Characterisation of IL-33/ST2 signalling and crosstalk in mast cells and their modulation by ES-62

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Abstract

In addition to their role in fighting infection, mast cells have long been implicated in the pathogenesis of allergic and autoimmune inflammatory diseases and cancers. Increasingly, however, there is recognition that these cells may also play a part in protecting against development of pathologies. Indeed, there is increasing evidence that mast cells comprise heterogeneous phenotypes that exhibit functional plasticity to allow them to play both pro- and anti-inflammatory roles during an immune response. This plasticity appears to reflect that immature mast cells are tailored by their particular microenvironment not only to trigger protective inflammatory responses but also to limit pathology by resolving inflammation and promoting wound healing and tissue repair. Mast cells can be activated by a range of stimuli including (pathogen-derived) antigen/allergen-mediated crosslinking of antibody-bound to Fc receptors, most notably FcεRI, pathogen-associated molecular patterns (PAMP) such as bacterial lipopolysaccharide (LPS) acting on TLR4, inflammatory cytokines such as IL-33 (via the IL-1R/TLR-like receptor ST2) and tissue-derived signals such as SCF (via cKIT). During infection these stimuli provoke a response optimised for pathogen clearance however in autoimmune or allergic disease such responses can initiate and exacerbate host pathology. Thus the challenge for therapeutic targeting of mast cells in inflammatory or malignant disease is to limit mast cells with pathogenic phenotypes whilst preserving those contributing to protective homeostatic and anti-pathogen responses.

Thus, as a first step, it was a core aim of these studies to generate *in vitro* mast cell models representing the phenotypic and maturational heterogeneity of mast cells *in vivo*, as these are difficult to isolate and purify due to their limited numbers in tissue. Distinct murine mast cell phenotypes, namely mature serosal peritoneal-derived mast cells (PDMC), connective tissue-like mast cells (CTMC) and mucosal-like mast cells (BMMC), the latter two subtypes both derived from bone marrow progenitors, were found to differentially respond, in terms of cytokine production and degranulation, to important immunoregulatory receptors in health and disease, namely FcεRI and TLR4. Consistent with their distinct functional responses, these mast cell

subtypes were also found to display differential calcium signalling profiles in response to FcεRI and TLR4 signalling, further highlighting the importance in investigating phenotypically relevant and microenvironment-specific (serosal versus mucosal) mast cells in drug discovery programmes.

Recently there has been great interest in IL-33, a pro-inflammatory cytokine increasingly recognised as playing an important role in a variety of mast cell responses associated with allergic inflammatory disorders and tumour pathogenesis. Consistent with this, whilst IL-33 can stimulate mast cell cytokine production, but not degranulation, via the IL-1R/TLR-like receptor ST2, responses to this cytokine are amplified following IgE sensitization and/or exposure to SCF or serum. Such augmented responses reflect increased calcium mobilization, PLD, SphK, ERK and NF-κB signalling and mTOR activation and can be suppressed by existing therapeutics targeting the costimulatory signal, for example, Imatinib or Dasatinib for SCF/cKIT and potentially Omalizumab for IgE/FcεR1. Moreover, IL-33/ST2 signalling can modulate mast cell responses resulting from antigen-mediated crosslinking of FcεRI and LPS-TLR4 signalling. Indeed, ST2 signalling can differentially modulate LPS/TLR4 responses depending on the presence (enhances) or absence (inhibits) of IL-33, as in the latter case ST2 acts to limit LPS cytokine production, potentially by sequestering MyD88. This receptor crosstalk is likely to occur under pathological conditions, thus targeting of such cooperative signalling may allow the downregulation of hyper-inflammatory responses, whilst leaving protective and homeostatic mast cell responses intact.

ES-62 is an immunomodulator produced by filarial nematodes to dampen immune responses in order to promote parasite survival and prevent tissue damage without immunocompromising the host to infection. As a serendipitous side effect of its anti-inflammatory actions, ES-62 exhibits therapeutic potential in both allergic and autoimmune inflammatory disease and thus to further explore the potential for safe, targeted downregulation of pathogenic mast cell responses, the parasite product was exploited in order to identify signals regulating mast cell activation. ES-62 was found to be able to induce hypo-responsiveness of all three mast cell phenotypes in terms of degranulation and cytokine production in response to stimulation of FcεR1-,

TLR4- or IL-33/ST2, either alone or in combination. ES-62 mediated these effects, at least in part, by mechanisms involving downregulation of PKCα (and in BMMC, MyD88) expression and calcium mobilisation and, in PDMC, potentially by subverting the negative feedback interactions of ST2 on TLR4. The precise mechanism of modulation varies both with receptor usage and mast cell phenotype as ES-62 exhibits differential effects in PDMC and BMMC. Nevertheless, collectively these data support the role of calcium-, PKCα- and MyD88- as key regulatory intersection sites in the functional crosstalk amongst these important immunoregulatory receptors and importantly, suggest they are potential targets for therapeutic intervention in pathogenic mast cell responses.

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Declaration

The work presented in this thesis represents original work carried out by the author. This thesis has not been submitted in any form to any other University. Where reagents, materials or technical support has been provided by others, due acknowledgement has been made in the text.

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Abbreviations

Abbreviation	Full Description
-/-	Knock out gene deletion
7-AAD	7-amino-actinomycin D
Ag	Antigen
AHR	Airway hyperresponsiveness
Akt	Serine/threonine protein kinase
Alum	Aluminium hydroxide
APC	Allophycocyanin
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage
BCR-Abl	Constitutively active tyrosine kinase
BMMC	Bone-marrow derived mast cell
BSA	Bovine serum albumin
Btk	Brutons tyrosine kinase
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
Ci	Curies
CIA	Collagen-induced arthritis
CIA	Collagen-induced arthritis
cKIT	Stem cell growth factor (SCF) receptor
CRAC	Calcium release activated channels
CTMC	Connective tissue– like mast cell
DC	Dendritic cell

DMF	Dimethylformamide
DMS	N,N-dimethylsphingosine
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
FACS	Flow cytometry
Fc	Fragment crystallizable [region]
FCS	Foetal calf serum
FcεRI	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
FYN	Src-family kinase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPC	Haematopoietic progenitor cells
HRP	Horseradish peroxidase
ICOS	Inducible T cell costimulator
IFN	Interferon
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL-	Interleukin
IL-1RAcP	IL-1 receptor accessory protein
IMDM	Iscove's modified Dulbecco's medium
JNK	c-Jun N-terminal kinase

KO	Knock out gene deletion
LFA1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LYN	Src-family kinase
M-CSF	Macrophage colony-stimulating factor
MAPK	Mitogen-activated protein kinase
MC	Mast cell(s)
MCP-1	Monocyte chemotactic protein-1
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCA	Passive cutaneous anaphylaxis
PDMC	Peritoneal-derived mast cells
PE	Phycoerythrin
PI3K	Phosphatidylinositide 3-kinases
PIP_2	Phosphatidylinositol biphosphate
PIP_3	Phosphatidylinositol triphosphate
PLCγ	Phospholipase C gamma
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
RA	Rheumatoid arthritis

Raf	Serine/threonine protein kinase
Ras	GTPase
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase
S1P	Sphingosine-1-phosphate
SCF	Stem cell factor
SD	Standard deviation
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SOS	Guanine nucleotide exchange factor
SphK	Sphingosine kinase
Src	Non-receptor tyrosine kinase
sST2	Soluble ST2
ST2	Membrane bound ST2
STIM1	Calcium sensor in the endoplasmic reticulum
SYK	Syk- family kinase
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper [cell]
TIR	Toll/interleukin 1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Vav1	Guanine nucleotide exchange factor
VEGF	Vascular endothelial growth factor
WT	Wild type

1 General Introduction

1.1 Mast cells

Mast cells are long-lived haematopoietic cells found in almost all vascularised organs [1]. They comprise an extremely heterogeneous cell population, varying in morphology, function and localisation, with individual subtypes exhibiting different protease expression, quantity and profile of eicosanoids and proteoglycan content [1-5]. Mast cells can be generated from multipotent haematopoietic stem cells [6, 7] but not the more differentiated granulocyte/ macrophage or common myeloid progenitors. Mast cell progenitors enter the blood where they migrate to and enter the tissues in an immature state [8-10]. They are then influenced by the local tissue resulting in the generation of mast cells with multiple innate immune system functions (Figure 1.1).

1.2 Mast cell development, location and phenotype

Mast cells reside throughout the body where they are in direct contact with a variety of cells including endothelial cells, nerve cells, smooth muscle cells and epithelial cells [11, 12]. However, they are often concentrated at locations exposed to the outside environment such as in the skin, airways and gut, allowing direct interactions with allergens and pathogens; this results in production of a range of pro-inflammatory mediators depending on the stimuli received [11, 13, 14], but which include SCF, IL-3 and IL-4, from the local microenvironment [1, 5]. Mast cell development is not limited to inflammatory situations and under homeostatic conditions the precise MC protease, proteoglycan and mediator release phenotype generated is driven by hormone and cytokine signals in the surrounding tissues [15]. In mice there appears to be two major MC subpopulations defined by their location and granule proteoglycan content; CTMC, connective tissue resident mast cells that have the MC proteases MMCP-4, MMCP-5, MMCP-6 and carboxypeptidase A and contain more heparin and histamine than BMMC, mucosal surface resident mast cells that contain MMCP-1 and MMCP-2. Interestingly,

development of mucosal MC *in vivo* has been proposed to be T cell dependent [1, 11]. In humans, mast cells present in the mucosal tissues contain primarily tryptase, whereas skin and submucosal MC contain chymase and carboxypeptidase [16].

1.3 Mast cell activation

The best-defined mechanism of mast cell activation is that resulting from crosslinking of antigen-specific IgE antibodies bound to the FcεRI receptor. FcεRI on mast cells is a high affinity receptor for IgE, constitutively expressed on mast cells throughout their development [17]. Surface expression of FcεRI is heavily regulated to ensure sufficient but controlled mast cell responses [18], with the receptor being downregulated quickly in the absence of bound IgE [17] and upregulated, both at the cell surface [19] and gene level [20] following FcεRI signalling. FcεRI is a tetrameric structure, comprising of α, ß and γ subunits ($\alpha\beta\gamma_2$). The α chain binds IgE, while the ß and γ subunits have roles of differing importance in signal transduction [21]. Indeed, the ß subunit is not essential for function but, along with γ, is important in translocation of the α chain to the surface [18] and maintenance of receptor expression [17].

FcεRI signalling has been comprehensively characterised and reviewed [17, 22-28] and the key components of IgE–mediated signalling and their relevant interactions are shown in Figure 1.2. In brief, crosslinking of antigen-specific IgE bound to FcεRI triggers initial signal propagation within plasma membrane-associated lipid rafts. Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the β and γ chains of FcεRI by a series of kinases including Lyn, Fyn and Syk, results in the recruitment of adaptor molecules and the construction of a signalling platform from which PI3K and PLCγ are activated resulting in calcium production, MAPK and PKC activation that drives degranulation and activation of NFκB to promote survival, growth, migration and cytokine release [26, 27, 29].

Although Ag-mediated activation via FcεRI is the best characterised system, the range of agonists known to induce mast cell activation are increasing

(Table 1.1). Mast cells can be directly activated by pathogens including bacteria, viruses and parasites, where pathogen-associated molecular patterns (PAMPs), including microbial nuclei acids, lipoproteins and carbohydrates, are recognised by receptors located on the MC surface, such as TLRs or cytosolic receptors such as NOD-like receptors. Mast cells can recognise pathogens specifically using pathogen-specific Ig bound to FcR and can be activated by anti-microbial peptides released by epithelial cells such as α and ß defensins and cathelicidins, complement coated bacteria as well as host cell components, such as ATP, released from damaged cells [11-13, 16, 28, 30-39]. Mast cells can also be directly activated by pathogen proteases including those from bee venom, house dust mites or parasites [40].

Alongside activation by direct recognition of pathogens, mast cell responses are defined by cytokines and chemokines. Cytokines such as TNFα, IL-33 and SCF are important in mast cell survival, maturation and under inflammatory conditions, they induce proinflammatory responses [41-43]. Likewise, chemokines including CCL2 (MCP-1) are crucial for mast cell recruitment to sites of inflammation and hence can tailor MC responses to further activation [1, 44] [45].

LPS-TLR4, SCF-cKIT and IL33-ST2 signalling appear to be particularly important in initiating and regulating responses in mast cells [16, 41, 46-49].

TLRs are type-1 transmembrane proteins containing leucine-rich repeats that recognize bacterial and viral PAMPs not only in the extracellular (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) but also in the intracellular environment within endo-lysosomes (TLR3, TLR7, TLR8, TLR9 and TLR10) [28]. TLR expression varies with cell type as for example, there is no TLR5 on murine mast cells or TLR8 on human mast cells [46]. TLR activation of MC induces mostly cytokine secretion rather than degranulation: however the exact signalling and consequent response(s) to each TLR is controversial as the different mast cell subsets and culture methods impact on the profile of TLR expression, calcium signalling and cytokines produced after TLR ligation [12, 16, 46, 50]. Thus, for peritoneal-derived MC (PDMC), lipopolysaccharide (LPS) induces IL-6, GMC-CSF, IL-10 and TNFα release whereas in BMMC, this TLR4 ligand releases

a far greater range of cytokines including IL-13, IL-5 and IL-1β [46]. Unusually, whilst TLR4 forms a complex with MD-2 and CD14 on BMMC to recognise LPS, it signals only via the MyD88-dependent but not the MyD88-independent, TRIF-dependent pathway [28, 48, 51]. Moreover, CD14 expression is only important for recognition of LPS species without *O*-chain linkages [52-54].

Individual stimuli can also interact to promote amplified responses. Thus, prolonged exposure to low levels of SCF-cKIT signalling allows MC degranulation at concentrations of Ag concentrations that would otherwise fail to induce activation [55, 56]. Similarly cKIT signalling is able to increase responses via other MC receptors including TLR4 [57] whilst TLR4 signalling can enhance mast cell responses including cytokines production to antigen [32, 49, 58, 59]. Reflecting this, in a murine model of asthma, co-administration of LPS with OVA increases airway hypersensitivity, eosinophil infiltration, cytokine and histamine levels in the lung [49]. Moreover, recent evidence has indicated the importance of IL-33-ST2 signalling in the increased activation of mast cells by antigen [60] and their subsequent, varied, role in disease [42, 61-64]. These receptors exhibit intricate crosstalk both at the receptor level and in downstream signalling, allowing modulated MC responses defined by the combination of ligands present. The interactions between TLR4 and ST2 will be discussed in more detail in Chapters 5 and cKIT and ST2 interactions in Chapter 6.

1.4 Mast cell responses

The wide range of stimuli interpreted by mast cells produce a specific and tailored response that can then influence the development, extent and duration of inflammation. Mast cells interact directly with tissues as well as innate and adaptive immune cells (Figure 1.3) thus allowing their involvement from the initial stages of inflammation where, due to their location at the body's surfaces, they can immediately detect and respond to breaches of epithelial barriers, through to the later stages of inflammation to promote the clearance of exhausted inflammatory cells and tissue healing.

MC mediator storage can adapt throughout a response as due to their longevity they can be present throughout an infection and remain at the site of inflammation after the response is terminated, providing a sort of innate memory cell. Although this reflects their roles in maintaining a healthy host via the clearance of infection, it also explains how they also perpetuate chronic inflammation, contributing to disease pathology.

When mast cells are activated by crosslinking of IgE bound to FcεRI the subsequent aggregation of FcεRI initiates signalling that results in the extracellular release by exocytosis (degranulation) of cytoplasmic granules (Figure 1.4). The precise content of granule release is time-dependent as these can contain preformed mediators, such as histamine, proteoglycans and proteases or those synthesised *de novo,* which include lipid mediators prostaglandins and cytokines, chemokines and growth factors (summarised in Table 1.2 [11, 13]). Such mast cell mediators can have both local and systemic effects, where they can be disseminated via the blood or packaged in microsomes, tiny granule-like structures, for delivery to lymph nodes, affecting the distant development of the adaptive immune response [65].

Involved in both acute and chronic conditions, mast cell-derived mediators modulate vasodilation, recruitment of innate and adaptive immune cells and tissue remodelling, all of which can negatively affect the function of the organ involved in the response via tissue destruction, fibrosis and reduced oxygen supply [66, 67]. However, in addition to driving inflammatory responses, mast cell-derived mediators have also been proposed to play roles in tissue homeostasis, where they appear to be important in homeostatic regulation of vasculature. Mast cell mediators such as heparin, tryptase and chymase all promote endothelial cell migration and new vessel formation [68-71] whilst VEGF and PDGF promote wound healing by inducing proliferation of epithelial cell and fibroblasts [11, 72, 73]. Importantly, they also play anti-inflammatory roles, as secretion of IL-10 and TGFß can suppress inflammation and promote tissue healing [36, 74-77]. Both pro- and anti-inflammatory responses may be occurring simultaneously or sequentially, potentially defining the level of pathology, and affecting the duration and resolution of inflammation.

