun N terminal kinase) and ERK1/2 (extracellular signal regulated kinase) which are involved in gene transcription and can promote cell proliferation (Ishizuka et al., 1998, Ronnstrand, 2004).

c-KIT-ligand interaction induces the activation of the phospholipase Cγ (PLCγ) signalling pathway by the interaction following phosphorylation of tyrosine residue Y^{930} by SCF (Gommerman et al., 2000). c-KIT/SCF interaction induces the activation of phosphoinositide 3 kinase (PI3K) through several signalling pathways following phosphorylation of tyrosine residue Y^{721} (Gilfillan and Rivera, 2009).

Moreover, the SCF-CD117 complex can induce other signalling pathways such as Janus kinase signal transducers and activators of transcription (JAK/STAT) pathways, in particular JAK2 (Brizzi et al., 1999). Other signalling molecules which are involved in c-KIT receptor phosphorylation include linker of activated T cells 2 (LAT2), Bruton's tyrosine kinase (Btk) and other secondary signalling events such as the molecular target of rapamycin (mTOR) (Gilfillan and Rivera, 2009).

Finally, the c-KIT-ligand crosslinking kinase activities can be reversed by several mechanisms. Under the influence of different pathways, protein kinase C (PKC) interacts with CD117 to detach the extracellular part of c-KIT by the phosphorylation of S^{741} and S^{746} residues of the kinase insert domain (Reber et al., 2006). c-KIT signalling can be down-regulated by the phosphatase SHP-1 and suppressor of cytokine signalling 1 and 6 (Socs-1 & 6) by binding to phosphorylated Y^{570} or to Grb2 and vav (human vaccinia virus oncogene) protein respectively (De Sepulveda et al., 1999). In addition, Casitas B-lineage protein (Cb1) is recruited to degrade phosphorylated c-KIT through the lysosome and proteasome pathways (Zeng et al., 2005).

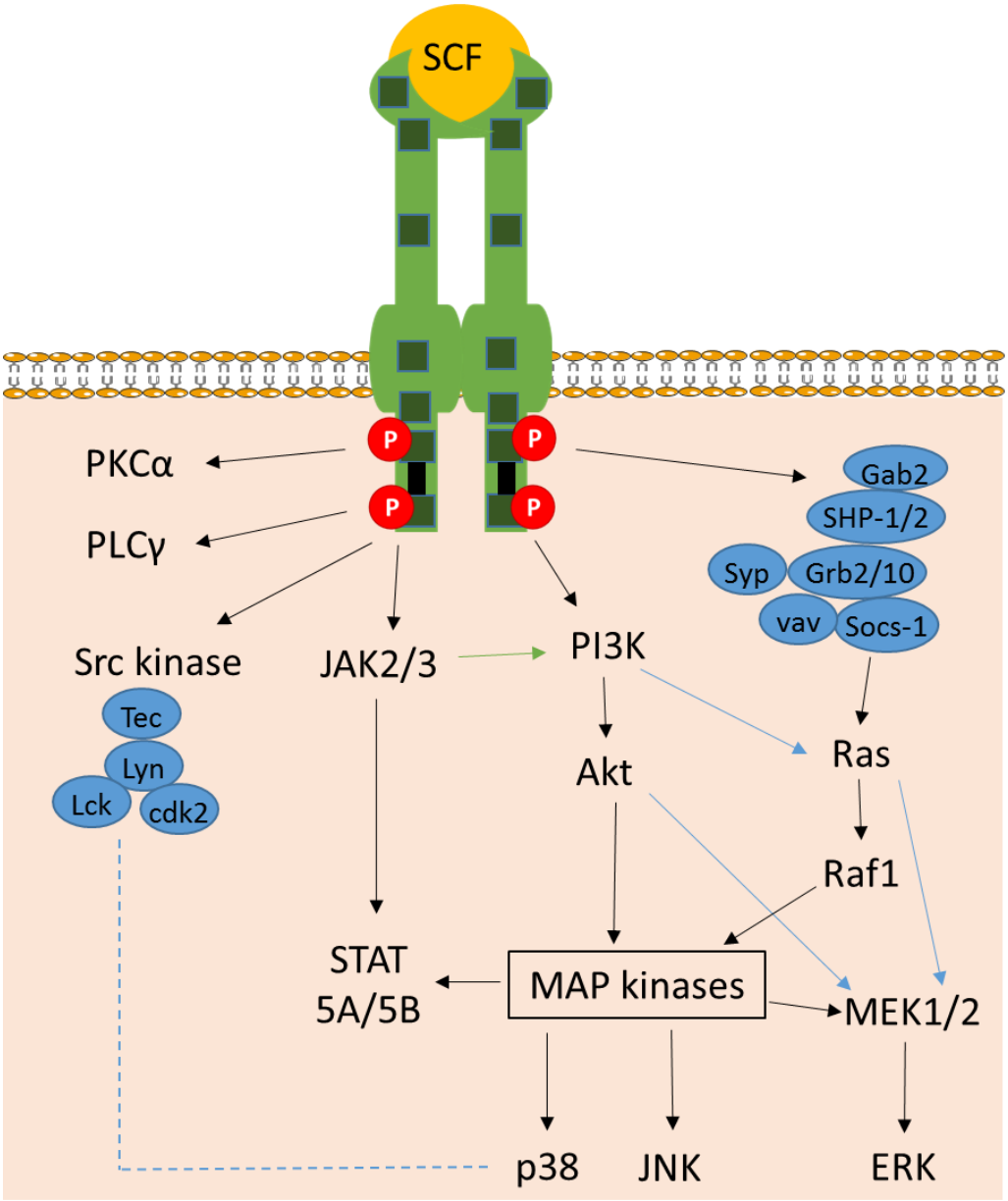


Figure.1.4 c-KIT receptor signal transduction pathways. This figure demonstrates the effect of SCF/c-KIT interaction which activates several signalling pathways by the phosphorylation of specific intracellular kinase domains. The activation of these pathways causes many biological effects such as proliferation, survival and mediator release. Diagram recreated from (Da Silva et al., 2006b) using Servier Medical ART.

1.6.4 c-KIT inhibitors

c-KIT inhibitors are used to reverse constitutive cell activation of the c-KIT receptor (von Mehren, 2006). Continuous activation of c-KIT is observed with mutations of the receptor such as D816V. Constitutive c-KIT receptor activation is considered to be involved in mastocytosis (see *Section 1.4.2*) (Jensen et al., 2008). There are several drugs, which inhibit c-KIT tyrosine kinase activity. Most important of these are imatinib (Gleevec or STI-571), dasatinib (Sprycel or BMS-354825) and nilotinib (Tasigna or AMN107). These drugs are mainly used to treat cancer but by interfering with c-KIT/SCF interactions the inhibitors can block signalling events to prevent continuous proliferation and activation of mast cells.

Imatinib prevents the interaction between adenosine triphosphate (ATP) and selective tyrosine kinase molecules such as Bcr-Abl, Abl, c-KIT and platelet-derived growth factor receptor (PDGRR) (von Mehren, 2006). Imatinib is a drug for patients with chronic myeloid leukaemia (CML) by inhibiting the formation of Bcr-Abl oncogene and preventing the expression of Bcr-Abl fusion protein (Buchdunger et al., 2002). Imatinib mainly targets the ATP binding site of c-KIT. However, imatinib resistance is an issue for the D816V mutation as the mutation changes the structure of the ATP binding site and prevents imatinib/c-KIT interactions (Jensen et al., 2008). Drug combination is a strategy that has been used to avoid resistance (Weisberg and Griffin, 2003).

Dasatinib is a Src kinase inhibitor but also inhibits the Abl and Bcr-Abl activity (von Mehren, 2006). *In vitro* studies illustrated that dasatanib has an inhibitory effect on several c-KIT mutants (D816V and V560G). However, clinical trials demonstrated that dasatinib is considered to be less specific than other c-

KIT inhibitors, as well as causing toxicity (Jensen et al., 2008). Dasatinib is over 300-fold more potent than imatinib by binding to the active and inactive kinase domains of Abl. Dasatinib works by blocking the signalling pathway of STAT5 and MAPK. Dasatinib is mostly used to treat CML patient who are resistant to imatinib (Breccia et al., 2013).

Nilotinib is also a potent second generation Bcr-Abl tyrosine kinase inhibitor (Breccia and Alimena, 2010). Nilotinib targets c-KIT receptor expressing the V560G mutation as well as wild type c-KIT, but nilotinib failed to show any inhibition of the D816V c-KIT mutation (Jensen et al., 2008). Nilotinib is about 30-fold more potent than imatinib despite the similarities between them. *In vitro* studies demonstrated that nilotinib when combined with other drugs could potentially reduce the tumour size of animal models of CML disease (von Mehren, 2006).

Collective data from *in vitro* and *in vivo* studies illustrated that dasatinib was the least effective c-KIT inhibitor in the context of systemic mastocytosis without D816V c-KIT mutation. Dasatanib has a very short half-life of about 5 h and has a tendency to cause pleural effusions and oedema. Nilotinib did not cause any significant inhibition of the D816V c-KIT mutant, however, it was effective against other c-KIT mutants such as D560D. Imatinib was effective in both cutaneous mastocytosis and systemic mastocytosis without the D816V c-KIT mutation. Imatinib was the only c-KIT inhibitor approved by the US Food and Drug Administration at treating adult patients with aggressive systemic mastocytosis (Ustun et al., 2011).

1.7 Mast cell mediators

Activation of mast cells through FcεRI leads to the induction of many signalling pathways (see *Section 1.5.3*). However, the consequent release of mediators is not exclusive to IgE-dependent activation, non-IgE dependent mechanisms can also contribute to mediator release. For example, secreted IL-33 from necrotic cells is known to activate mast cells. This activation plays an important role in tissue injury (Enoksson et al., 2011). In addition, brain injury, stress and related conditions such as post-traumatic stress disorder can also activate mast cells to release preformed and newly-synthesized mediators which suggests an important role for mast cell activation in these conditions (Kempuraj et al., 2017).

The activation of mast cells leads to the release of a variety of mediators, those that are preformed and stored within distinct secretory granules as well as those that are newly-synthesized such as the eicosanoids as well as other products such as cytokines and chemokines (see *Figure 1.5*). Biological events such as smooth muscle contraction, increases in vascular permeability, vasodilation, inflammation and bronchoconstriction can be caused by mast cell mediators (Bingham and Austen, 2000).

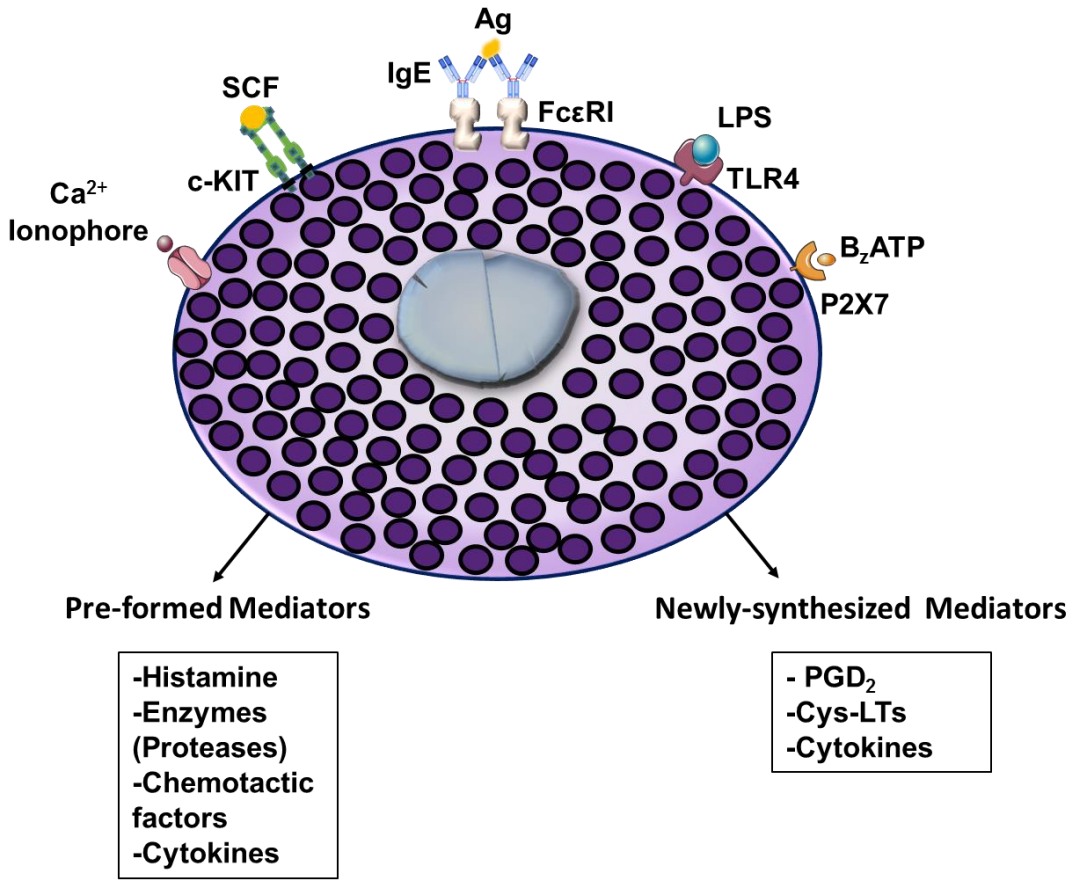


Figure.1.5 Mast cell activation by IgE-dependent and independent mechanisms. High affinity IgE receptors (FcεRI) are expressed all over the cell. The interaction between the antigen and corresponding IgE cross-links FcεRI and activates several signalling pathways. This activation then leads to the release of preformed and the newly synthesised mediators. Besides IgE-dependent processes, other receptors such as c-KIT, TLR4 and P2X7 may be involved in mast cell activation. Diagram created using Servier Medical ART.

1.7.1 Pre-formed mediators

There are several biogenic amines which are released as a result of mast cell degranulation such as serotonin, dopamine, polyamines and the well-recognised mediator, histamine (Riley, 1953). Histamine is synthesized by decarboxylation of histidine using histidine decarboxylase (HDC) which is expressed by mast cells. During mast cell maturation the levels of HDC expression increase (Rothschild and Schayer, 1959). However, HDC can be expressed by other cells such as basophils (Ringvall et al., 2008). Histamine causes several biological activities such as bronchoconstriction, increased vascular permeability, stimulating vasodilation and bronchial smooth muscle contraction (Lundequist and Pejler, 2011).

Proteoglycans are one of the major constituents of mast cell granules. Each proteoglycan has a specific protein backbone to which sulphated glycosaminoglycan (GAG) chains are attached (Lundequist and Pejler, 2011). The expression of mast cell peptidoglycan is species-dependent. For instance, in humans, mast cells have both heparin and chondroitin sulfate (CS) where GAG can be sulphated in both (Metcalfe et al., 1979, Thompson et al., 1988). In rodents, connective tissue-type mast cells (CTMCs) contain sulphated GAG chains of heparin whereas in mucosal mast cells (MMCs) over-sulphated GAG chains of CS are found (Yurt et al., 1977, Enerback et al., 1985).

In order to distinguish between the GAG subtypes and mast cells subclasses from stained granules, cationic dyes such as alcian blue, berberine sulfate and safranin are used. (Lundequist and Pejler, 2011). Differentially, these dyes bind to the peptidoglycans of the mast cell granules. For example, alcian

blue favourably binds to MMCs, both heparin-binding dyes safranin and berberine sulphate binds to CTMCs (Tsai et al., 1991, Enerback, 1974).

Mast cell proteases express their enzymes differently depending on the mast cell species and subclasses (Pejler et al., 2010). These enzymes include tryptase, chymase and carboxypeptidase A (MC-CPA). These proteases play a key role in allergic inflammation and defence against bacteria and parasites. Mast cell derived non-specific proteases such as serine protease (Granzyme B), and others might be involved in extracellular remodelling and apoptosis. MCc also plays an important role in facilitating the conversion of angiotensin I to angiotensin II which is involved in regulating blood pressure (Lundequist and Pejler, 2011).

Moreover, lysosome proteases are reported to be found in mast cell granules, such as cathepsins B, C and L (cysteine cathepsins) and cathepsins D and E (aspartic acid proteases) (Henningsson et al., 2005, Lundequist and Pejler, 2011). Cathepsin B, D and L are known to be released following IgE-mediated mast cell activation (Dragonetti et al., 2000). Mast cell cathepsins play an important role in allergic diseases especially Cathepsin E (Henningsson et al., 2005). Cathepsins are involved in extracellular matrix degradation which suggests a role in airway remodelling (Faiz et al., 2013).

Mast cells can produce pre-formed cytokines, growth factors and chemokines from the secretory granules, and these can be released suddenly after mast cell activation. A mouse study illustrated that cytokines such as TNF-α can be produced by rapid mast cell activation or by *de novo* synthesis (Galli et al., 1991). Moreover, several cytokines are pre-stored within the granules such as interleukin-4 (IL-4), IL-15, vascular endothelial growth factor (VEGF),

nerve growth factor (NGF), basic fibroblast growth factor-2 (bFGK-2), stem cell factor and transforming growth factor-β (TGF-β) (Lundequist and Pejler, 2011).

1.7.2 Lipid-derived mediators

Mast cells are a rich source of lipid-derived (newly synthesised) mediators such as eicosanoids. Eicosanoids are involved in the pathophysiology of several diseases such as asthma and other allergic diseases (Boyce, 2007). Eicosanoids cause alterations in smooth muscle constriction, vascular permeability, stromal cell activation and recruitment of effector cells (Boyce, 2007).

In mast cells, the principal eicosanoids generated are prostaglandin D_2 and the cysteinyl-leukotrienes, LTC_4, LTD_4 and LTE_4 (Peters et al., 1984). These mediators are produced *de novo* from arachidonic acid which is derived from the nuclear membrane phospholipids by the action of cytosolic phospholipase A_2 ($cPLA_2$) (Clark et al., 1991).

1.7.2.1 Prostaglandin D_2

PGD_2 is produced in large quantities by mast cells. Arachidonic acid is converted to PGH_2 by cyclooxygenase or prostaglandin H synthase (PGHS) which exists in mast cells as two isoforms PGHS-1 and PGHS-2 (Murakami et al., 1994). Then PGH_2 is converted to PGD_2 using either haematopoietic prostaglandin D_2 synthase (H-PGDS), a spleen type PGDS, or lipocalin prostaglandin D_2 synthase (L-PGDS) (Boyce, 2007). PGD_2 is synthesised and transported by PG transporter protein to be secreted into the extracellular space where it can interact with corresponding receptors (Lu et al., 1996). PGD_2 is non-enzymatically metabolized to 15-deoxy-δPGJ_2 (15-dPGJ_2) (Kliewer et al., 1995)

and to 9α,11β-PGF_2 (Bochenek et al., 2004). These products are used to detect mast cell activation by measuring serum and urine levels of the products (Boyce, 2007).

There are two specific G protein-coupled receptors (GPCRs), DP_1 and DP_2 that mediate the effects of PGD2 (Hawcroft et al., 2004). DP receptors are expressed differently in humans and mice (Boyce, 2007). DP_1 and DP_2 have opposite functions, induction of intracellular cyclic adenosine monophosphate (cAMP) by stimulating adenylate cyclase (Boie et al., 1995) and reducing the intracellular cAMP and adenylate cyclase suppression, respectively (Sawyer et al., 2002). When comparing PGD_2 with other mediators such as histamine, PGD_2 causes bronchoconstriction but is 10 times more potent than histamine (Bochenek et al., 2004).

1.7.2.2 Leukotrienes (LTs)

Leukotrienes are also synthesised by mast cells. Both 5-lipoxygenase (5-LO) and its activating protein, 5-lipoxygenase-activating protein (FLAP) are involved in converting arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (Mancini et al., 1993). 5-HPETE is converted into 5-hydroxyeicosatetraenoic (5-HETE) and then to 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) which induces basophilic and eosinophilic chemotaxis by interacting with specific GPCR oxoeicosanoid receptor (OXE) (Boyce, 2007). 5-HPETE can also be converted into LTA_4 by 5-LO. LTA_4 can then converted into LTB_4 by leukotriene A_4 hydrolase (LTA_4H) (Evans et al., 1985). Alternatively, LTC_4 is formed as a result of LTA_4 glutathione reduction by leukotriene C_4 synthase (LTC_4S) (Lam et al., 1994). LTC_4 is converted to LTD_4

and LTE_4 by different enzymes, the latter is more stable but biologically less active.

Cysteinyl-leukotriene (cys-LT) production can be measured by determining the levels of LTE_4 detected in urine (Boyce, 2007). LTE_4 is known to cause bronchoconstriction and induce neutrophilic and eosinophlic cell recruitment. LTD_4 has been linked to airway remodelling. LTB_4 is largely recognised as a potent chemotactic factor (Boyce, 2007).

The leukotrienes mediate their effects through interaction with specific GPCRs These receptors are divided into $CysLT_1$ and $CysLT_2$ receptors which mediate the effects of cysteinyl-leukotrienes and, BLT_1 and BLT_2 receptors which are linked to LTB_4 (Savari et al., 2014). LTE_4 binds poorly to both $CysLT_1$ and $CysLT_2$, whereas LTD_4 is more potent than LTC_4 at $CysLT_1$ receptors whereas LTC_4 and LTD_4 are equipotent at the $CysLT_2$ receptor. BLT_2 receptor binds to LTB_4 with higher affinity than BLT_1 (Boyce, 2007, Savari et al., 2014). *Figure 1.6* summarises the products of eicosanoid synthesis and their receptors.

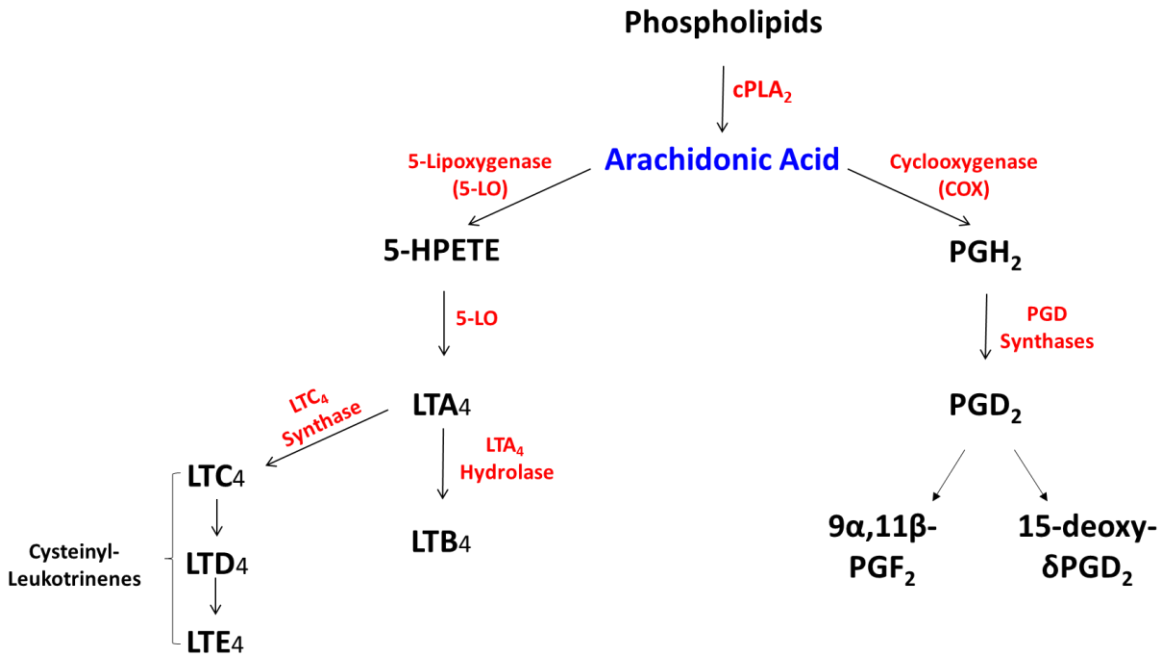


Figure.1.6 The biosynthesis of prostaglandin D_2 and leukotrienes. This figure illustrates the products of the fatty acid, arachidonic acid, which is metabolised through the COX and 5-LO pathways following mast cell activation to produce PGD_2 and the cys-LTs, respectively. These eicosanoids are involved in many allergic reactions.

1.7.3 Mast cell cytokines and chemokines

In addition to pre-formed and lipid-derived mediators, mast cells may generate many cytokines such as IL-4, IL-5 and IL-13 as well as chemokines such as IL-8. These cytokines contribute to allergic reactions such as asthma. For example, TNF-α, one of the main mast cell cytokines, activates macrophages and neutrophils to produce inflammatory mediators. Mechanistically, TNF-α increases E-selectin expression and other endothelial cell adhesion molecules and this leads to the recruitment of leukocytes to the site of inflammation. Moreover, TNF-α promotes migration and maturation of dendritic cells (Hart, 2001).

IL-5 is involved in the maturation and the survival of eosinophils. Eosinophils strongly contribute to late phase allergic reactions (Hart, 2001). IL-13 has an effect on smooth muscle function and airway remodelling. Moreover, IL-13 is involved in the production and synthesis of IgE antibody, in which IL-4 plays a similar role (Graber et al., 1998). IL-4 is also involved in orchestrating allergic reactions by influencing the phenotype of many inflammatory cells especially T-helper cells (Mosmann and Coffman, 1989).

Chemokines, in particular IL-8, is strongly chemotactic for eosinophils. IL-8 encourages neutrophils to secrete proteases and other inflammatory mediators. Other chemokines are secreted by mast cells such as monocyte chemotactic proteins 1, 3 and 4 which play an important role in asthma (Hart, 2001).

There is some controversy about the release of cytokines from mast cells, some studies have shown only limited generation of cytokines from mast cells

following activation (Gibbs et al., 1997). Cytokine generation might be species and phenotype specific. Church and Levi-Schaffer (1997) suggested that mast cells of the MC_T phenotype are immune system associated mast cells whereas MC_{TC} mast cells are non-immune related and involved in tissue remodelling and angiogenesis. MC_T mast cells are thought to be the main phenotype involved in allergic reactions, host defence and parasitic infections. They predominantly exist at mucosal surfaces. MC_T numbers increase at sites of Th2 activation. The activation of MC_T either causes the recruitment of T lymphocytes or the secretion of cytokines that contribute to allergic reactions (Church and Levi-Schaffer, 1997).

1.8 Concluding comments

In this introduction I have given an overview about mast cell origins and highlighted the physiological role of mast cells in innate immunity, adaptive immunity and immune tolerance. The potential pathological role of mast cells in several diseases such as allergy, mastocytosis and autoimmunity has also been discussed. I have deliberated about IgE, the FcεRI receptor and the classical way of activating mast cells through IgE-dependent mechanisms. Further discussion has centred on the potential role that SCF, acting through c-KIT, may have on mast cells. In this thesis, preliminary data are presented showing that contrary to expectations SCF is an effective direct activator of human lung mast cells. These data suggest that targeting the c-KIT receptor could be of benefit in respiratory diseases.

1.9 Aims and objectives

Aim: The principal aim of this project was to determine whether SCF is an effective activator of mast cells.

Hypothesis: SCF is a direct activator of human lung mast cells.

Objectives:

1. To investigate the effects of SCF on mediator release from mast cells.
2. To determine whether SCF is acting through the c-KIT receptor.
3. To explore the effect of SCF on eicosanoid generation.
4. To identify which COX isoform is responsible for PGD_2 generation.
5. To determine whether SCF promotes cytokine generation from mast cells.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Materials

Material	Company
Acetylsalicylic Acid (Aspirin)	Sigma, UK
Acrylamide, 99.9%	Bio-Rad Laboratories, UK
Agarose	Melford Labratories Ltd., UK
Alcian blue stain	Aldrich chemical company, inc., USA
Amersham Hybond-ECL Membrane	GH Healthcare, UK
Ammonium Persulfate (APS)	Sigma, UK
Anti-Actin Antibody produced in rabbit (A2066)	Sigma, UK
Anti-COX-1 goat polyclonal IgG (sc-1752)	Santa Cruz Biotechnology, USA
Anti-COX-2 goat polyclonal IgG (sc-1745)	Santa Cruz Biotechnology, USA
Anti-IgE	Stratech, UK
Bis N, N'-methylene-bis-acrylamide (BIS)	Bio-Rad Laboratories, UK
Blotting Grade Blocker Non-fat dry Milk	Bio-Rad Laboratories, UK
Bovine Serum Albumin (BSA)	Sigma, UK
Bromophenol Blue	Sigma, UK
$CaCl_2.2H_2O$	BDH, UK
Celecoxib	Bio Vision, USA
Collagenase from *Clostridium Histolyticum* Type IA	Sigma, UK
ColorPlus Prestained Protein Ladder, Broad Range (10-230 kDa)	New England BioLabs, UK
Complete, EDTA-free Protease Inhibitor Cocktail Tablets	Roche Applied Science, Germany

Cysteinyl-Leukotriene EIA Kit	Cayman Chemical Company, USA
Cysteinyl-Leukotriene express EIA Kit	Cayman Chemical Company, USA
Dasatinib	LC Laboratories, USA
Deoxyribonuclease I (DNase I) from bovine pancreas	Sigma, UK
Dexamethasone	Sigma, UK
Diclofenac (Sodium Salt)	Cayman Chemical Company, USA
Dimethyl Sulphoxide (DMSO)	Sigma, UK
DNA-free DNase Treatment & Removal	Ambion (Life Technologies), UK
Donkey anti-goat IgG-HRP (sc-2020)	Santa Cruz Biotechnology, USA
ECL Western Blotting Detection Reagent	GH Healthcare, UK
Ethanol	BDH, UK
Ethidium Bromide	Sigma, UK
Ethylenediaminetetraacetic acid (EDTA)	BDH, UK
FcR Blocking Reagent	Miltenyi Biotec Limited, UK
Fetal Bovine Serum (FBS)	Promocell, UK
FR122047 (Hydrate)	Cayman Chemical Company, USA
Fungizone	Invitrogen, UK
Gentamicin	Sigma, UK
Glacial acetic acid	Fisher scientific, UK
Glucose	Sigma, UK
Glycerol	Fisher scientific, UK
Glycine	Sigma, UK
GoTaq Flexi	Promega, Southampton, UK)
HEPES Buffer Solution (1M)	Lonza, BioWhittaker, Belgium

High Capacity cDNA RT Kit	Applied Biosystems, Paisley, UK
Histamine	Sigma, UK
Human CD117 Micro Beads	Miltenyi Biotec Limited, UK
Human Ready-Set-Go Cytokine ELISA	eBioscience, UK
Human Serum Albumin (HSA)	Sigma, UK
Human Stem Cell Factor (SCF)	Pepro Tech, London, UK
Hydrochloric Acid (12 M HCl)	Fisher scientific, UK
Ibuprofen	Sigma, UK
Indomethacin	Tocris, UK
Ionomycin	Tocris, UK
JW8-IgE	BioServ UK Ltd, UK
KCl	Sigma, UK
KH_2PO_4	Sigma, UK
Lipopolysaccharide (LPS) from *E.coli* serotype R515 (Re)	Enzo Life Sciences, UK
RPMI 1640 media with L-Glutamine	Lonza, BioWhittaker, Belgium
$MgCl_2.6H_2O$	BDH, UK
MACS separation Columns	Miltenyi Biotec Limited, UK
Methanol	BDH, UK
Na_2HPO_4	Sigma, UK
NaCl	Sigma, UK
Naproxen	Cayman Chemical Company, USA
Nilotinib	LC Laboratories, USA
NIP_{20}-HSA	Gift from Dr. Birgit Helm, University of Sheffield, UK

Nylon gauze (100 µm)	Incamesh Filtiration Equipment, UK
O-phthaldialdehde (OPT)	Sigma, UK
Penicillin-Streptomycin	Lonza, BioWhittaker, Belgium
Perchloric Acid 60%	BDH, UK
Percoll	Sigma, UK
Hematopoietic PGD synthase (sc-30066) rabbit polyclonal IgG (H-PGDS)	Santa Cruz Biotechnology, USA
Lipocalin PGD synthase (sc-14821) goat polyclonal IgG (L-PGDS)	Santa Cruz Biotechnology, USA
piperazine-N,N′-bis[2-ethanesulfonic acid](PIPES)	Sigma, UK
Polyclonal goat anti-rabbit IgG-HRP	DAKO, UK
Prostaglandin D_2 (PGD_2) MOX EIA Kit – Monoclonal	Cayman Chemical Company, USA
Proteome Profiler Human Cytokine Array Panel Kit	R&D Systems, UK
Quick load 100 bp DNA ladder	New England Biolabs, Hitchin, UK
RNase Inhibitor	Applied Biosystems, Paisley, UK
Sodium Dodecyl Sulfate (SDS)	Bio-Rad Laboratories, UK
Sodium hydroxide (10 M NaOH)	Sigma, UK
Sterile H_2O for injections	B.Braun, UK
Sulfuric Acid (1M)	Fisher scientific, UK
TaqMan Gene Expression Asssay	Applied Biosystems, UK
Tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, UK
TRI Reagent	Sigma, UK
Tris (hydroxymethyl) aminomethane	Fisher scientific, UK

(Tris Base)	
Triton™ X-100	Sigma, UK
Tween -20	Fisher scientific, UK
Wortmannin	Calbiochem, UK

2.2 Buffers and solutions

2.2.1 Mast cell isolation buffer

Lung digestion buffer

Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 25 mM hydroxyethyl piperazineethanesulfonic acid (HEPES), 10% foetal bovine serum (FBS), 1.5 mg/100 ml DNase I. For each gram of lung tissue, 1.2 mg Collagenase IA was added.

Washing buffer

RPMI 1640 medium supplemented with 25 mM HEPES and FBS (5%).

Mast cell culture Medium

RPMI 1640 medium supplemented with 25 mM HEPES and FBS (5%) and the following antibiotics: penicillin (10 U/ml), streptomycin (10 µg/ml), gentamicin (50 µg/ml) and fungizone (0.2 µg/ml).

2.2.2 Dyes for cell staining

Alcian blue stain solution

The following were dissolved overnight in 100 ml MilliQ H_2O:

0.076 g cetylpyridinium chloride

0.76 g lanthanum chloride ($LaCl_3$)

0.9 g NaCl

0.143 g alcian blue

20 µl Tween-20 (0.02 %)

0.9% EDTA/saline solution

The following were dissolved overnight in 100 ml MilliQ H_2O:

0.9 g NaCl

1 ml of 0.5 M EDTA

Erythrosin-B

A stock concentration (0.15%) was made of the following:

0.15 g Erythrosin-B

100 ml 1x PIPES

FBS (5%)

Saved in aliquots at -20^oC until required

2.2.3 Buffers for mediator release

Phosphate buffered saline (-PBS) buffer

The following were dissolved in 1 litre MilliQ H_2O:

8 g NaCl

2.16 g $Na_2HPO_4.7H_2O$

200 mg KCl

200 mg KH_2PO_4

pH to 7.4

Supplemented PBS (+PBS)

The following were dissolved in 100 ml PBS:

100 mg glucose

100 µl 1M $CaCl_2.2H_2O$

100 µl 1M $MgCl_2.6H_2O$

100 µl of 3% human serum albumin (HSA)

2.2.4 Buffers for mast cell purification

10x PIPES

The following were dissolved in 100 ml MilliQ H_2O:

7.6 g PIPES (free acid)

6.43 g NaCl

0.37 g KCl

pH to 7.4 with NaOH

This was diluted to 1x PIPES as appropriate to be used for mast cell purification.