1.4.1 *Mast cell involvement in the adaptive immune response.*

The immune system is composed of two branches; innate and adaptive immunity. Innate immunity is a localised, non-specific response to a variety of infectious pathogens and is the first line of defence. Adaptive immunity is more specific and robust, involving DC, T and B cells. This response can be further defined by the distinct cytokine patterns produced by the T cells, for example Th1, Th17, Th2 T cell responses. These categories are not fixed or exclusive and all display evidence of plasticity with interchanging T cell phenotypes. Th1 T cells, are driven by the presence of IL-12 in an immune response and are characterised by IFNγ and TNFα production. They have an important role in promoting cytolytic activity of CD8 T cells and innate immune cells such as macrophages, to combat infections by intracellular pathogens. Interestingly, therefore, Th1 T cells are also involved in the pathology of autoimmune disease such as rheumatoid arthritis and diabetes. By contrast, Th2 cells, thought to be induced by IL-4, produce IL-4, IL-5, IL-9, IL-10 and IL-13, important cytokines in the development of antibody-mediated responses, resistance to extracellular pathogens and mucosal immunity. Excessive Th2 activity, however, can lead to allergic disease and asthma. Whilst also important for fighting infection, Th17 cells are becoming increasingly recognised as playing important roles in the initiation and maintenance of pathology of both a variety of autoimmune disease such as multiple sclerosis, rheumatoid arthritis, psoriasis as well as allergic responses. Th17 T cells are driven by TGFß, IL-6 and IL-23 and produce IL-17A and IL-17F [78-81].

The balance between Th1, Th17 and Th2 phenotypes is important in determining the duration and outcome of autoimmune disease [82], parasitic infections [83] and allergies [84]. Thus, a key role for mast cells in immune responses is their ability to influence the development of each phenotype through rapid and robust cytokine responses at the onset of inflammation, driving DC maturation and/or directly influencing T cell maturation [30, 33, 39, 85]. Although mast cells are mostly thought to bias the development of Th2 responses, due to the variety of mast cell-derived mediators, they are also able to drive alternative phenotypes.

Mast cells and DC are found in close proximity at body surfaces allowing their direct interaction and cooperation in their activation. For example, use of a diptheria toxin-based MC deletion method demonstrated a crucial role for MC in the development of contact hypersensitivity where they drive DC maturation and migration [86]. Mast cells are also able to assist in antigen presentation by DC whereby they internalise antigen then apoptose, passing antigen on to antigen-presenting cells such as DCs [87]. In addition, they can modulate the T cell-priming/polarising effects of DC by increasing DC cytokine production and co-stimulatory molecule expression [88-94]. For example *in vitro*, histamine released by mast cells drives the maturation and phenotypic regulation of DC to promote the development of Th2 T cells [89].

Alongside their ability to modulate DC-mediated maturation of T cells, MC can also directly influence T cell activity [30, 33, 39, 95]. This is because MC constitutively express MHC Class I molecules whilst expression of MHC II can be upregulated following exposure to LPS, TNF or IFNγ [11, 96-98]. Thus, when coupled with OX40L, CD80 and CD86 expression this allows direct antigen-presentation to, and costimulation of, naïve T cells [12, 75, 95, 99]. MHC II and OX40L expression on BMMC has been shown to allow direct induction of Th2 T cells from naïve T cells [100], however others have found that this response still requires T cells to have been at least partially activated via TCR/CD3 by professional antigen-presenting cells such as DCs [101]. Moreover, mast cells can also enhance the proliferation and cytokine production of Th1, Th2 and Th17 T cells via their secretion of cytokines, including TNF, and/or direct cell contact. Thus, BMMC and PDMC were found to support activated T cell proliferation and function via interactions with MHC II, costimulatory molecules, such as ICAM-1, OX40L and ICOSL, and cytokine production [96, 97]. These effects appear to be strongest when MC have been activated via FcεRI.

MC can similarly influence development of immune responses via effects on B cells. Thus, MC-derived IL-4, IL-5, IL-6, IL-13 [39] and CD40L expression [102] support B cell development and function and where MC can drive the proliferation of naïve B cells [103] and subsequent class switching to IgE in the absence of T cells [104]. Mast cells can also directly migrate to lymph nodes, to promote lymph node enlargement and T cell recruitment [105]. All

of these interactions drive and shape the development of the adaptive immune response and are reciprocal, with DC, T cells and B cells influencing mast cell activation via the production of immunoglobulin and cytokines [106].

1.5 Mast cells in infection

Although mast cells are commonly associated with atopy and asthma, it should not be forgotten that their primary roles are to generate protective inflammation and subsequently, its safe resolution and wound healing. Due to their location in lining surfaces, which allows direct interaction with triggering factors such as LPS and parasite-derived molecules, mast cells are frequently the first to respond during infection [75]. This is evident by the important role played by mast cells in bacterial and parasitic infections [16, 107-109].

1.5.1 *Mast cells in bacterial infection*

Supporting their key role in fighting infection, mast cells express almost the full range of TLRs, including the receptor for bacterial endotoxin (LPS), TLR4 [46] and can initiate early responses to bacterial infections [12, 110, 111]. Consistent with this, mast cell-deficient mice are less efficient at clearing bacteria and have higher mortality [11]. Involvement of MC in the resolution of bacterial infection has been reported in multiple murine infection models including those involving peritonitis, either by cecal ligation and puncture or introduction of intraperitoneal bacteria as well as those involving intranasal or intracutaneous exposure to bacteria [77], indicating the role of MC in fighting bacterial infection at multiple invasive locations.

Although mast cells can directly kill such pathogens by phagocytosis and the consequent release of reactive oxygen species and antimicrobial peptides, the relatively low levels of these cells in tissues suggest this is not their predominant role in fighting infection [112]. Rather their activation in tissue is more likely to lead to recruitment of neutrophils, via TNFα, LTC_4 and LTB_4, [108, 113] as these are the cells responsible for the bulk of early phagocytosis

and mediator release. Histamine, which is released during early degranulation of mast cells, is an important vasodilator facilitating such inflammatory cell influx [112].

Many other mast cell mediators such as cytokines then act to promote activation of the adaptive immune system by recruiting DC and T cells, as well as their direct antigen presentation [112], to influence the Th phenotype of an immune response and consequently define the outcome of infection.

It is important to note that, while such mast cell responses may act to increase host defence locally at the site of infection, over-production of mast cell proteases, histamine and cytokines can also have deleterious effects on the outcome of infection by exacerbating inflammation and prolonging responses that increase tissue damage [77, 114].

1.5.2 *Mast cells in helminth infection*

Although the high IgE levels and strong Th2 responses induced by helminth infections suggest a role for mast cells in combating such infections, their precise role in immunity to nematodes, tapeworms and schistosomes, is unknown. However, during helminth infection, mast cell precursors are increased [12] and increases in Th2 cytokine production augment levels of non-specific IgE [38], increasing MC survival and enhancing their activation [115]. Nevertheless, the role of MC in parasite infection appears to vary depending on the location of infection and parasite species involved [77]. For example, mast cells are not essential for expulsion of *Nippostrongylus brasiliensis* [116]. By contrast, although MC are absent from tissue adjacent to *Heligmosomoides polygyrus* granulomas, structures created by the immune response to contain the parasite, increased mast cell numbers in the mucosa correlate with a more resistant phenotype to *H. polygyrus* [117]. Moreover, mast cells have been shown to be important in the expulsion of *Trichinella spiralis* infections by mechanisms thought to involve mast cell protease 1, TNFα and IL-4 signalling [11, 117-119].

In addition, although MC may not be essential for clearance of infection they act to promote immunity. Thus, during parasite infection, MC can be directly recruited to the gut or skin, in response to chemokines and via interactions with cell surface adhesion molecules [45], where they play a protective role via increased IL-5-mediated recruitment of eosinophils that specifically target the parasite [120] as well as IL-10 production, to limit destructive inflammation [75, 77]. Similarly, IL-13 release can increase smooth muscle contractions and mucus production to promote parasite expulsion [112]. Indeed, it appears that the mast cell response can be tailored to fight particular types of infection as indicated in a murine model of schistosomiasis which demonstrated differential localisation of individual mast cell subtypes relating to the length of infection, where mucosal MC are present during the acute phase and connective tissue MC during the chronic phase of infection [121].

1.6 Modulation of immune responses by ES-62

The immune response to helminth infections is the product of prolonged co-evolution between the host and parasite designed to create a cooperative relationship. Parasites need to survive within the host without being destroyed by the immune system but must allow the host to prevent itself being harmed by further infections. Conversely, the host has to generate an effective immune response to remove the parasite but not destroy host tissues in the process. This dynamic interaction has created a model whereby helminths manipulate immune responses to prevent hyperinflammatory responses and a beneficial side-effect of this immunoregulation is reflected by increasing evidence of low prevalence of autoimmune and allergic inflammation in areas of the world endemic for parasitic helminths. This inverse correlation of infection and inflammatory diseases has been supported by evidence where clearance of the helminth infection can result in a resurgence of these diseases [122-124]. Thus, in the search for development of novel therapies for autoimmune and allergic disease, the harnessing of such parasite-driven immunomodulation is being intensively

investigated with one potential candidate being ES-62, an immunomodulator secreted by the filarial nematode *Acanthocheilonema viteae* [125-135].

ES62 is the major secreted glycoprotein of this rodent filarial nematode and homologue of molecules found in filarial nematodes that parasitize humans (77% with that secreted by *Brugia malayi*). The molecule consists of a tetramer of identical monomers of 62kDa that contain a parasite-specific post-translational modification where phosphorylcholine (PC) is attached to the protein backbone via N-type glycans.

ES-62 has demonstrated ability to reduce pathology in murine models of Th1 and Th17-mediated inflammatory diseases such as arthritis. Rather surprisingly, given the Th2 responses exhibited towards helminths and ES-62 itself, ES-62 has also shown inhibitory effects in Th2-mediated diseases such as asthma (reviewed in [136]). This rather counter-intuitive finding has recently been shown to be mediated by targeting Th17 responses [135], consistent with increasing evidence that IL-17 can act as a master regulator of inflammation in asthma [137] as well as in autoimmune inflammation. ES-62 can affect a range of different cell populations, either directly or indirectly, depending on the varying context of the interaction, differing receptor usage by ES-62 and the individual signalling abilities of the cells. For example, studies in MyD88 -/- and TLR4 -/- macrophages and DCs indicate both molecules are important for ES-62 activity. Although ES-62's effects are dependent on TLR4 [138], ES-62 is still effective in macrophages obtained from C3H/HeJ mice that express a mutant TLR4 with a point mutation that prevents LPS-mediated pro-inflammatory signalling [132]. This indicates that ES-62 does not interact, or signal via TLR4 in the same way as LPS. Importantly, MC express TLR4 and respond to ES-62 [126]. Using human BMMC, ES-62 was found to inhibit degranulation, prostaglandin and cytokine production resulting from crosslinking of FcεRI. This effect was not due to modulation of FcεRI surface expression but was associated with a reduction in intracellular calcium mobilisation and NFκB activation. This was found to be due to reduced PLD and SPHK activation and the subsequent release of calcium from internal stores consequent to the sequestration of PKCα from FcεRI and its subsequent degradation via a proteasome-independent mechanism.

1.7 Mast cells in asthma and allergic disease

Mast cell recruitment and activation is clearly important during infection but increased numbers in the wrong location or incorrect and excessive activation can drive disease, as seen in asthma and allergy where, for example, elevated cell numbers are found in the bronchial lavage of asthmatic patients [139]. Mast cell-associated disease symptoms depend on the tissue affected and reflect the diverse characteristics of the mast cells in terms of their release of different mediators, the surrounding immune population phenotype as well as the responses from non-immunological cells in each organ affected.

MC crucially mediate responses to allergens. During initial exposure to allergen MC FcεRI receptors are sensitized by allergen-specific IgE produced by B cells responding to the elicited Th2 immune response. During secondary exposure, allergen binds to IgE, crosslinking FcεRI to stimulate degranulation of MC releasing mediators, cytokine and eicosanoid production. The symptoms of allergy depend on the location of allergen exposure and the mediators released, for example eicosanoids induce bronchoconstriction in the lungs but swelling and itching in the skin. Moreover, the pro-inflammatory mediators released can also recruit additional immune cells such as eosinophils, basophils and T cells to perpetuate and expand the response [30]. IgE-dependent reactions have been implicated in allergic asthma, rhinitis, conjunctivitis, atopic dermatitis, food allergies and anaphylaxis [16] and using an inducible mast cell deletion model, mast cells were found to be involved in passive cutaneous anaphylaxis, IgE-mediated passives systemic anaphylaxis and IgE-mediated chronic allergic inflammation, the latter where the absence of mast cells reduced airway hyper-responssiveness in an OVA-induced asthma model [140].

1.8 Mast cells in autoimmune disease

Although much interest has focused on allergy, the pathological responses of mast cells can also promote inflammation in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Autoimmunity occurs when the

immune system targets “self” tissues rather than pathogens, damaging the host. Autoimmunity is usually prevented by tolerising the immune system to host tissues during development of the adaptive immune system, which involves checkpoints ensuring potentially harmful self-reactive B and T cells are deleted. However, under conditions involving breakdown of such tolerance, innate cells are important in perpetuating the response and are responsible for much of the pathology and tissue destruction [101, 141-143].

For example, mast cells have been shown to be directly involved in the development of MS, a disease characterised by immune-mediated demyelination of nerves within the CNS. MS is a spontaneous disease; very little is known about its development and disease characteristics vary greatly between sufferers. [141]. However, the murine model, EAE, is driven by the development of immune responses to a myelin peptide and, although incompletely representing human disease, allows a useful tool for study. The exact role of MC in EAE has not been defined, however use of MC-deficient mice has demonstrated a clear role for MC in the development of disease [142, 144]. Mast cells normally reside adjacent to nerves and blood vessels with the brain and so are well placed to be involved in inflammation at this site [141]. In EAE, greater numbers of degranulating MC are observed within the brain [145] and it has been proposed that these may be responding directly to myelin protein [146] or may act, following activation by anti-myelin antibodies outside the areas of demyelination, to release cytokines such as TNFα and hence exacerbate inflammation within the brain [142, 147]. In humans, MC [148, 149] and MC-specific gene expression [150] are found to be elevated in CNS lesions. Consistent with this, tryptase, a potent MC protease released during degranulation, has also been identified in the cerebrospinal fluid of MS patients [151].

In rheumatoid arthritis, a condition characterised by inflammation in the synovial joints, MC are increasingly being implicated in disease pathology. For example, when arthritogenic, anti-collagen antibodies are directly introduced into murine joints, mast cell degranulation is observed [152], suggesting a role for mast cells in linking autoantibodies with an increase in proinflammatory mediators within the joint. Similarly, mast cells have been reported to accumulate and degranulate in joints of mice with collagen-

induced arthritis (CIA), a murine model of rheumatoid arthritis, [153]. They have also been found to be present in synovial fluid and joint tissue from human patients [154] where their production of TNFα and IL-1β can recruit further inflammatory cells and activate proinflammatory responses in surrounding tissues.

In autoimmunity that is driven by auto-antibodies there is a clear role for mast cells in driving pathology that has been proposed to result from either complement-mediated activation or via crosslinking of FcR by immune complexes. However MC activation can also result from exposure to inflammatory mediators, including cytokines such as IL-33 and neuropeptides released from the synovial membrane during RA, which can induce TNFα and IL-1 production from MC [155]. Moreover, cytokine production may drive autoantibody production by polarizing the phenotype of T and B cells [142] and indeed, mast cell-T cell interactions are well documented and may provide a potential crosstalk method of activation. Likewise, direct interactions of mast cells with tissues, via the release of proteases that induce tissue destruction, may contribute to the epitope spreading proposed to be involved in autoimmunity [142].

Finally, the release of tryptase, histamine and eicosanoids mediate an increase in vascular permeability and endothelial cell activation, allowing greater influx of immune cells including neutrophils, contributing to pathology. Moreover, the persistence of mast cells, normally crucial to the resolution of inflammation and tissue healing, may result in tissue reorganisation and fibrosis, leading to loss of function under conditions of autoimmune disease.