10% PIPES/FBS washing buffer

In 8 ml FBS, 80 ml 1x PIPES was dissolved.

Isotonic Percoll

Isotonic Percoll (100%) was prepared by adding 1 part of 10x PIPES to 9 parts of stock Percoll.

Purification Buffer

The following were dissolved in 50 ml PBS:

0.25 g BSA

200 µl 0.5 M EDTA (2 mM)

2.2.5 Protein lysis and Western blotting buffers

Western lysis buffer

The following were dissolved in 100 ml MilliQ H_2O:

0.61 g Tris base

0.8 g NaCl

Triton X100 (1% v/v)

Protease inhibitors cocktail (4% v/v)

pH to 8 with HCl

10x Tris-buffered saline (TBS)

The following were dissolved in 1 litre MilliQ H_2O:

121.1 g Tris

90 g NaCl

pH to 7.5 with HCl

5x Sample buffer

The following were dissolved in 100 ml MilliQ H_2O:

3.78 g Tris-HCl

10 g SDS

250 mg bromophenol blue

For sample preparation, 50 ml of this solution was prepared (pH 6.8) and glycerol was added at 50% v/v solution. This was diluted with MilliQ H_2O to 3x sample buffer.

10% SDS

10 g SDS was dissolved in 100 ml MilliQ H_2O.

1.5 M Tris-HCl

18.15 g Tris was dissolved in 100 ml MilliQ H_2O and titrated to pH 8.8

0.5M Tris-HCl

6 g Tris was dissolved in 100 ml MilliQ H_2O and titrated to pH 6.8

30% Acrylamide

0.8 g BIS and 30 g Acrylamide were dissolved in 100 ml MilliQ H_2O

10% Ammonium persulfate (APS)

0.1 g was dissolved in 1 ml MilliQ H_2O

10x running buffer

The following were dissolved in 1 litre MilliQ H_2O:

30.3 g Tris

190 g glycine

100 ml 10% SDS

pH to 8.3

This was diluted to 1x running buffer as appropriate to be used for SDS-PAGE running.

Transfer buffer

The following were dissolved in 1 litre MilliQ H_2O:

5.8 g Tris

2.9 g glycine

3.7 ml 10% SDS

200 ml methanol

pH to 8.3

5% TBS-milk

5 g non-fat powdered milk was dissolved in 100 ml 1x TBS

TBS (0.05%) Tween

125 µl Tween-20 was dissolved in 250 ml 1x TBS

2.2.6 Buffers for RT-PCR

50x Tris-acetate-EDTA (TAE) buffer

The following were dissolved in 1 litre MilliQ H_2O:

242 g Tris-base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA

This was diluted to 10x TAE as appropriate to be used for PCR experiments.

2.3 Preparation of compounds

Anti-human IgE was provided as a stock solution (2 mg/ml), was stored at -20 °C, and a working stock kept for a month at 4 °C. SCF stock solution (100 µg/ml) was reconstituted in dH_2O and stored at -20 °C. Ionomycin stock solution (10 mM) was reconstituted in DMSO and stored at -20 °C. Stock solutions (10 mM) of indomethacin, celecoxib, FR122047, aspirin, naproxen, ibuprofen, diclofenac, wortmannin, imatinib, dasatinib and nilotinib were prepared in ethanol or DMSO and stored at -20 °C. All stock solutions were divided into appropriate aliquots. +PBS buffer was used to dilute these stock solutions to the required concentrations.

2.4 Human lung mast cell isolation

Human lung tissue was collected from the Northern General Hospital (Sheffield, UK) from adult patients undergoing surgery. The study was approved by the National Research Ethics Service (REC reference: 15/NW/0657). All involved participants gave informed written consent. The ratio between male and female lung donors was almost equal. The age range of participants was between 28-89 years with a median age of 72.

The weight of the resected lung tissue was recorded. The tissue was subjected to both mechanical and enzymatic disruption to release the mast cells from the tissue. Initially, the tissue was chopped into small strips using scissors, then cut into small pieces (~ 1 mm^2). The chopped tissue was then washed in supplemented RPMI 1640 media and filtered over nylon gauze (100 µm) in order to remove the macrophages.

Following several cycles of chopping and washing (3 x ~ 50 ml), the tissue was reconstituted in supplemented RPMI 1640 media, 10% FBS, DNase I (1.5 mg per 100 ml of supplemented RPMI 1640 media) and collagenase type I was added (12 mg per 10 g of tissue). The processed tissue was then incubated in a 37°C water bath with gentle shaking for 90 minutes.

After incubation, the tissue was chopped into smaller pieces, before mechanical disruption and aspiration using a 50 ml syringe to dissociate the mast cells. The tissue was aspirated 10 times and filtered and washed over nylon gauze (100 µm). Aspiration was performed a total of 30 times with intervening washings (150 - 300 ml of supplemented RPMI 1640 media) over nylon gauze (100 µm). The filtered media containing mast cells was then divided into several 50 ml tubes and centrifuged at 480 x g for 10 minutes at room temperature (MSE Mistral 2000). The cell pellets were re-suspended in 100 - 200 ml of supplemented RPMI 1640 media. The cells were washed two times, and the cell pellet was re-suspended in 5 - 15 ml of RPMI 1640 media containing antibiotics.

Mast cell numbers were assessed by alcian blue staining method. Briefly, 20 µl of the cell suspension was mixed with 90 µl of an alcian blue stain solution and 90 µl of an EDTA/saline solution, and the cells were examined by light microscopy (Gilbert & Ornstein 1975). The cells were then reconstituted with an appropriate volume of RPMI 1640 culture media containing antibiotics. The freshly isolated mast cells were then seeded in 6-well plates at a concentration of about 5 x 10^6 mast cells per plate and incubated overnight at 37 °C in a 5% CO_2 incubator (Sanyo). The following day, cells were harvested, centrifuged at 480 x g for 10 minutes at room temperature, re-suspended in RPMI 1640 media

supplemented with antibiotics, re-counted and then used in experiments as appropriate.

2.5 Mast cell purification

2.5.1 Percoll density gradient

The Percoll density gradient technique separates and purifies mast cells up to 30 – 60%. Mast cells (3 – 5 x 10^6) were re-suspended in 5 ml 40% Percoll and filtered through a nylon gauze. The cells were then overlaid onto 5 ml 70% Percoll and centrifuged at 780 x g for 12 minutes at room temperature. The centrifuge brake was switched off to prevent disruption of the gradient interface layer. Following centrifugation, cells were then carefully aspirated from the interface layer, between the 40% and 70% Percoll solutions. 30 ml PIPES/FBS was added to the cells and the suspension was inverted 15 - 25 times. The suspension was then centrifuged at 780 x g for 12 minutes with the centrifuge brake on. Finally, the cells were washed in 5 ml PIPES/FBS, centrifuged at 480 x g for 5 minutes and then counted by light microscopy. Separated cells were either used in an experiment or further purified by immunomagnetic bead separation.

2.5.2 Immunomagnetic bead separation

For further mast cell purification of up to 85 to 99%, immunomagnetic bead separations were performed. The purification buffer (*see section 2.2.4*) was de-aerated for 25 - 30 minutes. The pelleted cells were resuspended in 500 µl of the purification buffer including 100 µl FcR blocking buffer and 100 µl anti-CD117 microbeads. The mixture was incubated at 4 °C for 20 minutes and mixed every

5 minutes. The mixture of cells and microbeads was filtered through nylon gauze. The cells were centrifuged at 480 x g for 5 minutes, and the pellet was reconstituted in 2 ml of the buffer and centrifuged again at 480 x g for 5 minutes. The supernatant was removed, and the pellet was re-suspended in 500 µl of the purification buffer. The re-suspended cells were then carefully loaded on to the LS column (Miltenyi Biotec) within the magnetic field (Midi MACS Separator, Miltenyi Biotec). The column was then washed with 3 ml of the purification buffer in order to elute non-adherent cells; this process was repeated two more times. The column was then removed from the magnetic field, and 5 ml of the buffer was forced through the column using a syringe to effectively dislodge the positively selected mast cells that were attached to the column. The dislodged cells were collected by centrifugation at 480 x g for 5 minutes. The cells were re-suspended and counted using the alcian blue staining method. Purified cells were either used in functional experiments or lysed for Western blotting analysis. Moreover, the anti-CD117 antibody binds to a specific epitope at the c-KIT which is different from the binding site of SCF. Havard et al. (2011) demonstrated that mast cell responses to either SCF or anti-IgE were unaffected by anti-CD117 positive selection.

2.6 Mediator release experiments

Mast cells were incubated at 37 °C for 5 - 10 minutes in +PBS. After that, mast cells were incubated for 25 minutes with a selection of stimuli including anti-IgE, SCF and ionomycin. After 25 minutes activation, -PBS (0.75 to 1 ml) was added. The cells were pelleted by centrifugation at 480 x g for 3 minutes and supernatants were collected and stored at -20 °C or -80 °C for the analysis of histamine, PGD_2 or cys-LTs release.

On occasion, mast cells were passively sensitized overnight with JW8-IgE (1/250) before challenging with JW8-IgE specific antigen (NIP-HSA) or other stimuli (anti-IgE or SCF).

For other experiments, mast cells were also pre-incubated overnight with SCF (100 ng/ml) before challenging with a stimulus such as anti-IgE, SCF or ionomycin.

When inhibitors were used, mast cells were pre-incubated for 10 - 15 minutes with the inhibitors before challenging with anti-IgE, SCF or ionomycin. The inhibitors included COX inhibitors: indomethacin, FR122047, celecoxib (10^{-12} to 10^{-6} M); other inhibitors such as NSAIDs aspirin, naproxen, ibuprofen and diclofenac (10^{-7} to 10^{-6} M); c-KIT inhibitors: imatinib, dasatinib and nilotinib (10^{-10} to 10^{-6} M); the PI3K inhibitor: wortmannin (10^{-6} M).

Following activation with stimuli, samples were processed as described above. All the mediator release experiments were done in duplicate. For these experiments 2 – 6 x 10^4 mast cells per sample were used.

2.7 Histamine analysis

In all experiments, some cell samples were also lysed with 8% perchloric acid in order to give an indication of total histamine content. Some cell samples were also incubated with buffer alone in order to get a measure of spontaneous histamine release. Histamine release was expressed as a percentage of the total histamine content and the spontaneous release was subtracted from values in order to express the results as net percentage histamine release.

The histamine analysis was determined using an automated fluorescence technique: fluorescence detector (LDC Analytical Fluoro Monitor™III) and a chart recorder (SE-120, BBC Goerz Metrawatt). The technique involves the condensation of histamine under basic conditions with o-phthaldialdehyde (OPT) to form a fluorescent adduct. The fluorescent adduct is stabilised in the presence of acid. The amount of fluorescence was proportional to the amount of histamine in the samples (Ennis, 1991).

2.8 Cysteinyl leukotriene assay

A commercially-available ELISA kit (Cayman Chemical Company) was used to detect all three forms of cys-LTs (leukotrienes C_4, D_4 and E_4) released from mast cells. The assay was carried out according to the manufacturer's instructions. In brief, the assay involved competition between cys-LTs in the sample and cys-LTs acetylcholinesterase (AChE) conjugate (cys-LT tracer) for a limited amount of cys-LT monoclonal antibody. The wells were pre-coated with a goat polyclonal anti-mouse IgG before incubation with the monoclonal antibody against cys-LT and the cys-LTs (sample and tracer). After 2 hours incubation at room temperature, wells were washed to remove the unbound reagents. Wells were developed with Ellman's reagent, which contains the substrate to the AChE, in the dark with on an orbital shaker for 60 – 90 minutes. The absorbance was then measured at 405 nm using a Thermo/Electron Corporation plate reader. The intensity of the colour (optical density) is proportional to the amount of cys-LTs tracer bound to the well, which is also proportional to the amount of cys-LTs loaded to the well. The readings were used to calculate the %Bound/Maximum Bound ($\%B/B_o$), where B_o is the maximum bound and B is the sample or standard bound.

Initially, samples were pre-tested by diluting the samples between 1:5 to 1:40, to ensure that they would fall on the linear portion of the standard curve [*Figure 2.1*].

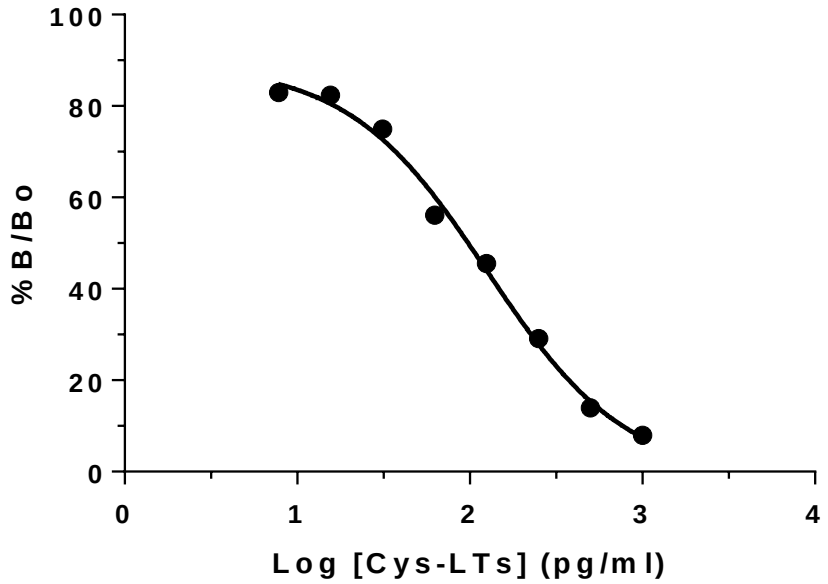


Figure 2.1: Representative Standard Curve for cys-LT assay. This standard curve was determined by diluting the bulk standard of cys-LTs with EIA buffer. The concentration range was 8.6 to 2500 pg/ml.

2.9 Prostaglandin D_2 assay

A commercially-available ELISA kit (Cayman Chemical Company), was used to determine the levels of PGD_2 released from mast cells. The assay was carried out according to the manufacturer's instructions. Before assay, samples were methoxylated using methoxylamine hydrochloride (MOX HCl). The methyloximating reagent (MOX) was prepared by mixing 1 ml of 10% ethanol with 0.1 g methoxylamine HCl and 0.082 g sodium acetate. The samples were diluted 1:1 with methoximate (20 µl of the MOX + 20 µl of the sample) this

complex was then incubated at 60 °C for 30 minutes. The diluted samples and standards were loaded to the coated plate, along with the PGD_2-MOX tracer and PGD_2-MOX anti-serum. The plate was then incubated overnight at 4 °C, washed and developed for 90 minutes. The plate absorbance was measured at 405 nm as described in section 2.8.

Before a definitive assay was performed appropriate dilutions of the samples were pre-tested by an initial EIA to ensure that samples were on the linear portion of the standard curve [*Figure 2.2*].

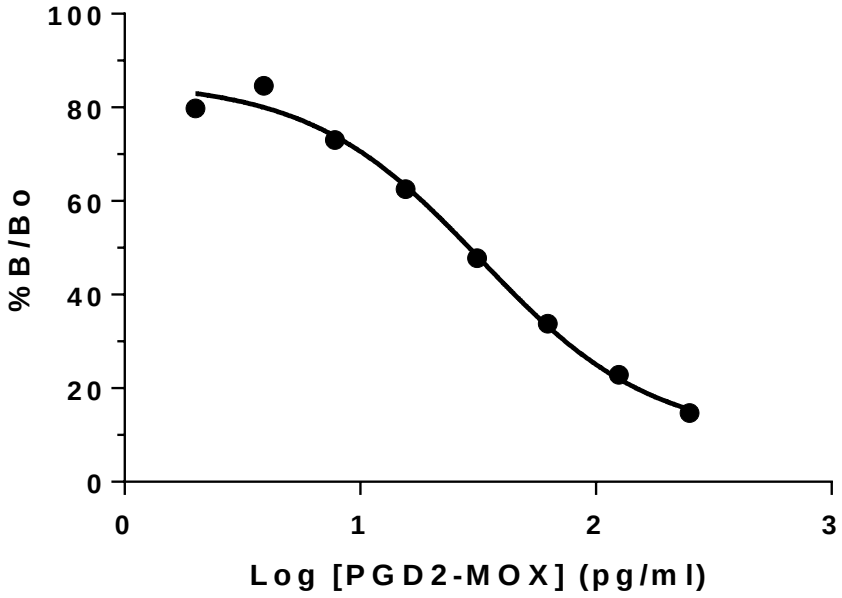


Figure 2.2 Representative Standard Curve for PGD_2 assay. This standard curve was determined by diluting the bulk methoximated PGD_2 standard with EIA buffer. The concentration range was 2 to 250 pg/ml.

2.10 Cytokine and chemokine release detection

Mast cells were incubated at 37 °C for 20 hours in RPMI supplemented with antibiotics in 6-well culture plate before activation with a selection of stimuli including anti-IgE, SCF, anti-IgE + SCF or LPS. After 20 hours of incubation, the

supernatant (1 ml) was collected and transferred to a 1.5 ml eppendorf. The cells were pelleted by centrifugation at 480 x g for 5 minutes and supernatants were transferred and stored in a new 1.5 ml eppendorf at -80 °C for further analysis. Cell viability was assessed to ensure that the stimuli would not affect cells ability to release cytokines after incubation for long.

2.10.1 Cell viability assessment

Cell viability was assessed by erythrosin-B exclusion. To assess viability of cells in the wells, 2 x 10^5 cells were challenged for 30 minutes or 4 hours with or without 100 ng/ml SCF or 2 µg/ml anti-IgE. Cells were suspended and transferred to 1.5 ml eppendorf tubes, centrifuged at 480 x g for 4 minutes and washed with 100 µl -PBS. 100 µl of the diluted erythrosin-B was added into each tube then incubated for 2 minutes at room temperature. Tubes were centrifuged at 480 x g for 4 minutes, the erythrosin-B was aspirated and washed with 500 µl -PBS then centrifuged at 480 x g for 4 minutes. The pelleted cells were re-suspended in 1 ml -PBS before visualizing under a light microscope (Motic, SFC-100FLA). Cell viability was determined by counting the number of non-viable mast cells that had taken up the pink dye as a proportion of total mast cells.

2.10.2 Proteome profiler array

To identify a range of cytokines presented in cell culture supernatants, proteome profiler human cytokine array kit (panel A) was used, which contains 4 nitrocellulose membranes dotted with 36 different capture antibodies, array buffers and detection antibody cocktail.

The Profiler array was performed as instructed in the manufacturer's booklet. In brief, each membrane was placed in the provided 4 well multi-dish and blocked for 1 hour on a rocking plate shaker at room temperature. Cell supernatants (2.2 - 3.5 x 10^6 cells/ml/condition), from three different preparations were pooled together (3 x 330 µl) and were supplemented with array buffer in a separate tube to acquire a final volume of 1.5 ml. After that, 15 µl of the reconstituted detection antibody cocktail was added to each diluted sample and the mixtures were incubated at room temperature for 1 hour. Array buffer was aspirated from each well and the sample-antibody mixtures were pipetted into the relevant wells. The dish was then incubated overnight at 4 °C on a rocking shaker. Next day, each membrane was placed in a separate container and the membranes were washed with 20 ml of 1 x wash buffer for 10 minutes, this process was repeated 2 more times on a rocking shaker at room temperature. The 4 separate wells were washed with milliQ H_2O and 2 ml of diluted streptavidin-HRP (1/2000) was added to each well, and incubated for 30 minutes at room temperature. After that, membranes were washed with 20 ml of 1 x wash buffer for 10 minutes three times on a rocking shaker at room temperature. The membranes were then exposed to ECL reagents and developed using BioRad Chemi Doc XRS^+ system, resulting in spots of different densities where cytokines and chemokines were detected. Semi quantitative analysis was carried out using Image Lab software v 5.2.1 (Bio Rad). Mean background adjusted pixel intensity (or density) was calculated.

2.10.3 Enzyme-linked immunosorbent assay (ELISA)

This technique is used to detect the cytokine levels in cell culture supernatant. 96-well high binding plates (Corning Costar 3590) were coated with capture

antibody (1/250 dilution) for different cytokines including TNF-α, IL-1β, IL-6 and IL-8 and incubated overnight at 4 °C. Wells were aspirated and washed 4 times with PBS containing 0.05% Tween-20, then blocked with 1x assay diluent at room temperature for 1 hour. Wells were aspirated and washed 4 times with PBS containing 0.05% Tween-20. Standards and samples were diluted in 1 x assay diluent, before being pipetted onto the plate (100 µl/well), the plate was then incubated at room temperature for 2 hours or overnight at 4 °C occasionally. Wells were aspirated and washed 4 times with PBS containing 0.05% Tween-20. The wells were incubated with detection antibody (100 µl/well) at room temperature for 1 hour; wells were aspirated and washed 4 times with PBS containing 0.05% Tween-20. The wells were incubated with avidin-HRP at room temperature for 30 minutes, and finally washed 4 times with PBS containing 0.05% Tween-20. The plate was developed with the addition of tetramethylbenzidine (TMB) substrate (100 µl/well) at room temperature for at least for 15 minutes. The reaction was stopped with 1 M H_2SO_4 (50 µl/well). The plate was read at 450 nm using a microplate reader (Thermo Multiskan EX). The limit of detection of TNF-α ELISA was 4 pg/ml. For the IL-1β, IL-6 and IL-8 ELISAs the limit was 2 pg/ml. Standard curves was produced and the concentration of the unknowns were determined by interpolation of the curves using GraphPad version 7 [*Figure 2.3*].

Before a complete assay was carried out, an initial experiment was assayed using a range of dilutions of a selected cell culture supernatants using the above protocol. This allowed a suitable dilution to be used to assay the whole of the experiment, and also to ensure that the levels of cytokines of each sample would be within the range of the standard curves.

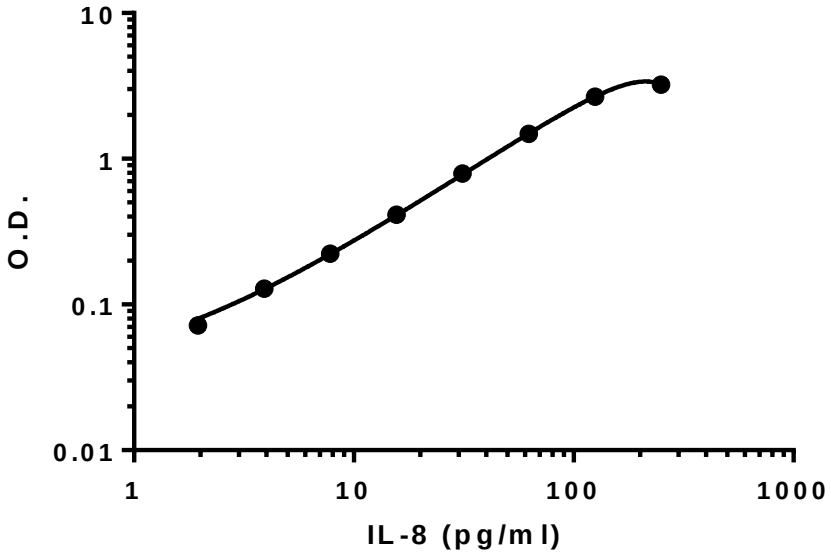


Figure 2.3: Representative cytokine ELISA standard curve. The above standard curve for an IL-8 ELISA was produced using non-linear regression and a third order polynomial (cubic) fit. The range of detection based on the linear section of the curve was determined as 1.95 – 250 pg/ml.

2.11 Gene expression

COX and *β-Actin* gene expression was determined by Polymerase Chain Reaction (PCR). RNA was extracted and synthesised to cDNA by Reverse Transcriptase PCR (RT-PCR). The cDNA was then amplified using specific primers for *β-Actin* and *COX* subtypes.

2.11.1 RNA extraction and quantification

RNA was extracted from mast cells (up to 3 x 10^6) by lysing the cells with 1 ml of TRI Reagent®. The lysate was used immediately or stored at -80 °C for long-term storage. 200 µl of chloroform was added to the samples, vigorously shaken for 15 seconds and left to stand for 10 minutes at room temperature before

centrifugation at 13000 x g for 10 minutes at 4 °C. The aqueous layer containing the RNA was transferred to a new microfuge tube. 500 µl of isopropanol was added and mixed well, the sample left to stand for 10 minutes. The samples were centrifuged at 13000 x g for 10 minutes at 4 °C. The supernatant was removed using a 21-gauge needle (BD Microlance™ 3) and 5 ml syringe (BD Plastipak™). The pellet was washed with 1 ml of 75% ethanol by inverting the tube and centrifuged at 7500 x g for 5 minutes at 4 °C. The supernatant was removed by a syringe and the pellet was left to air dry at room temperature. The pellet was suspended in 20 µl of sterile H_2O, this RNA solution was used immediately or stored at -80 °C for long-term storage.

The RNA was treated with DNase I digestion kit (DNA–free DNase treatment and removal, Ambion) to remove any DNA contaminant present in the extracted RNA. Briefly, 0.1 volume (2 µl) of 10x DNase I buffer was added to the RNA solution along with 1 µl of rDNase I and mixed by pipetting gently. The microfuge tube was incubated at 37 °C in a heat block for 25 minutes. The samples were vortexed and 0.1 volume (2 µl) of inactivation reagent was added and mixed by pipetting gently. The tubes were incubated for 1 minutes at room temperature, mixed by pipetting gently and incubated again for a further 1 minute before centrifugation at 10,000 x g for 1.5 minutes at room temperature.

The top clear layer containing the RNA was transferred to a new microfuge tube. The RNA concentration (ng/µl) was quantified using a NanoDrop ND - 1000 Spectrophotometer (Thermo Scientific) and the sample purity was determined by the 260/280 nm ratio. The RNA was stored at -80 °C unless proceeding to the next step.

2.11.2 Reverse transcriptase PCR (RT-PCR)

1 µg RNA was reverse transcribed to cDNA. The master mix of the required components for the PCR reaction prepared for each RNA sample is shown in *Table 2.1*. To ensure that no amplification had occurred from any genomic DNA in the sample, a negative control consisting of RNA and PCR master mix excluding the reverse transcriptase (RT) enzyme was run in parallel. The reverse transcriptase PCR conditions were, 1st cycle at 25 °C for 10 minutes, 37 °C for 2 hours and finally 85 °C for 5 minutes, followed by holding stage at 4°C (∞) (DNA Engine PTC-200 Peltier Thermal Cycler). The synthesised cDNA was then stored at -20 °C unless proceeding to the next step.

The cDNA was then amplified by RT-PCR to determine the gene expression of *COX-1*, *COX-2* and *β-Actin* (housekeeping gene). *Table 2.2* lists the components of the master mix that were used for all the PCR reactions. 24 µl of the master mix was added to 1 µl of cDNA and overlaid with 25 µl of mineral oil to avoid evaporation (Techne TC-312 Thermal Cycler). Primers were kindly designed by Dr Linda Kay and synthesized by Sigma (Poole, UK). *Table 2.3* lists the primer sequences and *Table 2.4* lists the PCR conditions for each gene.

Table 2.1 List of the master mix components for reverse transcriptase PCR

	Volume (µl) per PCR reaction	
	Positive	Negative
10 x RT buffer	4	4
25 x dNTPS mix (100 nM)	1.6	1.6
Multiscribe RT enzyme	2	-
RNase Inhibitor	2	2
10 x RT random Primers	4	4
H_2O	6.4	8.4

Table 2.2 List of the master mix components for standard RT-PCR

	Volume (µl) per PCR reaction
5 x GoTAQ® Flexi Green Buffer	5
dNTPs (10 mM)	0.5
Forward primer (2 µM)	2.5
Reverse Primer (2 µM)	2.5
$MgCl_2$ (25 mM)	2
GoTAQ® Flexi DNA polymerase	0.25
Sterile H_2O	11.25

Table 2.3 List of primer sequences for standard RT-PCR

Gene	Primer sequence in 5'-3' direction	Size (bp)
COX-1	5'-TGCCCAGCTCCTGGCCCGCCGCTT-3' (Forward) 5'-GTGCATCAACACAGGCGCCTCTTC-3' (Reverse)	304
COX-2	5'-TTCAAATGAGATTGTGGAAAAATTGCT-3'(Forward) 5'-AGATCATCTCTGCCTGAGTATCTT-3'(Reverse)	305
β-Actin	5'-ATATCGCCGCGCTCGTCGTC-3'(Forward) 5'-TAGCCGCGCTCGGTGAGGAT-3'(Reverse)	583

Table 2.4 List of conditions required for standard RT-PCR for 35 cycles

Gene	$MgCl_2$ concentration	Programme		
COX-1 *COX-2*	1.5 mM	Initial denaturation	95°C	2 minutes
		Denaturation	95°C	45 seconds
		Annealing	60°C	45 seconds
		Polymerisation	72°C	60 seconds
		Final elongation	72°C	10 minutes
β-Actin	1 mM	Initial denaturation	94°C	2 minutes
		Denaturation	95°C	30 seconds
		Annealing	60°C	45 seconds
		Polymerisation	72°C	35 seconds
		Final elongation	72°C	7 minutes

2.11.3 Agarose gel electrophoresis

PCR products were separated in 2% agarose gel. To visualise DNA bands, 10 µl of ethidium bromide was added to 50 ml agarose gel. The dissolved gel was cast in a cassette with an inserted well comb and left to set for ~ 30 minutes. The comb was removed and the cassette was placed into a gel tank covered with 1x TAE buffer. 100 base pair cDNA ladder (5 µl) and samples (6-9 µl) were loaded onto the gel. The gel was run at 100 V for ~ 1 hour (Bio-Rad PowerPac 300) and visualized using the Bio-Rad ChemiDoc XRS+ system. Image Lab software (version 5.2.1, Bio-Rad) was used to perform densitometry on the cDNA bands. In order to establish that the correct amplification had taken place, bands of amplified products were cut from the agarose gel and purified using MinElute Gel Extraction Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Genotypic analysis of the sample was performed in-house by automated sequencing (ABI 3730 DNA Analyser, Applied Biosystems, Carlsbad. CA, USA).

2.12 Protein expression

2.12.1 Protein lysis

Mast cell preparations (~ 90% purity) were lysed for Western blotting using a Western lysis buffer (WLB, see section 2.2.5). 10 - 15 µl of WLB was added per 1 x 10^6 cells. The cells were then incubated on ice for 20 minutes and vortexed every 5 minutes. After incubation, the lysates were cleared by centrifugation at 13000 x g for 5 - 10 minutes at 4 °C. The supernatant was then divided into appropriate aliquots, snap frozen by liquid nitrogen and stored at -80 °C.

2.12.2 SDS-PAGE and immunoblotting

10 - 12% resolving gel was prepared, poured and overlaid with H_2O saturated butanol and left to set for 30 minutes [see *Table 2.5* for details of gel preparation]. The butanol was decanted, and 4% stacking gel was poured and a comb was inserted and the gel left to set for 45 minutes. The comb was removed and the gel was placed in a tank full of 1 x running buffer. Protein lysate was mixed with 3 x sample loading buffer, DTT (10% v/v) and heated in a heat block at 95°C for 10 minutes along with an aliquot of the molecular weight markers (see section 2.1). The lysates were then centrifuged at 13,000 x g for 1 minutes. Samples and ladder were loaded into SDS-polyacrylamide gels and electrophoresis was allowed to proceed for 1 hour at 120 - 150 V (Mini-protean 3 cell, BioRad).

Following electrophoresis, the resolved proteins were subsequently transferred from the gel to nitrocellulose membranes by wet-transfer method (Mini-protean 3 cell, BioRad). Briefly, for each gel, 2 sponges, 1 nitrocellulose membrane, 2

pieces of filter paper and a cassette were soaked in a tray containing 1x transfer buffer. Each cassette was assembled in a tray with transfer buffer, a sponge was placed then the filter paper, membrane, gel, filter paper and lastly another sponge. The cassette was closed, ensuring no air bubbles. The cassette was placed into the tank with an ice pack, the tank was filled with 1x transfer buffer, and the transfer was carried out for 1 hour at 100 V.

After transfer, the nitrocellulose membranes were blocked with 5% milk-TBS and incubated on 1 hour at room temperature on a shaker. The membranes were washed twice with 0.05% TBS-Tween and then incubated with primary antibodies overnight at 4 °C on a rolling shaker. The membranes were then washed with 0.05% TBS-Tween. Secondary antibodies conjugated to horseradish peroxidase (HRP) were added to the membranes and incubated for 1 hour at room temperature on a rolling shaker [for details of antibodies used see *Table 2.6*].

The membranes were then washed with 0.05% TBS-Tween, blot dried before addition of enhanced chemiluminescence (ECL) detection reagent (Amersham), reagent A was mixed with reagent B at 1:1, the membranes were covered with ECL reagent for 1 minutes, and the excess ECL reagent was removed. The membranes were placed in a piece of plastic, and developed in order to visualise the proteins of interest (Chemi Doc XRS^{+}, BioRad).

Table 2.5 List of components used to cast the SDS-PAGE gels

	10% Resolving Gel	12% Resolving Gel	4% Stacking Gel
H_2O	6.1 ml	5.2 ml	6.1 ml
1.5 M Tris	3.75 ml	3.75	-
0.5 M Tris	-	-	2.5 ml
30% Acrylamide	4.95 ml	5.8 ml	1.3 ml
10% SDS	150 µl	150 µl	100 µl
10% APS	75 µl	75 µl	100 µl
TEMED	18 µl	18 µl	20 µl

2.12.3 Membrane stripping and re-probing

The same membrane was used to probe several proteins. Once the membrane had been analysed for one protein, the membrane was stripped by incubation with 0.2 M NaOH for 15 minutes, followed by washings with H_2O for 10 minutes, 1x TBS for 10 minutes and then blocked with milk-TBS for 1 hour before incubation with a different primary antibody specific for an alternative protein. The membrane was then processed as described above (*see section 2.12.2*).

Table 2.6 List of the antibodies used.

Primary antibody	***Dilution***	***Secondary antibody***	***Dilution***
COX-1 (sc-1752) (Santa Cruz)	1/200 - 400	donkey anti-goat IgG-HRP (sc-2020)	1/500
COX-2 (sc-1745) (Santa Cruz)	1/200 - 1/400	donkey anti-goat IgG-HRP (sc-2020)	1/500
β-Actin (A2066) (Sigma)	1/200	Mouse monoclonal anti-rabbit IgG-HRP (P0448)	1/500
H-PGDS (sc-30066) (Santa Cruz)	1/200	Mouse monoclonal anti-rabbit IgG-HRP (P0448)	1/500
L-PGDS (sc-14821) (Santa Cruz)	1/200	donkey anti-goat IgG-HRP (sc-2020)	1/500

13. Data analysis

Means and standard errors of the mean (SEM) were calculated. All data were tested for normality. Analysis of variance (ANOVA) or paired t test followed as appropriate by using Dunnett's or Tukey's multiple comparison test. A significant difference was considered as ($P< 0.05$). Confidence coefficients were determined in some experiments. All tests were determined using GraphPad version 7.

CHAPTER THREE

3. EFFECTS OF SCF ON MAST CELL MEDIATOR RELEASE

3.1 Introduction

Human mast cells play a pivotal role in allergic diseases (Stone et al., 2010). Mast cells are the main source of histamine, a potent mediator of immediate hypersensitivity reactions, in the immune system (Amin, 2012). In addition, mast cells express high affinity receptors for IgE (FcεRI) (Alber et al., 1991). IgE antibodies play an essential role in type-1-hypersensitivity allergic reactions and provide immunity against parasites (Platts-Mills, 2001, Mukai et al., 2016).

In sensitised mast cells, surface IgE to corresponding allergens is expressed. Engagement of this IgE by allergens activates mast cells. When mast cells are activated, they release histamine and other mediators such as PGD_2 and cys-LTs that cause allergic reactions (Frandsen et al., 2013). Mast cells are located throughout the body associated with the tissues. The impact of mast cell mediator release varies depending on the site of the mast cells. For instance, activated skin mast cells contribute to atopic dermatitis whereas activated lung mast cells participate in the development of asthma (Bradding, 2008, Liu et al., 2011).

The c-KIT receptor is expressed on a number of cells associated with the immune system to varying extents. However, a close association between c-KIT and mast cells has been recognized for some time. Importantly, mast cells express high levels of c-KIT that when it binds to its ligand, SCF, leads to mast cell proliferation, differentiation and survival (Edling and Hallberg, 2007, Ashman, 1999b). Asthmatic patients have been shown to have increased levels of SCF in the lung (Al-Muhsen et al., 2004, Da Silva et al., 2006a) and it has been proposed that when SCF binds to c-KIT receptor, this promotes mast cell

responses cells that can contribute to airway inflammation (Theoharides et al., 2012).

Studies, primarily in rodent mast cell systems and cultured human mast cells, indicate that SCF, although not a direct activator by itself, can enhance mast cell histamine release (Theoharides et al., 2012). The aims of this chapter were (a) to evaluate the regulation of human lung mast cells by SCF and (b) to determine whether any potential effects of SCF are mediated by c-KIT. The effects of SCF on human lung mast cell were compared to the effects of anti-IgE which mimics the effects of allergens and drives IgE-dependent activation. This comparison was made in an attempt to enhance our understanding of the potential mechanism by which SCF activates human lung mast cells.

3.2 Effects of SCF on mediator release from mast cells

3.2.1 Effect of SCF on histamine and PGD_2 release

Surveying the literature as a whole, there is no consistency as to whether SCF causes direct activation of mast cells or not. Most studies suggest that SCF does not activate mast cells directly (Gilfillan and Tkaczyk, 2006). Others demonstrated that SCF can induce histamine release from human lung mast cells (Frenz et al., 1997). However, our data suggests that, in 3 out of 9 mast cell preparations, SCF induces the release of substantial amounts of histamine (about 20-35%), [*Figure 3.1*].

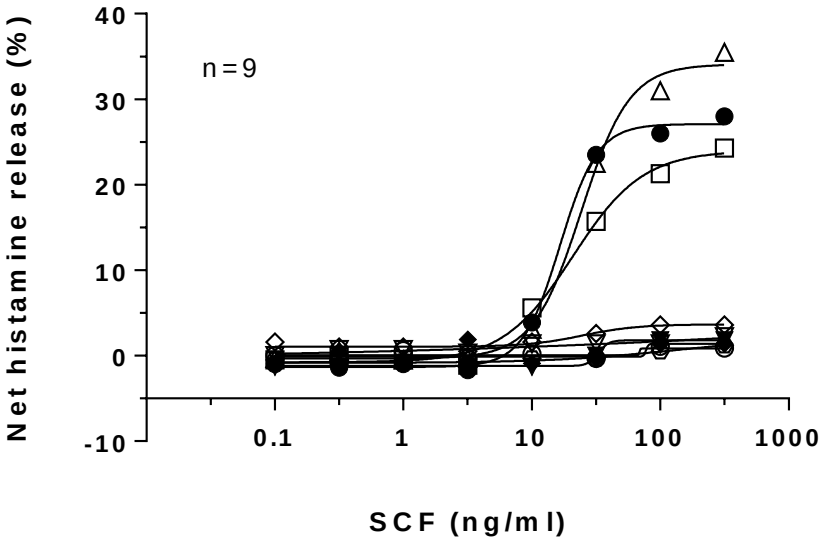


Figure 3.1 Concentration-response of the release of histamine by SCF from mast cells. Mast cells were incubated for 5 min at 37 ^{o}C before challenge with increasing concentrations of SCF for 25 min to induce histamine release. Each curve represents an individual experiment, n=9

We then decided to compare the effects of both anti-IgE and SCF on histamine release and PGD_2 generation from mast cell preparations. Overall, the levels of histamine released from mast cells challenged with anti-IgE were higher than when mast cells were challenged with SCF, the average release was 35.2 ± 5.0% and 5.3 ± 1.7%, respectively [*Figure 3.2A*]. In contrast, the levels of PGD_2 generated from mast cells activated with anti-IgE or SCF were the same, 59.4 ± 11.5 and 59.3 ± 11.4 ng PGD_2 generated per 10^6 mast cells, respectively [*Figure 3.2B*]. It is apparent from *Figure 3.2* that the release of both histamine and PGD_2 by both anti-IgE and SCF was variable among preparations.

Further studies were performed to determine whether lower concentrations of anti-IgE (0.02 - 2000 ng/ml) and SCF (0.001 - 100 ng/ml) could induce histamine release or PGD_2 generation. After activation with SCF (0.001 – 100 ng/ml), PGD_2 was only generated at the highest concentration (100 ng/ml) of SCF with an average (mean ± SEM) of 71.0 ± 25.8 ng PGD_2 generated per 10^6 mast cells compared to the spontaneous generation of 3.3 ± 1.4 ng per 10^6 mast cells [*Figure 3.3A*]. Similarly, histamine was only released when the highest concentration of SCF was used (100 ng/ml), the average of the release was about 15.0 ± 5.4% compared to 10.0 ± 3.0% spontaneous release [*Figure 3.3B*]. These data suggest that a high concentration of SCF (100 ng/ml) is required for optimum release of both PGD_2 and histamine by mast cells *in vitro*. On the other hand, mast cell activation by anti-IgE released mediators in a concentration-dependent manner [*Figure 3.4*]. When mast cells were activated with a high concentration (2000 ng/ml) of anti-IgE, PGD_2 was generated to a statistically significant extent with an average of 88.0 ± 44.0 ng PGD_2 generated per 10^6 mast cells compared to the spontaneous, 3.3 ± 1.4 ng PGD_2 generated

per 10^6 mast cells [*Figure 3.4A*]. When mast cells were activated with anti-IgE at either 200 or 2000 ng/ml, histamine was released to a statistically significant extent with an average (mean ± SEM) of 29.2 ± 8.8% and 36.3 ± 9.5% respectively compared to the spontaneous (10.0 ± 1.3%) [*Figure 3.4B*].

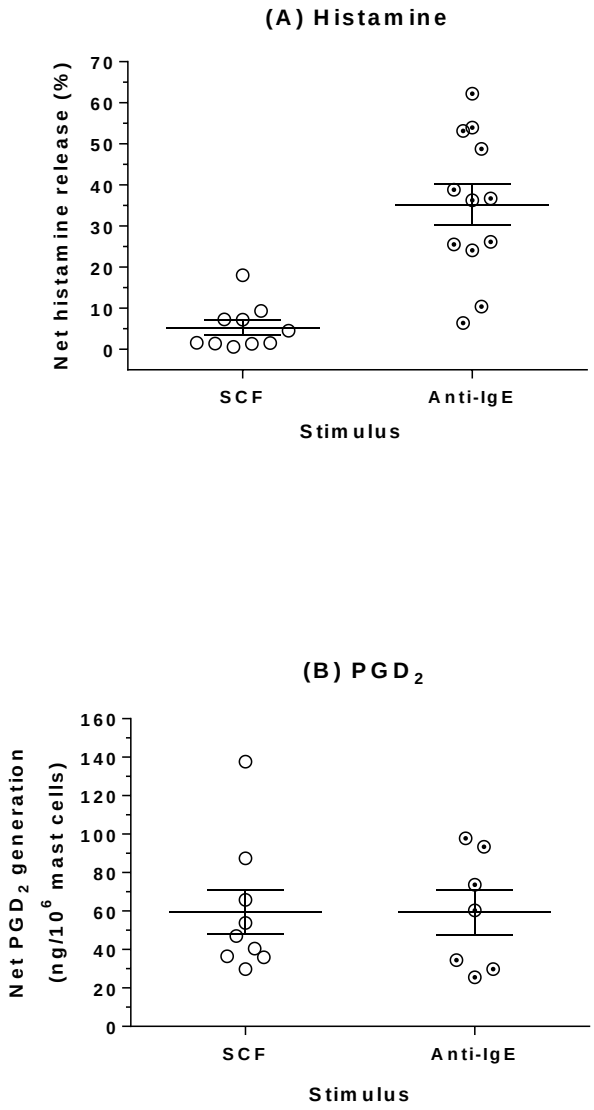


Figure 3.2 Effect of anti-IgE and SCF on the release of histamine and PGD_2 generation by mast cells. Cells were incubated at 37 ^oC before challenge with or without either SCF (100 ng/ml) or anti-IgE (2 µg/ml) for 25 min. The levels of (A) histamine release and (B) PGD_2 generation were determined from different data collected from different cell preparations. The mean spontaneous release for histamine was 2.6 ± 0.3% (A) and PGD_2 was 2.5 ± 1.8 ng/10^6 mast cells (B). Each circle represents an individual preparation, and means are shown in each figure as a horizontal bar. n=7-12.

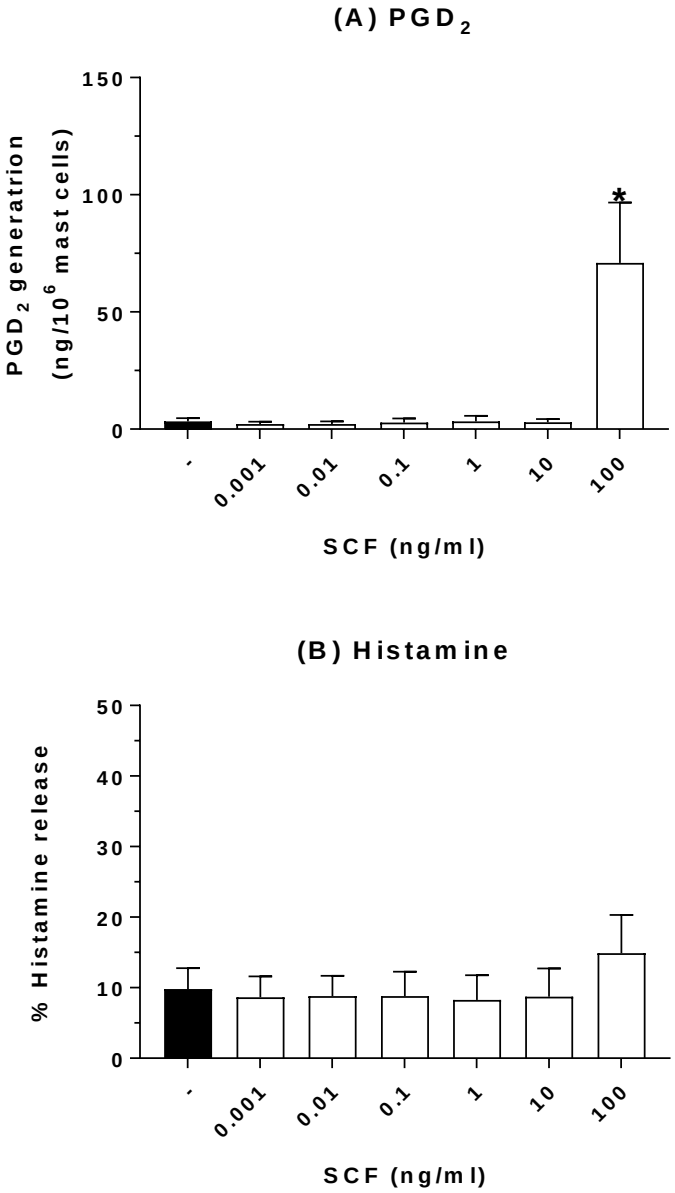


Figure 3.3 Concentration response of the generation of PGD_2 and histamine release by SCF from mast cells. Mast cells were incubated for 5 min at 37 ^{o}C before challenge with increasing concentration of SCF for 25 min to induce (A) PGD_2 generation and (B) histamine release. Values are means ± SEM, n=3-4. Statistically significant difference compared to control is indicated: * *P<0.05*.

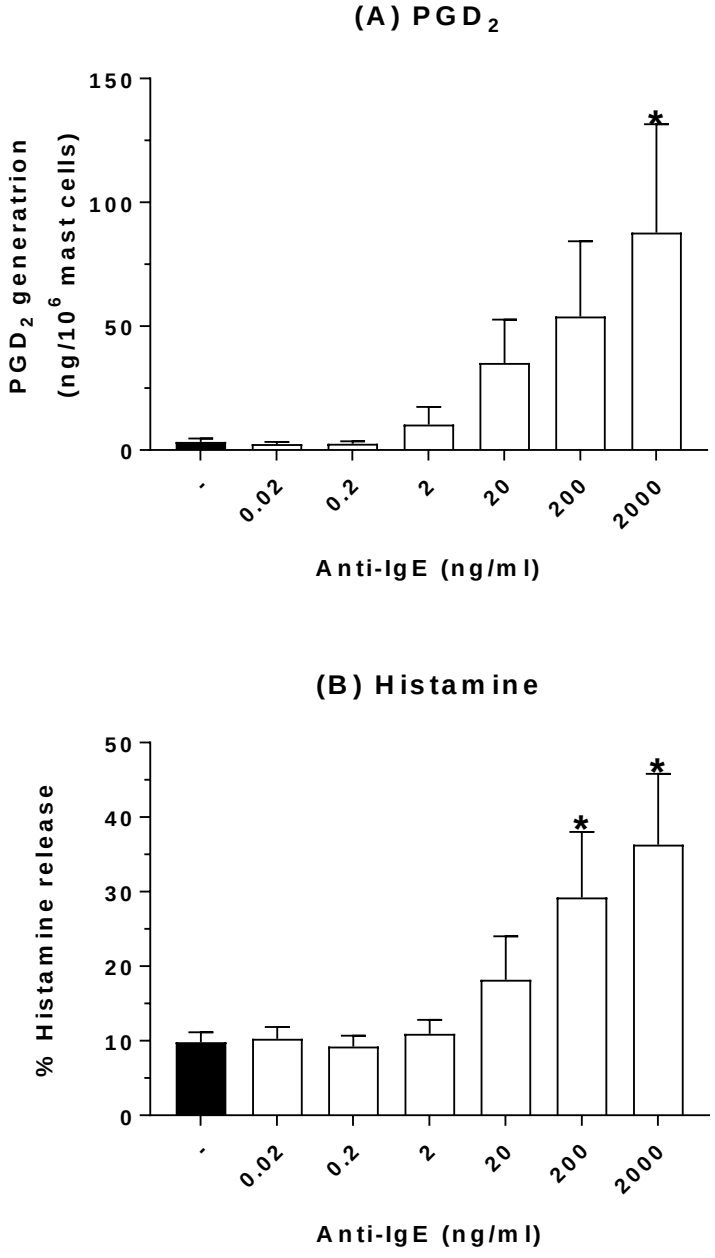


Figure 3.4 Concentration response of the generation of PGD_2 and histamine release by anti-IgE from mast cells. Mast cells were incubated for 5 min at 37 ^{o}C before challenge with increasing concentration of anti-IgE for 25 min to induce (A) PGD_2 generation and (B) histamine release. Values are means ± SEM, n=3-4. Statistically significant difference compared to control is indicated: * *P<0.05*.

It has been shown previously that SCF (100 ng/ml) can enhance histamine release when combined with anti-IgE (2000 ng/ml) (Theoharides et al., 2012). We investigated whether lower doses of SCF (0.1 – 10 ng/ml) can enhance both histamine release and PGD_2 generation when combined with a sub-maximal concentration of anti-IgE (200 ng/ml). When SCF (0.1, 1 or 10 ng/ml) was combined with 200 ng/ml of anti-IgE, PGD_2 generation was enhanced by SCF in a concentration-dependent manner but not to a statistically significant extent.

Anti-IgE induced 73.2 ± 27.5 ng PGD_2 generated per 10^6 mast cells, this was increased by SCF to 86.0 ± 30, 96.8 ± 34.0 and 117.4 ± 44.6 ng PGD_2 generated per 10^6 mast cells by increasing concentrations of SCF (0.1, 1 or 10 ng/ml) respectively. However, this effect was not statistically significant [*P=0.35; Figure 3.5A*]. Similarly, when looking at histamine release, release was enhanced by SCF in a concentration-dependent manner but not to a statistically significant extent. Anti-IgE induced 23.8 ± 6.7%, this was increased by SCF (0.1, 1 or 10 ng/ml) to 25.8 ± 7.0, 29.2 ± 6.6 and 35.3 ± 5.8% respectively, however, this effect was not significant [*P=0.17; Figure 3.5B*].

3.2.2 Time course of release of pro-inflammatory mediators

We also investigated a time-course of release of mediators by mast cells following activation with either anti-IgE (2 µg/ml) or SCF (100 ng/ml). Initially, time points studied ranged from 10 seconds up to 30 minutes [*Figure 3.6*]. After 1 minute, histamine release following anti-IgE activation was 16.6 ± 2.5% (half-maximal) and reached maximal levels of 33.2 ± 5.0% after 5 minutes [*Figure 3.6A*]. Histamine release following SCF activation was 3.1 ± 1.0 % (half-maximal) at 2 minutes, and reached maximal levels of 5.9 ± 1.4% after 20 minutes [*Figure 3.6B*].

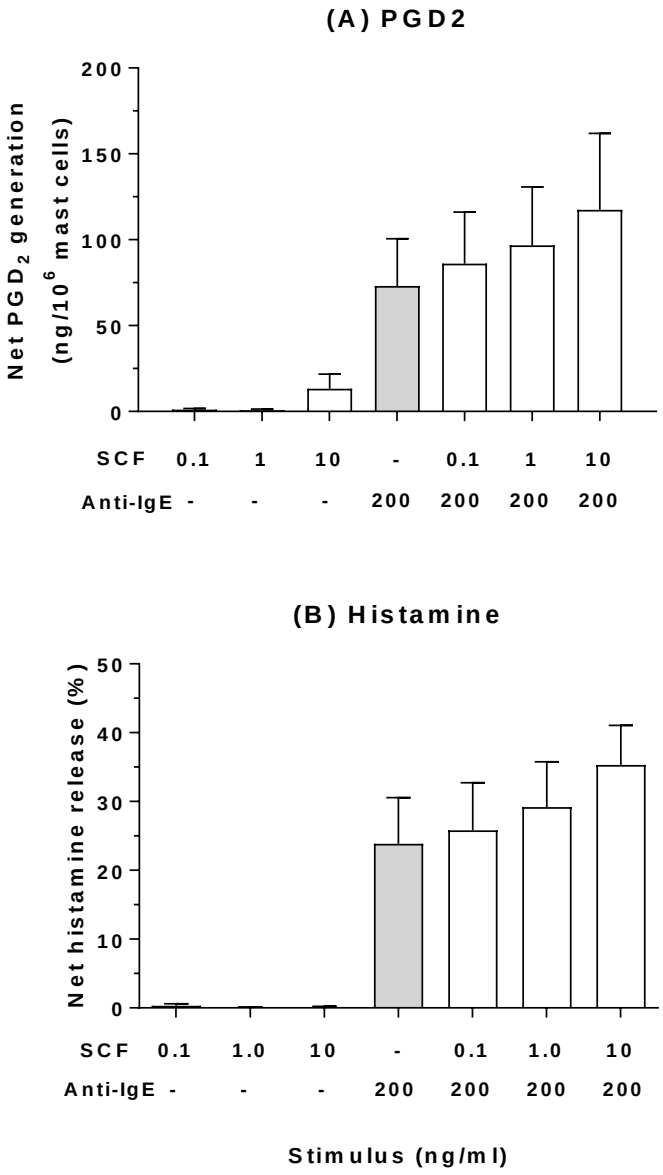


Figure 3.5 Effect of SCF and anti-IgE or both on the generation of (A) PGD_2 or (B) histamine by mast cells. Cells were incubated for 5 min at 37 ^{o}C before challenge with a half-maximal concentration of anti-IgE (0.2 µg/ml), different concentrations of SCF (0.1-10 ng/ml) or both stimuli for 25 min to induce mast cell mediator release. The mean spontaneous release for PGD_2 was 3.1 ± 1.0 ng/10^6 mast cells (A) and histamine was 10.4 ± 1.5% (B). Values are means ± SEM, n=5-6.

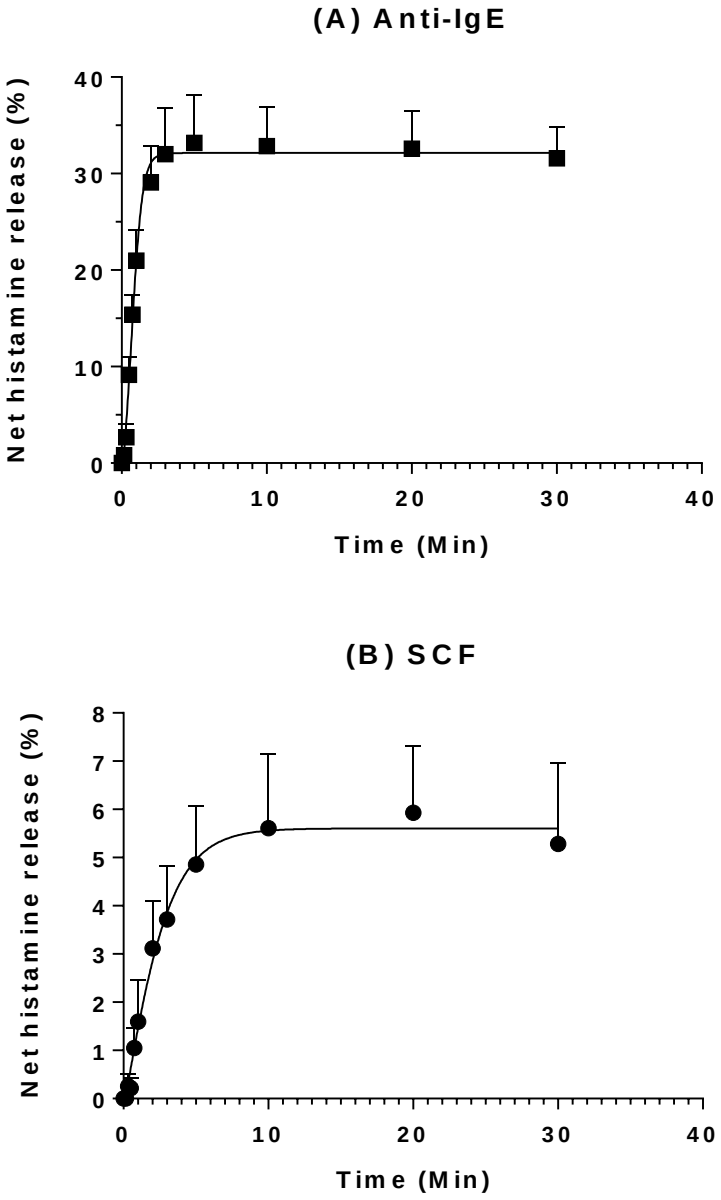


Figure 3.6 Time-course of the release of histamine by anti-IgE and SCF from mast cells. Cells were incubated for 5 min at 37 ^{o}C before being challenged with a maximal concentration of either (A) anti-IgE [2 µg/ml] or (B) SCF [100 ng/ml] for different time periods from 10 sec to 30 min to induce histamine release. The mean spontaneous release for histamine was 10.4 ± 1.2% (A) and 8.3 ± 0.6% (B). Values are means ± SEM, n=5-6.

Longer time points of activation of between 30 minutes up to 4 hours were investigated for effects on histamine release. When mast cells were incubated with anti-IgE (2 µg/ml) for 4 hours, the levels of histamine release were significantly higher (*P<0.05)* compared to levels obtained after 30 minutes of activation 44.8 ± 8% and 33.7 ± 6%, respectively [*Figure 3.7A*]. The same pattern was observed when mast cells were challenged with SCF for 30 minutes or 4 hours, there was a significant increase (*P<0.05*) in histamine release after 4 hours compared to 30 minutes [*Figure 3.7B*].

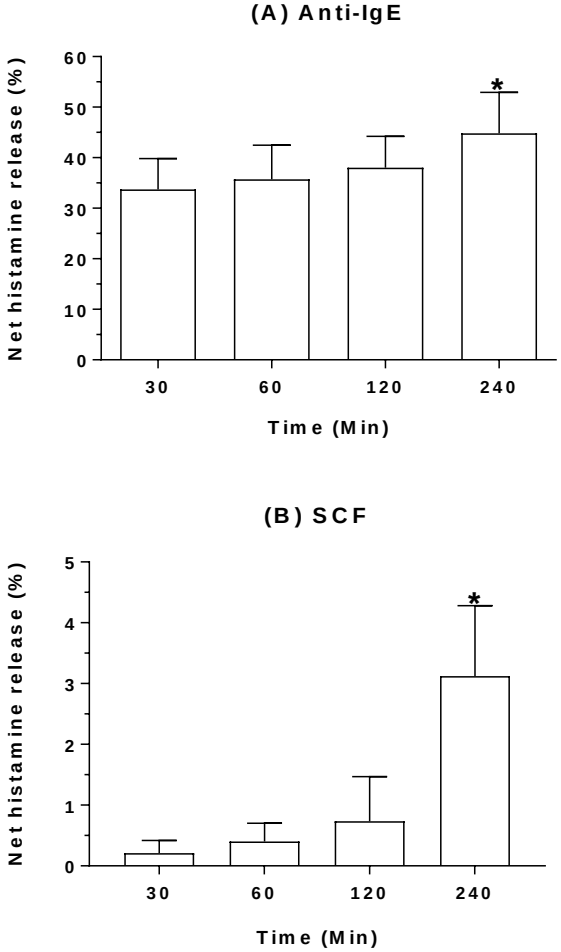


Figure 3.7 Time-course of the release of histamine by anti-IgE and SCF from mast cells. Cells were incubated for 5 min at 37 ^{o}C before being challenged with a maximal concentration of either (A) anti-IgE [2 µg/ml] or (B) SCF [100 ng/ml] for longer time periods from 30 min to 4 hours to induce histamine release. The mean spontaneous release for histamine was 9.3 ± 2.3% (A) and (B). Values are means ± SEM, n=5. Statistically significant difference compared to 30 min is indicated: * *P<0.05*.

PGD_2 generation was also investigated across a time-course similar to histamine release [*Figure 3.8*]. When mast cells were activated with anti-IgE (2 µg/ml), the levels of generated PGD_2 at 30 minutes or 4 hours were not different ($P>0.05$), the levels were 102.5 ± 28.8 and 104.5 ± 29.0 ng PGD_2 generated per 10^6 mast cells, respectively [*Figure 3.8A*]. However, PGD_2 generation following SCF (100 ng/ml) activation exhibited a time-dependent pattern; there was a significant increase (*P<0.05*) in PGD_2 release after 4 hours (70.7 ± 26.0 ng PGD_2 generated per 10^6 mast cells) compared to 30 or 60 minutes (32 ± 11 and 42 ± 14.8 ng PGD_2 generated per 10^6 mast cells, respectively) [*Figure 3.8B*].

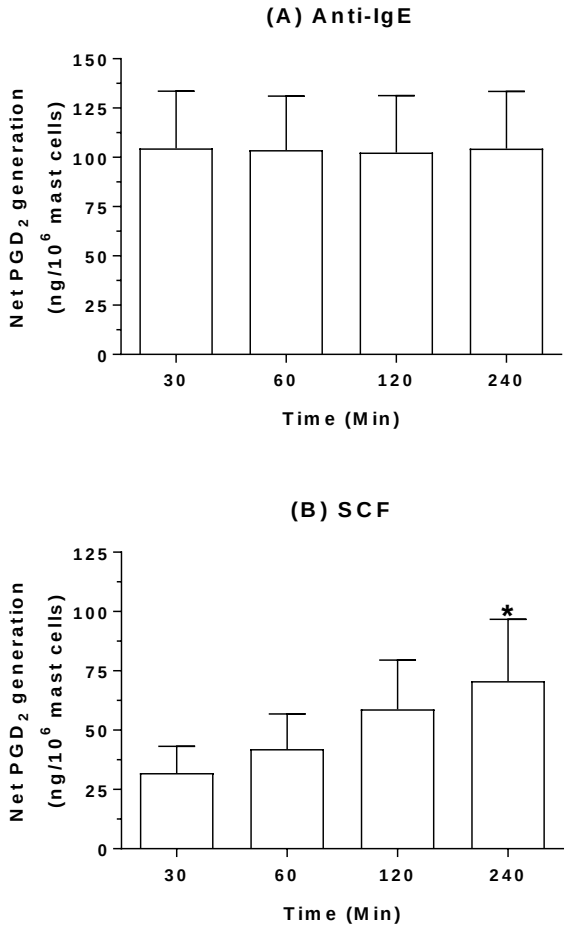


Figure 3.8 Time course of the generation of PGD_2 by anti-IgE and SCF from mast cells. Cells were incubated for 5 min at 37 ^oC before being challenged with a maximal concentration of either (A) anti-IgE [2 µg/ml] or (B) SCF [100 ng/ml] for longer time periods from 30 min to 4 hours to induce PGD_2 generation. The mean spontaneous release for PGD_2 was 4.9 ± 2.0 ng/10^6 (A) and (B). Values are means ± SEM, n=4. Statistically significant difference compared to 30 and 60 min is indicated: * *P<0.05*.

To investigate even longer time periods on the levels of histamine and PGD_2, mast cells were incubated with either anti-IgE (2 µg/ml) or SCF (100 ng/ml) for 20 hours [*Figure 3.9*]. When mast cells were activated with either anti-IgE or SCF for 4 hours or 20 hours, PGD_2 generation was decreased over time [*Figure 3.9A*]. Following anti-IgE activation, PGD_2 decreased significantly (*P<0.05*) from 159.6 ± 65.4 after 4 hours to 28.2 ± 9.9 ng PGD_2 generated per 10^6 mast cells after 20 hours. The same pattern was observed following SCF activation, PGD_2 significantly decreased from 152.6 ± 61.5 after 4 hours to 39.1 ± 17.3 ng PGD_2 generated per 10^6 mast cells after 20 hours.

By contrast, histamine release following mast cell activation enhanced over the time period [*Figure 3.9B*]. After 4 hours incubation, histamine levels were 655.4 ± 133.5 and 543 ± 87 ng of histamine per 10^6 mast cells when challenged with anti-IgE or SCF, respectively. After 20 hours incubation, histamine levels significantly increased (*P<0.05*) up to 1116.1 ± 171.6 and 1012.3 ± 137.8 ng of histamine per 10^6 mast cells when challenged with anti-IgE or SCF, respectively.

Taken together, these data suggest that histamine release increases over time following the activation of mast cells by either anti-IgE or SCF whereas PGD_2 reaches maximum generation 4 hours following mast cell activation, and then declines over time.

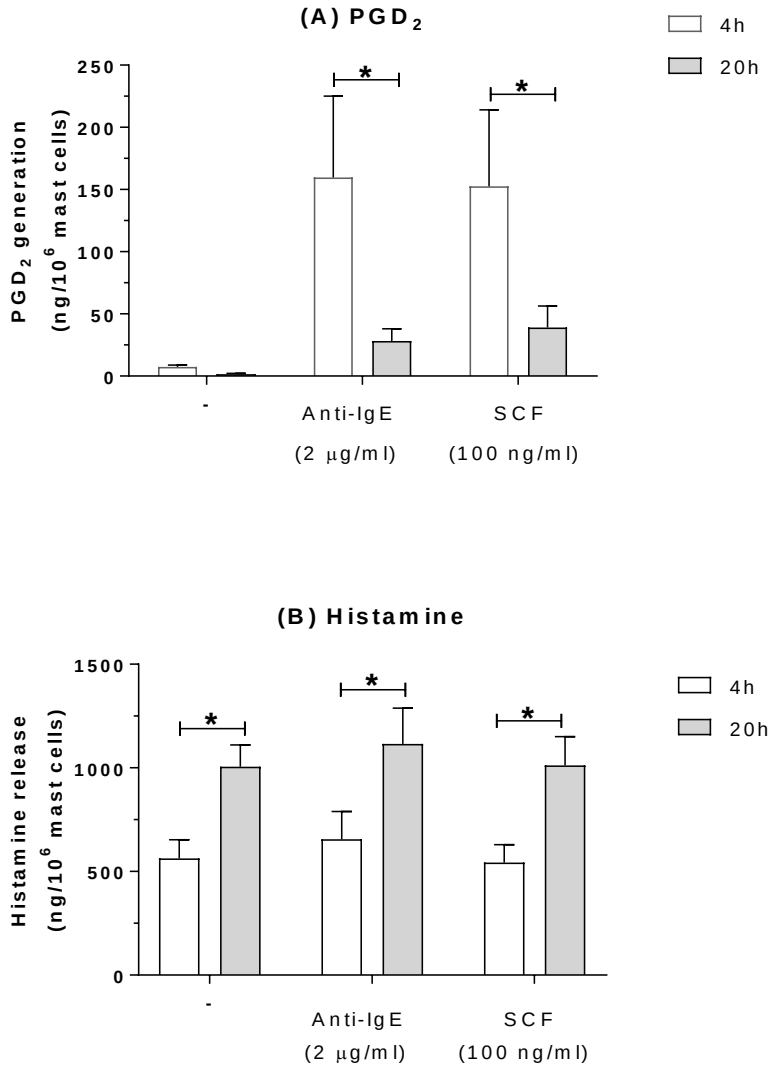


Figure 3.9 Time-dependence of SCF and anti-IgE on the generation of (A) PGD_2 or (B) histamine by mast cells. Cells were pre-incubated for 4 h (White) or 20 h (Gray) with or without anti-IgE (2 µg/ml) or SCF (100 ng/ml) to induce mast cell mediators. After this incubation the supernatants were collected and assayed for mediators. Values are means ± SEM, n=4. Statistically significant difference compared to control is indicated: * *P<0.05*.

3.2.3 Effect of overnight SCF treatment on mediator release

Mast cells were incubated with SCF (100 ng/ml) overnight (21 hours), and then challenged with anti-IgE (0.02 - 2 µg/ml), SCF (100 ng/ml) or ionomycin (10^{-6} - 10^{-8} M) and histamine release levels were determined [*Figure 3.10*]. There was no statistically significant difference in the levels of histamine release induced by any of the stimuli following incubation of mast cells with SCF overnight or control mast cells.