1.9 Aims of the Thesis

Mast cells are important for fighting infection, resolving inflammation and promoting wound healing: however, they are increasingly being implicated as playing pathogenic roles in cancer as well as in allergic and autoimmune diseases. These pleiotropic functions reflect that mast cells can exhibit

phenotypic and functional plasticity. It was therefore the core aim of this thesis to characterise the phenotype of distinct subtypes of murine mast cells (mature peritoneal-derived mast cells, PDMC; mucosal-like bone marrow-derived mast cells, BMMC and connective tissue-like bone marrow derived mast cells, CTMC) and their functional responses to stimulation via a range and combination of immunoregulatory receptors (FcεRI, TLR4, ST2 and c-KIT) as a first step to identifying key regulatory targets that might ultimately be exploited in the development of mast cell-targeted therapeutics.

Figure 1.1 Maturation and location of mast cells *in vivo*.

Mast cell progenitors derived from haematopoietic stem cells enter the blood to circulate throughout the body from where they can then enter the tissues. Signals from the microenvironment including SCF, IL-3 and IL-4 stimulate heterogeneous maturation into cKIT$^+$, FcεRI$^+$, ST2$^+$ mast cell populations with varying receptor, cytokine, protease and proteoglycan expression that can respond differentially to distinct stimuli. Mast cells in different locations can be developed to optimally target the potential pathogens within that site [1, 9, 38, 75].

Table 1.1 Common mast cell activatory receptors, their ligands and the responses induced.

Expression depends on species and maturational factors [38].

Figure 1.2 Kinetics of Mast cell activation and degranulation.

Cross-linking of FcεRI stimulates activation and degranulation of mast cells with defined kinetics. Binding of antigen to IgE-sensitized mast cells induces crosslinking of FcεRI; within seconds preformed mediators such as histamine are exocytosed from the cell in the process of degranulation. This does not affect cell viability and mast cells then begin synthesis of prostaglandins and leukotrienes and subsequently cytokines. Granule generation and exocytosis is maintained but not indefinitely. Under conditions of chronic mast cell activation, the constituents of the granules can alter to streamline a more specific response. This effect is driven by receptor ligation, "programming" the cell to target a specific pathogen. After the resolution of infection, some mast cells are removed by apoptosis while others persist within the site and can proliferate to form a store of "memory" mast cells. This activation process is not fixed and individual stimuli may regulate activation with alternative kinetics or without inducing degranulation [9, 26, 38, 75].

Table 1.2 Summary of mast cell mediators and their release mechanism.

Expression depends on species and maturational factors.

Figure 1.3 Mast cells interact with a variety of immune and non-immune cells.

Mast cells play both inflammatory and anti-inflammatory roles in an immune response. Depending on their location in tissues throughout the body, they can interact with T and B cells, antigen presenting cells and innate immune cells, as well as endothelial and epithelial cells and tissue cells including smooth muscle cells and fibroblasts. During infection these interactions result in pathogen clearance by enhanced recruitment of and phagocytosis by neutrophils, development of specific immune responses, increased vascular permeability to facilitate further immune cell recruitment and increased smooth muscle contraction to expel pathogens [75, 77, 156].

Figure 1.4 FcεRI signalling pathway that leads to mast cell activation.

Crosslinking by antigen initiates signalling via the Lyn mediated phosphorylation of ITAMs on the γ chain of FcεRI to recruit and activate, Syk. Syk phosphorylates LAT, which then serves as a scaffold for recruitment of Sos, Vav1 and PLCγ. Sos and Vav1 mediate Ras activation by promoting exchange of GDP for GTP and to initate activation of MAPK pathways resulting in ERK/JNK/p38 activation. Fyn and Syk recruit and activate PI3K that can maintain PLCγ activation and activate Akt and PLD to produce PA to initiate S1P production by SphK. S1P then induces an increase in cytoplasmic calcium concentration. PLCγ induces the catalysis of PIP_2 to DAG and IP_3. IP_3 induces the release of intracellular calcium and DAG, when coupled with Ca^{2+}, activates classical PKC isoforms, including PKCα. PKC can activate PLD and promote activation of NFκB via degradation of IκB by IKK. Lyn; src-family protein tyrosine kinase, ITAM; immunoreceptor tyrosine activatory motif, FcεRI; high affinity IgE receptor, Syk; tyrosine kinase, LAT; linker of activated T cells, Sos; guanine nucleotide exchange factor, Vav1; guanine nucleotide exchange factor, PLCγ; Phospholipase D gamma, Ras; GTPase, GDP; guanosine diphosphate, GTP; guanosine triphosphate, MAPK; serine/threonine specific protein kinase, ERK; extracelluar signal-regulated kinase, JNK; c-Jun N-terminal kinase, p38; mitogen-activated protein kinase, Fyn; protein tyrosine kinase, PI3K; phosphatidylinositide 3-kinase, Akt; serine/threonine protein kinase, PLD; phospholipase D, PA; phosphatidic acid, S1P; sphingosine-1-phosphate, SphK; sphingosine kinase, PIP_2; phosphatidylinositol 4, 5-biphosphate, DAG; diacylglycerol IP_3; inositol trisphosphate, Ca^{2+}; calcium, PKC; protein kinase C, NFκB; transcription factor, IKK; IκB kinase, IκB; inhibitor of NFκB [13, 17, 26-28].

2 Materials and Methods

2.1 Animals

Wild type (WT) BALB/c and C57Bl/6 mice were obtained from Harlan Olac (Bicester, Oxon, UK). CD200R1 gene knockout C57BL/6 were obtained from Professor Alan Mowat, GBRC, University of Glasgow, originally from I.Boudakov [157]. ST2 gene knockout BALB/c mice have a deletion within the ST2 gene that involves the majority of exons 4 and 5 [158]. These mice had previously been obtained from Professor Andrew Mackenzie, BBRC, University of Cambridge. Mice were maintained under standard specified pathogen-free animal house conditions with free access to both water and standard rodent pellets at the University of Glasgow Central Research Facilities in accordance with local and Home Office regulations.

2.2 Cell Culture

2.2.1 *Reagents*

All cell culture reagents were purchased from Invitrogen Life Technologies. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2.2 *Murine Mast Cell Culture*

During initial culture optimisation cells were grown in collaboration with Dr Hwee Kee Tay.

2.2.2.1 Isolation of peritoneal derived mast cells

After removing abdominal skin, the peritoneal cavity was filled with 5 ml sterile, cold RPMI 1640 using a 23G needle then vigorously massaged. The liquid was then removed using a 25G needle, ensuring minimum red blood

cell contamination. Any wash containing significant red blood cell volumes was discarded. Cells were then pelleted at 1250 rpm for 5 mins and resuspended in fresh complete RPMI before being incubated at $5x10^6$ cell/ml for 2 h at 37°C in tissue culture-treated dishes (Corning) to separate the adherent from the suspended cells. These cells were gated on SSC and FSC (Figure 2.1 A) then the relative mast cell population in each fraction was defined by their $CD117^+$ and $Fc\varepsilon RI^+$ expression (Figure 2.1 B), where there is very little difference in size and granularity. The non-adherent cells were slightly enriched for double positive mast cells (2.7%) (Figure 2.1 B, D) and had a high frequency (Figure 2.1 E) and level of ST2 expression (Figure 2.1 F). Light microscopy imaging of the cells with toludine blue (Figure 2.2 A) confirms the presence of mast cells in these cell fractions and further staining of the suspended cells using toludine blue (Figure 2.2 B) and tryptase staining (Figure 2.2 C) again demonstrates that there is a population of mature mast cells that can be harvested from the peritoneal cavity.
Malbec et al [159] derived their PDMC from the adherent cell population but in contrast to this, for the studies in this thesis, the suspension culture was enriched for mast cells after expansion for a further 28 days, resulting in a homogenous mast cell culture as defined by universal toludine blue (Figure 2.3 B) and tryptase staining (Figure 2.3 A).

2.2.2.2 Isolation of bone marrow–derived mast cells

Intact femurs and tibias were dissected from 6-8 week mice. Under sterile conditions the proximal and distal ends were removed and the shaft flushed with PBS through a 23G needle. A single cell suspension was made by passing through a 100 µm nylon monofilament gauze (Cadisch Precision Meshes, London). If the resulting cell pellet contained high levels of blood contamination, red blood cell lysis was performed for 1 minute at 22°C. Cells were washed once with PBS before culture.

2.2.2.3 Culture of primary murine mast cells

To prepare PDMC, the cells were cultured at $0.3x10^6$/ml in complete RPMI with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml

streptomycin, 1 mM sodium pyruvate, 100 µM non-essential amino acids and 50 µM ß-mercaptoethanol, supplemented with 4% KLS-C conditioned media.

To prepare BMMC the cells were cultured at $0.5x10^6$/ml in complete RPMI with 10% FCS, 2mM L-Glutamine, 100U/ml penicillin, 100ug/ml streptomycin, 1mM Sodium Pyruvate, 10mM Hepes and 50uM ß-mercaptoethanol, supplemented with 1% KLS-C conditioned media and 3% TOP3 conditioned media.

To prepare CTMC the cells were cultured at $0.8x10^6$/ml in RPMI with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 100 µM non-essential amino acids and 50 µM ß-mercaptoethanol, supplemented with 1% KLS-C conditioned media and 1 ng/ml recombinant murine IL-4 (Peprotech).

All cells were incubated at 37°C in a humidified incubator supplemented with 5% CO_2 in tissue culture-treated flasks (Greiner Bio-one) for 28 days. Non-adherent cells were retained for further culture whilst adherent and contaminating cells were discarded. All cell culture work was carried out under sterile conditions and wash steps were performed using warmed, sterile RPMI followed by centrifuge at 1250 rpm for 5 minutes (Jouan CR3i centrifuge) unless otherwise stated. A purity of >95% was identified by surface expression of CD117, FcεRI and ST2. Viability was confirmed using 0.1% Trypan Blue staining.

2.2.3 *Human Mast Cell Culture*

2.2.3.1 Culture of HMC-1

HMC-1s (human mast cell leukaemia cells) were generously donated by JH Butterfield. The cell line was cultured in IMDM (Lonza) supplemented with 10% FCS, 2 mM L-glutamine, 25 nM HEPES, 65 µl α-thioglycate, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, 5% CO_2. The population was passaged into clean flasks twice a week. Cells were kept for up to 3 months. Viability was confirmed using 0.1% Trypan Blue staining.

2.2.4 *Production of conditioned media*

2.2.4.1 TOP3 Culture

The cell line TOP3 is a hybridoma that produces IL-3 and was a kind gift from Dr Massimo Gadina, NIH. Cells were cultured at 37°C in RPMI (GIBCO) with 5% FBS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 50 µM ß-mercaptoethanol. IL-3-producing cells were selected using 0.4 mg/ml G418. IL-3- enriched supernatant was filtered to remove cell debris and the concentration determined by ELISA. For mast cell culture the stock concentration was adjusted with PBS to 550 ng/ml to be used at a final concentration of 16.5 ng/ml.

2.2.4.2 KLS-C Culture

KLS-C is a CHO (Chinese Hamster Ovary) cell line that produces SCF and was a kind gift from Dr Xiaoping Zhong, Duke University Medical Centre. Cells were cultured at 37°C in Minimum Essential Medium Alpha (MEMα) without nucleosides (GIBCO) with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. SCF-producing cells were selected with 2.5 µM methotrexate. SCF- enriched supernatant was filtered to remove cell debris and the concentration determined by ELISA. For mast cell culture the stock concentration was adjusted with PBS to 1000 ng/ml to be used at a final concentration of 10 ng/ml for BMMC and 40 ng/ml for PDMC.

2.3 Cell Stimulation

Unless otherwise stated, mast cell FcεRI were sensitized with 0.5 µg/ml murine anti-DNP IgE for 18 h prior to stimulation. In experiments where ES-62 was used, cells were incubated with 2 µg/ml for 18 h simultaneously with IgE. Cells were stimulated at $1x10^6$ cells/ml except where indicated. Addition of

0.5 µg/ml DNP-HSA cross-links the FcεRI to stimulate the cell. Unless otherwise stated IL-33 (Peprotech) was used at 10 ng/ml, LPS (*Salmonella minnesota*) at 0.5 µg/ml and SCF at 100 ng/ml (Table 2.1). Cell signalling inhibitors were used at the indicated concentrations (Table 2.2) unless otherwise specified. Media alone or PBS was used in control cultures. Each condition was replicated in duplicate or triplicate. After the desired culture period cells were centrifuged at 1250 rpm and supernatant was aspirated and stored separately to the remaining cell pellet. All samples were stored at -20^{o}C until analysed.

2.4 Preparation of ES-62

ES-62 was purified to homogeneity from spent culture medium of adult *A. viteae* using endotoxin-free reagents by Dr L Riyami and Dr J Rzepecka at the Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, UK as previously described [132]. In detail, ES-62 was prepared from 500ml of spent medium (endotoxin- free RPMI 1640; Invitrogen Life Technologies, Paisley, UK, with added endotoxin- free glutamine (2 mM), endotoxin- free penicillin (100 U/ml) and endotoxin- free streptomycin (100 µg/ml)) following culture of adult *A. viteae*. To remove larval forms (microfilariae) released by the adult female worms, the medium was passed through a 0.22 µm filter (Sigma, Poole, UK). It was then transferred to a stirred ultracentrifuge unit containing YM10 membrane (Amicon, Stonehouse, UK). After reducing the volume of the sample to 5-10 ml and transferring the holding medium to endotoxin-free PBS, pH 7.2 (Cambrex Bioscience, Berkshire, UK), it was further concentrated to 200-300 µl using Centricon microconcentrators with a 30kDa cut-off membrane (Amicon). The sample was applied to a 30 x 1 cm Superose 6 column (HR 10/30; Pharmacia, Milton Keynes, UK) fitted to an isocratic fast protein liquid chromatography system (Pharmacia) previously equilibriated with endotoxin-free PBS (pH 7.2) at room temperature. The column was eluted at a flow rate of 0.5 ml/min and monitored for absorbance at 280 nm. More than 95% of the protein elutes as a single peak that represents ES-62. Purity and identity of each batch was confirmed by a combination of SDS-PAGE and Western blotting, the latter

employing a rabbit antiserum specific for ES-62. Finally, the level of endotoxin in the ES-62 sample was confirmed using an Endosafe kit (Charles River Laboratories, Kent, UK). ES-62 is used at a working concentration that has an endotoxin reading of <0.003 endotoxin units/ml.

2.5 Enzyme–histochemical Staining and Light Microscopy.

2.5.1 *Toludine Blue staining*

Toludine Blue was used to identify heparin content, crucial to mast cell function as it is responsible for the stable expression and activation of many of the proteases present in mast cell granules [160]. $0.01x10^6$ cells were spun onto glass slides (Superfrost Plus) using a Shandon Cytospin3 (ThermoShandon, Runcorn, UK) at 500 rpm for 5 minutes. Slides were air dried before staining with 0.5% Toludine Blue in 0.5 M HCl for 10-15 minutes.

2.5.2 *Tryptase staining*

For tryptase staining, a colour solution (10 mM z-Gly-Pro-Arg-MNA in dimethylformamide (Bachem), 0.5 M Tris-HCl pH 7.5, 5 mg/ml Fast garnet GBC (SIGMA) and dH_2O (1:1:2:6) was added for 5-30 minutes. Excess stain was washed with dH_2O and the slides air dried before viewing by light microscopy. Images of cytospins obtained at x 10 and x 40 magnification using an Olympus BX41TF microscope.

2.6 Analysis of mast cell marker expression by flow cytometry

2.6.1 *Surface staining*

Cells were washed with cold PBS at 1500 rpm for 6min, 4°C. The cells were pre- incubated with 50 µl Fc receptor (FcR) blocking buffer (anti-CD16/32, clone 2.4G2, hybridoma supernatant, 10% mouse serum, 0.1% sodium azide) for 20 minutes at 4°C to reduce non-specific binding of antibodies to the high levels of FcR present on mast cells. The appropriate flurochrome-conjugated antibodies (2 µg/ml) (Table 2.3), suspended in 50 µl Fc Block, were added for 30 minutes, 4°C. Biotinylated antibodies required a two-step staining protocol. Firstly the biotinylated primary antibody was added for 30 minutes at 4°C, followed by a wash step before flurochrome-conjugated streptavidin was added for a further 30 minutes at 4°C. After labelling cells were washed twice with 3 ml FACS buffer (PBS containing 2% FBS and 2 mM EDTA) at 1500 rpm for 6 min, 4°C then resuspended in 100-300 µl FACS buffer. To enable exclusion of dead cells from the analyses cells were either stained with Live/Dead® Viability/Cytotoxicity Kit (Invitrogen) before commencement of staining or by the addition of 1 µl 7AAD (Ebioscience), a fluorescent dye with high affinity for DNA, immediately prior to data acquisition. Cellular fluorescence data was acquired using a Becton Dickinson LSR II or FACSCalibur™ flow cytometer and analysed using FlowJo software (Tree Star Inc, OR, USA) Analysis was performed on a minimum of 10 000 events and gating is demonstrated in Figure 2.4.