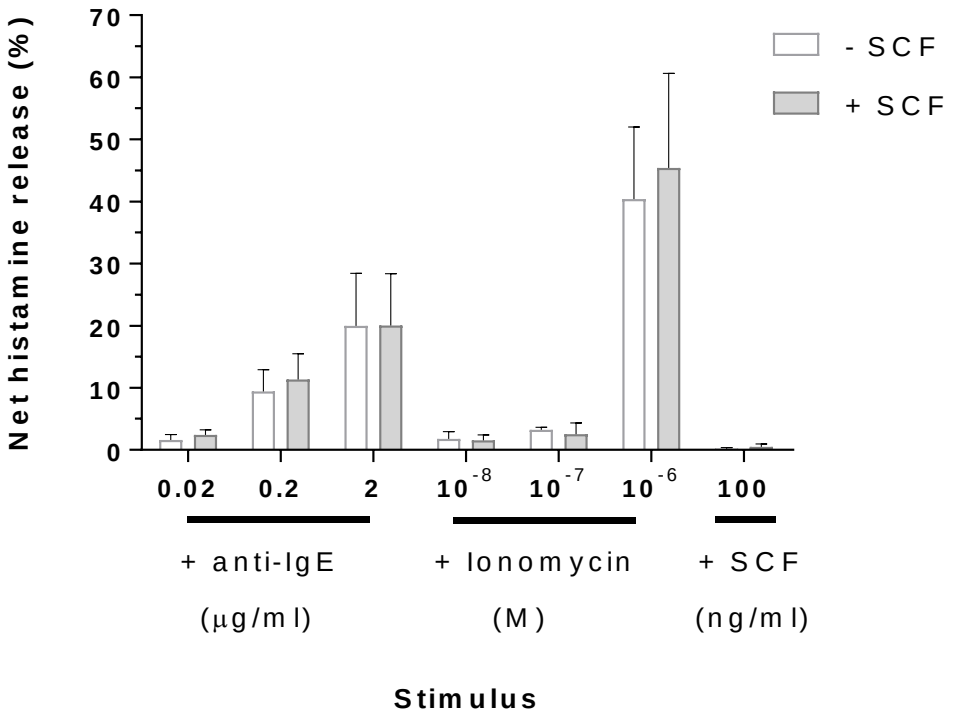


Figure 3.10 Effect of overnight SCF pre-treatment on histamine release. Cells were pre-incubated for 21 h with (Grey) or without (White) SCF (100 ng/ml). After this incubation the cells were washed and then incubated for 5 min at 37°C before challenge with increasing concentrations of anti-IgE (2 µg/ml), ionomycin (M) or a maximal concentration of SCF (100 ng/ml) for 25 min to induce histamine release. The mean spontaneous release for histamine was 10.2 ± 3.6% with or 11.3 ± 2.4% without SCF. Values are means ± SEM, n=3

3.2.4 Effect of passive sensitization on mediator release

Studies have shown that mast cells sensitised with IgE are more responsive to activation (Tauchi and Ohyashiki, 2006). To determine whether this might be the case in the present system, lung mast cells were passively sensitized with a NIP-specific IgE, JW8-IgE (1/250) overnight. After incubation with IgE, mast cells were washed and challenged with a range of concentrations of anti-IgE (0.02, 0.2 and 2 µg/ml), NIP-HSA (0.1, 1, 10 ng/ml) and SCF (1 - 100 ng/ml). Passive sensitization did not significantly enhance the responses of mast cells to anti-IgE or SCF [*Figure 3.11*]. However, the responses to NIP-HSA (10 ng/ml) were significantly (*P<0.05*) higher in sensitized mast cells compared to the control cells.

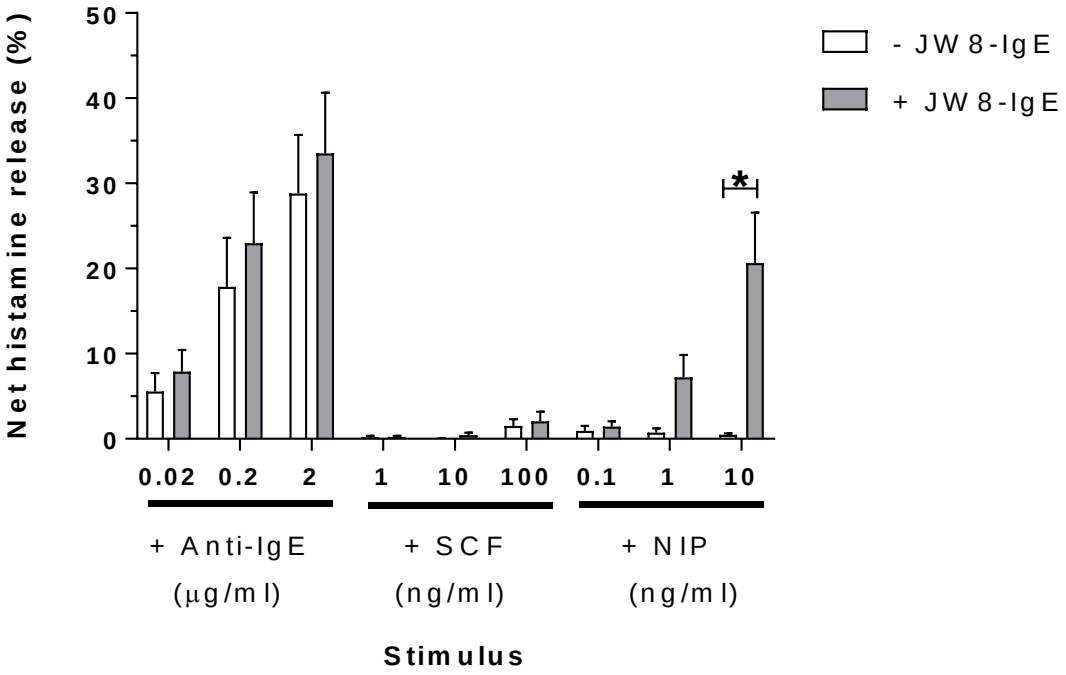


Figure 3.11 Effect of passive sensitization on responses. Cells were pre-incubated overnight with (White) or without (Gray) JW8-IgE (1/250) a NIP specific IgE. After this incubation the cells were washed then incubated for 5 min at 37 ^{o}C before challenge with increasing concentrations of anti-IgE (0.02 – 2 µg/ml) or NIP-HSA (0.1 - 10 ng/ml) and increasing concentrations of SCF (1 – 100 ng/ml) for 25 min to induce histamine release. The mean spontaneous release for histamine was 5.8 ± 0.8% with or 5.7 ± 0.5% without JW8-IgE. Values are means ± SEM, n=5. Statistically significant difference compared to control is indicated: * *P<0.05*.

3.3 The effect of c-KIT inhibitors on mediator release

3.3.1 Effect of c-KIT inhibitors on mediator release by SCF

We investigated the effect of the first generation c-KIT inhibitor, imatinib, on mediator release induced by SCF. Imatinib inhibited SCF induced histamine release in a concentration-dependent manner [*Figure 3.12A*]. The highest concentrations of imatinib, 10^{-6} and 10^{-5} M, almost abolished the levels of histamine released, the EC_{50} for imatinib was 1.3 x 10^{-7} M. Similarly, PGD_2 secretion was inhibited by the highest concentrations of imatinib [*Figure 3.12B*]. The EC_{50} for imatinib was 2.5 x 10^{-7} M. This suggests that imatinib inhibits the secretion of both histamine and PGD_2 to a similar extent.

Additional c-KIT inhibitors (10^{-5} M) were studied. These studies indicated that not only imatinib, but dasatinib and nilotinib had significant inhibitory effects on histamine released from mast cells activated by SCF (*P<0.05*) [*Figure 3.13A*]. These same inhibitors also completely blocked PGD_2 generation when mast cells were activated by SCF (*P*<0.05) [*Figure 3.13B*]. When a wider range of inhibitor concentrations were used (10^{-10} to 10^{-5} M) histamine release induced by SCF stimulation was blocked in a concentration-dependent manner [*Figure 3.14*]. Dasatinib (EC_{50} value; 8.3 x 10^{-10} M), demonstrated a profoundly significant inhibitory effect (*P<0.05*) on histamine release. Nilotinib and imatinib, (EC_{50} values; 9.2 x 10^{-8} M and 1.3 x 10^{-7} M, respectively), were roughly equipotent as inhibitors of SCF-induced histamine release at the highest concentrations used, completely blocked release (*P<0.05*).

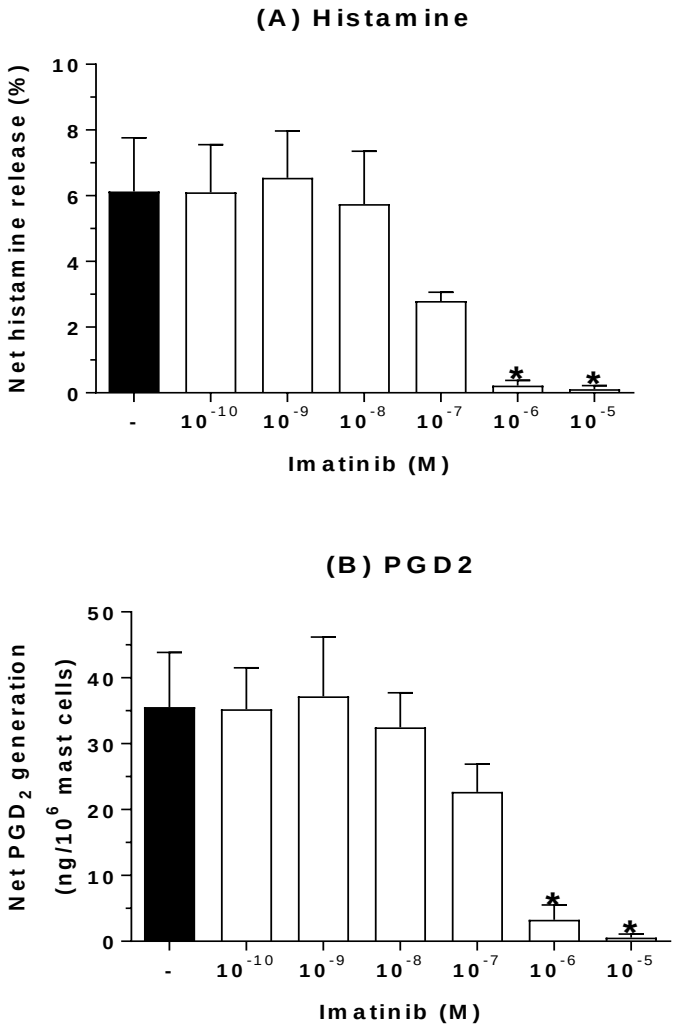


Figure 3.12 Effects of a c-KIT inhibitor (imatinib) on SCF-induced mediator release. Mast cells were incubated with or without (control) increasing concentrations of imatinib for 15 min before challenge with SCF (100 ng/ml) for a further 25 min. The effects of this inhibitor on (A) histamine release and (B) PGD_2 generation were determined. The mean spontaneous release for histamine was 3.1 ± 0.6% (A) and PGD_2 was 4.5 ± 3.0 ng/10^6 mast cells (B). Values are means ± SEM, n=5. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*.

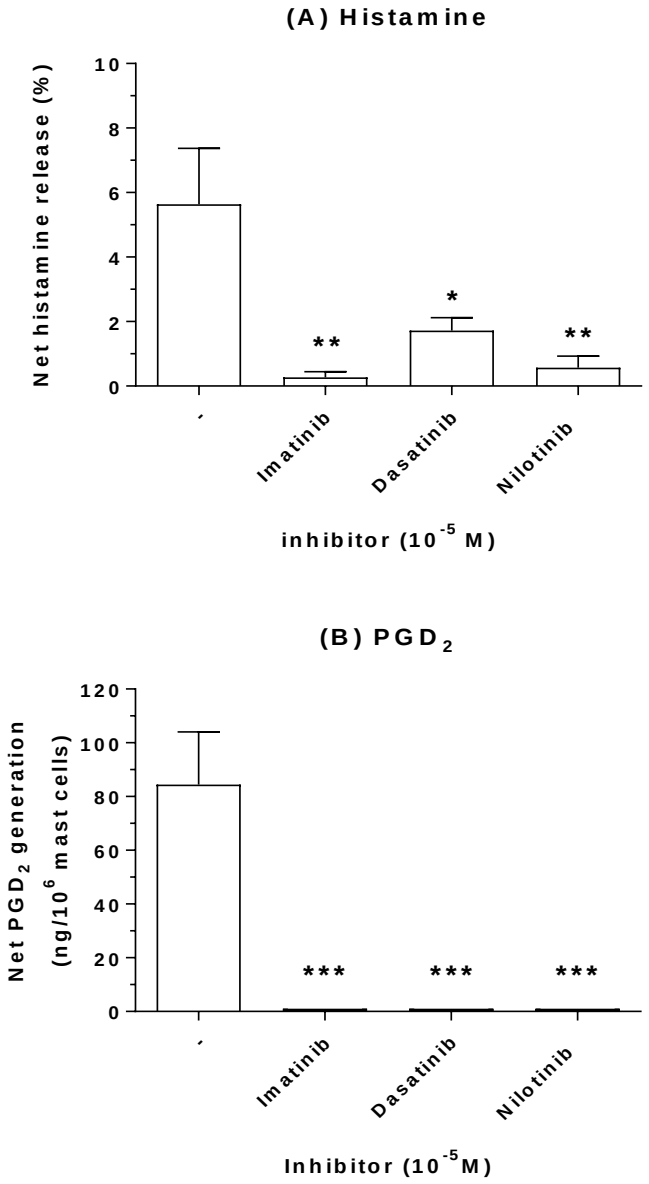


Figure 3.13 Effects of c-KIT inhibitors on SCF-induced mediator release. Mast cells were incubated with or without (-) imatinib, dasatinib or nilotinib for 15 min before challenge with SCF (100 ng/ml) for a further 25 min. The effects of these inhibitors on (A) histamine release and (B) PGD_2 generation were determined. The mean spontaneous release for histamine was 3.1 ± 0.8% (A) and PGD_2 was 0 ng/10^6 mast cells (B). Values are means ± SEM, n=4. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*, ** *P<0.01*, *** *P<0.001*

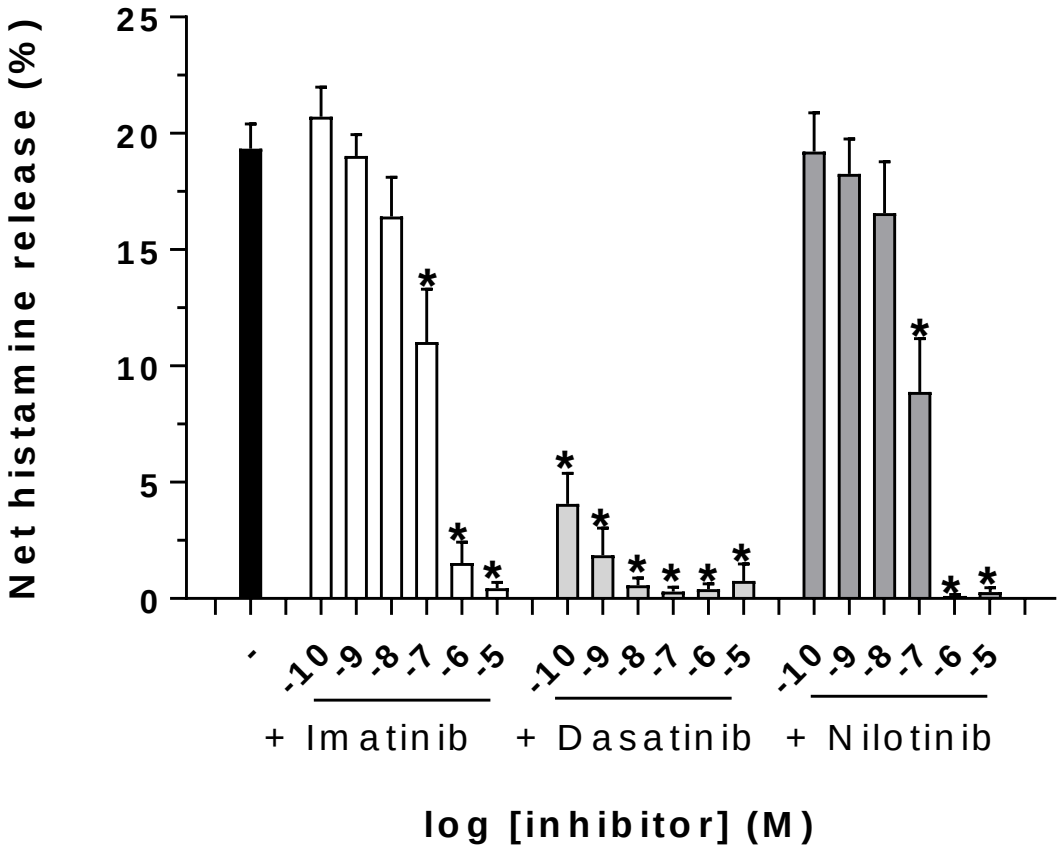


Figure 3.14 Effect of c-KIT inhibitors on SCF induced histamine release. Mast cells were incubated with or without (control) imatinib, dasatinib or nilotinib for 15 min before challenge with SCF (100 ng/ml) for a further 25 min. The effect of these inhibitors on histamine release was determined. The mean spontaneous release for histamine was 5.2 ± 0.5%. Values are means ± SEM, n=4. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*.

3.3.2 Effect of c-KIT inhibitors on histamine released by ionomycin

To investigate the effect of c-KIT inhibitors on histamine secretion induced by ionomycin (10^{-6} M), mast cells were incubated with imatinib, dasatinib and nilotininb before being challenged with the ionophore [*Figure 3.15*]. Imatinib and dasatinib did not inhibit histamine secreted by ionomycin. By contrast, nilotinib inhibited histamine release induced by ionomycin to a significant extent , (*P<0.05)*, at the highest concentrations ($\geq 10^{-6}$ M) used.

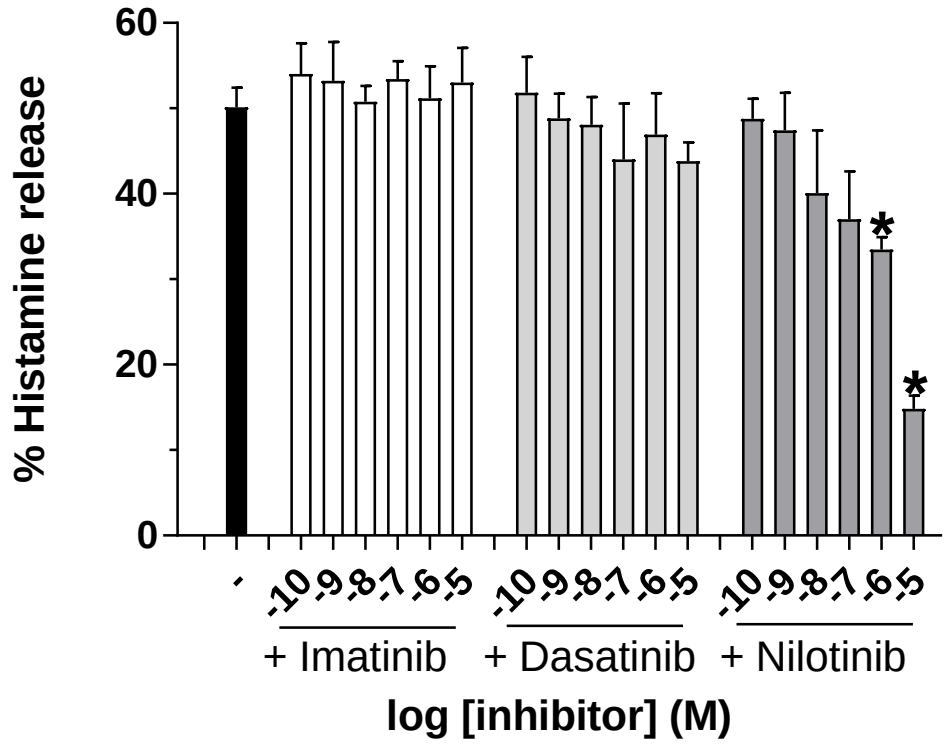


Figure 3.15 Effect of c-KIT inhibitors on ionomycin induced histamine release. Mast cells were incubated with or without (-) imatinib, dasatinib or nilotinib for 15 min before challenge with ionomycin (10^{-6} M) for a further 25 min. The effect of these inhibitors on histamine release was determined. The mean spontaneous release for histamine was 5.3 ± 0.9%. Values are means ± SEM, n=4. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*.

3.3.3 Effect of c-KIT inhibitors on mediators released by anti-IgE

Mast cells were incubated with c-KIT inhibitors dasatinib, nilotinib and imatinib before being challenged by anti-IgE [*Figure 3.16*]. The concentration (0.1 μM) of c-KIT inhibitors used in these experiments was considered to be maximally effective based on previously reported studies (see *Table I* in appendix) Dasatinib and nilotinib at (10^{-5} M) showed a significant inhibitory effect ($P<0.05$) on histamine release following mast cell activation by anti-IgE [*Figure 3.16A*]. Moreover, dasatinib significantly ($P<0.05$) inhibited PGD_2 generation following anti-IgE activation [*Figure 3.16B*].

The potential concentration-dependent effect of c-KIT inhibitors on anti-IgE-induced mediator release from mast cells was investigated [*Figure 3.17*]. Imatinib, at higher concentrations, inhibited histamine release moderately, while nilotinib significantly inhibited (*P<0.05*) histamine release at the highest dose. By contrast, dasatinib was an effective inhibitor.

The data in *Figure 3.17* were reworked to generate concentration-dependent inhibition curves [*Figure 3.18*]. From these curves, the EC_{50} values for both imatinib and nilotinib could not be determined, as the concentration-response had not reached a plateau. By contrast, since maximal inhibition was possible with dasatinib an EC_{50} value (2.8×10^{-8} M) could be calculated.

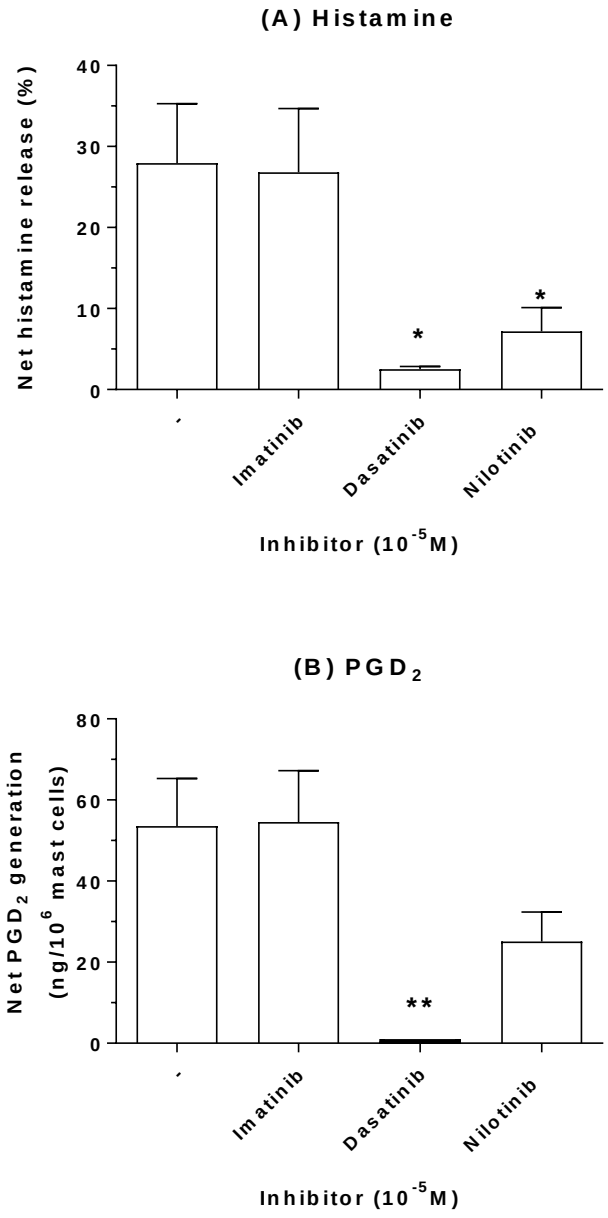


Figure 3.16 Effects of c-KIT inhibitors on anti-IgE-induced mediator release. Mast cells were incubated with or without (control) imatinib, dasatinib or nilotinib for 15 min before challenge with anti-IgE (2 µg/ml) for a further 25 min. The effects of these inhibitors on (A) histamine release and (B) PGD_2 generation were determined. The mean spontaneous release for histamine was 3.2 ± 0.6% (A) and PGD_2 was 0 ng/10^6 mast cells (B). Values are means ± SEM, n=6. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*, ** *P<0.01*.

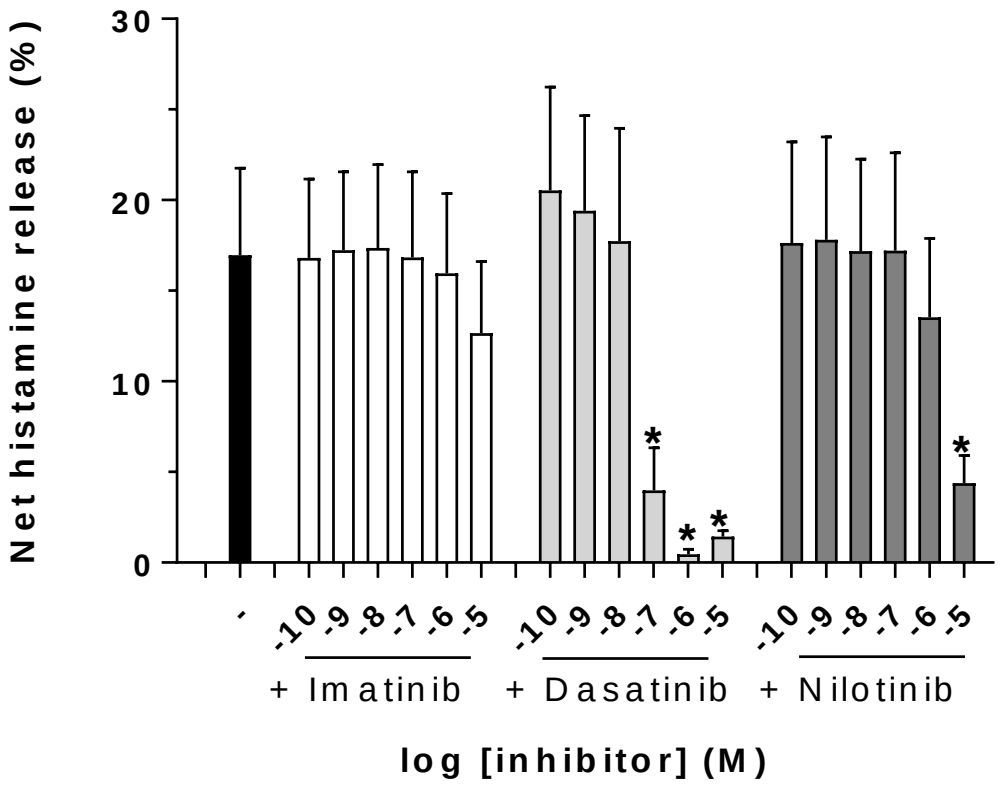


Figure 3.17 Effect of c-KIT inhibitors on anti-IgE induced histamine release. Mast cells were incubated with or without (control) imatinib, dasatinib or nilotinib for 15 min before challenge with anti-IgE (2 µg/ml) for a further 25 min. The effect of these inhibitors on histamine release was determined. The mean spontaneous release for histamine was 2.7 ± 0.3%. Values are means ± SEM, n=4. Statistically significant inhibition difference compared to control is indicated: * *P<0.05*.

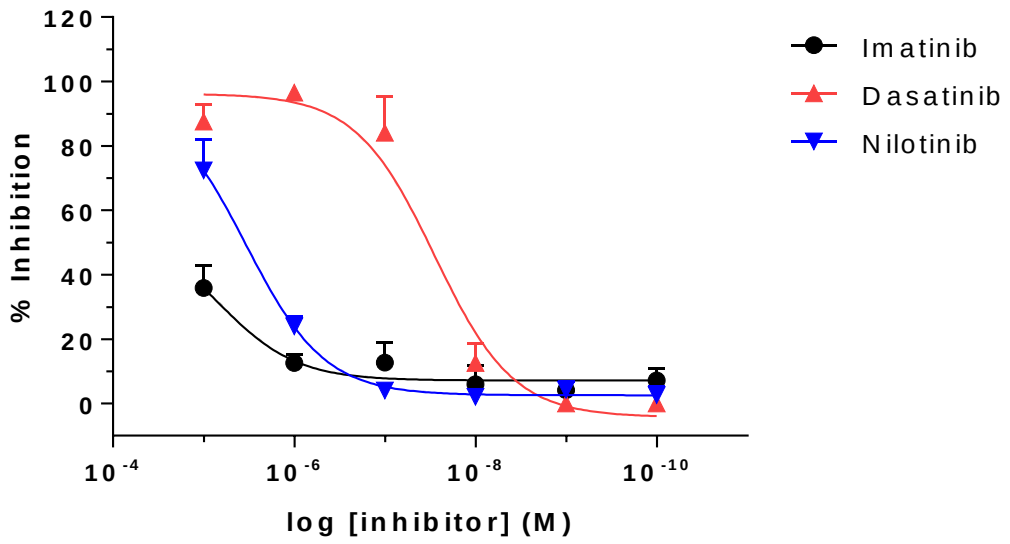


Figure 3.18 Effects of c-KIT inhibitors on anti-IgE. Mast cells were incubated with increasing concentrations of imatinib, dasatinib or nilotinib for 15 min before challenge with anti-IgE (2 µg/ml) for a further 25 min. The effects of these inhibitors were determined by percentage inhibition of the control histamine release which was 13.9 ± 4.6. Values are means ± SEM, n=4.

3.4 Discussion

3.4.1 Effects of SCF on mediator release

In this study, we investigated the effects of SCF on mediator release from mast cells. SCF, a growth factor, is involved in mast cell migration, survival, maturation and potentiates ongoing degranulation (Gilfillan and Tkaczyk, 2006, Columbo et al., 1992). In this study, low doses of SCF were not sufficient to induce histamine or PGD_2 generation by mast cells. However, SCF at a high concentration (100 ng/ml) was able to stimulate mast cells to release both histamine and PGD_2.

Previous work done by our group suggests a positive correlation between c-KIT expression and the amount of histamine release induced by SCF (Lewis et al., 2013). However, the response of mast cells to SCF was variable. About 30% of the mast cell preparations released a substantial amount of histamine in response to SCF whereas the majority of preparations released more modest quantities of histamine. To compare the effectiveness of SCF, mast cells were stimulated by anti-IgE in parallel and released mediators were measured. In paired studies, mast cells activated by anti-IgE released ~35% histamine, while mast cells activated by SCF released ~5% histamine (n=10-12). These data indicate that although SCF stimulates the release of histamine from mast cells it is less efficacious than anti-IgE. By contrast, PGD_2 generation, (around 60 ng of PGD_2 generated per 10^6 mast cells), was similar following mast cell activation by either, SCF or anti-IgE. This demonstrates that SCF is as effective as anti-IgE at generating PGD_2. Concentrations lower than 100 ng/ml SCF were unable to activate mast cells to generate PGD_2. Although lower concentrations (0.1 –

10 ng/ml) of SCF were unable to induce mediator release, lower concentrations of SCF tended to enhance mediator release by mast cells when combined with anti-IgE. Overall these findings suggest that SCF may be a physiologically relevant activator of human lung mast cells.

In further studies, we wanted to know whether there were any differences in the time-course of histamine release induced by SCF or anti-IgE. The maximal responses of mast cells activated by anti-IgE were observed at 5 minutes after activation and half maximal responses at 1 minutes. SCF-induced histamine release was rather slower as the maximal response to SCF was seen at 10 minutes and the half maximal response was at 2 minutes. These data imply that anti-IgE induces histamine release faster than SCF. However, it would be interesting to repeat these experiments using a lower concertation of anti-IgE that gives similar levels of histamine release compared to SCF to be able to provide a definitive description of the kinetics.

Reddy and Herschman (1997) suggested that activated murine mast cell lines generate PGD_2 in two stages, an early stage that reaches maximum within 30 minutes while a later stage is maximal after about 4 hours. For this reason, we have looked at longer activation time points of 30 minutes to 4 hours, to establish whether there is any difference in the extent of histamine release. Histamine release showed a modest increase in response to anti-IgE and SCF at 4 hours compared to 30 minutes. By contrast, mast cells incubated with anti-IgE generated almost the same PGD_2 levels at all-time points. When mast cells were incubated with SCF, PGD_2 levels were generated in a time-dependent manner; higher levels were observed at 2 hours compared to 30 minutes (*P=0.055*), and even greater increases were observed at 4 hours compared to

30 minutes (*P<0.05*) A possible explanation for this might be that SCF can activate c-KIT continuously and thereby drive histamine release and PGD_2 generation over at least 4 hours.

Given these findings, we wondered whether longer incubation periods of up to 20 hours might affect the generation of PGD_2 and histamine release. Our data suggest that 20 hours incubation with anti-IgE (2000 ng/ml) or SCF (100 ng/ml) induced greater levels of histamine release compared to 4 hours (*P<0.05*). These findings suggest that histamine can be continuously secreted by mast cells in a time-dependent manner. However, the levels of PGD_2 generated after 20 hours were significantly decreased (*P<0.05*). The reasons for this are not immediately apparent. However, it is well-known that prostanoids are labile and may be prone to degradation and this may provide an explanation for the decreased levels of PGD_2 seen after 20 hours.

Since SCF is known to be an important growth factor for mast cells, we investigated the effects of overnight incubation of SCF on mast cell responses. Our results suggest that there is no increase in the levels of histamine release in response to stimulation (ionomycin, anti-IgE and SCF) after treatment with SCF overnight. These data indicate that long-term incubation of mast cells with SCF failed to enhance stimulus-induced histamine release. Whether eicosanoid generation by stimuli is affected following overnight priming with SCF was not explored and could warrant investigation in the future.

Previous studies have shown that increasing IgE on rodent mast cells leads to an increase in mast cell activity not just to IgE-dependent activators like antigen (Tauchi and Ohyashiki, 2006). Therefore, we wondered whether passively sensitizing mast cells with IgE might increase responses to SCF. Mast

cells were incubated overnight with JW8-IgE, which is a NIP specific IgE. As responses to the antigen, NIP-HSA, were observed at the highest concentration used this shows that the cells had taken up the JW8-IgE. Following the passive sensitization, responses to anti-IgE also increased moderately and this might be expected if there is an increase in IgE at the surface. However, the treatment did not increase mast cell responses to SCF unlike the situation in alternative mast cell systems. In conclusion, increasing IgE on the surface of human lung mast cells did not lead to an increase in responses to non-IgE-dependent activators.

3.4.2 The effect of c-KIT inhibitors on mediator release

To confirm that SCF is working at the c-KIT receptor to activate mast cells, we have studied a number of c-KIT inhibitors including imatinib, dasatinib and nilotinib. Imatinib (Gleevec®) is a first generation tyrosine kinase inhibitor which has been successfully used as a first line therapy to treat chronic myeloid leukaemia (CML) and gastrointestinal stromal tumours (GISTs). Imatinib targets tyrosine kinases including Bcr-Abl oncogene, platelet-derived growth factor receptors (PDGFRA) and most importantly c-KIT (Iqbal and Iqbal, 2014). Our results indicate that imatinib significantly (*P<0.05*) blocked histamine release induced by SCF in a concentration-dependent manner and completely abrogated PGD_2 generation (*P<0.05*) when a single concentration (10^{-5} M) was used. These findings provide initial evidence that SCF acts at the c-KIT receptor to activate mast cells.

Dasatinib (Sprycel®) and Nilotinib (Tasigna®) are both second generation tyrosine kinase inhibitors used as a first line therapy to treat chronic myeloid leukaemia (CML) in patients resistant to imatinib or in those that show side

effects to imatinib (Tauchi and Ohyashiki, 2006). Dasatinib and nilotinib are more potent than imatinib against Bcr-Abl kinase by blocking the mechanism of activation (Wei et al., 2010). Our results indicate that dasatinib completely blocked histamine release induced by mast cells activated by SCF, while nilotinib significantly (*P<0.05*) blocked the release in a concentration-dependent manner. In addition, a single concentration (10^{-5} M) of both inhibitors completely abrogated PGD_2 generation (*P<0.05*). These data provide further evidence that SCF acts through the c-KIT receptor to drive both histamine and PGD_2 generation by mast cells.

By contrast, imatinib did not work against anti-IgE induced release, whereas nilotinib was moderately effective and dasatinib worked well. As anti-IgE does not work through the c-KIT receptor, it is quite possible that imatinib is the only selective c-KIT inhibitor because it worked against SCF but not anti-IgE. Dasatinib and nilotinib are probably acting at other tyrosine kinases, as well as c-KIT, which may be induced when mast cells are activated by anti-IgE. The relative lack of effect of these inhibitors against ionomycin suggests that ionomycin does not use protein tyrosine kinases targeted by these inhibitors.

3.4.3 Concluding comments

The main goal of this chapter was to determine whether SCF can activate human lung mast cells. SCF was found to be an effective activator of human lung mast cells, and was particularly effective at generating PGD_2. These effects appeared to be mediated through the c-KIT receptor as a selective inhibitor of c-KIT, imatinib, worked against SCF. Since high concentrations of SCF have been found in the lungs of asthmatics, these studies suggest that SCF could be important in the context of asthma.

CHAPTER FOUR

4. CHARACTERIZATION OF THE ISOFORM OF COX REGULATING PGD_2 GENERATION FROM HUMAN LUNG MAST CELLS

4.1 Introduction

Mast cells are a very rich source of prostaglandin D_2 (PGD_2) which is a product of arachidonic acid cyclooxygenation (Lewis et al., 1982). Very few other cells such as alveolar macrophages and lymphocytes T helper cell 2 (Th2) have the capacity to produce PGD_2. Mast cell activation can be assessed by the presence of PGD_2 or its metabolite 9α,11β-PGF_2 (Dahlen and Kumlin, 2004, Bochenek et al., 2004). PGD_2 mediates bronchoconstriction and is largely pro-inflammatory (Beasley et al., 1987, Matsuoka et al., 2000). PGD_2 and other mast cell derived mediators such as histamine and cysteinyl-leukotrienes (cys-LTs) contribute to allergic type reactions (Bingham and Austen, 2000, Bradding et al., 2006). It is well documented that human lung mast cells produce PGD_2 (Schleimer et al., 1985), however the pathway of PGD_2 generation needs greater elucidation.

The COX enzyme is essential to the generation of prostanoids (Rouzer and Marnett, 2009). COX converts arachidonic acid to a highly labile intermediate, PGH_2. Specific synthases then act to convert PGH_2 into prostanoids. Whereas COX is found widely, synthases tend to show more restricted distribution (Ueno et al., 2005). Other than PGD_2, important prostanoids include PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 which tend to be secreted by discrete cell types (Woodward et al., 2011). These display varying profiles of activity. For example, PGE_2 is known to inhibit acid secretion from parietal cells in the gut, and it is considered important in mediating inflammation and sensitizing sensory neurones to pain (Tilley et al., 2001, Peskar et al., 2003).

Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and ibuprofen, target COX by attenuating prostanoid generation; they are useful for

the treatment of certain types of inflammation and pain (Cryer and Feldman, 1998). COX is known to exist as two isoforms COX-1 and COX-2. COX-1 is recognised as the isoform that is expressed constitutively whereas COX-2 is thought to be the form that is induced during inflammation (Mitchell et al., 1993, Warner et al., 1999, Chan et al., 1999). Aspirin and ibuprofen are to some extent non-selective drugs and affect both isoforms of COX (Mitchell et al., 1993, Warner et al., 1999, Chan et al., 1999, Cryer and Feldman, 1998). Any anti-inflammatory benefit of non-selective NSAIDs may therefore be accompanied by off-target effects including inhibition of COX-1 in the gut, leading to a reduction in the production of PGE, increased acid secretion and potential gastropathy (Warner et al., 1999). Since many NSAIDs show limited selectivity for either COX-1 or COX-2, this has led to the development of COX-2 selective drugs such as celecoxib that are less likely to target COX-1 and are gut-sparing (Warner et al., 1999, Chan et al., 1999, FitzGerald and Patrono, 2001).

Despite the undoubted value of NSAIDs in the treatment of certain types of inflammation and moderate pain, the use of aspirin and related compounds is largely contraindicated in individuals with asthma and respiratory diseases. This is because aspirin can induce exacerbations in a sizeable proportion of asthmatics (Kowalski et al., 2011). The mechanism behind this underlying susceptibility to aspirin and related drugs has not been elucidated but aspirin intolerance has been linked to increased production of both cys-LTs and PGD_2 (Cahill et al., 2015, O'Sullivan et al., 1996). The production of PGD_2 suggests that mast cells are central to the process. While an expectation would be that NSAIDs are likely to inhibit PGD_2 generation from human lung mast cells, there are suggestions that aspirin might activate mast cells directly by mechanisms that are not clearly understood (Steinke et al., 2014).

To date, no systematic evaluation of the effects of NSAIDs on human lung mast cells has been performed. The present study aims to redress this. Studies in rodent cell systems and in cultured human mast cells suggest that both COX-1 and COX-2 may contribute to PGD_2 production (Murakami et al., 1995, Reddy and Herschman, 1997, Obata et al., 1999). The principal aim of this component of my PhD project therefore, was to identify which isoform(s) of COX mediates PGD_2 generation from human lung mast cells. The findings of this study may help to explain the actions and behaviour of NSAIDs in the context of respiratory disease.

4.2 Effects of COX inhibitors on PGD_2 generation

Our data shows that mast cells following the activation by anti-IgE or SCF released approximately the same amount of PGD_2 [see *Section 3.2.1*]. To investigate this further, we attempted to identify which COX isoform is responsible for the generation of PGD_2. To establish this, we investigated the effects of a variety of COX inhibitors on mast cells, different ELISAs was performed to determine the effect of those inhibitors on PGD2, cys-LTs beside histamine release. Western blotting was also performed to confirm which COX isoforms is dominantly expressed.

4.2.1 Effect of COX inhibitors on mediator release by anti-IgE

We investigated the effects of range of commonly used NSAIDs (1 µM) including aspirin (ASP), naproxen (NAP), ibuprofen (IBU), diclofenac (DIC), indomethacin (INDO) on PGD_2, cys-LTs and histamine release from human lung mast cells. The concentration (1 µM) of NSAIDs used in these preliminary experiments was considered to be maximally effective based on previously reported studies (see

Table II in appendix). These COX inhibitors were investigated when mast cells were activated by anti-IgE (2 µg/ml).

All the NSAIDs (1 µM) significantly ($P<0.05$) inhibited PGD_2 generation, except aspirin which was ineffective at inhibiting PGD_2 generation [*Figure 4.1A*]. We also looked at the effect of these COX inhibitors on the release of cys-LTs and histamine from activated mast cells. None of the COX inhibitors tested had any effect on cys-LTs generation [*Figure 4.1B*] or the release of histamine [*Figure 4.1C*].

However, when a higher concentration of aspirin was used (10 µM), PGD_2 generation by activated mast cells was inhibited [*Figure 4.2A*], but the inhibition was far from complete (~36% inhibition) unlike the alternative NSAIDs studied which essentially abolished PGD_2 generation [*Figure 4.1A*].

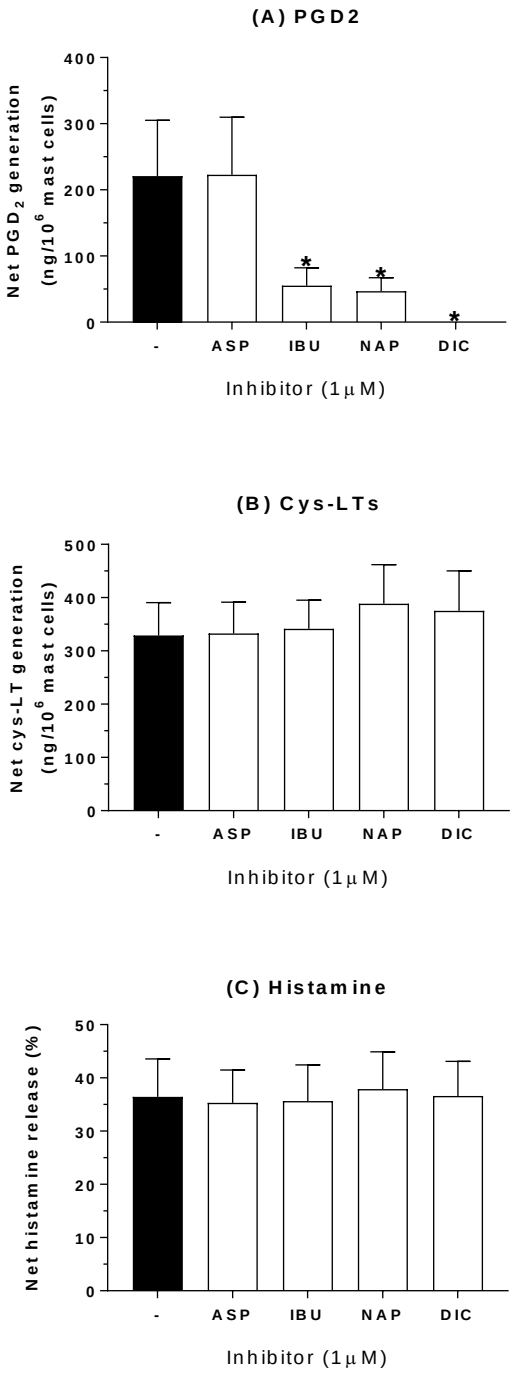


Figure 4.1 Effects of NSAIDs on mediator release. Mast cells were incubated (15 min) without (-) or with either aspirin (ASP), ibuprofen (IBU), naproxen (NAP) or diclofenac (DIC) [1 μM] before challenge for a further 25 min with anti-IgE (2 μg/ml) for mediator release. Effects of NSAIDs on (A) PGD_2, (B) cys-LT and (C) histamine generation were evaluated. The mean spontaneous release for PGD_2 was 9.9 ± 2 ng/10^6 mast cells (A), cys-LT was 10.3 ± 3 ng/10^6 mast cells (B) and histamine release was 9.8 ± 1.8% (C). Values are means ± SEM, n= 4-5. Statistically significant reductions in PGD_2 generation, difference compared to control (-) are indicated; *P<0.05.

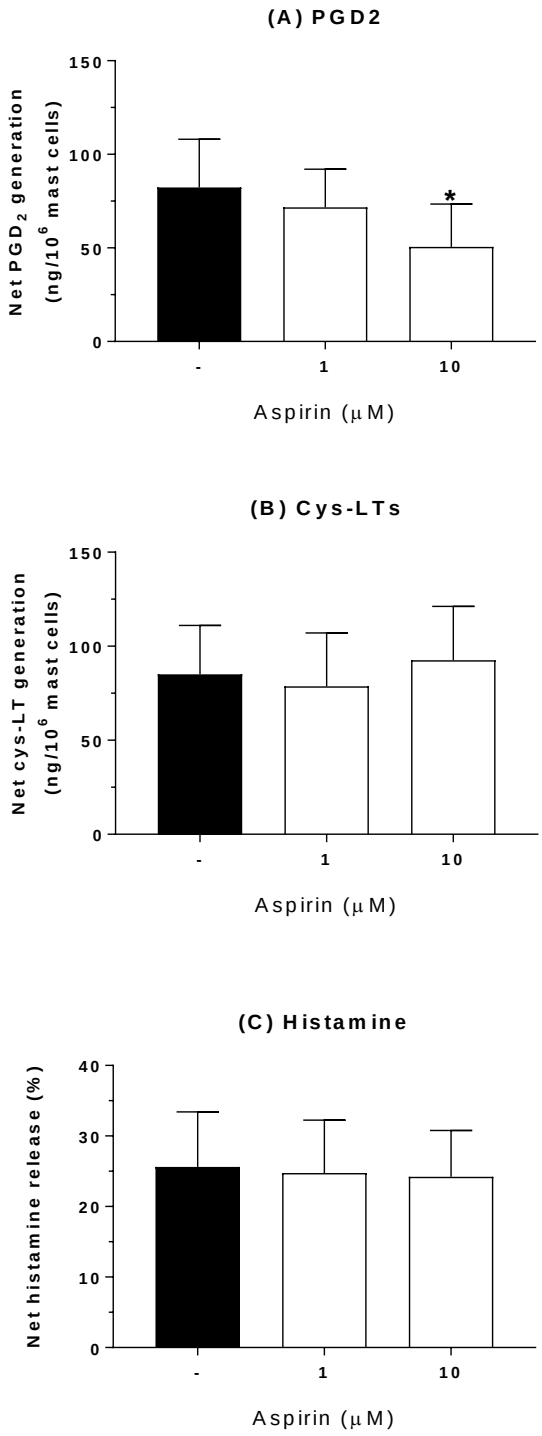


Figure 4.2 Effects of Aspirin on mediator release. Mast cells were incubated (15 min) without (-) or with aspirin [1 or 10 μM] before challenge for a further 25 min with anti-IgE (2 μg/ml) for mediator release. Effects of NSAIDs on (A) PGD_2, (B) cys-LT and (C) histamine generation were evaluated. The mean spontaneous release for PGD_2 was 7.4 ± 2 ng/10^6 mast cells (A), cys-LT was 7.0 ± 1.7 ng/10^6 mast cells (B) and histamine release was 8.5 ± 1.8% (C). Values are means ± SEM, *n*= 5. Statistically significant reductions in PGD_2 generation, difference compared to control (-) are indicated; *P<0.05.

We also examined the effect of the following inhibitors: FR122047, a COX-1 selective inhibitor, and celecoxib, a COX-2 selective inhibitor, and indomethacin, non-selective COX inhibitor, on PGD_2 generation from activated human lung mast cells. In these experiments, the PI3K inhibitor, wortmannin, was used as a positive inhibitory control. Both indomethacin and FR122047 significantly (P<0.05) inhibited PGD_2 generation from mast cells induced by anti-IgE, whereas celecoxib was ineffective [*Figure 4.3A*]. We also looked at the effect of these inhibitors on the release of cys-LTs and histamine from mast cells. None of the COX inhibitors tested had any inhibitory effect on cys-LTs generation [*Figure 4.3B*] or the release of histamine [*Figure 4.3C*]. The positive inhibitor control, wortmannin, was effective (P<0.05) against histamine release, PGD_2 and cys-LTs generation from activated mast cells [*Figure 4.3*].

4.2.2 Effect of COX inhibitors on mediator release by SCF

A previous study has demonstrated increased levels of the mast cell growth factor, SCF, in asthma (Makowska et al., 2009). A recent study of ours has shown that SCF is also an effective driver of mediators release from human lung mast cells, especially PGD_2 generation (Lewis et al., 2013).

To investigate the effects of COX inhibitors on PGD_2 generation by mast cells activated by SCF, we incubated human lung mast cells with selective (FR122047 and celecoxib) and non-selective (indomethacin) COX inhibitors followed by SCF (100 ng/ml).

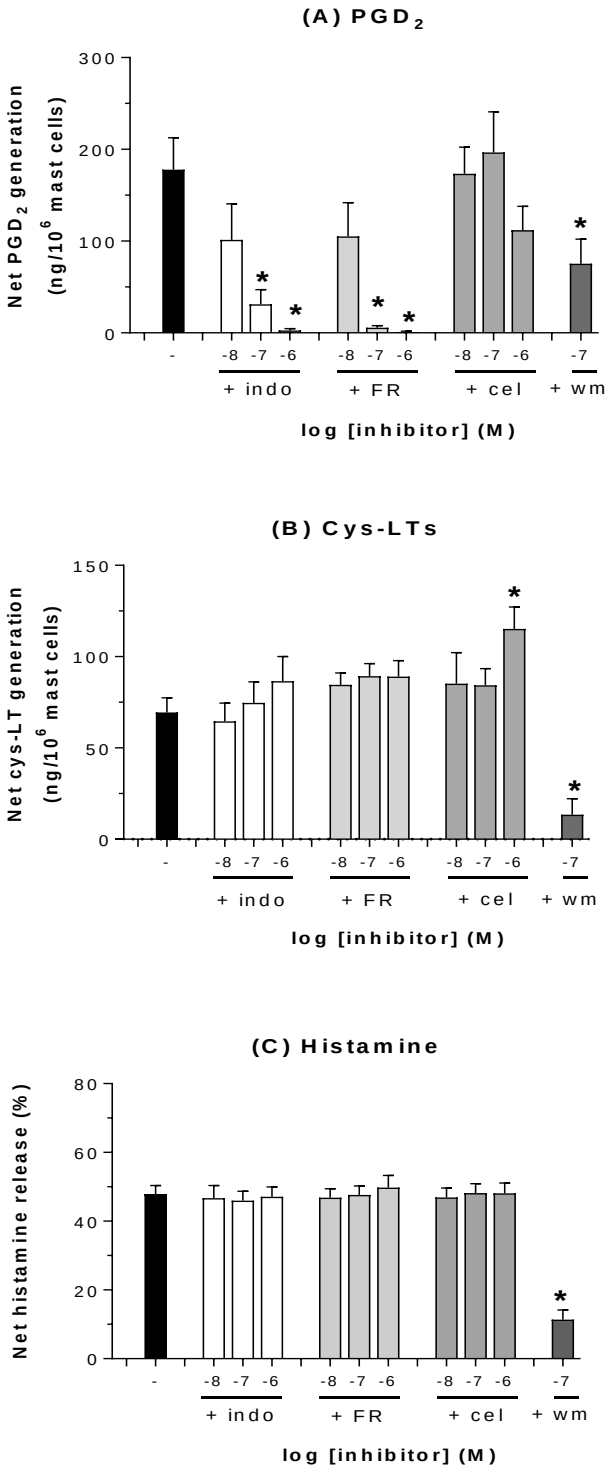


Figure 4.3 Effect of COX inhibitors on anti-IgE mediated mast cell responses. Mast cells were incubated with or without (control) Indomethacin, FR122047, celecoxib or wortmannin for 15 min before challenge with anti-IgE (2 µg/ml) for a further 25 min. The effect of these inhibitors on (A) PGD_2 generation, (B) cys-LT generation and (C) histamine release were determined. Values are means ± SEM, n=4-5. Statistically significant inhibition, difference compared to control is indicated; * $P<0.05$.

Both FR122047 (COX-1 selective) and indomethacin (non-selective COX), abolished (P<0.001) PGD_2 generation at all concentrations used, whereas celecoxib (COX-2 selective) was less potent. We also looked at the effect of these inhibitors on the release of cys-LTs and histamine from mast cells. None of the COX inhibitors tested had any inhibitory effect on cys-LTs generation [*Figure 4.4B*] or the release of histamine [*Figure 4.4C*]. The positive inhibitor control, wortmannin, was effective (P<0.05) against histamine release, PGD_2 and cys-LTs generation [*Figure 4.4*].

Further studies were performed with FR122047 using an extended concentration range (10^{-12} – 10^{-6} M). FR122047 inhibited PGD_2 generation by mast cells activated by either anti-IgE or SCF in a concentration-dependent manner [*Figure 4.5*]. FR122407 was slightly more potent as an inhibitor of PGD_2 generation induced by SCF (EC_{50} of 9 nM) than anti-IgE (EC_{50} of 38 nM). FR122047 had no effect on histamine released by mast cells activated by either anti-IgE or SCF [*data not shown*].

4.2.3 Expression of COX-1 and COX-2 in mast cell preparations

To determine whether human lung mast cells express mRNA for COX-1 and COX-2, RT-PCR was performed [*Figure 4.6*]. The data show that mast cells express mRNA for both COX-1 and COX-2. Further studies were performed to confirm COX expression at the protein level in human lung mast cells. Western blot analysis was performed on lysates of purified mast cells (n= 21).

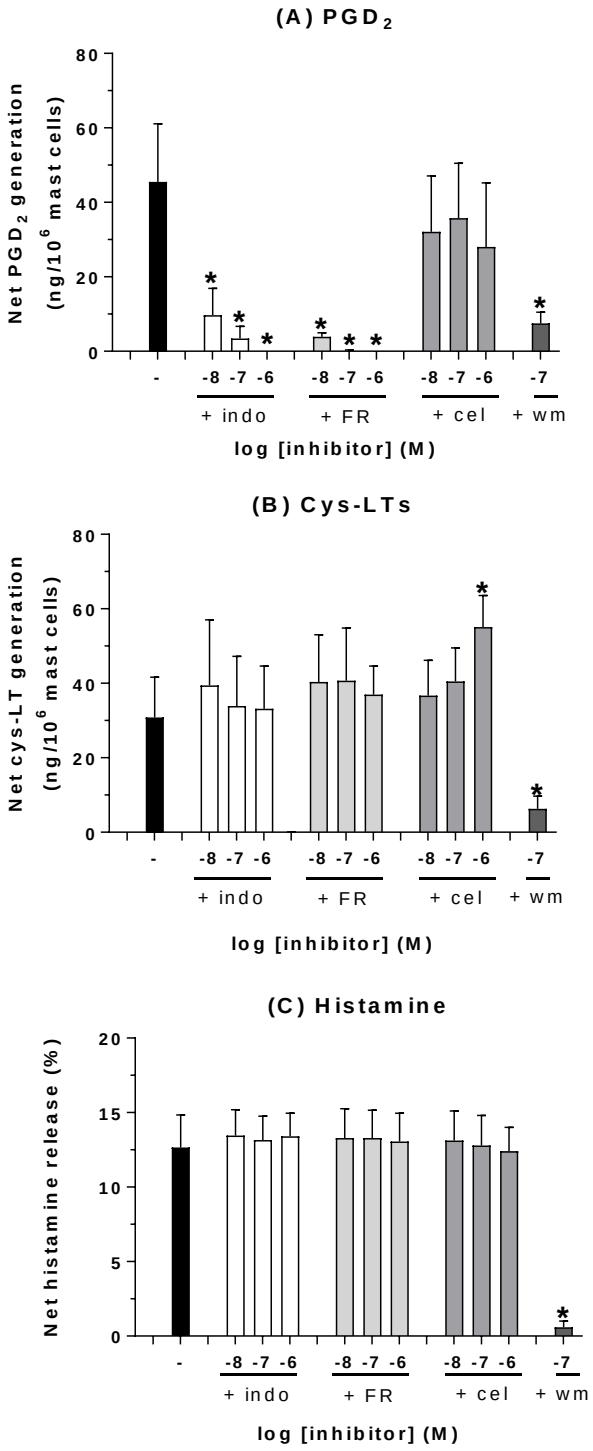


Figure 4.4 Effect of COX inhibitors on SCF mediated mast cell responses. Mast cells were incubated with or without (control) indomethacin, FR122047, celecoxib and wortmannin for 15 min before challenge with anti-IgE (2 µg/ml) for further 25 min. The effect of these inhibitors on (A) PGD_2 generation, (B) cys-LT generation and (C) histamine release were determined. The mean spontaneous release for PGD_2 was 1.9 ± 0.6 ng/10^6 mast cells (A), cys-LT was 17.3 ± 10.2 ng/10^6 mast cells (B) and histamine release was 5.3 ± 0.9% (C). Values are means ± SEM, n=5-7. Statistically significant inhibition, difference compared to control is indicated: * $P<0.05$.

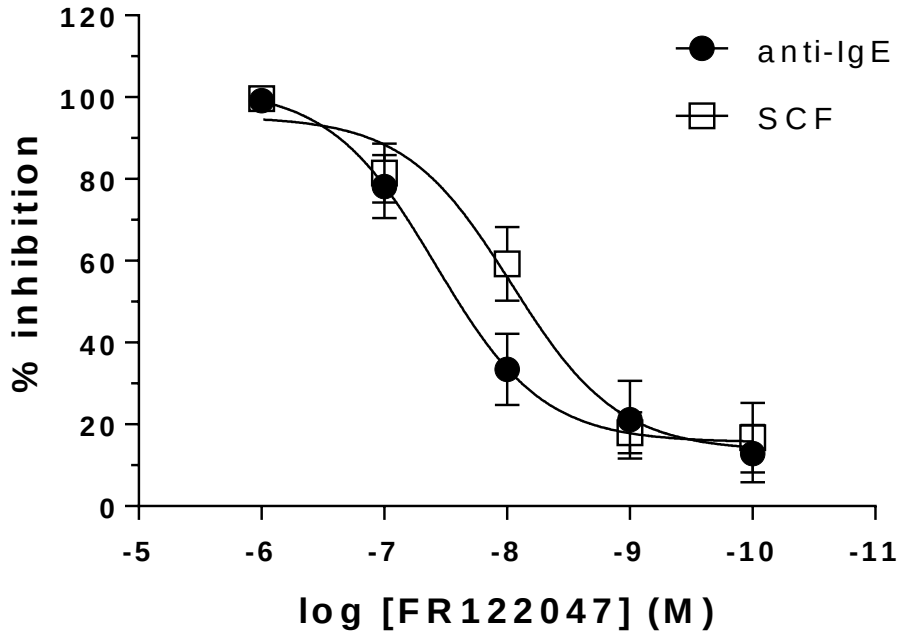


Figure 4.5 Effect of FR122047 on PGD_2 generation. Mast cells were incubated without (-) or with FR122047 for 15 min before challenge for a further 25 min with either anti-IgE (2 µg/ml) for SCF (100 ng/ml) or PGD_2 generation. Results are expressed as the % inhibition by FR122047 of the control unblocked PGD_2 generation which was 190.5 ±30.32 ng of PGD_2 per 10^6 mast cells for anti-IgE and 84.17 ± 23.5 ng/10^6 ng of PGD_2 per 10^6 mast cells for SCF. Values are means ± SEM, n= 6.

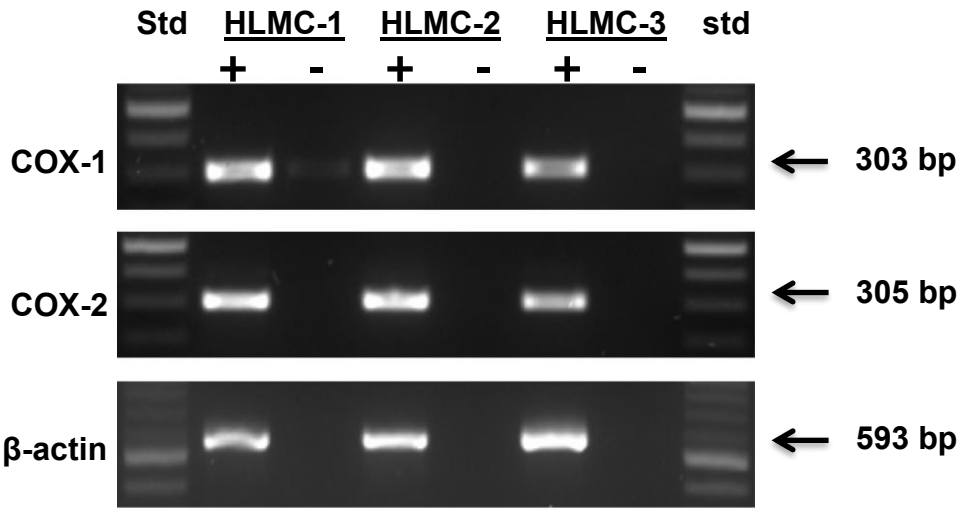


Figure 4.6 COX isoform expression in human lung mast cells. Isolated RNA was converted to cDNA by reverse transcriptase (+) and this step was also carried out in the absence of reverse transcriptase for control purposes (-). Amplification of cDNA was performed using primers for COX-1 and COX-2 and for β-actin. Expression profiles for 3 mast cell preparations (HLMC-1, HLMC-2 and HLMC-3) are shown. Data are representative of a total of 6 different mast cell preparations in excess of 95% purity. Lanes at either end of each gel represent standards (std) comprising a 100 bp ladder.

Our results show that COX-1 was dominantly expressed in all mast cells preparations whereas COX-2 was more variably or weakly expressed [*Figure 4.7A*]. In the 21 mast cell preparations that showed a positive actin signal, COX-1 was expressed [*Figure 4.7B*]. However, out of all the preparations that expressed COX-1, COX-2 was also observed in a few preparations

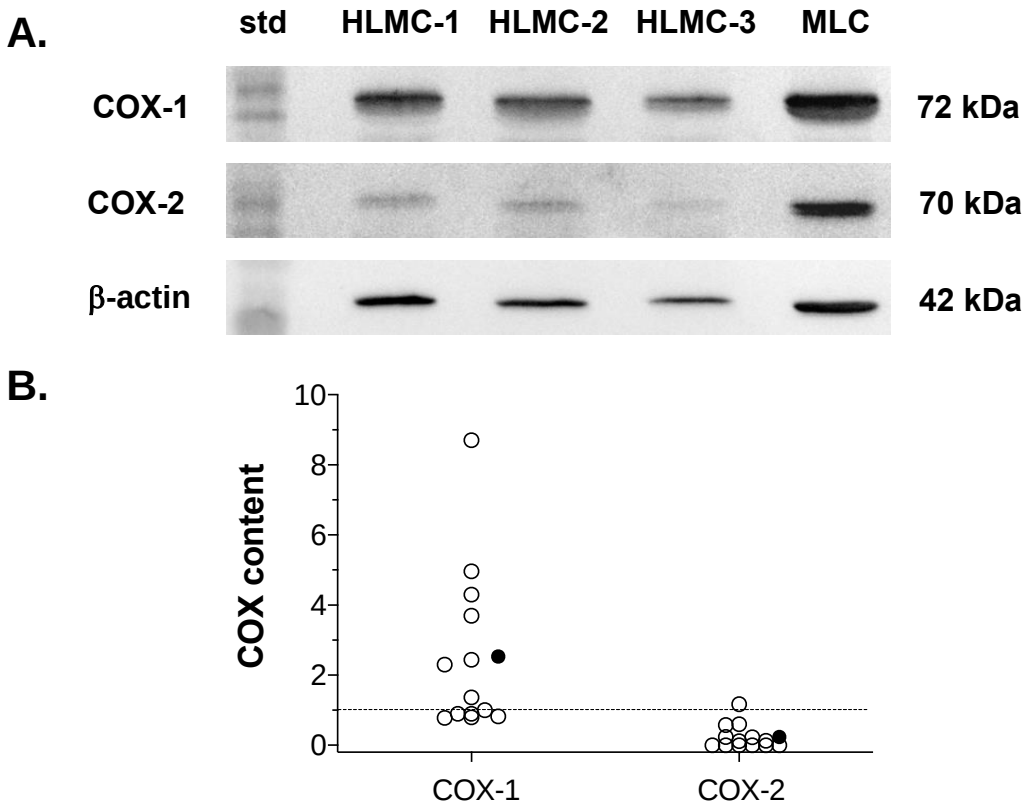


Figure 4.7 Immunoblot for COX isoform expression in human lung mast cells. (**A**) Three mast cell preparations (HLMC-1, HLMC-2 and HLMC-3) at purities of 85 ± 2% were solubilized and subjected to SDS-PAGE along with a mixed lung cell (MLC) preparation. Following electrophoretic transfer to a nitrocellulose membrane, the membrane was probed with an antibody to COX-2, the membrane stripped and probed with antibody to actin and the membrane stripped again and probed with antibody to COX-1. std stands for mw standards. (**B**) COX-1 and COX-2 content in mast cells was determined by densitometry. Content is expressed relative to the same MLC preparation that was used as a control in all blots and normalised relative to cell actin. Open symbols represent the COX content in individual mast cell preparations, filled symbols the mean value. Data are for 13 different mast cell preparations.

4.2.4 Effect of SCF on COX expression in mast cells

Previously we investigated whether longer-term activation of mast cells with either anti-IgE or SCF influenced PGD_2 generation. When mast cells were activated with SCF (but not anti-IgE), PGD_2 generation levels were significantly (P<0.05) higher, almost double the amount, after 4 hours compared to 30 minutes (see Section 3.2.2). To investigate whether this effect of SCF might be related to the up-regulation of COX isoforms, mast cells were treated with SCF for 4 hours or 20 hours. These treatments had no effect on the expression of either COX-1 or COX-2 [*Figures 4.8 and 4.9*]. We also investigated whether LPS was able to affect COX expression in mast cells since our own studies demonstrated that LPS is a strong inducer of COX-2 in lung macrophages [*Figure 4.11*]. However, our data show that LPS has no effect on COX expression in human lung mast cells [*Figure 4.9 A*].

We also investigated whether long-term activation of mast cell might up-regulate prostaglandin-D synthases (PGDS) expression. Mast cells expressed hematopoietic prostaglandin-D synthase (H-PGDS) but not lipocalin-type prostaglandin-D synthases (L-PGDS) [*Figure 4.10*]. Following activation with SCF for 4 hours or 20 hours, or LPS for 20 hours, there was no increase in expression of either of these synthases [*Figures 4.9A and 4.10*].

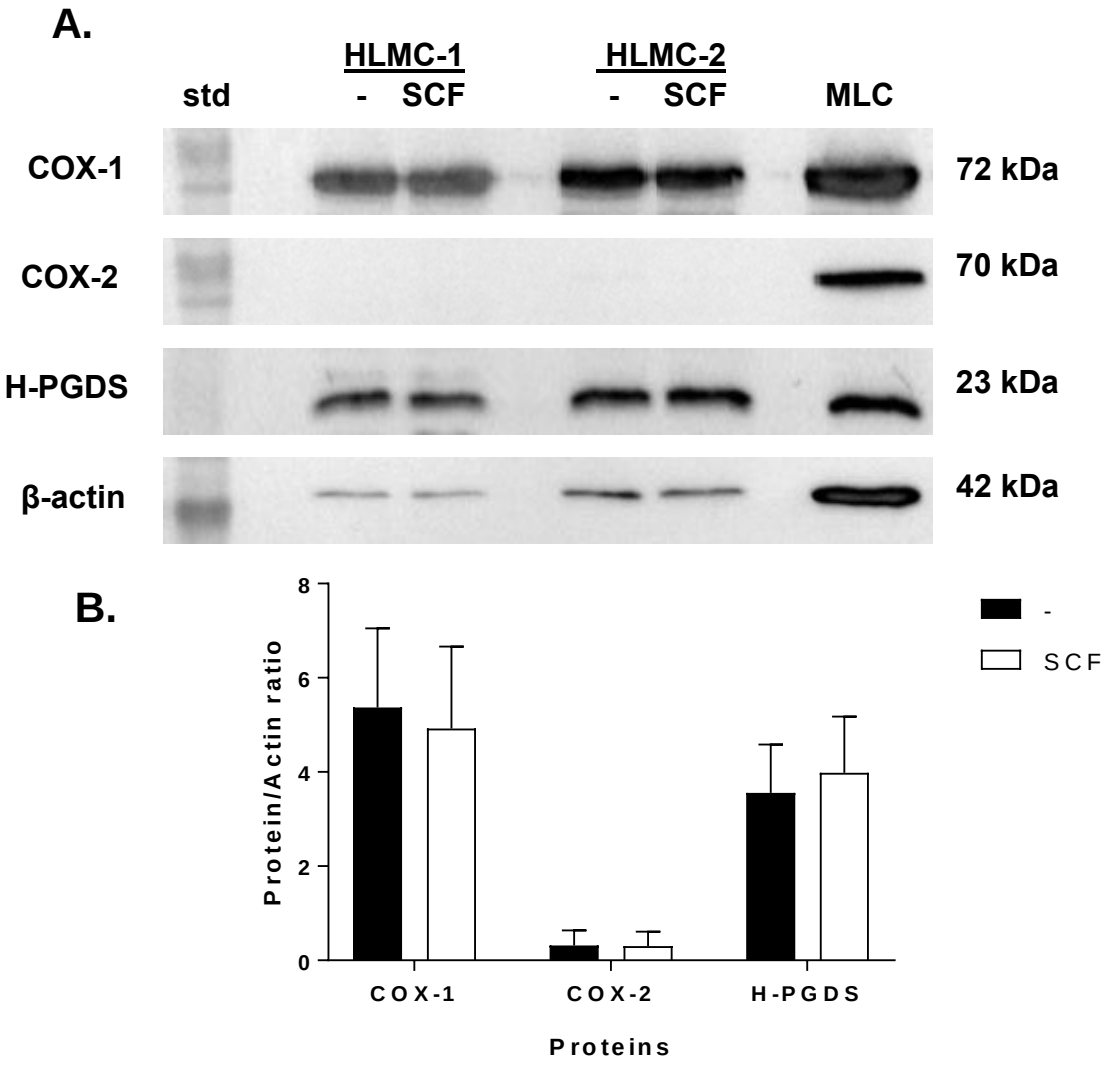


Figure 4.8 Effects of SCF on COX and H-PGDS expression. (**A**) Two mast cell preparations (HLMC-1 and HLMC-2) were incubated (4 hours) without (-) or with SCF (100 ng/ml) and expression of COX-1, COX-2, H-PGDS and β-actin evaluated. A mixed lung cell (MLC) preparation was also included. (**B**) COX-1, COX-2 and H-PGDS content in mast cells was determined by densitometry. Content is expressed relative to the same MLC preparation that was used as a control in all blots and normalised relative to cell actin. Mast cell purities were 81 and 90%, n=3; std stands for mw standards.