2.6.2 *Intracellular staining*

Cells were prepared as for surface staining except that after surface labelling the cells were fixed for 10 minutes at RT with 200 µL fixation buffer (Ebioscience) before washing twice with 1X permeabilisation buffer (Ebioscience). All antibodies for intracellular staining were added in permeabilisation buffer for 30 minutes at 4°C. After labelling, cells were

washed twice in the permeabilisation buffer before being resuspended in 100-300 µl FACS buffer immediately prior to analysis.

2.7 Calcium Mobilisation

Cells were loaded with 5 µM of the fluorescent calcium-sensing dye Fura-2/AM (Invitrogen) in HBSS buffer (145 mM NaCl, 5mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM) supplemented with 0.18% (w/v) D-glucose (Sigma) and 0.2% (w/v) BSA (Sigma) for 30 minutes at 37°C in the dark. Fura-2 AM quantifies intracellular calcium concentration by emitting light after being excited with light of a varying wavelength depending on whether it is in a bound or unbound state (Figure 2.5). Where necessary, removal of intracellular calcium was performed by the addition of 10 mM BAPTA during Fura-2/AM treatment. Cells were washed twice to remove excess dye at 1250 rpm for 5 minutes and resuspended in a volume of buffer with 1 mM Ca^{2+} before stimulation. For removal of extracellular calcium Ca^{2+} free buffer, HBSS without 1 mM $CaCl_2$ supplemented with 100 µM EGTA, was used. Cells were used at $0.75x10^6$ cells/ml. From this 1.5 ml was added to a stirred glass cuvette in a Hitachi F-700 fluorescence spectrophotometer at 37°C. Stimulation was added at 50 s and measurements acquired for a total of 180 s. Calcium levels were detected every 500 ms using excitation-emission ratios of 340/380 nm to correct for potential variables in Fura-2 AM concentration or cell volume and for a decline in fluorescent signal over time [161]. Following each experiment Rmax values were calculated by the addition of 1% Triton-X. Rmin was subsequently determined by the addition of 20 mM EGTA (pH 7.4). These values allowed removal of background signal but were omitted from the final graphs. All treatments were performed in triplicate.

2.8 Cytokine and Prostaglandin D_2 ELISA

ELISAs were performed according to the suppliers' recommendations (Table 2.4). Briefly, COSTAR Immunlon plates were incubated overnight at 4°C in

buffer recommended in the manufacturer's protocol. Plates were blocked for 1 h with assay buffer. Samples and standards, diluted in assay buffer, were incubated for 2 h at room temperature or overnight at 4°C. Detection antibodies and avidin or steptavidin (supplied with the kits) were incubated for 1 h and 30 minutes respectively at room temperature. Plates were washed at least four times between stages. Finally, plates were developed using TMB substrate and absorbances determined using TECAN Sunrise Microplate reader. Concentrations were determined using a standard curve. The S1P ELISA was performed using single wells. All other ELISAs were performed in at least duplicate.

2.9 Analysis of Mast Cell Degranulation

2.9.1 *Measurement of β-hexosaminidase release*

The level of degranulation was determined using a modified colorimetric assay to assess release of ß-hexosaminidase. $0.2x10^6$ cells were suspended in 200 µL Tyrodes buffer supplemented with 1% FCS. Stimuli were added for 30 minutes at 37°C. Cells were centrifuged at 2000 rpm and 50 µL of supernatant (released ß-hexosaminidase) aliquoted in triplicate in a 96 well plate. The cell pellets and remaining supernatant were lysed by the addition of 150 µL 1% Triton-X 100 (Sigma) in dH_20. 50 µL of lysate (total cell content) was then aliquoted in the same manner as the supernatant. 200 µL of 1 mM *p*-nitrophenyl-*N*-acetyl-ß-d-glucosamine (NAG, Sigma) substrate solution (1 mM in 0.05 M citrate buffer, pH 4.5) was added. After incubation in the dark at 37°C for 1 h the reaction was quenched by the removal of 62.5 µL of the reaction mix into a clean well and the addition of 125 µL/well 0.1 M sodium bicarbonate buffer. Optical density was read on Tecan Sunrise microplate reader at 405 nm. Degranulation was calculated as a percentage of released ß-hexosaminidase compared to total cell content.

2.9.2 *Measurement of Lamp1 surface expression*

An alternative method of quantifying degranulation based on the Lamp1 expression method developed for CD8+ T-cells [162] was adapted for mast cells. This method works on the principle that Lamp1, usually expressed on lysosomes and intracellular vesicles, is presented on the cell membrane surface during exocytosis. By quantifying the geometric mean fluorescence intensity (GMFI) of Lamp1 on the cell surface, a level of cell degranulation can be determined similar to ß-hexosaminidase release. Lamp1 expression is biphasic, with the high expression peak to the right representing Lamp1 after degranulation (Figure 2.6). The advantages of this technique include the increased sensitivity to low-level degranulation and the ability to distinguish exocytosis that perhaps does not results in ß-hexosaminidase release.

After sensitization cells were washed with PBS at 1250rpm for 5 mins, 4^{o}C and resuspended in complete RPMI. The cells were allowed to equilibriate at 37^{o}C before stimuli was added for 30 mins. The cells were then washed and pre-incubated with 50 µL Fc receptor (FcR) blocking buffer (anti-CD16/32, clone 2.4G2, hybridoma supernatant, 10% mouse serum, 0.1% sodium azide) for 20 minutes at 4^{o}C to reduce non-specific binding of antibodies to the high levels of FcR present on mast cells. The appropriate flurochrome-conjugated antibodies (2 µg/ml), suspended in 50 µl Fc Block, were added for 30 minutes at 4^{o}C. Cells were washed twice with 3 ml FACS buffer (PBS containing 2% FBS and 2 mM EDTA) at 1500rpm for 6 min, 4^{o}C then resuspended in 100-300 µl FACS buffer. Immediately prior to data acquisition 1 µL 7AAD (Ebioscience) was added to each sample to enable exclusion of dead cells from the analysis. Cellular fluorescence data was acquired using a Becton Dickinson FACSCalibur™ flow cytometer and analysed using FlowJo software (Tree Star Inc, OR , USA). Analysis was performed on a minimum of 20000 events and gating is demonstrated in Figure 2.6.

2.10 Western Blotting

In order to determine the involvement of downstream molecules ERK and NFκB in receptor signalling, ERK phosphorylation and IκB degradation was determined by Western blotting. ERK marks the terminal end of the MAPK pathway and phosphorylation results in its activation, where it then in turn is able to phosphorylate and activate a variety of transcription factors. The activation of NFκB requires the targeted degradation, via ubiquitination, of two inhibitor molecules IκBα and IκBß. After this NFκB is free to translocate to the nucleus where it can act directly on the DNA to initiate transcription of genes important for proinflammatory responses.

2.10.1 *Whole Cell Lysate Preparation*

Either murine mast cells or HMC-1 cells were stimulated in $2x10^6$/ml as indicated. The reaction was then terminated and washed by the addition of ice-cold PBS 1250 rpm, 4^oC, 5 minutes. Lysis was performed by the addition of 50 µL ice-cold, modified RIPA lysis buffer (50 mM Tris buffer, pH 7.4 containing 150 mM sodium chloride, 2% (v/v) NP40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium othovanadate, 0.5 mM phenylmethylsulfonylfluoride, chymostatin (10 µg/ml), leupeptin (10 µg/ml), antipain (10 µg/ml) and pepstatin A (10 µg/ml)). After vortexing the cells were incubated on ice for 30 minutes before centrifugation of lysates at 12000 rpm for 15 minutes. The resulting supernatants (whole cell lysate) were stored at -20^oC before being used for Western Blot analysis.

2.10.2 *Gel Electrophoresis*

Equal protein loadings of whole cell lysate (30-40 µg protein per lane), determined by BCA protein assay (Thermo Pierce), were resolved on the XCell *SureLock* Mini-Cell kit with NuPAGE Novex high-performance pre-cast Bis-Tris gels and NuPAGE buffers and reagents (Invitrogen). Lysates were diluted in

lysis buffer to a constant final volume and the appropriate volume of 4 x NuPAGE LDS sample buffer and 10 x NuPAGE reducing agent were added prior to heating samples to 70^{o}C for 10 minutes. Samples were resolved using NuPAGE MOPS running buffer supplemented with NuPAGE antioxidant at 150V for 70 minutes. The gel was then transferred onto nitrocellulose (Amersham) or PVDF membrane (pre-activated in methanol for 30 seconds, washed in dH_20 for 1 minute then washed in transfer buffer for 5 minutes) using NuPAGE transfer buffer with 20% (v/v) methanol at 30V for 120 minutes.

2.10.3 *Western Blot Analysis*

Following transfer, the presence of protein on the membranes was checked using Ponceau Red (Sigma). Membranes were washed in Tris-buffered saline (TBS) (0.5 M NaCl and 20 mM Tris pH7.5) with 0.1% (v/v) Tween-20 (TBS/Tween) and blocked for 1 h in TBS/Tween with 5% non-fat milk (Marvel). Membranes were then incubated overnight at 4^{o}C with the appropriate primary detection antibody (Table 2.5). All antibodies were diluted in TBS/Tween with either 5% non-fat milk or 5% BSA. Following incubation with primary antibody the membranes were washed (3 x 8 minutes) with TBS/Tween and incubated in the appropriate horseradish peroxidise (HRP)-conjugated secondary antibody for 2 h at room temperature. Membranes were then washed (3 x 8 minutes) with TBS/Tween and protein bands visualised using the ECL detection system. This involved incubation of membranes with a mixture of equal volumes ECL solution A (2.5 mM luminol, 0.4 mM p-coumaric acid and 100 mM Tris pH8.5) and ECL solution B (0.002% hydrogen peroxide and 100 mM Tris pH8.5) for 1 minute before exposing membranes to Kodak X-Ray film. Densitometry was performed using Image J software.

2.10.4 *Stripping Western Blots*

Membranes were sometimes stripped between alternate primary antibodies. Membranes were stripped at room temperature for 1 h in stripping buffer

(100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris pH 6.7) then washed thoroughly in TBS/Tween before re-starting the Western Blotting protocol.

2.11 Measurement of Phospholipase D Activity

The PLD assay involves loading the cells with a radiolabelled fatty acid that is then incorporated into the lipid compartments of the cells allowing quantitation of PLD activity via scintillation counting of the radiolabelled product. PLD activity is measured by a transphosphatidylation assay which exploits the finding that primary alcohols preferentially act as acceptors, relative to water, for the PLD product and hence in the presence of ethanol, phosphatidylethanol (PE) rather than phosphatidic acid is generated. This phosphatidylethanol accumulates within the cells as it cannot be metabolised and can be quantitated as it will contain the radiolabelled fatty acids in its diacyl-side chains. Cellular lipids are obtained by the Bligh-Dyer two-phase separation and PE identified relative to standards using thin-layer chromatography. PLD activity can be specifically and directly quantified with the levels of PE in each sample being normalised to the total cell incorporation of the fatty acid radiolabel, thus determining the relative production of PE for each cell treatment.

Murine mast cells were incubated with 2 µCi/ml ^{3}H palmitic acid overnight at 37^{o}C. Following labelling cells were washed twice in HBSS (pH 7.4) supplemented with 1 g/L BSA and 1 g/L D-glucose. Cells were then resuspended at 2x10^{6}/150 µL/treatment in HBSS buffer with 1% ethanol for 20 minutes at 37^{o}C. Where used, inhibitors were added prior to the ethanol for 20 minutes. The cells were then aliquoted into glass tubes. A control sample was kept untreated and immediately lysed after treatment with 1% ethanol. Cell stimulation over the indicated time course was initated by the addition of stimuli and incubation at 37 ^{o}C. Reactions were terminated by the addition of 0.75 mL ice-cold chloroform: methanol (1:2 v/v) for 10 minutes on ice followed by 0.25 mL of chloroform and 0.3 mL 1M NaCl (final ratio 0.9:1:1 aqueous: methanol: chloroform). To achieve phase separation, the samples were vortexed and centrifuged at 2000rpm for 5 minutes.

Whatman silica gel (G-150) TLC plates were pre-run in solvent (2,2,4 trimethylpentane: ethyl acetate: dH_2O: acetic acid (5: 11: 10: 2) in a TLC tank for approximately 2 h then the plates air dried before being activated at 110^{o}C for 15 mins. An aliquot (180 µL) of the lower chloroform phase of each sample was removed into clean glass tubes, spiked with 25 µg/ml phosphatidylethanol and dried by vacuum centrifugation. A further aliquot (180 µL) was removed for direct liquid scintillation counting (total cell incorporation). Each dried sample was re-dissolved in 50 µL chloroform:methanol (19:1) and applied to a pre-absorbent strip of the TLC plate. The plate was then developed in a tank with the solvent mix until the solvent front reached the top of the plate. Phosphatidylethanol was located by exposure of the plate to iodine vapour and the radioactivity of each sample was determined by scraping 1 cm segments and combined with OptiPhase Supermix Cocktail (Perkin Elmer). Liquid scintillation counting was performed using Perkin Elmer 1450 MicroBeta TriLux LSC and Luminescence counter. The activity of PLD was determined by the relevant percentage of ^{3}H palmitic acid incorporation into phosphotidyethanol compared to the total cell incorporation. Each treatment was performed in triplicate.

2.11.1 *Optimization of PLD assay in mast cells.*

Several steps were taken to optimise the experiment to determine the best protocol for mast cells. The cellular incorporation of several candidate radiolabelled fatty acids was analysed in order to optimise the assay in mast cells including oleic acid, myristic acid, arachidonic acid and palmitic acid. [3H] Palmitic acid showed the highest total levels of incorporation as determined by scintillation counting of the cells directly (Figure 2.7 A), presumably reflecting the predominance of di-palmityl PtdCho in these cells. Nevertheless, although the other fatty acids were only poorly incorporated in the cells lipid compartments, stimulation with PMA, a pharmacological activator of PLD, after the percentage of PE produced and thus the activity of PLD was calculated, cells labelled with [3H] arachidonic acid showed the highest production of PE as a percentage of total incorporation (Figure 2.29 B). Although PMA did not induce detectable PE production by myristate-

labelled cells, it also stimulated PE production from oleate- and palmitate-labelled cells (Figure 2.29 C). Thus, although the % production of PMA-stimulated PE was low in palmitate-labelled cells, this assay was most robust due to the high total cell incorporation (cpm). Prior studies with many cell types, including mast cells, had used butan-1-ol as the alcohol acceptor. The inactive butan-2-ol is used as a control as comparison of functional responses in cells treated with these primary and secondary alcohols allows for probing of the role of a (not necessarily PtdCho-specific) PLD signal in such responses and also controls for the effect of adding alcohols to cells. However, in these experiments, no FcεRI stimulation of PE could be detected in the presence of butan-1-ol. However, ethanol is also routinely used as an acceptor, and under these conditions, FcεRI-coupled PLD activity could be detected (Figure 2.7 D). Interestingly from these results an increase in basal activity was noted between 10 minutes and 60 minutes, and a further experiment demonstrated significant PLD activity in unstimulated cells (Figure 2.7 E) after 60 minutes hence in order to determine the level of constitutive PLD activity and its possible effects, experiments incorporated both "zero" time (Control) and "unstimulated" (Basal) controls.

2.12 Measurement of Sphingosine Kinase Activity

To determine sphingosine kinase activity in mast cells an assay was used that exploits a fluorescently-labelled sphingosine analogue JP02 as a SphK substrate, which when phosphorylated by SphK alters its fluorescence emission characteristics, allowing the levels of kinase activity to be determined by quantifying the levels of fluorescence. This assay protocol does not allow the determination of particular isoform involvement however using this method it was possible to identify the activation kinetics of SphK.

Mast cells at 2 ml of $1x10^6$ cells/ml were stimulated with DNP (0.5 µg/ml) or IL-33 (10 ng/ml) for selected timepoints at 37^{o}C. The reaction was terminated with 1 ml ice-cold PBS and washed at 1250 rpm for 5 minutes, 4 ^{o}C. The resulting pellet was dried thoroughly to remove all trace of PBS. Lysis was performed by the addition of 100 µl ice-cold modified RIPA buffer

and the solution passed through a 25 G needle 5 times. After vortexing the cells were incubated on ice for 30 minutes before centrifugation of lysates at 12000rpm for 15 minutes. The protein quantity of the resulting supernatants was analysed by BCA and 160 µg/sample was combined with assay buffer (20 mM Tris-HCl, 20% glycerol, 1 mM ß-mercaptoethanol, 1 mM EDTA and Halt Protease and Phosphatase Inhibitors) to make a final volume of 90 µl. The substrate JF02 (15-NBD-Sph-BSA) (20 µM) followed by 1 mM ATP was added and vortexed well. A sample with cell lysate omitted was used as a control. The samples were then incubated for 30 minutes at 37 ^{o}C. The reaction was stopped with 100 µl 1M potassium phosphate (pH 8.5) followed by 500 µl chloroform:methanol mix (2:1) and the phases separated at 5000 rpm for 5 minutes. The upper phase was removed and 75 µl aliquots made in triplicate in a black 96 well plate (Greiner Bio-one). Equal volumes of the fluorescence enhancer, dimethylformamide (DMF) were added to each well before 3 minutes of gentle shaking. Fluorescence was determined using an EnVision Multilabel reader (Perkin Elmer) at excitation 485 nm, emission 535 nm and gain 300.