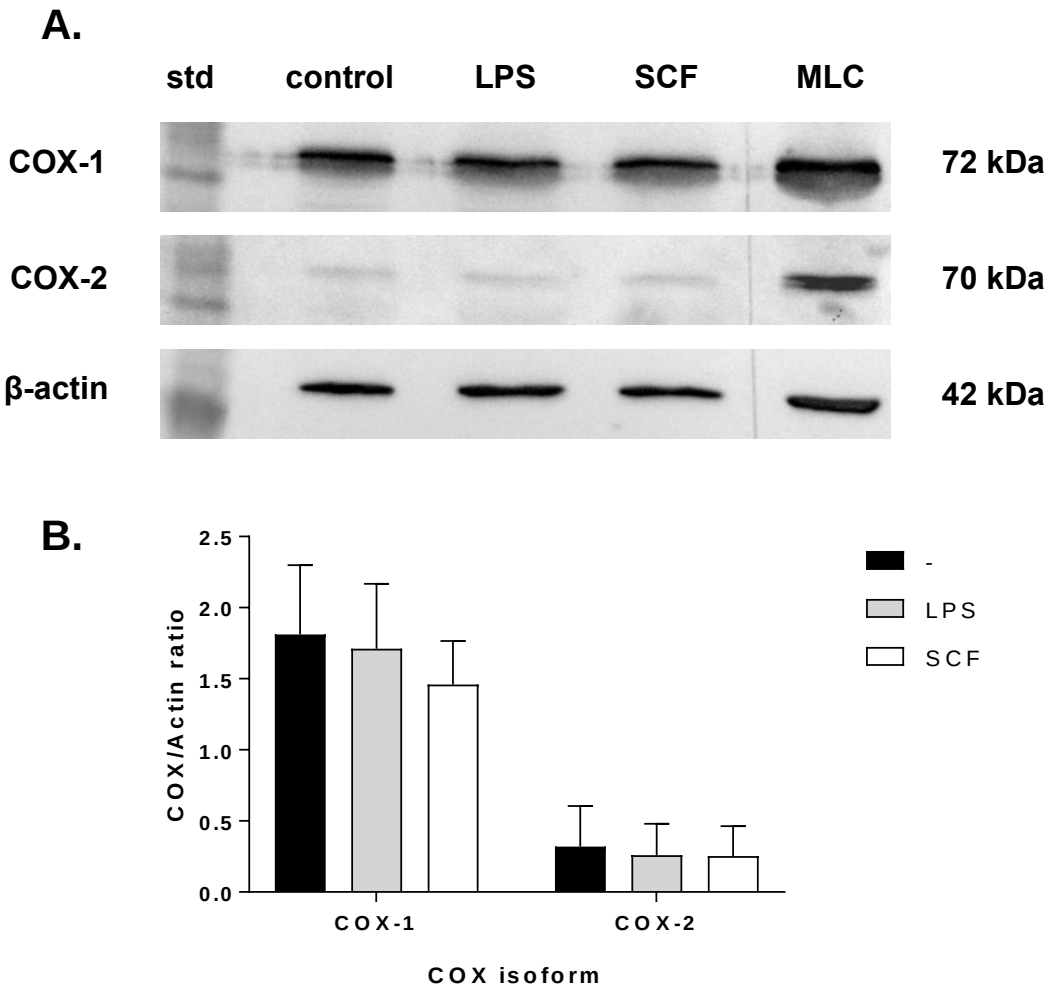


Figure 4.9 Effects of LPS and SCF on COX expression. (**A**) A single mast cell preparation was incubated (20 hours) in buffer alone (-), LPS (100 ng/ml) or SCF (100 ng/ml) and then expression of COX-1, COX-2 and β-actin was evaluated. A mixed lung cell (MLC) preparation was also included. (**B**) COX-1 and COX-2 content in mast cells was determined by densitometry. Content is expressed relative to the same MLC preparation that was used as a control in all blots and normalised relative to cell actin. Experiments were performed 3 more times. Mast cell purity was 87%; std stands for mw standards.

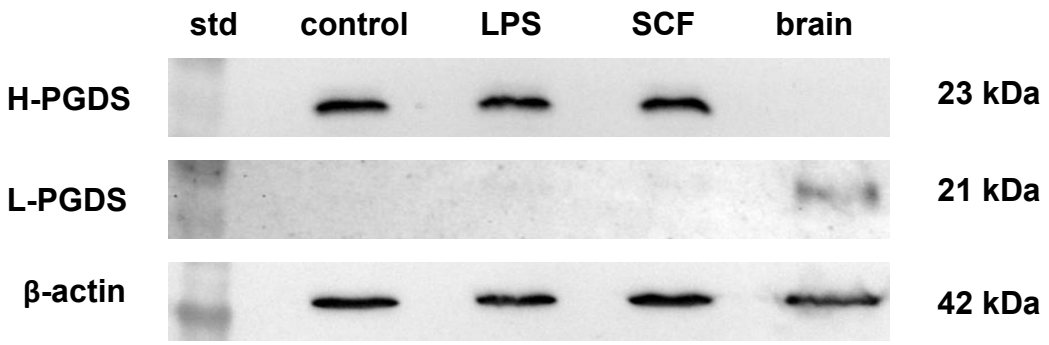


Figure 4.10 Effects of LPS and SCF on PGDS expression. A single mast cell preparation was incubated (20 hours) in buffer alone (-), LPS (100 ng/ml) or SCF (100 ng/ml) and then expression of H-PGDS, L-PGDS and β-actin was evaluated. A solubilized mouse brain preparation (20 µg) was also included as a control for L-PGDS. Experiments were performed 3 more times. Mast cell purity was 87%; std stands for mw standards.

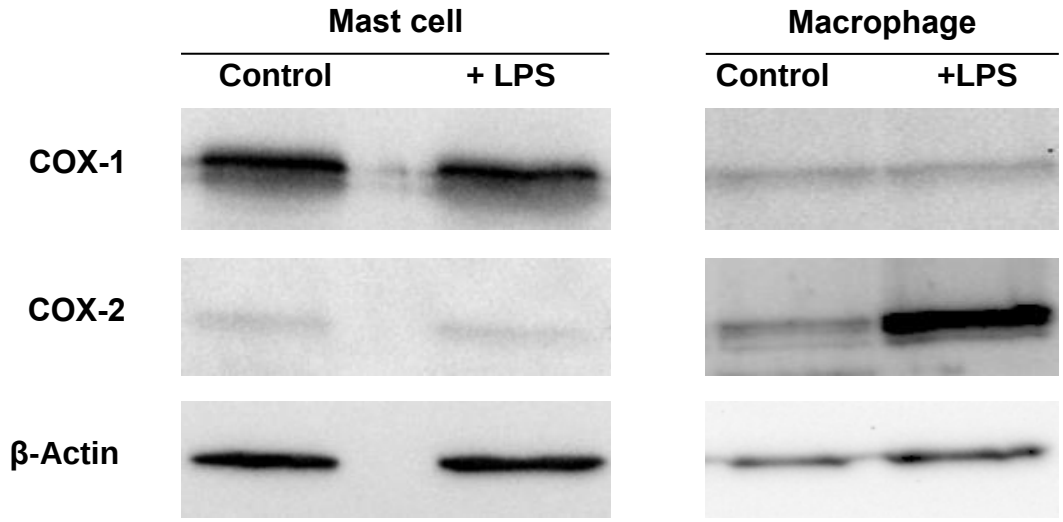


Figure 4.11 Induction of COX-2 by LPS in lung macrophages but not mast cells. Human lung mast cells and macrophages were incubated in buffer (control) or with LPS at 10 ng/ml (macrophages), or 100 ng/ml (mast cells) for 20 hours after which the cells were solubilised and expression of COX-1, COX-2 and β-actin evaluated. The purity of the mast cell and macrophage preparation was 80% and 90% respectively. The data are representative of 3-4 other experiments of similar design; std stands for mw standards.

4.3 Discussion

In this present study, we have assessed the effects of a variety of COX inhibitors and investigated COX expression in human lung mast cells. Our results demonstrated that COX-1 is the principal isoform responsible for PGD_2 generation in human lung mast cells.

Studies with COX-selective inhibitors were particularly informative. FR122047 abolished IgE-dependent PGD_2 generation; this provided strong evidence that COX-1 is responsible for the production of PGD_2 in human lung mast cells. FR122047 inhibits human recombinant COX-1 with an IC_{50} of ~30 nM and is about 2000 fold more potent against COX-1 than COX-2 (Ochi et al., 2000). The potency of FR122047 (EC_{50} ~25 nM) as an inhibitor of PGD_2 generation from human lung mast cells was very much in keeping with an effect at COX-1. By contrast, that celecoxib was relatively unable to inhibit PGD_2 argues against a role for COX-2. It should be noted that although celecoxib shows some modest selectivity for COX-2 over COX-1, it is possible that at higher concentrations, celecoxib may target COX-1 to some extent (Warner et al., 1999, Chan et al., 1999). Nonetheless, the effects of celecoxib overall were moderate. Collectively, these data suggest that IgE-dependent PGD_2 generation from human lung mast cells is driven by COX-1.

In further studies, we investigated the effects of COX inhibitors on SCF driven PGD_2 generation from mast cells. Elevated concentrations of the mast cell growth factor, SCF, have been observed in asthma (Al-Muhsen et al., 2004, Da Silva et al., 2006a, Kowalski et al., 2005) and our recent work has shown that SCF is unexpectedly effective at stimulating PGD_2 generation from mast

cells (Lewis et al., 2013). Previous studies have shown that mouse bone marrow derived mast cells express both COX-1 and COX-2; whereas IgE-dependent PGD_2 generation mobilises COX-1, cytokine driven PGD_2 generation activates COX-2 (Murakami et al., 1995). However, in the present study, SCF-induced PGD_2 generation was inhibited effectively by FR122047 whereas celecoxib demonstrated only modest inhibitory effects. Overall, these findings suggest that COX-1 is the primary isoform that mediates both IgE- and SCF-dependent PGD_2 generation. These findings strongly support clinical studies showing that COX-1 is likely to be the main driver of PGD_2 generation in asthma (Daham et al., 2011).

The effects of commonly used NSAIDs on PGD_2 production from human lung mast cells were instructive in as much as these compounds are largely considered non-selective, although data from a number of studies indicate some preference for either COX-1 or COX-2 (Cryer and Feldman, 1998, Mitchell et al., 1993, Warner et al., 1999, Chan et al., 1999). Selectivity for a given isoform may be dependent on the enzyme system studied. Taking the literature collectively, indomethacin and aspirin appear to be predominantly COX-1 selective, diclofenac is to some extent COX-2 selective, naproxen and ibuprofen overall display only fractional selectivity for one or other isoform. It is of interest that all the NSAIDs studied inhibited PGD_2 generation very effectively with the notable exception of aspirin, which was an unexpectedly weak inhibitor. The reasons for this are not really apparent. If an assumption is made that COX-1 is the primary isoform present in mast cells, then aspirin is at least as potent at COX-1 as some of the other NSAIDs studied such as ibuprofen, yet ibuprofen was effective at blocking PGD_2 generation. Why aspirin was relatively ineffective is yet to be elucidated. Potentially, there may be difficulties with aspirin accessing and/or inactivating mast cell COX.

Expression of COX isoforms was determined both by RT-PCR and by Western blotting. Strong mRNA expression was observed for both COX-1 and COX-2. However, at the protein level, the picture was rather different and it was evident that human lung mast cells strongly express COX-1 and only weakly express COX-2 in the majority of mast cell preparations. These findings are at odds with studies in murine systems as well as cultured human mast cells wherein both COX-1 and COX-2 are thought to co-exist (Murakami et al., 1995, Reddy and Herschman, 1997, Obata et al., 1999).

Based on previous studies in a mouse mast cell line (Reddy and Herschman, 1997), the possibility was considered that discrete COX isoforms might be mobilised in a time-dependent fashion. Mast cells were activated for time intervals of 30 minutes to 4 hours with either anti-IgE or SCF. It was of interest that the extent of PGD_2 generation induced by anti-IgE was the same after 30 minutes or 4 hours whereas when SCF was used as a stimulus, over twice as much PGD_2 was generated after 4 hours compared to 30 minutes [Section 3.2.2]. These data suggest that SCF can drive PGD_2 generation continuously over at least 4 hours.

We considered whether this continuous release of PGD_2 by SCF might be due to the induction of COX-2. However, our data showed that COX-2 levels did not change following longer-term activation of mast cells with SCF. In point of fact a well-recognised inducer of COX-2, LPS, was also investigated to determine whether this would induce COX-2 (Hempel et al., 1994). However, LPS failed to induce up-regulation of COX-2 in mast cells but readily did so in isolated human lung macrophages. The possibility that SCF might induce PGDS was also considered but, similarly, there was no indication that SCF induced

PGDS. Presumably, the nature of the SCF/c-KIT interaction transduces signals that permit continuous ongoing activation of the pathway leading to PGD_2 generation.

In the clinical context, these studies may have some bearing on mechanistic aspects related to aspirin-intolerant asthma. Aspirin-intolerant asthma has been shown to be accompanied by increases in cys-LTs and PGD_2 (O'Sullivan et al., 1996, Cahill et al., 2015). This association with PGD_2 has led to suggestions that aspirin directly activates mast cells by an unidentified mechanism (Steinke et al., 2014). While the possibility exists that mast cells in asthmatic lung or discrete subsets of mast cells found in particular areas of the lung may behave differently in response to aspirin, we were unable to demonstrate that aspirin can activate human lung mast cells directly.

An alternative possibility that has been put forward to explain aspirin-intolerant asthma is that COX inhibition by aspirin may reduce PGE_2. Whereas in general PGE_2 is known to have pro-inflammatory effects, paradoxically in the lung, PGE_2 is a bronchodilator and inhibits human lung mast cells (Kay et al., 2013, Buckley et al., 2011, Gauvreau et al., 1999, Hartert et al., 2000). The loss of an endogenous stabiliser of mast cells in PGE_2 could permit an increase in the release of mast cell derived mediators. If, as we have shown in the present study, aspirin was also ineffective at inhibiting mast cell COX, this might then be reflected by even greater levels of PGD_2 generation. Thus, an increased elaboration of PGD_2 by aspirin may reflect an inability to contain PGD_2 generation rather than direct activation of the mast cell.

In summary, we have shown that PGD_2 generation by mast cells is driven by COX-1. We have been unable to demonstrate any supporting role for COX-

2. While the possibility cannot be excluded that mast cells in different disease states may behave differently, our findings strongly support the notion that COX-1 is the principal isoform driving PGD_2 from human lung mast cells.

CHAPTER FIVE

5. CYTOKINE GENERATION BY HUMAN LUNG MAST CELLS

5.1 Introduction

Mast cells can be activated by IgE and non-IgE dependent mechanisms. However, there seems to be some so controversy in the literature as to whether mast cells can generate cytokines. Cross-linking of IgE by antigen leads to mast cell activation and the release of inflammatory mediators such as histamine and eicosanoids (Wajdner et al., 2017). Mast cells may also potentially be a source of multiple pro-inflammatory cytokines which can be either pre-stored and generated soon after cell activation or synthesised *de novo* (Gordon et al., 1990). Cytokines are not exclusively generated by IgE-dependent mechanisms and can also apparently be generated by IgE-independent mechanisms such as SCF/c-KIT activation (MacNeil et al., 2014).

The release of cytokines after mast cell activation may play an important role in IgE synthesis, leukocyte recruitment, chemotaxis, cell activation and survival, upregulation of adhesion molecules on endothelial cells which are all involved in defence against bacteria and parasites (Aggarwal and Pocsik, 1992). It has been suggested that cytokine generation may vary among mast cells dependent on the species and the site at which mast cells are found (Kobayashi et al., 2000).

The first evidence that mast cells can generate cytokines was demonstrated in the Abelson murine leukemia virus (A-MuLV)–transformed tumorigenic mouse mast cell lines, as they constitutively produce granulocyte–macrophage colony–stimulating factor (GM-CSF) and IL-4 mRNA and protein (Galli et al., 1991). Other studies suggested that murine primary mast cells or mast cell lines have the potential to generate different cytokines such as IL-1,

IL-5, IL-6, IL-8, TNF–α and many more (Bradding and Holgate, 1999). Murine studies demonstrated that mast cells do generate cytokines. However, this does not seem to be universally accepted as work by Gibbs et al. (1997) showed that human lung mast cells barely generate cytokines such as IL-4 and TNF-α.

We thought it would be a worthwhile endeavour to determine whether human lung mast cells can generate any cytokines when activated using a profiler array to provide an initial indication of cytokine generation followed by confirmatory studies using ELISAs. The aim of this chapter, therefore, was to establish whether human lung mast cells can generate any cytokines when challenged with a range of different stimuli.

5.2 Cytokine generation determined by proteomic profiler array

In addition to preformed and lipid-derived mediators, mast cells might potentially generate cytokines. To determine whether human lung mast cells can generate cytokines, mast cells (2.2 to 3.5 x 10^6 per ml per condition) were challenged with or without anti-IgE (2 µg/ml), SCF (100 ng/ml), anti-IgE and SCF together (same concentrations) or LPS (100 ng/ml) for 20 hours. After activation, the supernatants were collected and processed according to the manufacturer's instructions. A total of six mast cell preparations were studied and supernatants from these experiments harvested and stored. These six preparations were then randomly split and supernatants from three preparations were pooled to provide two pooled samples which were then assayed using the profiler array.

5.2.1 Array analysis of human lung mast cells challenged with anti-IgE

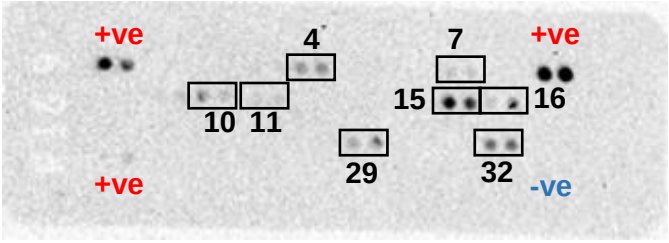
We investigated the effects of anti-IgE on cytokine generation by array and compared this to a corresponding control array (incubation in buffer only). The first pool showed some cytokines which were only spontaneously generated. These were, GM-CSF, IL-1β, MIF, sCAM-1, IL-6, IL-8 and Serpin E1. However, the only cytokines that were released over and above spontaneous levels by anti-IgE were GM-CSF, IL-1β, MIF, IL-1α, IL-1ra (receptor antagonist) and IL-5 [*Figure 5.1C and 5.1D*]. The second pool showed a slightly different pattern of expression to the first pool. Although the second pool spontaneously generated the same cytokines as the first pool, a few more cytokines were spontaneously expressed. These were, CXCL1, MIP-1α, IL-1α and IL-1ra. Some cytokines

were detected over and above spontaneous following anti-IgE challenge. These were of a similar profile of cytokine expression as the first pool (GM-CSF, IL-1 family, IL-5 and MIF) plus IL-16, MCP-1, MIP-1α, Serpin E and TNF-α [*Figure 5.2C and 5.2D*]. The differences between the pools show that there is some heterogeneity among mast cell preparations. The pattern of expression for spontaneous and challenged samples between both pools was slightly different. Although they might have expressed similar cytokines, the levels of expression for the second pool were higher than the first pool.

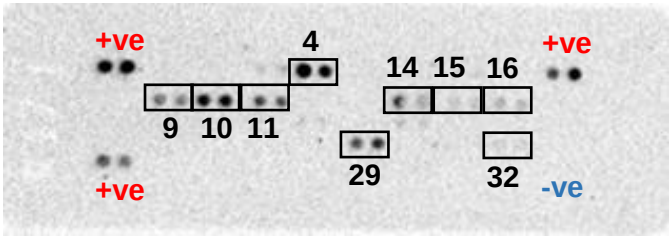
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (1st pool)



(**C**) Anti-IgE (1st pool)



(**D**)

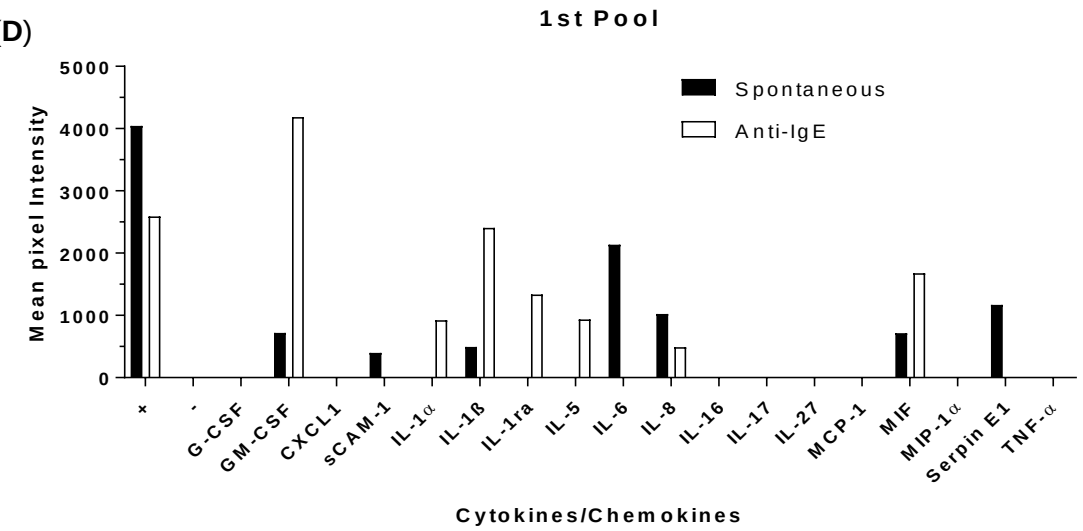
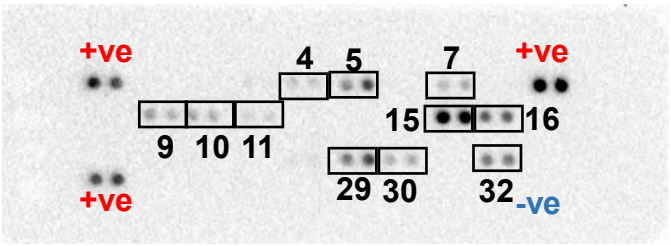


Figure 5.1. Proteome profiler human cytokine array analysis of anti-IgE challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) anti-IgE (2 µg/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the first pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

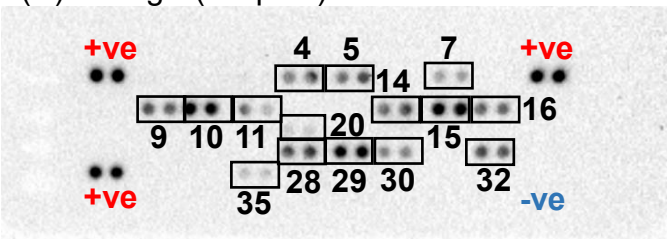
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (2nd pool)



(**C**) Anti-IgE (2nd pool)



(**D**)

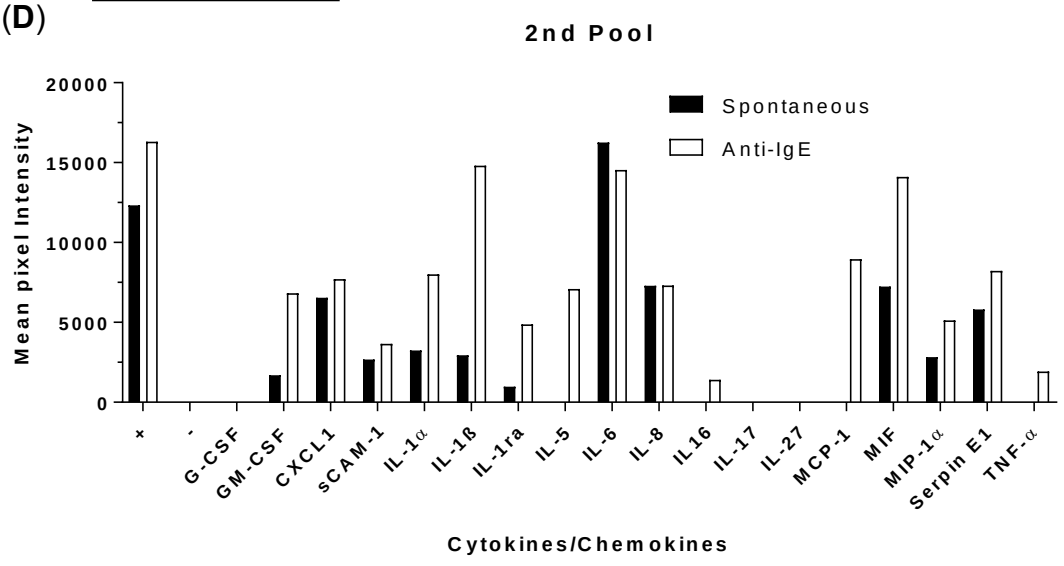


Figure 5.2. Proteome profiler human cytokine array analysis of anti-IgE challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) anti-IgE (2 µg/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the second pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

5.2.2 Array analysis of human lung mast cells challenged with SCF

We investigated the effects of SCF on cytokine generation by array and compared this to a corresponding control array (incubated in buffer only). The first pool showed a similar profile of spontaneous cytokine expression as seen before (GM-CSF, IL-1β, MIF, sCAM-1, IL-6, IL-8 and Serpin E1). However, cytokines that were released over and above spontaneous by SCF were GM-CSF, IL-1 family, MIF, G-CSF and TNF-α [*Figure 5.3C and 5.3D*]. The second pool showed a similar pattern of spontaneous cytokine expression to that seen before (GM-CSF, IL-1β, MIF, sCAM-1, IL-6, IL-8, Serpin E1 CXCL1, MIP-1α, IL-1α and IL-1ra). However, additional cytokines detected over and above spontaneous following SCF challenge were G-CSF, GM-CSF, IL-1 family, MCP-1, Serpin E1, MIF, TNF-α and IL-16 [*Figure 5.4C and 5.4D*]. SCF challenged samples showed a somewhat similar pattern of cytokine expression to anti-IgE challenged samples.

5.2.3 Array analysis of human lung mast cells challenged with both anti-IgE and SCF

We investigated the effects of anti-IgE and SCF on cytokine generation by array and compared this to a corresponding control array (incubation in buffer only). The first pool showed as indicated previously the following generation of cytokines spontaneously (GM-CSF, IL-1β, MIF, sCAM-1, IL-6, IL-8 and Serpin E1). The only cytokine that was generated over and above spontaneous by anti-IgE plus SCF was IL-5 [*Figure 5.5C and 5.5D*]. The second pool showed a similar pattern of spontaneous cytokine expression as seen before (GM-CSF,

IL-1β, MIF, sCAM-1, IL-6, IL-8, Serpin E1 CXCL1, MIP-1α, IL-1α and IL-1ra). Additional cytokines were detected over and above spontaneous levels following challenge with anti-IgE plus SCF. These were G-CSF, GM-CSF, IL-1 family, IL-5 and TNF-α [*Figure 5.6C and 5.6D*]. Although both pools spontaneously expressed similar cytokines, the first pool failed to express any cytokines after anti-IgE plus SCF stimulation.

5.2.4 Array analysis of human lung mast cells challenged with LPS

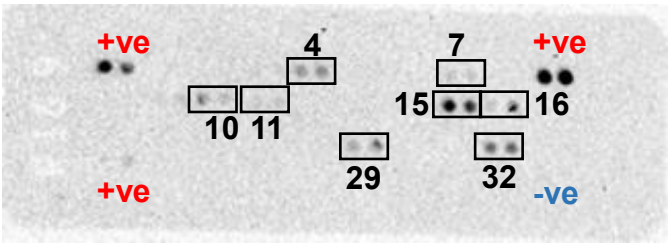
We investigated the effects of LPS on cytokine generation by array and compared this to a corresponding control array (incubation in buffer only). The first pool showed some cytokines which were only spontaneously generated. These were, G-CSF, sCAM-1, IL-1 family, IL-6, IL-8, IL-27, TNF-α, GM-CSF and IL-17 [*Figure 5.7C and 5.7D*]. However, none of these cytokines were released over and above spontaneous by LPS. The second pool showed a completely different pattern of expression to the first pool. Spontaneously, these generated the same cytokines (sCAM-1, IL-1 family, IL-6, IL-8, IL-27, GM-CSF and IL-17) plus CXCL1 which was exclusive to the second pool. However, additional cytokines were detected over and above spontaneous following challenge with LPS. These were G-CSF, CXCL1, sCAM-1, IL-1 family, IL-6, IL-8, GM-CSF, MIP-1α, MIF, MCP-1 and Serpin E1 [*Figure 5.8C and 5.8D*]. The second pool generated more cytokines after LPS challenge. These data confirm the heterogeneity between the pools.

Table 5.1 summarises all the profiler array data showing the presence and absence of the expression of cytokines and chemokines and their signal strengths following challenge with all the stimuli compared to the spontaneous release.

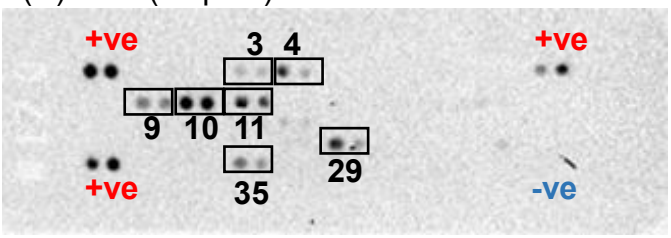
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (1st pool)



(**C**) SCF (1st pool)



(**D**)

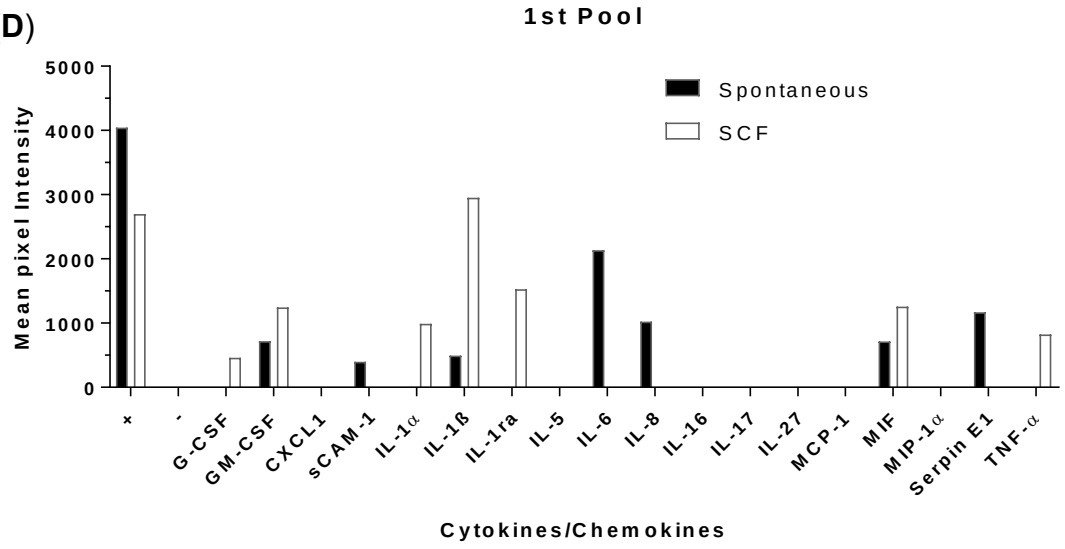
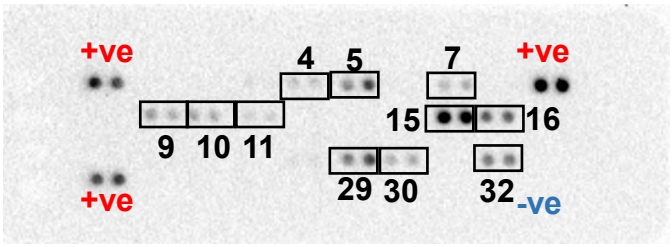


Figure 5.3. Proteome profiler human cytokine array analysis of SCF challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) SCF (100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the first pool of 3 different mast cell preparations of 90% purity or greater (+ve, positive control; -ve, negative control).