2.13 Measurement of DNA synthesis using ^{3}H-Thymidine

To quantify proliferation the level of thymidine incorporation into newly synthesised DNA was determined. 0.2x10^{6} cells were cultured in triplicate in round bottom microtitre plates in the presence of the indicated stimuli for 24 h at 37 ^{o}C. ^{3}H-thymidine (0.5 µCi/well, Amersham) was added for 12 h at 37 ^{o}C before cell harvesting with an automated cell harvester (Perkin Elmer). Incorporated label was analysed by liquid scintillation analysis (Microbeta Trilux, Perkin Elmer).

2.14 Ovalbumin-induced asthma

The ovalbumin-induced asthma model was induced and maintained by Dr Jennifer Coltherd, Department of Immunology, University of Glasgow. Briefly, 6-8 week old female BALB/c mice were sensitized to whole ovalbumin (OVA) by intraperitoneal injection of 100 µg OVA in 1% alum (Alhydrogel; Brenntag Biosector, Fredriksund, Denmark) on days 0 and 14. On day 14 mice were additionally challenged intranasally with 50 µg aerosolised OVA in phosphate buffered saline (PBS) (after anaesthesia was induced with isopentanol). On days 25, 26 and 27 mice were anaesthetised and rechallenged with 50 µg aerosolised OVA in PBS, administered intranasally. Control mice received PBS in place of OVA, Mice were euthanized on day 28. Blood was obtained from each mouse by cardiac puncture. This was centrifuged for 10 minutes to separate serum and blood cells. The serum was transferred to a separate tube and stored at -20^{o}C. Bronchoalveolar lavage was perfomed on the mice by flushing the airways with 1 ml PBS. The BAL was centrifuged and the BAL fluid stored at -20^{o}C.

2.15 S1P ELISA

S1P concentrations were analysed from murine serum and BALf obtained from Dr Jennifer Coltherd, cell lysates and supernatants using a S1P ELISA (Echelon Biosciences) according to manufacturers protocol.

2.15.1 *Treatment of cells for S1P ELISA.*

PDMC were sensitized overnight at 2x10^{6} cells/ml. Cells were stimulated with 100 ng/ml IL-33 in RPMI supplemented with 1% FCS for the described time course. To terminate reaction cells were centrifuged 1250 rpm, 5 minutes at 4^{o}C. The supernatant and cell pellet were retained separately and stored at -20^{o}C.

2.15.2 *Preparation of samples for S1P ELISA*

Murine serum, BALf and cell supernatant was diluted 1:10 with delipidized human serum before use. Cells were lysed using specified lysis buffer (20 mM PIPES, 150 mM NaCl, 1 mM EGTA, 1% v/v Triton X-100, 1.5 mM $MgCl_2$, 0.1% SDS, 1 mM sodium sorthovanadate, 1X Halt Protease Inhibitor without EDTA, pH 7 (Thermo Pierce). Protein concentration was determined by BCA protein assay (Thermo Pierce) and adjusted to 3 mg/ml. The lysate was then diluted 1:10 with delipidized human serum.

2.16 Statistical Analysis

All statistical analysis was performed using Graphpad Prism 5.0 (Graphpad Software Inc). Statistical analysis was by paired or unpaired t-test or one-way ANOVA with Tukey's post-test and p is significant at * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$ and **** $p< 0.0001$.

Figure 2.1 Phenotyping peritoneal wash.

Exemplar plots of mast cell phenotyping by flow cytometric analysis (A-C). Peritoneal washes were performed on 10 week old Balb/C mice. The resulting wash (Initial Wash) was incubated for 2 h at 37^{o}C on tissue-culture treated plates. Cells that adhered to the plate were gently scraped free (Adherent) and cells that remained in suspension (Suspended) were harvested. FSC and SSC parameters of the peritoneal wash (A) and gating (relative to isotype controls, not shown) of the CD117^{+} FcεRI^{+} cells (B) prior to analysis of ST2^{+} expression (C) are shown. Isotype controls represented by grey shaded plots. Frequency of CD117^{+} FcεRI^{+} cells was determined using isotype controls (D), these cells were then further analyzed to determine the % frequency of ST2^{+} cells (E) and the geometric mean fluorescence of ST2 (F). Data is representative of 2 independent experiments.

Figure 2.2 Histochemical phenotyping of peritoneal wash.

Exemplar plots of mast cell phenotyping by toluidine blue (A-B) and tryptase staining (C). Peritoneal washes were performed on 10 week old Balb/C mice. The resulting wash (Initial Wash) was then incubated for 2 h at 37^{o}C on tissue-culture treated plates. Cells that adhered to the plate were gently scraped free (Adherent) and cells that remained in suspension (Suspended) were harvested. The cells were stained with toluidine blue and imaged at 10 x magnification (A). The Suspended population was stained for tryptase (C) alongside toluidine blue (B) and imaged at 10 x and 40 x magnification (B). Data is representative of 2 independent experiments.

Figure 2.3 Histochemical phenotyping of cultured peritoneal-derived mast cells.

Exemplar plots of mast cell phenotyping by toluidine blue (A) and tryptase (B) staining. The suspended cell fraction was prepared from peritoneal washes of 10 week BALB/c mice and the cells cultured *in vitro* for 28 days in RPMI supplemented with 4% SCF-containing KLS-C conditioned media to generate PDMC. Toludine blue and tryptase staining of these cells was performed as described and imaged at 10 x and 40 x magnification. Data is representative of 3 independent experiments.

Table 2.1 Compounds used for stimulation of murine mast cells.

Table 2.2 Inhibitors used for stimulation of murine and human mast cells.

Table 2.3 Antibodies used for fluorescence assisted cell sorting of murine and human mast cells.

Figure 2.4 Exemplar plotting scheme for flow cytometric analysis of murine mast cells

Exemplar plots of mast cell phenotyping by flow cytometric analysis. Size of cells, defined by forward scatter (FSC), and granularity, defined by side scatter (SSC) was used to place an initial gate to exclude cell debris and particles. Single cells were then identified using FSC-A and FSC-H and the viability of these cells determined using 7AAD staining. A positive control of heat-killed cells was used to identify gating position. 7AAD negative cells were then gated for CD117 and FcεRI expression with gates placed using isotype staining. Double positive cells were then further analysed as described for individual experiments.

Figure 2.5 Activity of Fura-2 AM in the detection of calcium mobilisation.

Fura-2 AM works on the assumption that fluorescent light emitted following excitation at two wavelengths reflects the intracellular calcium concentration. Cells loaded with Fura-2 AM are exposed to light altering between two excitation wavelengths. Fura 2-AM is excited by light of two wavelengths depending on its bound (340 nm) or unbound (380 nm) status and emits light at 510 nm [163]. These wavelengths are used to form a ratio (340/380 nm) which reduces the effects of experimental variation such as concentration of Fura-2 AM loading, cell volume or loss of fluorescence over time. The fluorospectrophotometer used for the experiments calculates the concentration of free calcium within the cytoplasm from the levels of light emitted after each excitation as a ratio of bound and unbound. Fura 2 AM has a high affinity and is able to detect changes in concentration occurring slower than 10 ms meaning any changes in calcium detection are clearly visualised [161]. A problem when performing fluorescence-based calcium studies is that too large a concentration of a high affinity indicator, such as Fura-2 can buffer cytosolic calcium [161]. In this thesis a concentration was used that minimised this effect. Similarly the Fura-2 AM can be hydrolysed within the cell, passively or actively be removed from the cells, sequestered in non-cytoplasmic compartments (organelles) or photobleached.

Table 2.4 Cytokine and prostaglandin ELISAs for murine and human mast cells.

Figure 2.6 Exemplar plotting scheme for fluorescent Lamp1 staining of murine mast cells for quantitation of degranulation.

Exemplar plots of mast cell phenotyping by flow cytometric analysis. Size of cells, defined by forward scatter (FSC), and granularity, defined by side scatter (SSC) was used to place an initial gate to exclude cell debris and particles. The viability of these cells was determined using 7AAD staining. A positive control of heat-killed cells was used to identify gating position. 7AAD negative cells were then gated for ST2 and FcεRI expression with gates placed using isotype staining. Double positive cells were then further analysed for Lamp1 expression, positive expression was defined by the peak displaying higher expression.

Table 2.5 Primary and secondary antibodies used in Western blots for the detection of signalling molecules.

Figure 2.7 Optimisation of PLD assay.

PDMC were treated with 5 μCi [^{3}H] palmitate, [^{3}H] oleic acid, [^{3}H] myristic acid or [^{3}H] arachidonic acid and murine anti-DNP IgE (0.5 μg/ml) overnight. Cells were loaded with ethanol (1%) (B-E), butan-1-ol (0.3%) or butan-2-ol (0.3%) (D) for 20 mins at 37^{o}C. After alcohol loading a sample was taken and the reaction terminated (0 mins) (E). Cells were stimulated with PMA (1 μM) (B-D) or unstimulated (E) for 60 mins or the indicated time course. Results represent scintillation counting (A) or a percentage of scintillation count activity of the samples run by TLC to the corresponding total cell incorporation count (B-E). (C) represents treatments with Arachidonic acid omitted. Data represents a single experiment performed in triplicate (A-C). For (D) results were normalized to determine the fold increase using 0 min as a basal value which was assigned the value "1". Data is representative of a single (A-C), at least 2 (D) or 8 (E) independent experiments performed in triplicate.

3 Mast cell subsets and their functional modulation by the *Acanthocheilonema viteae* product ES-62.

3.1 Introduction

3.1.1 *Mast cell development and function*

Whilst mast cells are recognised as playing important roles in initiating inflammation resulting in immunity or driving pathogenesis in a variety of allergic and autoimmune diseases [156, 164], there is increasing evidence of their participation in tissue repair and resolution of inflammation [12, 75, 159], as well as for them exhibiting protective roles in cancer [36].

Such contradictory reports relating to mast cell function most likely reflects that mast cells, which are haematopoietic cells found in all vascularised organs [1, 11, 165], constitute a heterogeneous cell population [3] varying in morphology, function and location with subpopulations being characterised by their differential protease, eicosanoid and proteoglycan content [2-4, 11, 38]. Such heterogeneity arises because bone marrow-derived mast cell progenitors [6, 166], arrive in tissue before they are fully matured [8] allowing the different cytokines, hormones and reactive oxygen and/or nitrogen species produced by various microenvironments to essentially create 'custom-made' site-specific mast cells [3, 11]. Moreover, the functional response of mast cells depends on the stimuli received; for example following classical activation of the IgE receptor, FcεRI, mast cells degranulate rapidly (within seconds) to exocytose prostaglandins and leukotrienes as well as preformed cytokines, tryptase, histamine, heparin and platelet activating factor (PAF) whilst *de novo* synthesised cytokines exhibit a more delayed (hours) release [36, 43].

Mast cells can also be activated independently of FcεRI by cytokines or other pro-inflammatory mediators, reflecting direct interaction with triggering factors such as LPS, parasite molecules or allergic stimuli in the skin or mucosa. Due to their placement at environmental barriers, mast cells are

frequently the first cell type to respond during inflammation [75], as evidenced by the important roles played by mast cells in bacterial and parasitic infections [16, 107-109] where they are able to influence disease progress, both directly via release of pro-inflammatory mediators, and indirectly via their effects on other immune cells, including DC, T and B cells and macrophages [75].

3.1.2 *In vitro mast cell culture*

The development of individual mast cell populations with the ability to interpret subtle signals to mould their response is crucial, allowing the immune response to be tailored in individual areas, increasing efficiency and reducing unwanted or excessive responses, thereby limiting tissue damage and conserving energy. This heterogeneity is mirrored *in vitro* by the different responses generated by mast cell populations created by varying culture conditions. Although most protocols for generating mast cells are similar, even subtle changes have dramatic functional effects, especially when related to *in vivo* mast cells that have their maturation intricately tailored by their environment.

However, isolation of *in vivo* mast cells for direct study can be complicated and expensive. They represent a numerically negligible population in tissues so retrieval of MC, both mechanically and to obtain a suitable purity, is complex [159] and so several techniques have been optimized to generate larger yields with fewer experimental difficulties [167]. However in order to perform larger experiments this quantity still needs to be improved and thus, the current best solution remains the use of *in vitro* differentiated MC.

Further development of *in vitro* culture methods is essential, not just to increase quantity but to select the phenotype most appropriate to the disease model. Mast cells occupying different tissues have distinct characteristics and it is thus important to generate cells comparable to the *in vivo* population of interest. To date, studies are most commonly performed using mast cell lines such as RBL-2H3 and transformed BMMC (tBMMC). As with all transformed cell lines, these cells have limitations as their biology can be

far removed from the primary cells they are meant to represent. Thus, a better solution is to develop the *in vitro* culture and differentiation of primary mast cells. Currently there are several protocols employed to culture primary murine mast cells that produce mast cell populations with distinct phenotypic and functional characteristics. For example, whilst each of these populations can be defined as mast cells by their expression of CD117, FcεRI and ST2, differences in their granular phenotype and response to stimulation indicate their functional heterogeneity.

Protocols developed in the 1980s allowed the differentiation of bone marrow-derived mast cells (BMMC), which can be generated in large numbers and hence are commonly used to study mast cell activation and signaling [168]. These are often used to represent mucosal mast cells [1] although phenotypically, they more closely resemble immature cells and have no identifiable physiological equivalent in tissues [11, 159]. Such BMMC can repopulate both mucosal and serosal mast cell compartments when given to mast cell deficient mice [3]. This latter property further indicates that BMMC may represent precursors of mature tissue mast cells that, given the correct maturation signals, can undergo additional development, that would be difficult to achieve *in vitro* as the signals required are complex and individual to the location [11].

Connective tissue mast cells (CTMC) are traditionally produced by co-culture of BMMC with fibroblasts [169] and are used as a model system to represent serosal MC [1]. More recently, it has been shown that they can also be developed by differentiation from bone marrow precursors using SCF and IL-4 [3, 170]. Although not as frequently studied as BMMC, CTMC have been implicated as being involved in autoimmunity [142] and contact hypersensitivity [171].

Finally, although they constitute <5% of the peritoneal cell population, when cultured with SCF, mature mast cells can be expanded *in vitro* [159]. These cells, peritoneal-derived mast cells (PDMC), are fully mature, serosal-type mouse mast cells that retain most of the morphological phenotypic and functional features of freshly isolated mast cells [159]. PDMC differ in their functional responses when compared to BMMC, producing less lipid mediators,

chemokines and cytokines but exhibiting greater degranulation responses [159].

Culture of all these mast cell models *in vitro* requires SCF, consistent with this chemokine being essential for MC development and survival *in vivo* [172]. BMMC require IL-3, as this is crucial for their development into a uniform population [173, 174], whereas CTMC do not require IL-3 but rather need IL-4 for their differentiation [175]. SCF also ensures the generation of MC rather than basophils, as the latter are also induced as a result of IL-3 treatment [176].

3.1.3 ***ES-62***

The phosphorylcholine (PC)-containing excretory-secretory filarial nematode product, ES-62 exhibits broad anti-inflammatory properties including desensitisation of FcεRI-mediated mast cell responses. Consistent with this, ES-62 displays therapeutic potential in associated mucosal allergic inflammatory disorders such as asthma [126, 177]. As ES-62 is also protective in autoimmune and allergic connective tissue inflammatory pathologies such as arthritis and contact hypersensitivity [126, 177], we have therefore investigated the effects of ES-62 on mature PDMC and also on both connective tissue (CTMC)- and mucosal (BMMC)-type mast cell function in order to better understand mast cell biology as a first step to producing disease tissue-targeted therapeutics based on ES-62 action.

3.2 Aims

Characterisation of phenotypic, functional and signalling differences between three *in vitro* mast cell subtypes.

Identify potential modulation of mast cell responses to FcεRI and LPS by ES-62 and investigate the method of inhibition.