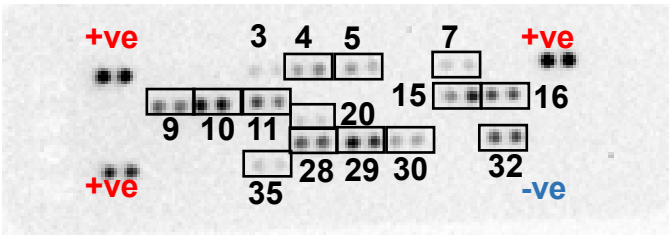
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (2nd pool)



(**C**) SCF (2nd pool)



(**D**)

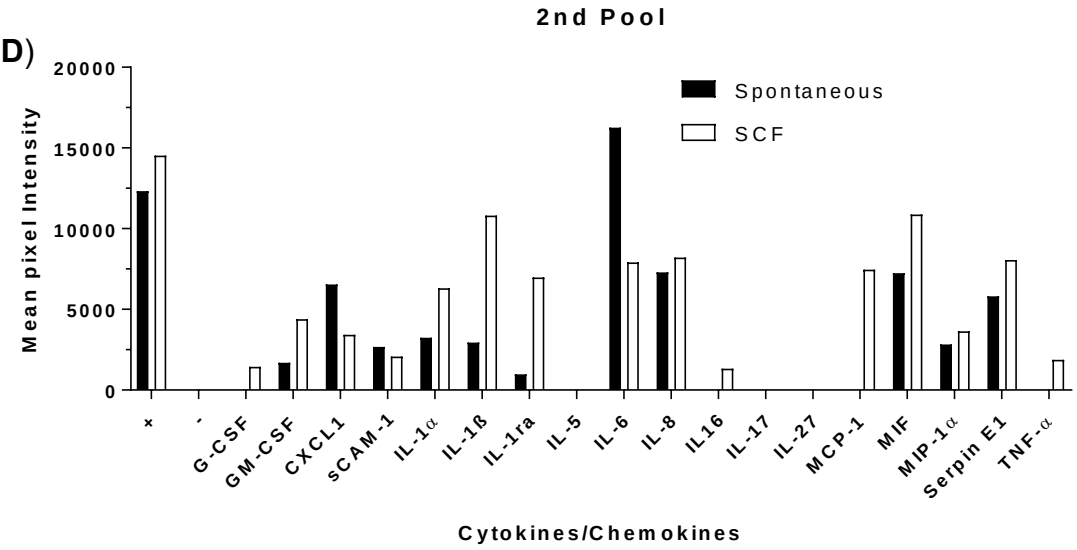
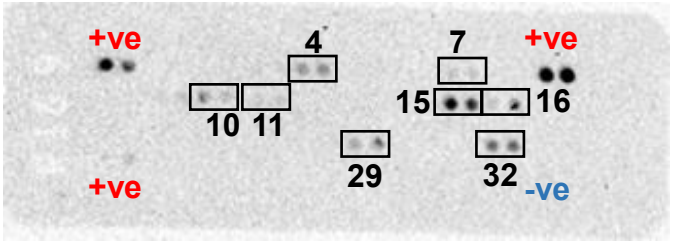


Figure 5.4. Proteome profiler human cytokine array analysis of SCF challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) SCF (100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the second pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

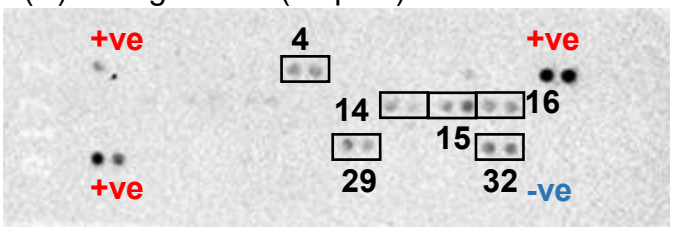
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (1st pool)



(**C**) Anti-IgE+SCF (1st pool)



(**D**)

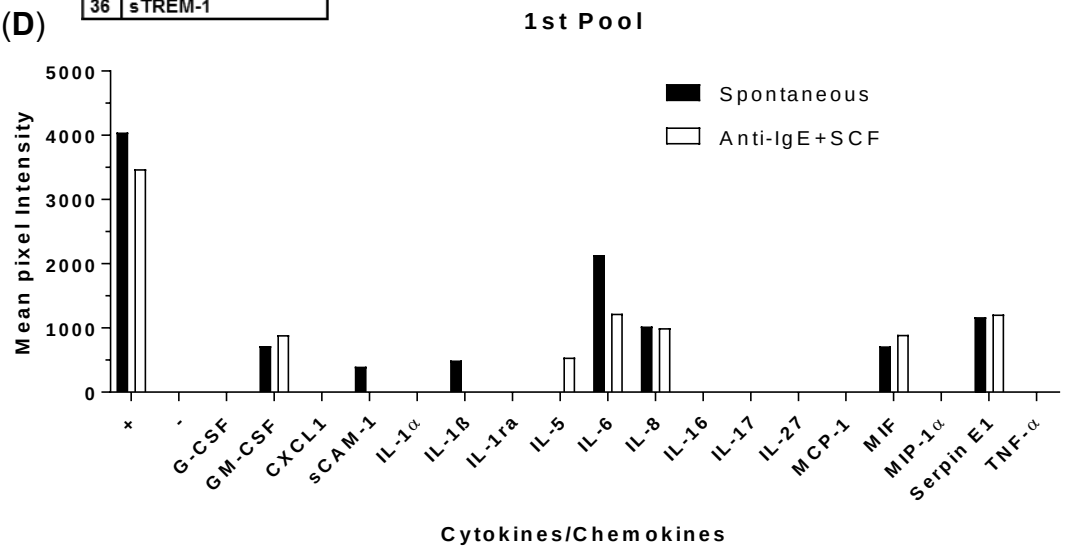
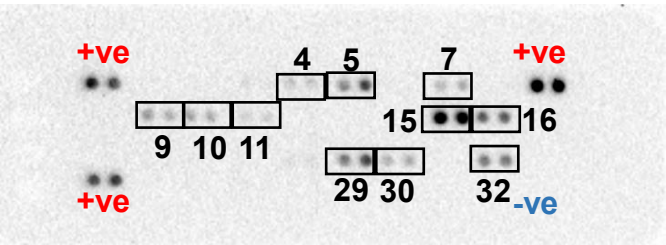


Figure 5.5. Proteome profiler human cytokine array analysis of anti-IgE and SCF challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) anti-IgE and SCF (2 µg/ml, 100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the first pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

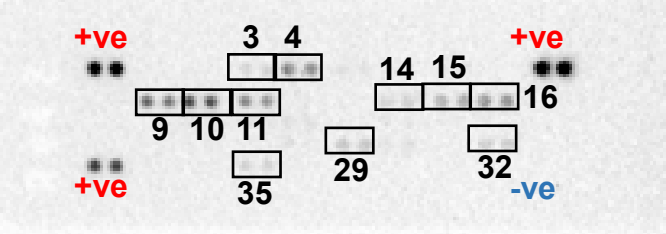
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (2nd pool)



(**C**) Anti-IgE+SCF (2nd pool)



(**D**)

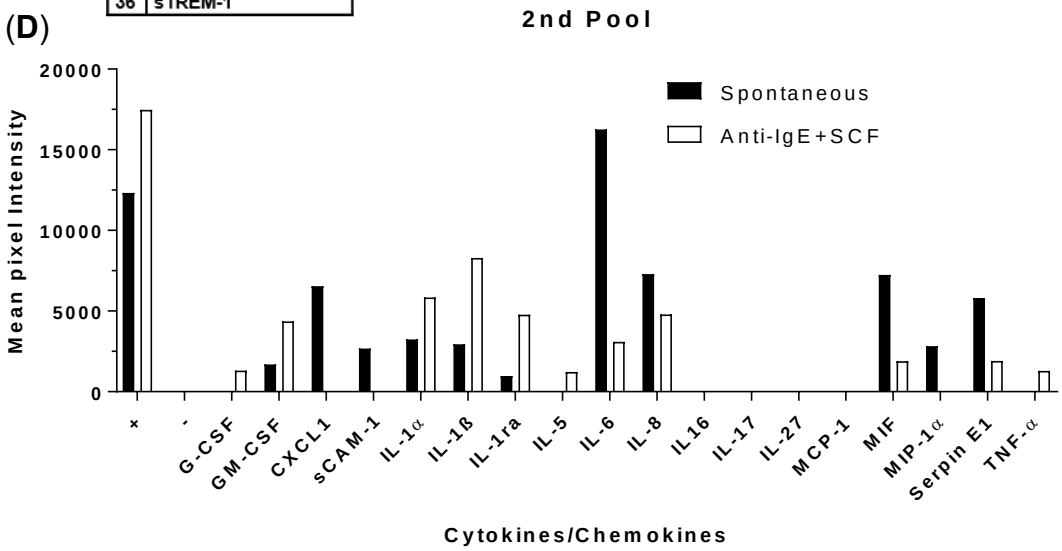
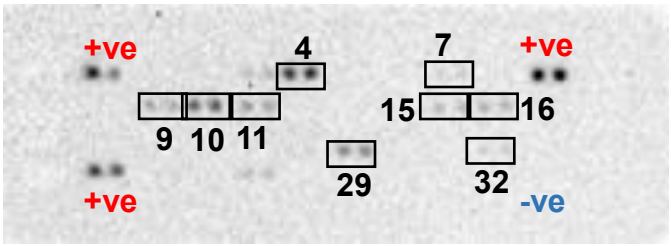


Figure 5.6. Proteome profiler human cytokine array analysis of anti-IgE and SCF challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) anti-IgE and SCF (2 µg/ml, 100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the second pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

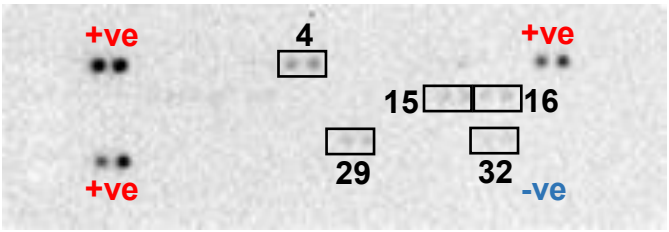
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (1st pool)



(**C**) LPS (1st pool)



(**D**)

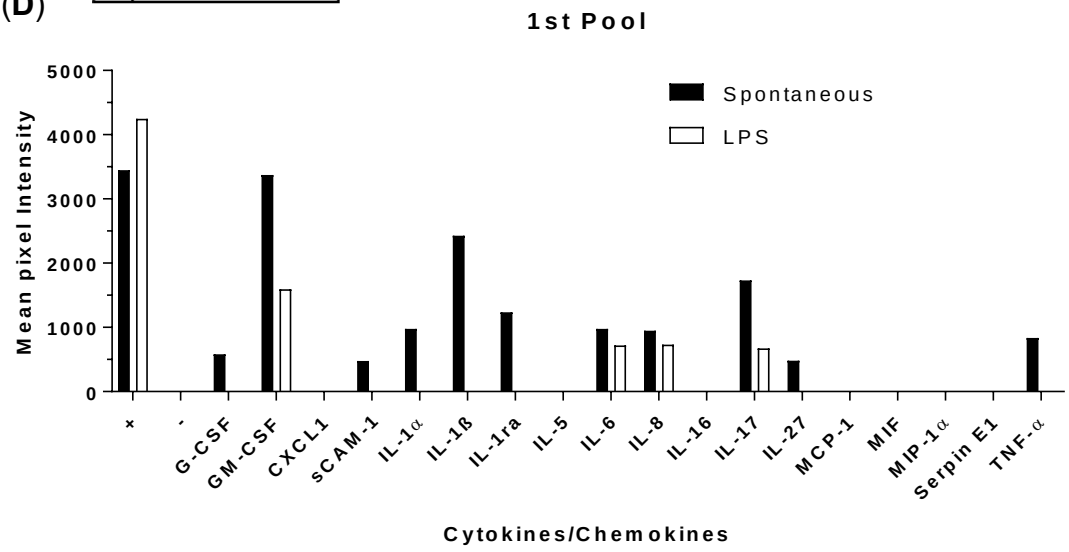
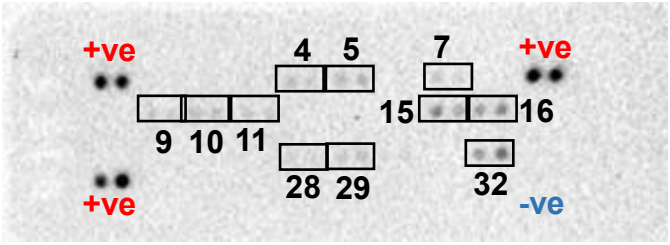


Figure 5.7. Proteome profiler human cytokine array analysis of LPS challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) LPS (100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the first pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

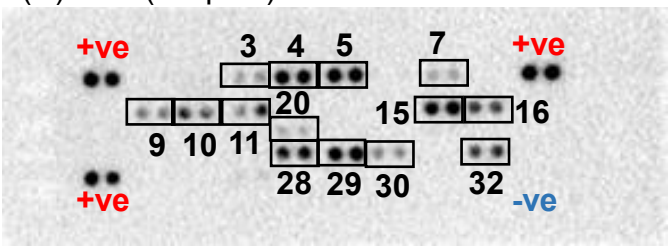
(**A**)

	Cytokine/Chemokine
1	C5/C5a
2	CD40 Ligand (CD154)
3	G-CSF
4	GM-CSF
5	CXCL1 (GROα)
6	CCL1 (I-309)
7	sICAM-1 (CD54)
8	IFN-γ
9	IL-1α
10	IL-1β
11	IL-1ra
12	IL-2
13	IL-4
14	IL-5
15	IL-6
16	IL-8
17	IL-10
18	IL-12p70
19	IL-13
20	IL-16
21	IL-17
22	IL-17E (IL-25)
23	IL-23
24	IL-27
25	IL-32α
26	CXCL10 (IP-10)
27	CXCL11 (I-TAC)
28	MCP-1 (CCL2)
29	MIF (GIF)
30	MIP-1α (CCL3)
31	MIP-1β (CCL4)
32	Serpin E1 (PAI-1)
33	RANTES (CCL5)
34	SDF-1 (CXCL12)
35	TNF-α
36	sTREM-1

(**B**) Spontaneous (2nd pool)



(**C**) LPS (2nd pool)



(**D**)

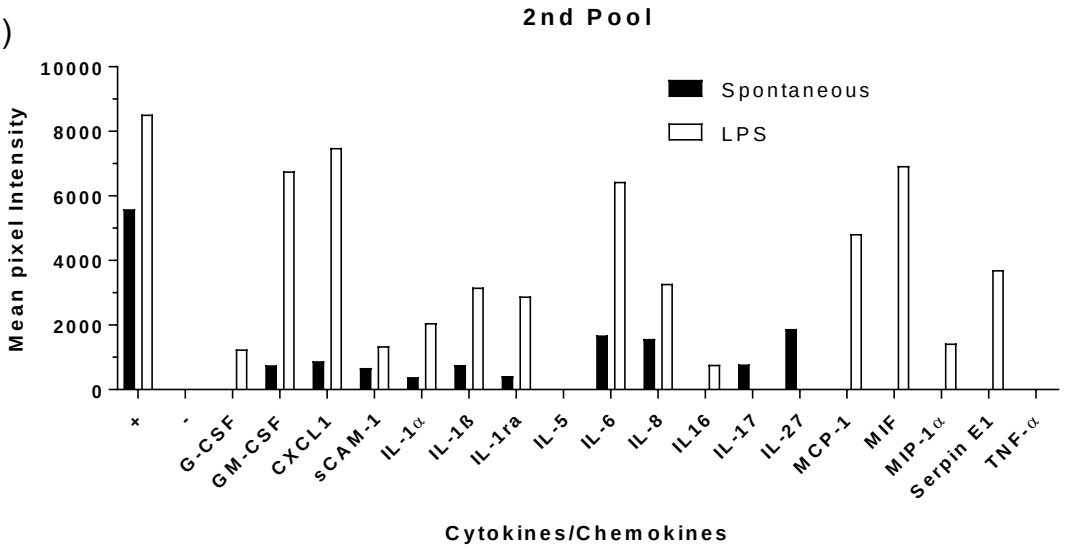


Figure 5.8. Proteome profiler human cytokine array analysis of LPS challenged mast cells. Potential expression of (**A**) 36 cytokines/chemokines was assessed in cell culture supernatants of human lung mast cells, incubated without [spontaneous] (**B**) or with (**C**) LPS (100 ng/ml) for 20 hours. (**D**) Quantification of the pixel intensity of the detected spots. Data are representative of the second pool of 3 different mast cell preparations of 90% purity or greater, (+ve, positive control; -ve, negative control).

		Anti-IgE		SCF		Anti-IgE + SCF		LPS	
#	Cytokine /Chemokine	Pool 1	Pool 2	Pool 1	Pool 2	Pool 1	Pool 2	Pool 1	Pool 2
1	G-CSF	-	-	+	+	-	+	-	+
2	GM-CSF	+++	++	+	+	+	++	-	+++
3	CXCL1	-	-	-	-	-	-	-	+++
4	sICAM-1	-	-	-	-	-	-	-	+
5	IL-1α	+	++	+	+	-	+	-	+
6	IL-1β	++	+++	+++	+++	-	++	-	++
7	IL-1ra	+	+	++	++	-	++	-	++
8	IL-5	+	++	-	-	+	+	-	+++
9	IL-6	-	-	-	-	-	-	-	++
10	IL-8	-	-	-	-	-	-	-	+
11	IL-16	-	+	-	-	-	-	-	+
12	MCP-1	-	++	-	++	-	-	-	++
13	MIF	+	++	+	+	+	-	-	+++
14	MIP-1α	-	+	-	-	-	-	-	+
15	Serpin E1	-	+	-	-	-	-	-	++
16	TNF-α	-	+	+	+	-	+	-	-

Table 5.1 Cytokine and chemokine generation from human lung mast cells stimulated with anti-IgE, SCF, anti-IgE + SCF or LPS. The expression of cytokines/chemokines was determined in 2 different mast cell pools. The symbol (-) indicates cytokine not present whereas the symbol (+) indicates the cytokine is present. The strength of the cytokine signal is indicated by the number of (+) presented.

5.3 Cytokine generation determined by ELISA

The six preparations used in the proteomic profiler array were subsequently tested for TNF-α, IL-1β, IL-6 and IL-8 by ELISA to determine the reliability of the profiler array data. The amount of TNF-α and IL-6 generated by LPS, SCF, anti-IgE and SCF + anti-IgE challenged cells, was about the same as the spontaneous generation of TNF-α (5834 ± 3672 pg/10^6 mast cells) and spontaneous generation of IL-6 (19138 ± 6884 pg/10^6 mast cells). [*Figure 5.9A and 5.9C*].

LPS and SCF had no effect on IL-1β generation. However, IL-1β generation was significantly (*P<0.05*) higher (6871 ± 3047 pg/10^6 mast cells) after anti-IgE stimulation than spontaneous (5390 ± 2279 pg/10^6 mast cells) yet significantly (*P<0.05*) lower (5257 ± 2151 pg/10^6 mast cells) than spontaneous after anti-IgE + SCF stimulation [*Figure 5.9B*].

The amount of IL-8 generated after anti-IgE, LPS and anti-IgE + SCF stimulation was significantly (*P<0.05*) higher than the spontaneous generation (5390 ± 2279 pg/10^6 mast cells) [*Figure 5.9D*]. However, SCF alone had no effect on IL-8 generation.

The histamine release of these samples was measured to confirm mast cell activation. The amount of histamine release after 20 hours challenge was modest using the 3 stimuli and only achieved significance when SCF + anti-IgE were used together. Histamine release with SCF + anti-IgE (1903 ± 430 ng of histamine per 10^6 mast cells) was significantly (*P<0.05*) higher than both the spontaneous (686 ± 99 ng of histamine per 10^6 mast cells) and compared to anti-IgE alone (1258 ± 251 ng of histamine per 10^6 mast cells) [*Figure 5.10*].

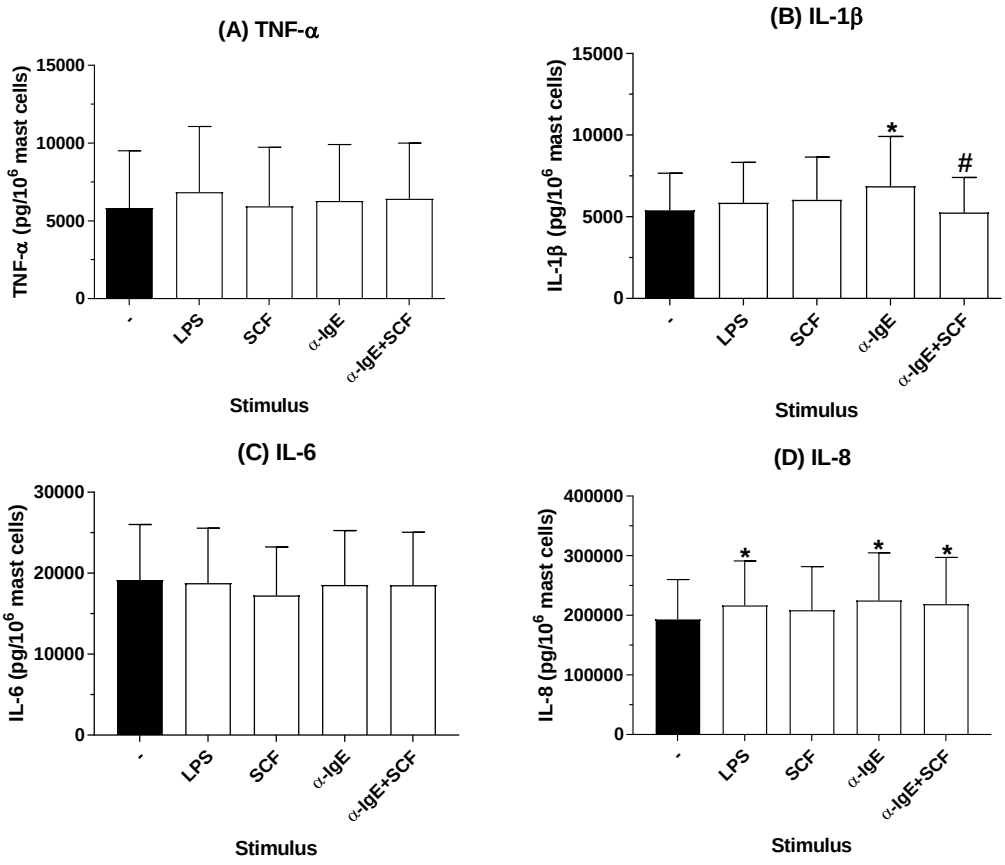


Figure 5.9 Effect of different stimuli on (**A**) TNF-α, (**B**) IL-1β, (**C**) IL-6 and (**D**) IL-8 generation by human lung mast cells. Cells were incubated without (-) or with LPS (100 ng/ml), SCF (100 ng/ml), anti-IgE (2 μg/ml) and anti-IgE + SCF at 37 ^{o}C for 20 hours to induce mast cell mediators before supernatants were collected for analysis of cytokines. Values are means ± SEM, n=6. Statistically significant difference compared to control is indicated: * *P<0.05*. # *P<0.05* compared to anti-IgE.

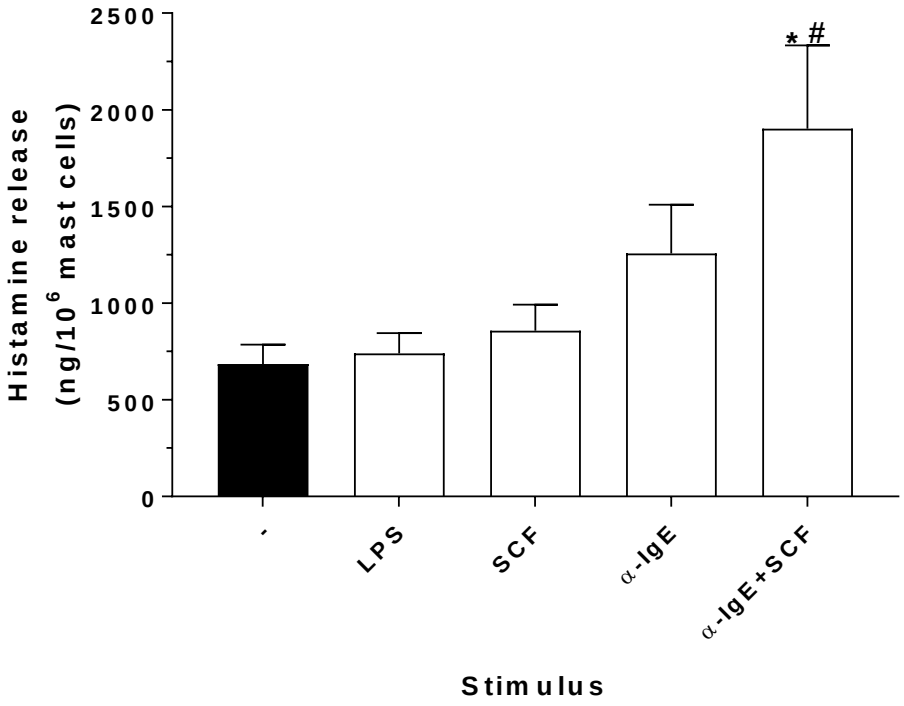


Figure 5.10 Effect of different stimuli on histamine release from human lung mast cells. Cells were incubated without (-) or with LPS (100 ng/ml), SCF (100 ng/ml), anti-IgE (2 µg/ml) and anti-IgE + SCF at 37 ^{o}C for 20 hours to induce mast cell mediators before supernatants were collected for histamine release analysis. Values are means ± SEM, n=6. Statistically significant difference compared to control is indicated: * *P<0.05*. # *P<0.05* compared to anti-IgE.

5.4 The effect of BzATP on cytokine generation by mast cells

In these experiments we were unable to demonstrate much cytokine release with the stimuli. However, in macrophages, it has been shown that BzATP can facilitate IL-1β generation after long-term (22 hours) LPS challenge (Ferrari et al., 1997, Ward et al., 2010). In this system BzATP is thought to act through purinergic (P2) receptors in order to generate cytokines. The interaction between BzATP and certain related P2 receptors is thought to initiate signalling through G_q and/or G_i family proteins which activate phospholipase C, inducing Ca^{2+} flux and inositol phosphate accumulation (Communi et al., 2000). For this reason, the effects of B_zATP on mast cells were investigated.

The amount of IL-1β generated by mast cells after B_zATP or B_zATP + LPS stimulation was significantly (*P<0.05*) higher compared to spontaneous IL-1β generation [*Figure 5.11A*]. Since LPS failed to generate IL-1β, this data shows that B_zATP alone can drive this IL-1β generation.

By contrast. the generation of TNF-α was significantly (*P<0.05*) lower than the spontaneous TNF-α generation after B_zATP or B_zATP + LPS stimulation [*Figure 5.11B*]. The amount of IL-6 and IL-8 generated was more or less the same for B_zATP, LPS and LPS + B_zATP challenged mast cells compared to the spontaneous IL-6 and spontaneous IL-8 generation [*Figure 5.11C and 5.11D*].

We also looked at the effect of anti-IgE or SCF with or without BzATP on IL-1β, TNF-α, IL-6 and IL-8 generation. The data show that neither anti-IgE nor

SCF had any effect on cytokine generation and that any observed increases in cytokines generated were due to BzATP alone *[Figure 5.12 and 5.13].*

Interestingly, our data also show that BzATP alone can drive histamine release from mast cells. The amount of histamine release after BzATP stimulation was significantly (*P<0.05*) higher than the spontaneous histamine release [*Figure 5.14*]. There was no indication that BzATP enhanced histamine release following long-term incubation with LPS, anti-IgE or SCF

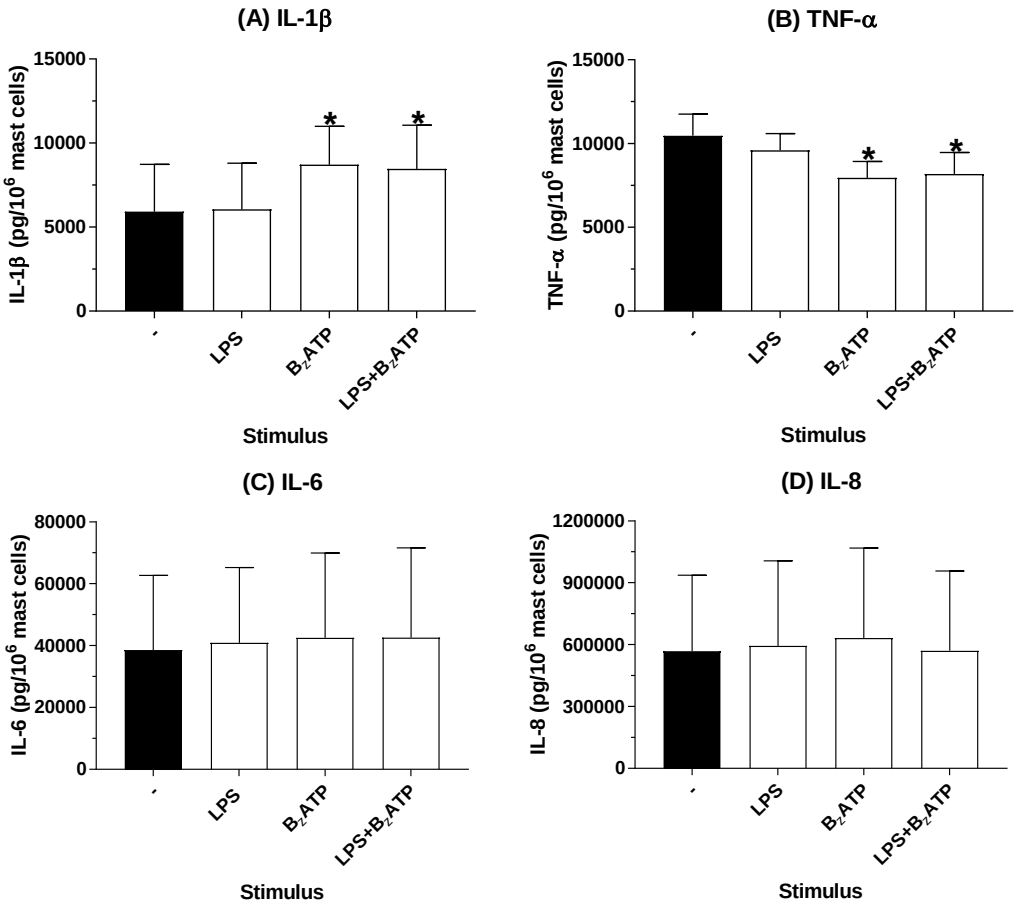


Figure 5.11 Effect of BzATP on LPS induced (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6 and (**D**) IL-8 generation by human lung mast cells. Cells were incubated without (-) or with LPS (100 ng/ml) at 37 ºC for 22 hours before challenge with BzATP (350 µM) for 30 min to induce mast cell mediators release and supernatants collected for cytokine generation analysis. Values are means ± SEM, n=3-4. Statistically significant difference compared to control is indicated: * *P<0.05*.

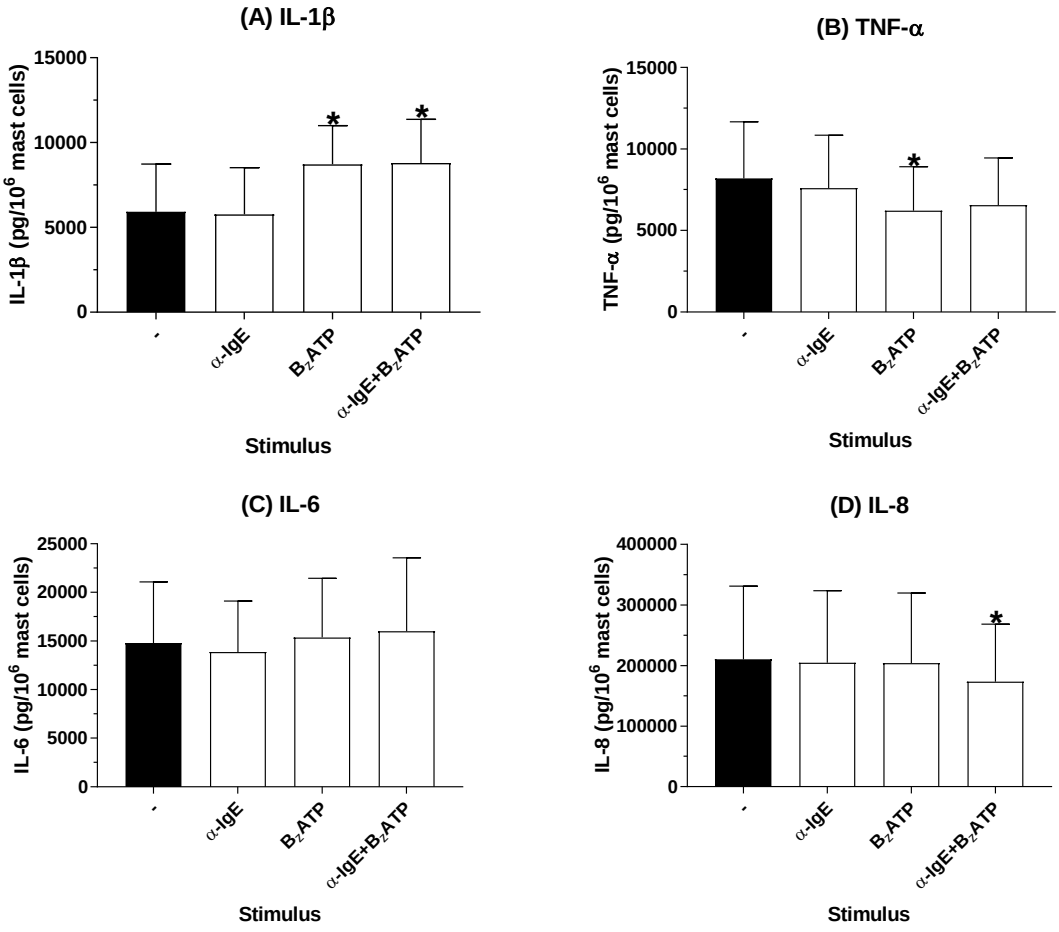


Figure 5.12 Effect of B_zATP on anti-IgE induced (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6 and (**D**) IL-8 generation by human lung mast cells. Cells were incubated without (-) or with anti-IgE (2 µg/ml) at 37 °C for 22 hours before challenge with B_zATP (350 µM) for 30 minutes to induce mast cell mediators and supernatants collected for cytokine generation analysis. Values are means ± SEM, n=3. Statistically significant difference compared to control is indicated: * *P<0.05*.

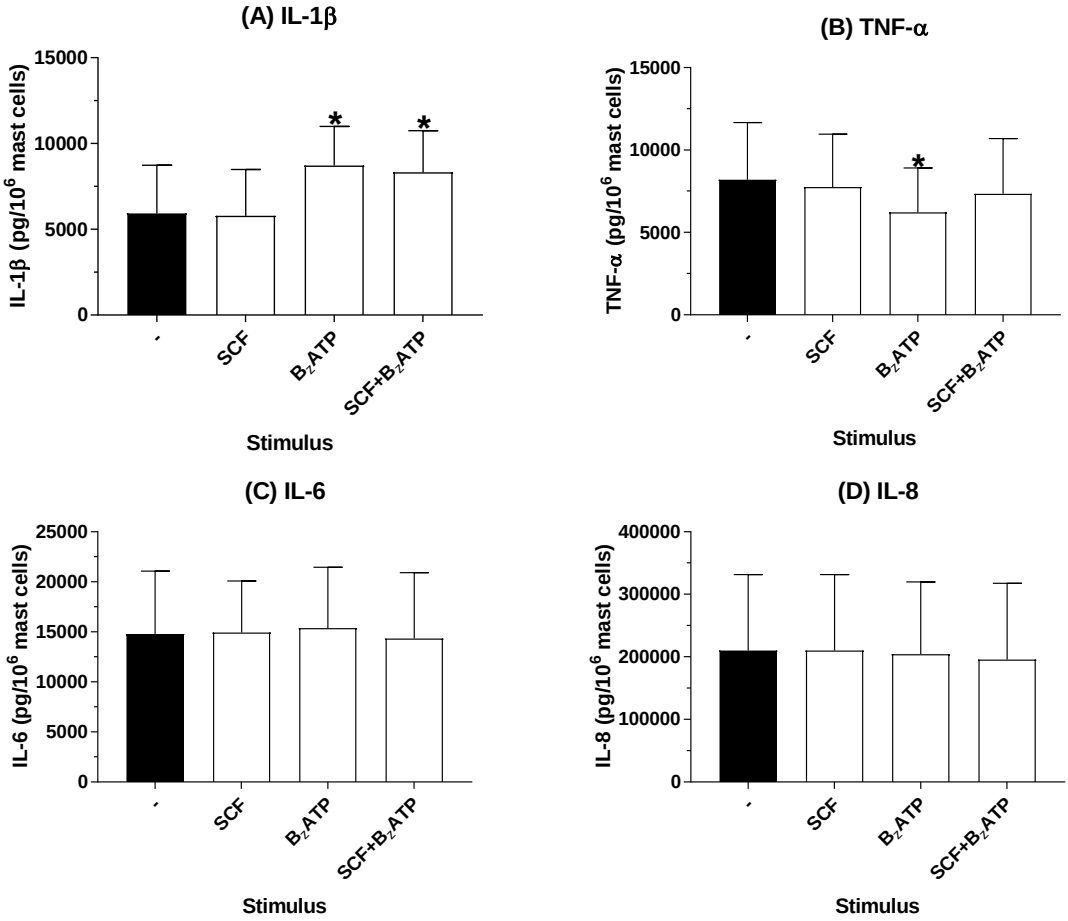


Figure 5.13 Effect of B_ZATP on SCF induced (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6 and (**D**) IL-8 generation by human lung mast cells. Cells were incubated without (-) or with SCF (100 ng/ml) at 37 ^oC for 22 hours before challenge with B_ZATP (350 μM) for 30 minutes to induce mast cell mediators and supernatants collected for cytokine generation analysis. Values are means ± SEM, n=3. Statistically significant difference compared to control is indicated: * *P<0.05*.