3.3 Results and Discussion

3.3.1 *Mast cell subtype comparison*

As previously discussed there is an increasing requirement for *in vitro* cultured MC. This chapter will investigate the differences observed between BMMC, CTMC and PDMC, MC derived from three culture methods described in the Materials and Methods (Chapter 2.2). After an initial identification of the phenotypic differences between the subsets, the functional responses of differing mast cells subsets to two key stimuli, LPS and antigen-mediated crosslinking of FcεRI, were characterised. Signalling via TLR4 and FcεRI are key activation mechanisms for mast cells and demonstrate the diversity of potential stimulation received by mast cells in disease. To compare the different responses to these stimuli in each mast cell subset, degranulation and cytokine production were analysed. Components released by mast cells including chemokines such as MCP-1 and cytokines such as IL-6 and IL-13, are responsible for the optimum recruitment and activation of innate cells, including mast cells, and adaptive immune cells during infection and inflammation. The relative release of each mediator can vary the immune response to individual pathogens ultimately influencing disease outcome. To provide a mechanism for the differential responses between subtypes, calcium signalling, crucial for the initiation of degranulation and cytokine release in mast cells, and downstream PKCα expression were analysed. The differential response to inhibition by the filarial nematode product, ES-62, was also investigated to highlight and compare the differences between the subtypes and their responses.

3.3.1.1 Phenotypic analysis of mast cell subtypes.

Flow cytometric analysis revealed that all three mast cell phenotypes display a similar size, as defined by forward scatter (FSC), and granularity, as defined by side scatter (SSC), although the CTMC population appear slightly less granular (Figure 3.1 A). FACs analysis of the surface marker expression of the different mast cell populations revealed that essentially all (> 98%) of the cells express both FcεRI and CD117 (cKIT), the classic mast cell markers, as

homogeneous populations (Figure 3.1 B). Similarly, all the mast cell subtypes express the LPS signalling receptor, TLR4-MD2 (Figure 3.1 C), PDMC, peritoneal-derived mast cells, have increased expression when compared to the bone marrow derived mast cells, BMMC and CTMC. ST2, the IL-33 receptor, is also expressed on all cell types, however CTMC appear to have the greatest expression (Figure 3.1 D). This expression of both ST2 and TLR4-MD2 is interesting when the recently proposed inhibitory effect of ST2 on TLR4 signalling is considered [178, 179]. The interaction between these two receptors is discussed further in Chapter 5.

3.3.1.2 Mast cell subtypes exhibit differential granule phenotypes.

After mast cells are stained with toludine blue their granularity, size and morphology is easily identified with clear staining of heparin-containing granules in all the different mast cell phenotypes (Figure 3.2). Staining of the PDMC is predominantly uniform while BMMC, although all positively stained, exhibit varying levels of staining. Mast cells are extremely heterogeneous *in vivo* and their maturation within tissues is driven by micro-environmental factors with, for example, tracheal MC expressing a different protease profile to skin MC, and this is defined by the surrounding tissues [15]. Similarly, this variation in BMMC phenotype is most likely a reflection of the SCF used in cell culture, previously shown to increase heparin expression and granularity in BMMC that normally express little or no heparin [41]. Such plasticity allows BMMC to further differentiate to repopulate both serosal and mucosal compartments *in vivo* [38]. CTMC granule content was found to be even more heterogeneous than that of BMMC. Unlike PDMC and BMMC that have small, dense, strongly stained granules, a population within the CTMC have large, open, vacuole-like, unstained granules giving them a slightly ‘swiss cheese’ appearance. This visible phenotypic difference may explain the slightly reduced granularity seen in the FACs staining (Figure 3.1 A). As the granule profile of mast cells is highly variable and affected by multiple factors it is possible that they are granules that contain different proteases to those found in heparin-containing granules such as carboxypeptidase A [160].

3.3.1.3 Mast cell subsets display different functional responses.

To examine the relationship between subtypes and ensure that variation in MC progenitors, culture and experimental conditions amongst experiments were not affecting responses, BMMC, CTMC and PDMC were derived from the same cohort of mice and mast cell degranulation investigated. Mast cell degranulation in response to FcεRI crosslinking (and PMA/Ionomycin) is observed in all mast cell phenotypes (pooled data from multiple experiments are shown in Table 3.1) whilst LPS does not induce degranulation by any phenotype. Overall, PDMC display greater degranulation responses, both to PMA/Ionomycin a pharmacological stimulus that is independent of receptor signalling, and to Ag-induced crosslinking of FcεRI (FcεRI-XL), when compared to bone marrow-derived MC, BMMC and CTMC. This perhaps suggests that PDMC have received a maturational signal *in vivo* that licences recruitment of degranulation-associated signalling pathways by the PDMC that are uncoupled in bone-marrow derived-MCs.

The observed differential degranulation responses are unlikely to be a result of reduced granularity as CTMC, which have the lowest granularity and toluidine blue staining (Figure 3.1 and 3.2) exhibit higher levels of degranulation than BMMC. It should be noted, however, that the granule constituents are not completely defined for each subtype and may vary. In addition, degranulation here is being assessed on the basis of a single granule component, ß-hexosaminidase, rather than exocytosis in general. Although this does allow a general idea of the ability of cells to exocytose in response to a specific stimuli it does not reveal the nature or relative levels of individual granule components. This is important in a cell type that tailors mediator release depending on the environment and stimulus. Indeed, a method that determines exocytosis [162] by quantitating Lamp1 expression at the cell surface after granule-plasma membrane fusion may give a better overall assessment of the level of degranulation and has been adapted for MC and employed later in this thesis.

MC degranulation stimulates prostaglandin production [180]. Prostaglandins, including PDG2, are synthesised from arachidonic acid released by the cytosolic phospholipase A2 (cPLA2)-mediated breakdown of membrane

phospholipids. PDG2 is one of the initial pro-inflammatory mediators released by MC and, as it is rapidly degraded *in vivo,* its effects, although potent, are often short-lived [181]. PGD2 is produced at high levels by all subtypes, particularly the BM-derived MC, even in the absence of stimulus. FcεRI-XL fails to initiate a strong PDG2 response above basal from PDMC or CTMC compared to that seen with BMMC. CTMC respond better to LPS than FcεRI-XL and produce greater levels of PDG2 than BMMC under these conditions. Collectively, these data suggest that *in vitro*, PGD2 production by mouse MC subtypes may be largely constitutive and not closely coupled to degranulation.

IL-6, IL-13, TNFα and MCP-1 are cytokines important for both homeostatic and inflammatory functions of mast cells. Similarly to what was observed for PDG2 production, PDMC produce less cytokines than either CTMC or BMMC, the later of which produce the greatest overall cytokine responses. Moreover, whilst cytokine production by PDMC and CTMC is greatest after LPS treatment, FcεRI XL of BMMC induces the strongest cytokine responses. BMMC, but not PDMC or CTMC, also produce high levels of TNFα, both spontaneously and after stimulation via FcεRI. It has been reported that BMMC and freshly isolated PDMC derived from C57BL/6 mice exhibited higher levels of degranulation and had lower levels of cytokine production than those from BALB/c mice [182] but this trend was not found to be significantly reproducible in this study (data not shown).

BMMC can differentiate into mucosal or serosal cells [38] and perhaps consistent with this, CTMC appear to be an intermediate between BMMC (mucosal) and PDMC (serosal) as evidenced by their intermediate cytokine and degranulation profiles. BMMC seem to exhibit a functional phenotype more skewed towards cytokine and prostaglandin production whilst degranulation appears to be a more specialised function restricted to the more mature PDMC. As immature MC have been suggested to play a regulatory role in localised immune responses [183], this functional bias may allow their involvement in the early immune response where strong cytokine responses act to recruit inflammatory cells and coordinate the subsequent immune phenotype [45].

3.3.2 *Effect of ES-62 on mast cell function*

3.3.2.1 ES-62 modulates degranulation in mast cell subsets

As each MC subset appears to exhibit differential functional responses, it was important for the development of potential therapeutics to define any variation in their responses to ES-62. ES-62 was found to significantly inhibit the degranulation of BMMC and PDMC resulting from FcεRI- XL (Figure 3.3 A). CTMC degranulation was unaffected however FcεRI- XL does not stimulate a strong response in this subtype. Likewise, degranulation responses to LPS were consistently and uniformly very low, preventing detection of any significant ES-62 effect (Figure 3.3 B). By contrast, PMA/Ionomycin responses were significantly reduced by ES-62 in PDMC. BMMC and CTMC degranulation was reduced but as this was only performed as a single experiment, the reproducible significance of this could not be determined (Figure 3.3 C).

3.3.2.2 ES-62 modulates cytokine production in mast cell subsets

ES-62 was found to significantly inhibit the production of FcεRI-induced IL-6, IL-13 and MCP-1 from BMMC (Figure 3.4 A). By contrast, for LPS stimulation of BMMC, IL-13 release was reduced although ES-62 only significantly inhibited IL-6 production. Neither TNFα nor MCP-1 production was inhibited by ES-62 (Figure 3.4 B) in 2 and 3 independent experiments respectively. Similarly, PMA/Ionomycin-induced IL-6 and IL-13 from BMMC is unaffected by ES-62 treatment (Figure 3.4 C), although this may reflect that ES-62-mediated inhibition of FcεRI signalling occurs further upstream than that mimicked by PMA/Ionomycin stimulation.

Rather similar effects were seen with CTMC although in this case only FcεRI-mediated production of IL-13 (single experiment) (Figure 3.5 A) and LPS-stimulated IL-6 and TNFα production were significantly inhibited by ES-62 (Figure 3.5 B). Although MCP-1 production appears to be routinely inhibited, this is not significant perhaps due to the strong spontaneous production of this chemokine.

Interestingly, although the responses are very low, ES-62 significantly inhibits FcεRI-mediated IL-13 and IL-6 production by PDMC (Figure 3.6 A) and all detectable cytokine responses to both LPS (Figure 3.6 B) and PMA/Ionomycin-induced cytokines (Figure 3.6 C) are significantly inhibited by ES-62.

Unlike with macrophages and DCs, where ES-62 can directly stimulate IL-6, IL-12 and TNFα production [184], ES-62 does not stimulate cytokine production by any MC subtype. Moreover, despite ES-62 differentially inhibiting cytokine production by MC in response to the described stimuli, it does not completely ablate such pro-inflammatory mediator release. This preservation, although at reduced levels, of signalling and the subsequent functional responses indicates that rather than being completely suppressed, MC can still perform functions during an immune response but with an ES-62-tailored phenotype.

3.3.2.3 ES-62 does not affect receptor surface expression

As a first step to investigating the mechanism(s) of action of ES-62 in suppressing degranulation and cytokine production by MC subsets, the effects of ES-62 on surface expression of FcεRI, CD117 and ST2 were determined by flow cytometry. Sensitized (Figure 3.7 B) and non-sensitized (Figure 3.7 A) PDMC were stained for these receptors, crucial for full mast cell function, after treatment with ES-62. Regardless of the sensitization status there was no obvious effect on their surface expression after an 18 h treatment with ES-62. Although TLR4 expression was not analysed, these results suggest that the modulation of MC responses by ES-62 is not a consequence of downregulation of these key mast cell receptors but rather via targeting of their downstream signalling.

3.3.3 *ES-62 targets calcium and PKCα signalling in mast cells.*

As ES-62 does not affect receptor expression, the effect of the parasite product on calcium mobilisation and PKCα expression was investigated. These signalling parameters were examined as both signalling components have previously been identified as important in both MC cytokine and degranulation signalling [185, 186] as well as targets of ES-62 in its inhibition of antigen receptor-mediated B cell [187] and FcεRI-driven human MC [126, 177] responses.

3.3.3.1 Calcium mobilisation in mast cell subsets.

One of the major regulators of mast cell signalling is calcium; intracellular mobilisation of calcium ions (Ca^{2+}), a universal second messenger, is crucial to the response of FcεRI- XL. By contrast, the TLR4 ligand, LPS has previously been reported to fail to stimulate calcium mobilisation in BMMC [31, 188]. Most MC functions have been reported to rely on calcium mobilisation, including proliferation, gene expression, secretion, adhesion and migration of mast cells [189-191].

FcεRI-mediated activation of calcium mobilisation has been reported to involve several components highlighted in Figure 3.8. Following its generation by phospholipase C (PLC) activation, IP_3 binds IP_3R on the membrane of the endoplasmic reticulum (ER) and activates SOCE (store-operated calcium entry) channels, releasing luminal ER calcium. PKC can also activate IP_3R directly, bypassing IP_3, to induce Ca^{2+} release [190]. As a result of this calcium depletion from the ER, STIM1, normally associated with the ER membrane, translocates to the plasma membrane to associate with Orai/CRACM1 [192]. The channel formed by this complex allows an influx of extracellular Ca^{2+} to maintain the intracellular calcium concentration and helps replenish the ER stores [190]. CRAC are not the only SOC in mast cells but are the most dominant [193].

In addition to IP_3, S1P can also initiate calcium mobilisation, however the precise role of S1P is controversial [194, 195]. Whilst S1P can be exported from the cell to trigger calcium influx via cell surface S1P receptor signalling [196, 197], there is also evidence for ER membrane-associated SphK generating S1P that can directly mediate calcium release from the ER [198-200], although it is currently thought that S1P plays a more important role in influx rather than ER release [190, 201].

Although producing equivalent maximum calcium responses, S1P stimulates a more transient calcium signal whereas IP_3 induces more sustained responses reflecting additional influx. Inhibition of SphK suppresses the calcium response but leaves IP_3 signalling intact indicating that S1P is signalling alongside and cooperating with, rather than instead of, IP_3. [194].

The regulation of the concentration, duration, location and pattern of calcium responses [190, 191], is extremely important in determining the outcome of the response in mast cells. In MC, degranulation is dependent on intracellular Ca^{2+} release via SOCE [202] whereas cytokine release is independent of intracellular calcium mobilisation [192, 203] indicating that the phenotype of the calcium response directly affects the subsequent signalling outcomes.

To investigate the role of calcium signalling in all three mast cell subtypes, the cells were stimulated by cross-linking of FcεRI (XL) (Figure 3.9 A) or addition of LPS (Figure 3.9 B). The profiles observed for all subtypes demonstrated similar kinetics after cross-linking (XL), with a rapid increase in intracellular calcium levels within seconds of administration of DNP, the crosslinking agent, at 50s. This is followed by a further, steadier increase to a maximum concentration after a further 50 s before the intracellular calcium signal begins to return to basal levels. Although the maximum cytosolic calcium concentration for each subtype is different (typically, PDMC> BMMC> CTMC), direct comparison is hazardous as the maximum calcium signalling response can be affected by a variety of factors including culture and experimental conditions.

Ca^{2+} movement within the cells comes in many forms; puffs, waves and oscillations. Calcium microdomains are pockets of Ca^{2+} in the cytoplasm formed in the immediate area of release from either ER or influx. Immediately after release these initial localised "puffs" at receptor sites are followed by transduction of the signal in "waves" through the cytosol facilitated by release from the ER. Oscillations are formed as the ER luminal stores replenish using influxed calcium, allowing successive and repeating Ca^{2+} release from ER triggered by IP_3 [190, 204].

The dynamics of calcium fluctuations may define functional responses [204], for example in RBL-2H3, oscillations are linked to degranulation [205, 206]. Although this rapid fluctuation still obeys a pattern of increasing then decreasing levels of total intracellular calcium the amplitude and duration of these oscillations add another level of variation to the calcium response and its outcome. Oscillations reduce the overall threshold of calcium required to activate NFκB, allowing activation even after low levels of stimulation. The frequency of the oscillations also dictates the activation of individual transcription factors; NFAT is only activated after rapid oscillations, whereas NFκB can also respond to lower frequency oscillations. This variety in transcription factor activation is translated into cytokine production [207]. These oscillations cannot be determined using this experimental method and require single cell analysis, a potential future experiment.

Stimulation with LPS (Figure 3.9 B) induces a different profile of calcium mobilisation as the increase in intracellular calcium concentration is slower and does not reach the same maximum levels observed following crosslinking of FcεRI (XL). Moreover, the different mast cell subtypes clearly have varied responses as demonstrated by PDMC that display only a weak response to LPS relative to that seen with CTMC and particularly, BMMC. Degranulation is regulated by calcium [190, 191], so the weak calcium profile seen in response to LPS stimulation compared to XL may be inadequate to initiate degranulation (Table 3.1).

To determine the relative contribution of intracellular and extracellular calcium sources in the responses induced by XL and LPS, the involvement of each was inhibited by BAPTA and EGTA respectively as these ion-chelators

have extremely high affinity with Ca^{2+} [161]. BAPTA-AM, a potent cell permeable calcium chelator, binds and sequesters intracellular calcium ions. Removal of this calcium signal ablates the calcium responses to stimulation with either LPS or XL in all subtypes, resulting in nothing more than basal calcium movement. This demonstrates the essential role of the initial release of calcium from cytosolic stores, such as the ER, in initiating the subsequent influx of extracellular calcium in mast cells.

To remove extracellular calcium sources, calcium can be omitted from the buffer and a calcium chelator, EGTA, added. Stimulation of cells under these conditions preserves the initial release of calcium from intracellular stores and consistent with this, after XL, in the absence of extracellular calcium, the initial rapid peak of calcium is observed but not the secondary steady increase, resulting in a rapid return to basal levels. This initial peak represents the involvement of intracellular calcium release, which is rapid and transient in mast cells after XL. The different subtypes have a maximum concentration produced by the intracellular stores of 100-200 μM, typically <50% of the concentration observed in the presence of extracellular calcium. By contrast, when cells were stimulated with LPS in the absence of extracellular calcium, calcium mobilisation was essentially abrogated with only a small intracellular calcium mobilisation signal remaining in the BMMC.

The variation in calcium responses to FcεRI (XL) between subtypes is unlikely to be due to variable levels of receptor expression between the subtypes (Figure 3.1 B) but is potentially a reflection of the differing expression of calcium mobilisation machinery or the effects of regulatory mechanisms on signal strength propagated upstream of calcium release.

Although LPS has not previously been reported to stimulate calcium in BMMC [31, 188], it has been reported to increase FcεRI-driven calcium mobilisation by upregulating the Orai1 and Stim1 subunits of the SOC channel RBL-2H3 [49]. Here LPS was found to induce varying levels of calcium amongst the mast cell subtypes. As with FcεRI signalling, the strength of LPS-induced calcium mobilisation is perhaps unlikely to be regulated by TLR4 expression as the weak calcium profile observed in PDMC does not reflect the rank order of TLR4-MD2 expression (Figure 3.1 C). The ability of BMMC to respond most

strongly to LPS and to exhibit a greater involvement of mobilisation of intracellular calcium may be a reflection of their stage of maturation. As BMMC appear to represent a more immature MC, perhaps there is a bias towards TLR4 signalling which allows, for example, shaping of the mast cell activity in response to bacterial infection as occurring in a physiological setting where they represent MC as they first enter tissues. During infection MC are one of the first cell types to respond and progenitors are further recruited during infection [45]. Perhaps these freshly recruited cells are hardwired to be more sensitive to TLR ligands so they can respond immediately, before full maturation [183].

Consistent with the key role played by calcium mobilisation in MC responses, both FcεRI- and LPS-mediated calcium mobilisation can be inhibited by ES-62 pre-treatment (18h; Figure 3.10). In both cases, this inhibition appears to target the influx of extracellular calcium and this may reflect that whilst LPS/TLR4 signalling induces SOCS, ES-62 in subverting TLR4 signalling acts to suppress assembly of this calcium mobilisation machinery. ES-62 does not affect the loading of Fura 2/AM or basal calcium levels in PDMC as represented by the equivalent calcium concentration before addition of treatment observed without baseline adjustment.

The "classical" PKC, PKCα has been reported to be involved in the coupling of Fc receptors to calcium mobilisation via PLD (Figure 1.4) [126, 208] and in addition, Ca^{2+} and DAG act to further recruit and activate PKCα [209]. ES-62 has previously been shown to desensitise human FcεRI-mediated MC responses and consistent with this, it was found that ES-62 could induce downregulation of PKCα (Figure 3.11 A and HK Tay and MM Harnett, unpublished) in PDMC, BMMC and CTMC [134], confirming the results seen in human mast cells [126] and the generality of this effect of ES-62 in MC, regardless of subtype.

During activation PKC translocates to plasma membrane where it is able to phosphorylate membrane-localised substrates. This response only lasts 2-5 minutes before PKC is returned to the cytosol [209]. Prolonged activation of PKC happens at a more peri-nuclear location, perhaps at the ER or in recycling endosomes [209, 210]. The downregulation of PKCα activity and expression has been investigated in rat intestinal epithelial cells where PKCα

activity can be reduced by two methods, potentially acting simultaneously. Firstly, caveolar internalisation to a peri-nuclear location, PKCα dephosphorylation and subsequent proteasome-independent degradation using a lysosomal route and alternatively, ubiquitination of phosphorylated PKCα at the plasma membrane then degradation by the 2G S proteasome [211].

Previous work suggests that ES-62 uses the lysosomal route to modulate PKCα signalling. Thus, in human MC, ES-62-mediated PKCα downregulation could be prevented by nystatin, an inhibitor of caveolae/lipid raft trafficking [126] whilst in B cells, the cysteine protease inhibitor, leupeptin, preserved PKCα expression after ES-62 treatment indicating lysosomal involvement [212]. To identify the regulatory mechanism(s) involved in ES-62-mediated downregulation of PKCα in the three mouse MC subsets, inhibitors of PKCα localisation and processing were used to investigate both regulation pathways (Figure 3.11 B). To exclude inhibitor toxicity influencing the results, FACs analysis of cell viability was determined. Overall cell death, as determined by % of cells incorporating 7AAD, did not exceed 6% regardless of the inhibitor concentration or the duration of incubation (Figure 3.12).

With CTMC, similar results to human mast cells were observed as nystatin and the lysosomal inhibitors, E64d plus pepstatin A, but not lactacystin (proteosomal) preserved PKCα expression, preventing ES-62 downregulation. However, with PDMC and BMMC, the picture was more complicated with both routes involved in BMMC and incomplete involvement in PDMC.

This differing response between subtypes, summarised in Figure 3.11 C, confirms previous reports that PKCα regulation can occur using both pathways simultaneously, even in response to a single ligand. The relative role of each pathway is suggested to be defined by temporal and spatial factors [209, 211] and perhaps for MC the level of maturity and culture conditions. The different PKCα regulatory profiles seen in each MC phenotype provide a mechanism for the fine-tuning of MC responses and allow PKCα to be a point whereby receptor signal strength could dictate downstream signal strength.

3.3.4 *Is CD200R a potential target of ES-62 in mast cells?*

Preliminary data from a microarray screen of targets of ES-62 suggested that CD200R was upregulated by small molecular analogues (SMAs) of the parasite product that mimicked it's anti-inflammatory actions (J Rzepecka & W Harnett, unpublished) and so to address whether ES-62 was mediating its desensitising effects on FcεRI- and LPS-coupled responses in MC cells via this receptor, functional responses of MC from WT and CD200R-deficient mice were compared. CD200R is a member of the Ig-superfamily, generally restricted to cells of myeloid lineage [213] and perhaps consistent with its potential as a mediator of the effects of ES-62, CD200R -/- mice [157] display increased symptoms of EAE and CIA due to over-activation of monocytes and macrophages [214, 215]. However, although these mice respond to LPS with slightly elevated cytokine responses, they did not display uncontrolled inflammatory responses indicating that CD200R is non-essential in TLR4 signalling regulation. Corroborating studies demonstrated that prevention of CD200R ligation in CD200-/- mice allows for TLR2 and TLR4 upregulation and an increase in LPS-induced cytokines [213]. Moreover, and of particular interest to the present studies, mouse mast cells grown *in vitro* express CD200R, [216] and CD200 ligation of CD200R was found to inhibit FcεRI-mediated mast cell degranulation without the need for co-ligation [217, 218]. Overall, therefore, these studies suggested a role for CD200R in limiting both FcεRI- and LPS-mediated responses in mast cells and hence suggested that CD200R could be a potential molecular target for ES-62.

3.3.4.1 PDMC from WT and CD200R KO exhibit comparable functional and signalling responses

As a first step to determining whether CD200R was important in the ES-62-mediated regulation of either TLR4 or FcεRI signaling in mast cells, phenotyping of PDMC from WT and CD200R -/- C57BL/6 mice was performed to rule out any developmental or phenotypic effects. PDMC from CD200R -/- mice show comparable expression of FcεRI, ST2 and TLR4 expression to WT cells (Figure 3.13), indicating that any effects seen in CD200R -/- cells are not a consequence of modulation of such receptor expression.

Analysis of FcεRI- and LPS -mediated functional responses of PDMC derived from WT and CD200R -/- mice showed no significant differences in terms of degranulation (Figure 3.14 B) or cytokine secretion (Figure 3.14 A). This was somewhat surprising given the postulated negative regulatory role of CD200R in these cells but these findings were supported by analysis of calcium mobilisation after FcεRI XL (Figure 3.14 E) or LPS treatment (Figure 3.14 F) and PLD activation, which showed negligible effects of CD200R deficiency on these responses induced by FcεRI-signaling (Figure 3.14 C, D). The only clear effect was an increase in PMA/Ionomycin-induced IL-13 production in the CD200R -/- cells. Although these data are somewhat at odds with previously published studies they may be restricted to the PDMC phenotype.

An alternative explanation for the seemingly redundant role for CD200R in MC is that the effects of CD200R may be very subtle in mast cells. BMMC were found to require an overexpression of CD200R and for the receptor to be crosslinked, preferably by membrane bound CD200, to inhibit responses [218, 219]. Moreover, MC *in vivo* may be more susceptible to CD200R inhibition as freshly isolated skin MC have more CD200R than cultured MC and in a model of PCA, activation of the receptor using anti-CD200R antibodies inhibited IgE-dependent vascular permeability [218]. Nevertheless, the results presented here suggest that ES-62 is unlikely to mediate its effects, at least on PDMC, via CD200R-signaling.

3.4 Conclusions

Mast cells are extremely heterogeneous and their maturation within tissues is driven by micro-environmental factors. In this study, the three murine mast cell subtypes display strong variation in receptor expression, functional response and signaling profiles (Table 3.2) reflecting previous findings that the mast cell phenotype is very flexible and can change in response to helminth infection [15]. Consistent with this, ES-62, derived from helminths, has been shown to inhibit MC responses. Collectively, ES-62 induces a hyporesponsive functional response in both serosal and mucosal MC after a variety of stimuli (Table 3.2). Both immature MC, represented by BMMC and

mature MC, PDMC and CTMC, can be modulated by ES-62. The ability to inhibit even immature MC presents the possibility that ES-62 can alter MC function during maturation, allowing ES-62 to alter MC phenotype early in their development regardless of the potentially proinflammatory maturation signals in the microenvironment. This mirrors the response previously seen in bone-marrow progenitors of macrophages and dendritic cells exposed to ES-62 *in vivo*, under normal or inflammatory conditions, which can develop an anti-inflammatory phenotype *in vitro*, even after ES-62 withdrawal. [131, 135]. It would be interesting to determine if ES-62 is able to modulate immature MC throughout their maturation and to establish whether any changes in phenotype are stable.

ES-62 does not appear to act by modulating the cell surface expression of these receptors key to MC function. Rather, together with the differential effects of ES-62 on calcium and PKCα responses, these data indicate that the effects of ES-62 are on downstream signaling elements, which depend not only on the cell but also on the stimulus. The differing effect of ES-62 on each response may reflect that ES-62 has selective actions, important if being used in a therapeutic setting where it is crucial to preserve homeostatic MC activation. [37]

The ability of mast cells to differentiate into two functionally distinct subsets from the same progenitors with the only difference in culture conditions being the presence of IL-3 (BMMC) or IL-4 (CTMC) highlights their delicate interpretation of surrounding influences not only in their maturation but also in their subsequent responses. This makes MC both an excellent target for therapeutics, but also a tricky cell to manipulate into a particular, anti-inflammatory phenotype. Although mast cell targeting drugs are limited in both their specificity and efficacy, there is huge potential for further development. ES-62 may provide a starting block for a tailored therapeutic that preserves some function while removing pro-inflammatory features of their response.

Figure 3.1 Phenotyping of mast cell subsets

Exemplar plots of mast cell phenotyping by flow cytometric analysis (A-D). FSC and SSC parameters of PDMC, BMMC and CTMC cultured *in vitro* for 28 days (A) and gating (relative to isotype controls, not shown) of the $CD117^+$ $Fc\varepsilon RI^+$ cells (B) prior to analysis of TLR4-$MD2^+$ expression (C). Expression of ST2 on the $CD117^+$ $Fc\varepsilon RI^+$ cells was performed in parallel (D). Isotype controls are represented by grey shaded plots. Data are representative of 3 independent experiments.

Figure 3.2 Histochemical phenotyping of mast cell subsets.

Exemplar images of mast cell phenotyping by toluidine blue staining. BMMC, CTMC and PDMC derived by *in vitro* culture for 28 days, were stained with toluidine blue. Representative images were taken at 10 x and 40 x magnification. Data are representative of 3 independent experiments.

Table 3.1 Summary of mast cell subset responses to FcεRI, LPS and PMA/Ionomycin

Statistical analysis between treated cell values and basal values determined using unpaired, two-tailed t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 3.3 ES-62 modulates degranulation of mast cell subsets.

PDMC, BMMC and CTMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) then stimulated with either DNP (0.5 μg/ml) to induce FcεRI cross-linking (XL) (A), LPS (0.5 μg/ml) (B) or PMA (1 μM) plus Ionomycin (1 μM) (C) for 30 mins at 37^{o}C. Degranulation was determined as the % β-hexosaminidase released relative to the total activity of the cells. Results are from a single experiment representing at least 2 independent experiments. Error bars represent SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$ and *** $p < 0.001$.

Figure 3.4 ES-62 modulates cytokine production from BMMC.

BMMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) then stimulated with either DNP (0.5 μg/ml) to induce FcεRI cross-linking (XL) (A), LPS (0.5 μg/ml) (B) or PMA (1 μM) plus Ionomycin (1 μM) (C) for 24 h at 37^{o}C. Release of IL-13, IL-6, MCP-1 or TNFα was measured by ELISA. Results are from a single experiment representative of at least 2 independent experiments. Error bars represent SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.5 ES-62 modulates cytokine production from CTMC.

CTMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) then stimulated with either DNP (0.5 μg/ml) to induce FcεRI cross-linking (XL) (A), LPS (0.5 μg/ml) (B) or PMA (1 μM) plus Ionomycin (1 μM) (C) for 24 h at 37^{o}C. Release of IL-13, IL-6, MCP-1 or TNFα was measured by ELISA. Results are from a single experiment representative of at least 2 independent experiments. Error bars represent SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. ** $p < 0.01$ and *** $p < 0.001$.

Figure 3.6 ES-62 modulates cytokine production from PDMC.

PDMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) then stimulated with either DNP (0.5 μg/ml) to induce FcεRI cross-linking (XL) (A), LPS (0.5 μg/ml) (B) or PMA (1 μM) plus Ionomycin (1 μM) (C) for 24 h at 37^{o}C. Release of IL-13, IL-6, MCP-1 or TNFα was measured by ELISA. Results are from a single experiment representative of at least 2 independent experiments. Error bars represent SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 3.7 Effect of ES-62 on mast cell phenotype.

Plots of mast cell phenotyping by flow cytometric analysis (A-D). CD117, FcεRI and ST2 expression by resting PDMC, BMMC and CTMC (A) or mast cells sensitized (B) with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) are shown. Isotype controls are represented by grey shaded plots.

Figure 3.8 Role of calcium signaling in FcεR1-mediated responses.

Following FcεRI crosslinking, PLCγ converts PIP_2, associated with the plasma membrane, to IP_3 and DAG. IP_3 binds IP_3R on the endoplasmic reticulum (ER) and activates SOCE (store-operated calcium entry), releasing luminal ER calcium. PKC can also activate IP_3R directly, inducing Ca^{2+} release, bypassing IP_3 [190]. Calcium depletion from the ER activates STIM1, normally associated with the ER membrane, to translocate to the plasma membrane to associate with Orai/CRACM1 [192]. The channel formed by this complex allows an influx of extracellular Ca^{2+}.

S1P can also initiate calcium mobilisation, however the role of S1P is controversial. [194, 195]. S1P can be exported from the cell to trigger calcium influx via S1P-R signaling [196, 197] and/or directly stimulate calcium release from the ER [198-200], although recent data suggest that S1P plays a more important role in mobilising influx rather than ER release[190, 201].

To regulate intracellular calcium concentrations, CRAC channels are inactivated by a calcium-dependent mechanism and define PMCA, found associated with the plasma membrane in mast cells, pumps Ca^{2+} back out into the extracellular space, returning cytosolic levels to basal. Cytosolic calcium is also removed by define SERCA on the ER, which replaces depleted luminal ER stores. Ca^{2+} is also released and taken up by mitochondria that remove cytosolic calcium adjacent to ER and CRAC channels promoting further ER store depletion and increasing the duration of CRAC activation [189, 190].

Figure 3.9 Calcium mobilisation in mast cell subsets.

Fura-2/AM loaded resting or IgE-sensitised (murine anti-DNP IgE; 0.5 µg/ml) sensitized BMMC, CTMC and PDMC were stimulated at 50s with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (A) or 0.5 µg/ml LPS to stimulate TLR4 (B). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Error bars represent SD.

Figure 3.10 ES-62 modulates calcium mobilisation in mast cells.