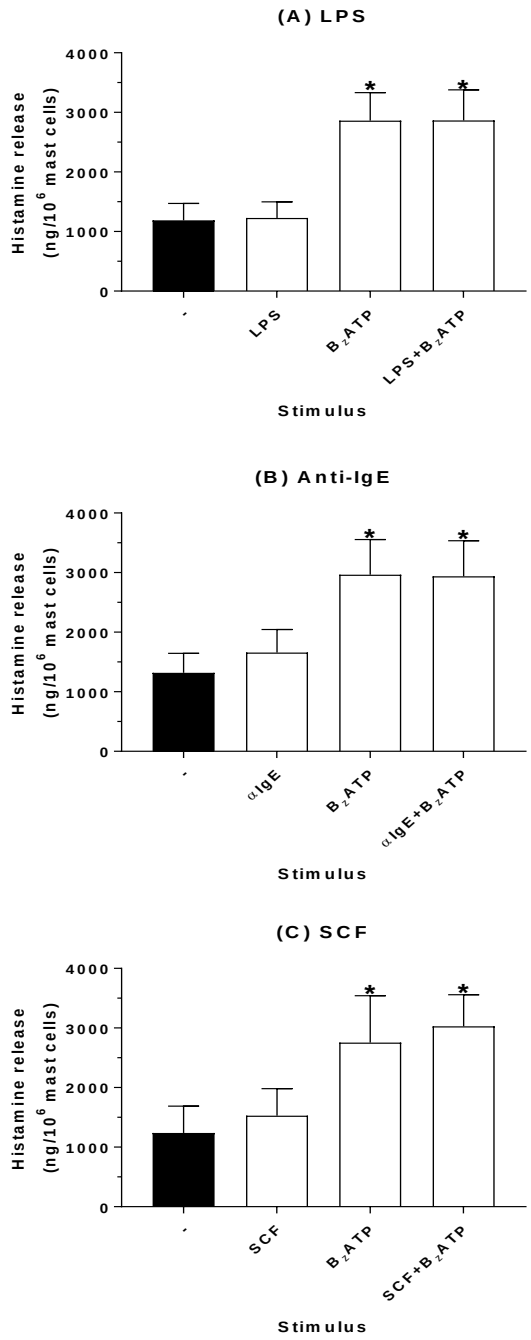


Figure 5.14 Effect of B_zATP on (**A**) LPS, (**B**) anti-IgE and (**C**) SCF induced histamine release by human lung mast cells. Cells were incubated without (-) or with LPS (100 ng/ml), anti-IgE (2 µg/ml), SCF (100 ng/ml) at 37 ^oC for 22 hours before challenge with B_zATP (350 µM) for 30 minutes to induce mast cell mediators and supernatants collected for cytokine generation analysis. Values are means ± SEM, n=3-5. Statistically significant difference compared to control is indicated: * $P<0.05$.

5.5 The effect on mast cells of long-term BzATP incubation

In the previous section (see Section 5.4) we have looked at a short incubation (30 minutes) with BzATP as a mechanism which could drive cytokine generation from mast cells. Given these data, we wondered what effect overnight (22 hours) activation with BzATP might have on mast cells.

Surprisingly, long-term (22 hours) incubation of mast cells with BzATP inhibited the levels of TNF-α, lL-6 and IL-8 while BzATP still stimulated IL-1β generation [*Figure 5.15*]. The extent of histamine release after BzATP challenge for 22 hours was significantly (*P<0.05*) higher than the spontaneous histamine release but no different to the amount of histamine released after 30 minutes challenge *[Figure 5.16].*

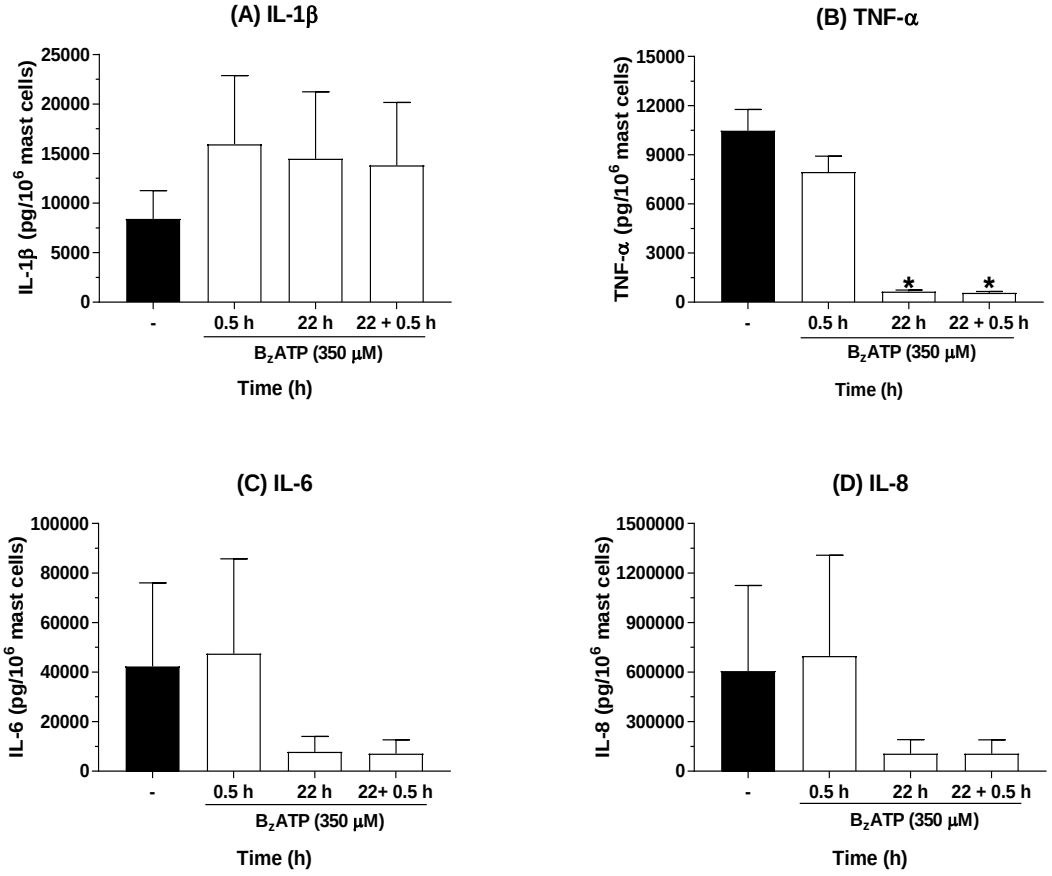


Figure 5.15 Effect of B_ZATP on (**A**) IL-1β, (**B**) TNF-α, (**C**) IL-6 and (**D**) IL-8 generation by human lung mast cells. Cells were incubated without (-) or with BzATP (350 μM) for 30 minutes and 22 hours at 37 ^oC for 22 hours before challenge again with B_ZATP (350 μM) for 30 minutes to induce mast cell mediators and supernatants collected for cytokine generation analysis. Values are means ± SEM, n=3. Statistically significant difference compared to control is indicated: * $P<0.05$.

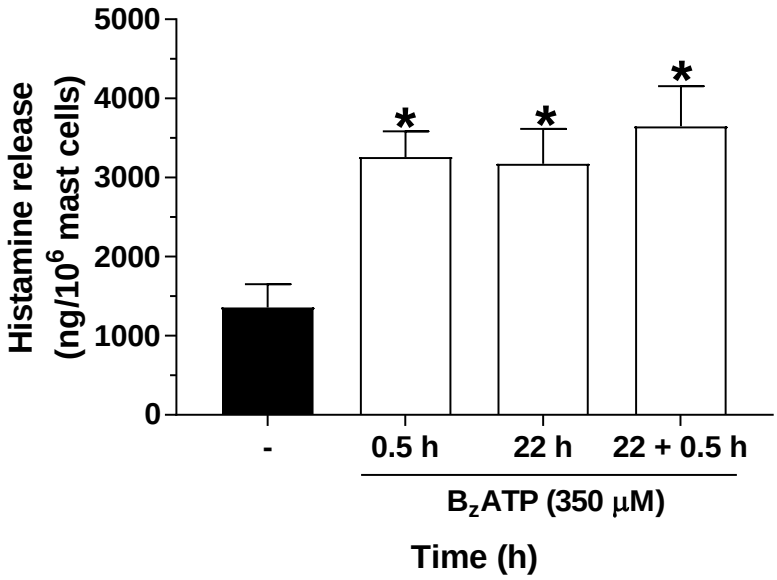


Figure 5.16 Effect of B_ZATP on histamine release by human lung mast cells. Cells were incubated without (-) or with B_ZATP (350 μM) for 30 minutes and 22 hours at 37 ^oC before challenge again with B_ZATP for 30 minutes after 22 hours to induce mast cell mediators and supernatants collected for histamine release analysis. Values are means ± SEM, n=4. Statistically significant difference compared to control is indicated: * P<0.05.

5.6 Discussion

The principal approach that we have selected to characterize the profile of cytokines in the supernatant samples of challenged mast cells is proteomic cytokine array. This array allows us to detect many cytokines simultaneously. It was the most suitable approach to screen for any changes in multiple biological factors which might be secreted by human lung mast cells. Six purified mast cell preparations were allocated randomly to two pools of three preparations and the two pools were then assessed separately by array.

The proteomic array data show that a few cytokines of those measured were generated spontaneously or after mast cell stimulation. When mast cells were stimulated with either anti-IgE (2 µg/ml) or SCF (100 ng/ml), both pools secreted similar cytokines but the second pool seems to have generated a wider selection of additional cytokines. In addition, when mast cells were challenged with both anti-IgE (2 µg/ml) and SCF (100 ng/ml), noticeably the second pool generated IL-1 family members (IL-1α, β and ra) and not the first pool. Interestingly, when mast cells were stimulated with LPS (100 ng/ml) neither pool showed a different pattern of expression. These data demonstrate that there is a variation between the cytokine expressions of the first and second pool suggesting that mast cells are heterogeneous in cytokine generation. The relative changes in the expression of the cytokine profiler array might be a good initial test but to be sure a quantitative test such as ELISA needs to be performed to confirm these preliminary results.

Based on the profiler array data of the cytokines detected from mast cells, IL-1 (most importantly IL-1 β), Il-6, IL-8 and to some extent TNF-α were identified. The profiler array also highlighted others cytokines such as GM-CSF,

IL-4 and IL-13, IL-5, MCP-1 and MIF which are important for mast cell proliferation, immunoglobulin secretion, eosinophil activation, chemotaxis and innate immunity regulation.

In parallel to the array data, ELISAs were performed using the same supernatant samples of the proteomic cytokine array to confirm the expression of IL-1 β, IL-6, IL-8 and TNF-α. Walsh et al. (1991) illustrated that dermal human mast cells are a principal source of TNF-α which can be released *in vitro* and *in vivo* after mast cell stimulation. Another study by Grabbe et al. (1994) demonstrated that mast cells cultured from peripheral blood monocytic cells (PBMC) and the human mast cell line (HMC-1) spontaneously generates decent levels of TNF-α and IL-1 β after 24 hours. However, these levels were way lower than the spontaneous generation of TNF-α and IL-1 β from human lung mast cells observed in the present study. The data imply that human lung mast cells might pre-store substantial amounts of pro-inflammatory cytokines (IL-1 β, IL-6, IL-8 and TNF-α) which are readily secreted spontaneously.

While our data show that human lung mast cell might have the potential to pre-store substantial amounts of cytokines, no significant TNF-α or IL-6 generation was observed when mast cells were challenged with LPS, SCF, anti-IgE and SCF + anti-IgE. The observation of high spontaneous generation of cytokines from mast cells is supported by alternative data from our lab. Spontaneous histamine release has been followed for up to 24 hours and interestingly while levels released are low (<6%) up to about 4 hours, there is a progressive increase in spontaneous histamine release with time with as much as ~ 35% released after 24 hours without the addition of any stimulus. This study needs further investigation in order to confirm that human lung mast cells have

the capacity to store these cytokines and simultaneously generate them without stimulation.

Gordon and Galli (1990) demonstrated that freshly purified mouse peritoneal mast cells spontaneously generate twice as much TNF-α as mouse peritoneal macrophages when stimulated with LPS. The same authors also illustrated that mouse peritoneal mast cells can contains up to 100-fold more TNF-α than various *in vitro*– derived mouse mast cells. These findings suggest that spontaneous generation of TNF-α and other cytokines may be a general property of certain types of mast cells. However, we cannot assume that human lung mast cells could pre-store the same amount of TNF-α or other cytokines as mouse mast cells. These data suggest that HLMCs have the potential to secrete cytokines spontaneously.

LPS is a classical activator of macrophages. The interaction between bacterial endotoxin (LPS) and the Toll-like receptor 4 leads to cell activation and the release of several pro-inflammatory cytokines such as IL-1 β, IL-6, IL-8 and TNF-α. TNF-α in particular plays a protective role in host defence against bacterial infection (Okayama et al., 2004). The question is whether human mast cells express TLR4 or not. Human cord blood-derived mast cells do not express TLR4 but TLR1, TLR2 and TLR6 (McCurdy et al., 2003). However, Okayama et al (2004) illustrated that human peripheral blood-derived cultured mast cells express functional TLR4 when exposed to IFN-γ. Kulka and Metcalfe (2006) demonstrated that HLMCs strongly express mRNA for TLR2 and TLR3 besides TLR4, TLR7 and TLR10. Our data show that LPS failed to generate any of the selected cytokines from human lung mast cells over and above spontaneous generation with the possible exception of IL-8.

Following on from this point, mast cells generated a significant (*P<0.05*) amount of IL-8 when challenged with all stimuli except for SCF. Also the amount of IL-1β generated when challenged with anti-IgE was significantly (*P<0.05*) higher than spontaneous. However, when mast cells were stimulated with both anti-IgE and SCF, SCF failed to enhance IgE-dependent mediator release and if anything reduced the amount of cytokine generated by anti-IgE alone. This observation is hard to reconcile but it could be due to the fact that both stimuli have different mechanisms of action and they might have interacted negatively with each other. This was unexpected since previous data have shown that SCF generally enhances the responses of mast cells, such as histamine release, to anti-IgE. None of the stimuli tested caused any effect on TNF-α and IL-6 generation.

Many monocyte and macrophage studies have demonstrated that ATP induces the release of IL1-β after LPS stimulation (Wilson et al., 2004, Ferrari et al., 1997, Ward et al., 2010). LPS may act to synthesise the cytokine, whereas ATP is needed to induce release. Therefore, in this part of the study it was investigated whether a similar process might happen in mast cells. Rather than enhancing cytokine generation induced by LPS or other stimuli such as anti-IgE and SCF, it was found that 30 minutes stimulation with BzATP alone initiated IL-1β generation. This effect of BzATP did not translate to the secretion of alternative cytokines, TNF-α, IL-6 and IL-8. This was an unexpected result and indicated that ATP alone could independently drive IL1-β generation selectively from HLMCs.

By contrast, long-term incubation (20 hours), as opposed to short-term (30 minutes) incubation, of mast cells led to the *inhibition* of TNF-α, IL-6 and IL-

8 generation but not IL-1β. As reported by Duhant et al. (2002), polyclonal stimulation of human T lymphocytes by BzATP occurs through P2 receptor which stimulates cAMP accumulation. This stimulation induces the inducible cAMP early repressor (ICER) expression in human lymphocytes. ICER blocks the binding between transcription factors (NF-AT and AP-1) and their targeted promoter sites which suppress the expression of and other cytokines (Bodor and Habener, 1998). It is possible that a similar mechanism might explain the inhibitory effects of BzATP in mast cells.

HLMCs do express functional P2 receptors such as P2X1, P2X4 and P2X7 (Wareham et al., 2009). In this study, Wareham et al. (2009) demonstrated that BzATP is an agonist which interacts with the functional P2X7 receptor in HLMCs and LAD2 cells. ATP and the related P2 receptor initiates signalling through G_q and/or G_i family proteins which activates phospholipase C, inducing Ca^{2+} flux and inositol phosphate accumulation (Communi et al., 2000). This is the process that is generally believed to lead to the secretion of histamine and other cytokines such as IL-1β from HLMCs.

In summary, although the current study is based on a small sample of participants, the findings suggest that human lung mast cell have the potential to store and generate cytokines. Other researchers have reported that different mast cell phenotypes express the same or different kind of cytokines and chemokines. Overall, the expression data observed with the profiler array do not exactly confirm the ELISA. The reasons for this discrepancy are not immediately apparent.

The ELISA data indicate that mast cells did not generate cytokines particularly in response to different stimuli over and above the spontaneous

cytokine generation. One unanticipated finding was the high spontaneous release of cytokines. This suggests that HLMCs might have the potential to pre-store enormous amounts of cytokines and secrete these spontaneously. If similar processes were operative *in vivo*, then this spontaneous generation of cytokines is liable to contribute to inflammatory responses in the lung. However, the levels of cytokines generated were very much higher than the anticipated. This could be due to an artefact, anomaly with the kit, some procedural difficulties we are unaware of or an experimental design issue. There was insufficient time for further investigations. In these experiments, we have used the same supernatants assessed by proteomic array in ELISA determinations. It is possible that experiments should have been repeated using fewer mast cells per condition for analysis of cytokines by ELISA.

Finally, this study surprisingly highlighted a dual effect of BzATP on HLMCs. BzATP induced significant ($P<0.05$) generation of IL-1β and histamine release after a short incubation (30 minutes) and significant ($P<0.05$) inhibition of TNF-α after long-term incubation (22 hours). Further studies could be profitably directed at identifying the receptors involved in mediating this dual effect of BzATP.

CHAPTER SIX

6. GENERAL DISCUSSION

Mast cells may play an important role in the lung in the context of host defence. Situated at sites where airborne pathogens might first gain entry into the body, mast cells may act as sentinels. However, inappropriate activation of mast cells, principally in an allergic context, can lead to the release of mediators that can cause inflammation, induce bronchoconstriction and initiate airway remodelling (Hofmann and Abraham, 2009). A greater understanding of the mechanism of activation and the phenotype involved in these events may help to clarify how mast cells might participate in processes involved in respiratory diseases.

The classical mechanism of activation of mast cells in an allergic context involves IgE-dependent processes. However, there might be other ways in which the cell can be activated. One possibility could involve SCF which is recognised as an important regulator of mast cell proliferation and function. Although a broad spectrum of knowledge exists concerning the response of murine mast cells to SCF, very little is known about how human lung mast cells respond. Whether murine mast cells or indeed mast cell lines are appropriate models reflecting the responses of primary human mast cells is questionable. This is because of the functional heterogeneity that is known to exist among mast cells derived from different sites and from different species (Kumar and Sharma, 2010). It follows that data generated from the present study involving primary human lung mast cells are far more likely to reflect relevant responses in the lung and how these responses might impact on respiratory diseases.

Most studies in murine systems have illustrated that SCF works in a way that might enhance mast cell proliferation and development (Sette et al., 2000, Yoshida et al., 2001). Moreover, SCF was found to enhance IgE-mediated secretion in murine mast cells (Theoharides et al., 2012). While similar

properties might apply to the responses of human lung mast cells to SCF, the most striking finding from the present study was that SCF can *directly* activate mast cells to release mediators.

In this study, we confirmed that SCF is working through the c-KIT receptor. This was determined by evaluating the effects of putative c-KIT inhibitors, imatinib, dasatinib and nilotinib on the responses of SCF. Our data, indicated that imatinib was probably the only *selective* c-KIT inhibitor as it only worked against SCF whereas dasatinib and nilotinib were likely to be less selective as they were effective against both SCF and anti-IgE. This suggests that dasatinib and nilotinib might act to inhibit other tyrosine kinases in addition to c-KIT.

These c-KIT inhibitors are used clinically for conditions such as chronic myeloid leukaemia and mastocytosis in which unregulated tyrosine activity is recognised as a driving force for disease development (Jensen et al., 2008, Buchdunger et al., 2002, Breccia et al., 2013, von Mehren, 2006). However, more recently, imatinib has been recognised as an effective therapeutically in the context of severe asthma. Imatinib was found to inhibit mast cell counts, tryptase release and reduce airway hyper-responsiveness in patients with severe asthma (Cahill et al., 2017). This study is in keeping with our own work and suggests that c-KIT can potentially serve as a therapeutic target for asthma.

Our data demonstrated that SCF is effective at directly activating human lung mast cells. While the effects of SCF on histamine and cys-LTs generation were variable amongst mast cell preparations what was really striking was the capacity of SCF to generate PGD_2. While anti-IgE was, overall, more effective

than SCF at generating histamine and cys-LTs, SCF and anti-IgE were equally efficacious at generating PGD_2. Even in situations where SCF caused very little histamine release it could still induce the generation of high levels of PGD_2. This could be important in the context of asthma and other respiratory diseases.

That SCF was very effective at causing PGD_2 generation from mast cells was a surprising finding. Compared to other mediators such as histamine and cys-LTs, which are potent bronchoconstrictors (Felix et al., 1991, Kondeti et al., 2013), PGD_2 is thought to be more important at causing inflammation (Ricciotti and FitzGerald, 2011). PGD_2 works principally through the prostanoid receptors, DP1 and DP2 (Hawcroft et al., 2004). The interaction between PGD_2 and DP1 increases intracellular cAMP and has been shown to have a chemotactic effect on inflammatory cells. Conversely, the interaction between PGD_2 and DP2 has the opposite effect, suppression of adenylate cyclase and reduction in intracellular cAMP levels which can contribute to bronchoconstriction in the airway (Kupczyk and Kuna, 2017, Monneret et al., 2001). It is possible therefore that SCF-induced generation of PGD_2 from human lung mast cells could be a mechanism contributing to both inflammation and bronchoconstriction in respiratory diseases such as asthma.

The generation of PGD_2 is catalysed by the enzyme COX. Two isoforms of COX have been identified, COX-1 and COX-2, and we questioned which COX isoform might be responsible for PGD_2 generation from lung mast cells (Murakami et al., 1994). Studies in rodent mast cell systems suggest that IgE and cytokine-dependent PGD_2 generation may be mediated by COX-1 and COX-2, respectively (Ashraf et al., 1996, Bando et al., 2017). However, our own data using COX-selective inhibitors, FR122047 (COX-1 selective) and celecoxib

(COX-2 selective), unequivocally indicate that COX-1 is the principal isoform that mediates both IgE and SCF-dependent PGD_2 generation. Further experiments by Western blotting confirmed the dominant expression of COX-1 in all mast cell preparations.

An interesting aspect of the response of mast cells to SCF is that SCF can drive PGD_2 generation continuously over at least 4 hours, a property not shared by anti-IgE. Therefore, we questioned whether this generation of PGD_2 over the longer-term is mediated by induction of COX-2. Our data show that long-term incubation of SCF failed to induce COX-2 expression. We also considered whether SCF might induce the enzyme PGDS, which facilitates the conversion of PGH_2 to PGD_2, but similarly no induction of the synthase was observed. Further studies using LPS, which is known to be a strong inducer of COX-2 in many systems and which we have shown to be a strong inducer of COX-2 in human lung macrophages, failed to induce COX-2 expression in mast cells. Collectively, these data indicate that PGD_2 generation from human lung mast cells is catalysed exclusively by COX-1.

In allied studies, we also looked at the effects of a number of NSAIDs which act as non-selective COX inhibitors. An interesting aspect of this work was the finding that all the NSAIDs tested (indomethacin, ibuprofen, naproxen and diclofenac) were highly effective at preventing PGD_2 generation from mast cells with the striking exception of aspirin.

Despite an expectation that NSAIDs might be useful in inflammatory conditions like asthma, aspirin and related NSAIDs, are contraindicated in asthma. This is because aspirin can induce exacerbations in a large proportion of asthmatics (Varghese and Lockey, 2008). The underlying mechanism behind

this susceptibility to aspirin has not been identified but aspirin intolerance is associated with increases in cys-LTs and PGD_2. Since PGD_2 is essentially mast cell specific, this suggests an important role for mast cells. The suggestion has been made that aspirin can directly activate mast cells. However, we were unable to demonstrate that this occurs in human lung mast cells.

One possible explanation for aspirin-intolerant asthma is that aspirin inhibits COX which may result in the reduction of an important stabiliser of mast cells, PGE_2. PGE_2 does not act as a pro-inflammatory mediator in the lung, instead PGE_2 is both a bronchodilator and can also inhibit human lung mast cells (Kay et al., 2013, Buckley et al., 2011, Gauvreau et al., 1999, Hartert et al., 2000). Inhibiting the production of a mast cell stabiliser such as PGE_2 may increase the release of mast cell-derived mediators. Our data show that, since aspirin was ineffective at inhibiting COX from mast cells, this might lead to even greater levels of PGD_2 generation and worsening asthma. An increase in PGD_2 levels observed in aspirin-intolerant asthma may indicate an inability of aspirin to contain PGD_2 generation from mast cells rather than direct activation of mast cells by aspirin.

Another possible explanation for aspirin-intolerant asthma might be connected to a possible shunting of arachidonic acid metabolism from COX to the 5-LO pathway following blockade of COX by a NSAID. This shift in the pathway could lead to an increase in bronchoconstrictor cys-LT production contributing to exacerbations (Szczeklik, 1990). In the present study, we were unable to demonstrate an increase in cys-LT production from mast cells following treatment with NSAIDs arguing against such a mechanism in the lung mast cell context.

As well as pre-stored mediators and lipid-derived mediators, this study also investigated the effect of SCF and other stimuli on cytokine generation from human lung mast cells. There is some controversy between mast cell studies investigating cytokine generation. For example, Gordon and Galli (1990) demonstrated that mast cells can generate cytokines whereas Gibbs et al. (1997) reported that mast cells barely generate any cytokines. Our data show that neither SCF nor other stimuli were particularly effective at generating any cytokines from human lung mast cells which is in agreement with the Gibbs study (1997).

A striking finding was the surprisingly high spontaneous cytokine generation from mast cells. The reason for this high spontaneous cytokine generation is unknown. However, one possible explanation is that when mast cells are purified and so artificially removed from their normal microenvironment within the tissue any natural restraints on the cells might have been removed and this might have allowed the cells to be activated. However, the possibility of inappropriate activation of mast cells by endotoxin contamination *in vitro* cannot be excluded. However, this comment is tempered by the knowledge that LPS is relatively inert as an activator of human lung mast cells.

It is of interest that data generated by other members of the lab show that a similar situation occurs for histamine release over time. Thus histamine release steadily increases over 24 hours but, interestingly, mast cell histamine content remains the same suggesting that the loss is replenished. Similarly, if mast cells are activated to release histamine then the lost histamine is rapidly replenished over 24 hours (Lewis et al., 2017). This suggests that the mast cell is a cell that, far from being quiescent, is still active even in the absence of direct

activation by a stimulus. Again, whether this is because the cells have been removed from an environment that naturally restrains activity is not known and will require further investigation.

As part of our studies on cytokine generation from mast cells we investigated alternative strategies to determine whether mast cells might be a source of cytokines. To this end we investigated the effects of ATP which has previously been shown to induce the release of IL-1β after LPS challenge in monocytes and macrophages (Wilson et al., 2004, Ferrari et al., 1997, Ward et al., 2010). Therefore, we wondered if we could observe a similar effect on human lung mast cells.

Our data showed a dual effect of BzATP on human lung mast cells. Interestingly, BzATP significantly generated IL-1β from mast cells after a short incubation (30 minutes) indicating that BzATP alone could independently drive IL-1β generation selectively from human lung mast cells. One possible explanation for this secretion could be an interaction between BzATP and certain related P2 receptors which initiate signalling through G_q and/or G_i family proteins which activate phospholipase C, inducing Ca^{2+} flux and inositol phosphate accumulation (Communi et al., 2000). Clearly, further experimental work would be needed to confirm whether this was the case.

By contrast, a longer incubation (22 hours) with BzATP alone inhibited TNF-α, IL-6 and IL-8 but not IL-1β generation from mast cells. The mechanism by which TNF-α, IL-6 and IL-8 are inhibited by B_zATP has not been determined and warrants further investigation. One possible explanation could be the stimulation of cAMP accumulation by a BzATP/P2 receptor interaction which induces the ICER expression and blocks the binding between transcription

factors (NF-AT and AP-1) and their targeted promoter sites which suppress the expression of certain cytokines (Duhant et al., 2002)(Feng et al., 2004).

These findings with BzATP may have some physiological relevance. ATP can be released when cells are damaged or stressed. This can occur most commonly at sites of injury and inflammation (Kouzaki et al., 2011). Extracellular ATP can thereby act as a 'danger' signal mediating effects by activating purinergic receptors. In asthma and other respiratory diseases, elevated concentrations of ATP in the lungs are likely because these diseases are inflammatory in nature. Consequently, extracellular ATP could act to modulate cytokine generation from human lung mast cells as well as other cells (Riteau et al., 2010, Idzko et al., 2007).

An important question that needs to be addressed is the potential pathophysiological relevance of this work. It has been reported that normal concentrations of SCF that are found in the serum are about 3.3 ng/ml (Hsu et al., 1997). Although this concentration does not directly activate human lung mast cells, these concentrations of SCF are capable of enhancing responses to IgE-mediated activation. In the context of asthma, much higher concentrations of SCF have been demonstrated (Al-Muhsen et al., 2004, Da Silva et al., 2006a) in keeping with the range of concentrations of SCF used in the present study. Makowska et al. (2009) also demonstrated that patients with severe asthma have significantly higher levels of SCF in serum (1010 ± 37 pg/ml) compared to the control group (799 ± 33 pg/ml). This suggest that SCF could contribute to inflammatory events and exacerbated responses in disease situations.

Overall, this study has highlighted the importance of SCF as a *direct* activator of human lung mast cells that is particularly adept at generating PGD_2.

These findings suggest that targeting the SCF/c-KIT axis could be of benefit in the therapeutic management of respiratory diseases such as asthma.

CHAPTER SEVEN

7. BIBLIOGRAPHY

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8. APPENDIX

Table I. Comparison of IC_{50} values for c-KIT inhibitors on c-KIT and/or c-KIT mutants in different studies.

Table II. Comparison of IC_{50} values for NSAIDs on COX-1 and COX-2 activity in different studies.

Study/ c-KIT inhibitors	**Imatinib**	**Dasatinib**	**Nilotinib**
Merchant et al. (2002)	c-KIT (0.1 -0.5 μM)	-	-
Schittenhelm et al. (2006)	c-KIT (50 - 100 nmol/L)	c-KIT (1 - 10 nmol/L)	-
Shah et al. (2006)	c-KIT [WT] (1550 nM) c-KIT [D816V] (>10000 nM)	c-KIT [WT] (79 nM) c-KIT [D816V] (37 nM)	-
Weisberg et al. (2005)	c-KIT [K462E] (84 – 126 nM) c-KIT [D816V] (25 – 29 nM)	-	c-KIT [K462E] (148 – 213 nM) c-KIT [D816V] (24 – 30 nM)
Kitagawa et al. (2013)	c-KIT [WT] (137 nM) c-KIT [V560G] (6.9 nM) c-KIT [D816V] (8100 nM)	c-KIT [WT] (3.3 nM) c-KIT [V560G] (0.36 nM) c-KIT [D816V] (2.4 nM)	-
Agaram et al. (2008)	c-KIT [WT] (3132 nM) c-KIT [V559D] (63 nM)	c-KIT [WT] (316 nM) c-KIT [V559D] (27 nM)	c-KIT [WT] (35 nM) c-KIT [V559D] (44 nM)

Table I. Comparison of IC50 values for c-KIT inhibitors on c-KIT and/or c-KIT mutants in different studies.

Study/ Cox inhibitors	**Indomethacin**	**Diclofenac**	**Aspirin**	**Ibuprofen**	**Naproxen**	**FR122047**	**Celecoxib**
Cryer and Feldman (1998)	COX-1 (0.21 μM) COX-2 (0.37 μM) Gastric Mucosa (0.85 μM)	COX-1 (0.26 μM) COX-2 (0.01 μM) Gastric Mucosa (0.23 μM)	COX-1 (4.45 μM) COX-2 (13.88 μM) Gastric Mucosa (0.03 μM)	COX-1 (5.9 μM) COX-2 (9.9 μM) Gastric Mucosa (0.7 μM)	COX-1 (32.01 μM) COX-2 (28.19 μM) Gastric Mucosa (0.52 μM)	-	-
Mitchell et al. (1993)	COX-1 (0.009 – 0.011 μg/ml) COX-2 (0.53 – 0.68 μg/ml)	COX-1 (0.3 – 0.7 μg/ml) COX-2 (0.2 – 0.5 μg/ml)	COX-1 (0.1 – 0.5 μg/ml) COX-2 (40 – 60 μg/ml)	COX-1(0.93 – 1.07 μg/ml) COX-2 (9.7 – 20.3 μg/ml)	COX-1 (1.3 – 3.1 μg/ml) COX-2 (0.5 – 2.1 μg/ml)	-	-
Chan et al. (1999)	COX-1 (0.17 – 0.21 μM) COX-2 (0.37 – 0.51 μM)	COX-1 (0.11 – 0.19 μM) COX-2 (0.04 – 0.06 μM)	-	-	-	-	COX-1 (5.3 – 6.3 μM) COX-2 (0.72 – 1.2 μM)
Warner et al. (1999)	COX-1 (0.013 μM) COX-2 (0.13 - 1 μM)	COX-1 (0.075 μM) COX-2 (0.02 – 0.038 μM)	COX-1 (1.7 μM) COX-2 (7.5 - >100 μM)	COX-1 (7.6 μM) COX-2 (7.2 - 20 μM)	COX-1 (9.3 μM) COX-2 (28 - 35 μM)	-	COX-1 (1.2 μM) COX-2 (0.34 – 0.83 μM)
Ochi et al. (2000)	COX-1 (0.19 – 0.25 μM) COX-2 (0.35– 0.81 μM)	-	-	-	-	COX-1 (0.019 – 0.037 μM) COX-2 (46– 84 μM)	-

Table II. Comparison of IC50 values for NSAIDs on COX-1 and COX-2 activity in different studies.