PDMC were sensitized (murine anti-DNP IgE; 0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml). Following Fura-2/AM loading the PDMC were stimulated at 50s with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI (A) or 0.5 μg/ml LPS to stimulate TLR4 (B). The intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Error bars represent SD. Statistical analysis using paired t-test. **** $p < 0.0001$.

Figure 3.11 ES-62 modulates PKCα expression in mast cell subsets.

CTMC, BMMC and PDMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight then cultured with ES-62 (2 μg/ml) for the indicated times and expression of PKCα analysed by Western blotting (A). Sensitized CTMC, BMMC and PDMC were preincubated for 1 h with inhibitors of proteosomal degradation (10 μM lactacystin), caveolae/lipid raft trafficking (50 μg/mL nystatin) and lysosomal degradation (E64d + pepstatin A, both 10 μg/mL), before culture with ES-62 (2 μg/ml) for the indicated times and expression of PKCα analysed by Western blotting (B). β-actin was used as a loading control. Results summarised are representative of at least 2 independent experiments (C).

Figure 3.12 Endosomal and Proteosomal Inhibitors do not affect mast cell viability within 18 hours of culture.

PDMC viability following treatment with indicated concentrations of inhibitors for 6 h and 18 h, was assessed by 7AAD uptake using flow cytometric methods as described in Materials and Methods 2.6. Data are from a single experiment.

Figure 3.13 CD200R expression does not affect FcεRI, ST2 or TLR4 expression of murine mast cells.

PDMC derived from WT or CD200R -/- C57BL/6 mice was phenotyped by flow cytometric analysis. FcεRI (A), ST2 (B) and TLR4 (C) expression are shown. As TLR4 expression was very low in these cultures, the TLR4+ gate is also shown on an expanded scale to allow comparative analysis (D). Isotype controls are represented by grey shaded plots and data are from a single experiment.

Figure 3.14 CD200R expression does not affect FcεRI or TLR4-induced responses or PLD and calcium signaling in murine mast cells.

PDMC derived from WT or CD200R -/- C57BL/6 mice sensitized with murine anti-DNP IgE (0.5 µg/ml) were stimulated with DNP (0.5 µg/ml) to induce FcεRI cross-linking (XL), LPS (0.5 µg/ml) or PMA (1 µM) plus Ionomycin (1 µM) for 24 h (A) or 30 min (B) at 37^{o}C. IL-13 release in culture supernatants was analysed by ELISA (A). Degranulation was determined as the % β-hexosaminidase released relative to the total activity of the cells (B). PDMC treated overnight with murine anti-DNP IgE (0.5 µg/ml) and 5 µCi [^{3}H] Palmitate (C, D) were incubated with 1% ethanol for 20 mins at 37^{o}C then stimulated with DNP (0.5 µg/ml) to induce cross-linking (XL) or LPS (0.5 µg/ml) for the indicated time course. Results represent the level of [3H]PtdEtOH expressed as a percentage of the total [3H] palmitate incorporated into cells. PtdEtOH was resolved by TLC relative to standards (C) then baseline adjusted for each timepoint (D). Results from a single experiment (A-D). Error bars represent SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. **** $p < 0.0001$. Fura-2/AM loaded, murine anti-DNP IgE (0.5 µg/ml) sensitized WT or CD200R -/- PDMC were stimulated at 50s with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (E) or 0.5 µg/ml LPS (F). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (-Ca^{2+}). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples (baseline calcium values subtracted) from a single experiment representative of at 2 independent experiments. Error bars represent SD.

Table 3.2 Murine mast cell characterisation.

A summary of mast cell subtype generation and responses to FcεRI crosslinking and LPS treatment.

Table 3.3 ES-62 modulation of murine mast cell responses.

A summary of functional response modulation in mast cell subtypes by ES-62 treatment.

4 IL-33 signaling in murine mast cells and the involvement of IgE sensitization.

4.1 Introduction

Recently identified as the ligand for the orphan receptor ST2, the cytokine IL-33 is a member of the IL-1 superfamily, alongside IL-1α, IL-1ß and IL-18 [220]. Despite its relatively recent emergence, IL-33 is recognised as being involved in many diseases including allergy, cardiovascular disease and rheumatoid arthritis, where it generally plays a proinflammatory role [221, 222].

4.1.1 *Production of IL-33*

IL-33 is produced by a wide variety of cells. Thus, in mice, IL-33 (mRNA) is detected in resting DC, resting and activated macrophages [223] as well as in epithelial cells of the stomach, lung, gastrointestinal tract, skin and in the spinal cord and brain [220]. Lower quantities are found in lymphoid tissue, spleen, pancreas, kidney and heart [220, 224]. Similarly, in humans, IL-33 mRNA expression has been recorded in smooth muscle cells, endothelial and epithelial cells, activated dermal fibroblasts and keratinocytes with lower quantities in activated DC and macrophages [220]. Although IL-33 mRNA is expressed by many cells, the processing and secretion mechanism(s) required to generate the bioactive cytokine have still not been defined. Initially it was speculated [225, 226] that caspase 1[220], caspase 8 and calpain [227] mediated the appropriate cleavage of IL-33, in a similar manner to their processing of other IL-1 family members. However, although IL-33 can be cleaved by caspase 1 *in vitro* [220], recent studies using knockout mice and caspase inhibitors indicate that the processing and release of IL-33 are independent of caspases, which rather inactivate IL-33 and prevent its signaling via ST2 [223, 224]. Indeed, the full-length form of IL-33 has now been confirmed to exhibit biological activity [228-231]. Interestingly, therefore, although cleavage by caspases and calpain inactivate IL-33,

processing at different sites by neutrophil-derived proteases such as cathepsin G or elastase, has been demonstrated to increase IL-33-mediated ST2 activation.

IL-33 is found in high levels in conditioned medium from necrotic structural, but not haematopoietic, cells [232]; this led to the proposal that IL-33 was released from necrotic or damaged cells as an “alarmin”, a warning that barriers had been breached and that an inflammatory response is required. Full length IL-33 appears to be localised and stored within the nucleus, from where it can be released after necrosis and cell damage [224]. Recently, however, the release of IL-33 from fibroblasts has been associated with mechanical stress rather than necrosis, indicating that living cells can also secrete IL-33: this mechanism involves export via nuclear pore complexes and trafficking through the cytoplasm in vesicles prior to being released from a viable cell [233] and appears to be preceded by signaling to increase calcium mobilisation.

4.1.2 *IL-33 and Disease*

IL-33 interacts with many cells of the immune system, including Th2 cells, eosinophils, basophils, DCs, macrophages, NK cells and mast cells [64, 234] but due to the nature of its release from damaged cells, IL-33 can initiate proinflammatory responses, inducing cytokine release from innate cells such as MC, which express ST2, as evidenced by this response being dramatically reduced in ST2-/- mice [232]. Consistent with this, in mucosal inflammation, IL-33 plays a dominant role in the initial response prior to the involvement of the adaptive immune response [235].

Thus, in situations where epithelial barriers are breached by pathogens, the release of IL-33 from damaged cells may be one of the first signals in mobilisation of immune responses. Indeed, IL-33 release from damaged epithelia is involved in the clearance of parasites, including helminths, as such IL-33 can act on nuocytes [236] and mast cells to induce the production of IL-13, crucial for activating smooth muscle hypercontractability and mucus

production from goblet cells allowing physical expulsion of the parasite [237]. This role is seen with *Trichuris muris* where IL-33 administration in a model of the intestinal parasite infection induces worm expulsion and prevents a Th1 response, reducing pathology [238]. In a non-mucosal setting, IL-33-ST2 signaling has also been shown to be important for protection against *Toxoplasma gondii*-induced encephalitis [239].

Although IL-33 function can be beneficial to the host in parasite responses, the enrichment of IL-33 at epithelial surfaces can exacerbate pathology at these sites as evidenced by increased eosinophil recruitment and induction of IL-13 (mRNA) that promotes remodelling of the epithelium and development of cutaneous fibrosis following subcutaneous administration of IL-33 [240]. Consistent with this, levels of IL-33 are found to be increased in human psoriatic plaques and administration of IL-33 induces skin lesions in murine models, and these are characterised by the presence of mast cells, neutrophils and increase serum levels of IL-5, IL-13, SCF and MCP-1 [241]. Moreover, in terms of lung pathology, *in vivo* administration of IL-33 to C57BL/6 mice induced eosinophilia, IgE and IgA accumulation, epithelial hyperplasia and mucus hypersecretion [220] and reflected the well documented role of IL-33 in a variety of different experimental models of asthma, allergy and lung inflammation [64, 220, 221, 242-252]. Interestingly, therefore, IL-33 has also been implicated in the initiation and development of autoimmune disease where it has been reported to exacerbate pathology in models of arthritis, ulcerative colitis, Crohns disease and SLE [61, 64, 234, 253-255].

As indicated by its role in parasite expulsion, expression of IL-33 can also be protective and this has recently been seen in inflammatory disease, where its ability to reduce adipose tissue development and tissue inflammation demonstrated a cardio-protective function of IL-33 in atherosclerosis. Similarly the fatal hyper-activation of the immune response associated with sepsis is reduced by IL-33 [64, 234, 253]. Relating to this latter effect in sepsis, IL-33 has been shown to be protective with respect to infection with a range of diverse pathogens [256-258] and consistent with this, ST2 -/- mice are more susceptible to polymicrobial sepsis via reduced bactericidal activity of macrophages and neutrophils [259, 260].

4.1.3 *IL-33 and Mast cells*

Mouse mast cells express ST2 [220, 261-263] at higher levels than other leukocytes [261]. ST2 is expressed on mouse mast cells throughout development from the earliest stages and preceding FcεRI expression [6]: however, it is thought that ST2 signaling is unlikely to be involved in transducing differentiation of mast cells, rather it most likely has a role in enhancing survival and modulating function [261]. By contrast, in humans, ST2 does not appear to be expressed on mast cells throughout their development [62].

There are several potential splice variants of ST2, including membrane bound and soluble forms (sST2): normally, mast cells express ST2 at their surface but upon stimulation with calcium ionophore, to mimic IgE crosslinking, production is switched to the soluble form [42, 262, 264, 265]. Soluble ST2 acts as an antagonist decoy receptor by preventing IL-33-mediated ligation of surface ST2 [266] and hence this process is proposed to limit mast cell activation via a negative-feedback loop mechanism. In contrast, when mast cells are stimulated with IL-33, surface expression of ST2 is upregulated [267], creating a positive feedback loop to amplify the signal and thus the response.

There is increasing evidence that IL-33 activates mast cells with reports of induction of IL-6, IL-13, IL-1ß, GM-CSF, MCP-1 and MIP1α release from BMMC [42] in the absence of IgE sensitization and degranulation [42, 61, 62, 268]. IL-33 is also able to induce MCP6 release, the murine homologue of tryptase, an important mediator in many mast cell processes [269]. These responses promote inflammation by inducing leukocyte infiltration and activation *[61].* Interestingly, IgE sensitization appears to enhance IL-33-mediated survival [62], eicosanoid and cytokine production of human mast cells, perhaps reflecting that IgE sensitization increases ST2 expression[268]. However, as with murine mast cells treated with IL-33 *in vitro* [42, 60, 268] or *in vivo* [155], no degranulation has been observed in human mast cells treated with IL-33 [62].

4.1.3.1 IL-33 signaling in mast cells

IL-33 mediates its effects by binding to, and signaling through the receptor ST2 [220]. Initiation of signaling after IL-33 ligation of ST2 is dependent on the recruitment of IL-1RAcP [267, 270-272] which forms a complex with ST2 via their TIR domains [272], allowing signal propagation from the plasma membrane (Figure 4.1). The importance of this coreceptor is seen *in vivo* when the effects of IL-33 on cytokine expression are reduced by the expression of a dominant negative form of IL-1RAcP. Recently in human and mouse mast cells, ST2 has also been found to associate with cKIT, the SCF receptor, and it has been proposed that IL-1RAcP and cKIT are constitutively associated and complex with ST2 after its ligation by IL-33 [273]. Although the forming of this complex is not essential for IL-33 to induce a reaction from mast cells, it allows a full response. In this way IL-33 can synergize with SCF to amplify chemokine and cytokine production and perhaps increase survival [261, 273, 274].

In vitro studies using BMMC demonstrated that MyD88 is essential for IL-33 signaling [268] and results in coupling, via IRAK1 and IRAK4 and TRAF6/TAK1, to the subsequent activation of NFκB and ERK, JNK and p38 MAPKs [60, 220, 273] (Figure 4.1). Similarly, it has been reported that, in human mast cells, IL-33 activates ERK, JNK and p38 MAPKs as well as protein kinase B (PKB) and NFκB [62, 273, 274].

4.1.4 *IL-33 and IgE sensitization*

It has been reported that although MC can be directly activated by IL-33, such responses are enhanced by sensitization with IgE [42, 61, 62, 268].

Classical FcεRI-mediated activation of mast cells involves antigen-mediated crosslinking of IgE-bound FcεRI, resulting in receptor aggregation and initiation of signaling cascades, required for production of cytokines and eicosanoids, as well as degranulation [156]. The initial binding of IgE to FcεRI has traditionally been thought to be a “silent” interaction with no biochemical outcome for the MC. This idea has been increasingly challenged

by data suggesting that the interaction between IgE and its receptor increase development and survival of mast cells as well as triggering induction of cytokine production in the absence of receptor crosslinking [275-278].

The precise mechanism of this IgE-mediated activation, which appears to involve components of classical FcεRI signaling such as Lyn and Syk [279], is unclear with three potential mechanisms described; (1), FcεRI signaling after ligation by IgE; (2), low levels of FcεRI aggregation within the membrane triggered by binding of IgE initiates signaling [17, 277]; (3) signaling by an as yet undefined receptor that binds IgE, either via the Fab portion of IgE or a non-specific interaction [276]. The low-affinity IgE receptor FcεRII (or CD23) is not expressed in BMMC [164]. Interestingly, therefore, whilst treatment of BMMC with monomeric IgE stimulates production of IL-4, IL-13, IL-6 and TNFα mRNA, this induction is not mirrored by cytokine secretion [277], suggesting that IgE may prime mast cells but that antigen-induced crosslinking is required to provide signals for release of mediators from the cell. Relating to this, *In vivo* experiments demonstrated IgE binding to FcεRI increases IL-6, MCP-1 and IL-1β mRNA [171], irrespective of the specificity of the IgE. It has been proposed [171] that this low level of IgE sensitization is crucial for normal immune function by priming mast cells for the subsequent antigen response, with repeated low level sensitizations such as in allergic responses or parasite infections perhaps further altering the subsequent MC response.

4.2 Aims

To investigate the role of IgE sensitization in IL-33-ST2 responses in murine mast cells

To identify further potential key signaling components, including PLD and SphK involved in IgE sensitization and priming of mast cells as well as their potential roles in FcεRI- and ST2-mediated activation of murine mast cells.

4.3 Results and Discussion

4.3.1 *Murine mast cells respond to IL-33 to produce cytokine; signaling is ST2-dependent and increased by IgE sensitization.*

It has been widely demonstrated that mast cells express ST2; PDMC, BMMC and CTMC all demonstrate ST2 expression as shown earlier (Figure 3.1) with PDMC exhibiting the greatest expression, perhaps reflecting their increased maturation. Consistent with their expression of ST2, all subtypes demonstrated a dose-dependent increase in cytokine production, either IL-6 or MCP-1, in response to IL-33 treatment that plateaus at 50-100 ng/ml (Figure 4.2). This response is dependent on ST2 expression as IL-33-stimulated cytokine production is abrogated in both ST2 -/- PDMC (Figure 4.3 A) and BMMC (Figure 4.3 B), findings expected as no other receptor has been identified for IL-33 [220, 267].

4.3.1.1 Sensitization affects IL-33-induced cytokine production.

The cytokine responses reported above were assayed from mast cells sensitized with IgE to reflect the physiologically relevant state of mast cell activity. To determine whether IgE sensitisation modulates the functional responses of IL-33 signaling, the effects of such priming on IL-33-mediated degranulation and cytokine production were investigated.

Although IL-33 is able to induce cytokine production in the absence of IgE sensitization, sensitization increases this production, particularly at higher concentrations of IL-33. For example, for IL-33 at 100 ng/ml (Figure 4.4 B), the addition of IgE significantly increases IL-6 and IL-13 production after IL-33 stimulation compared to that from non-sensitized cells.

4.3.1.2 Sensitization does not license IL-33-induced degranulation.

As different phenotypes of mast cells exhibit differential degranulation responses (Chapter 3), both PDMC (Figure 4.5 A, C) and BMMC (Figure 4.5 B, D) were analysed for their ability to degranulate in response to IL-33, with. PMA/Ionomycin treatment used as a positive control as it induces strong degranulation responses and is not affected by pre-treatment with IgE. As expected sensitization is essential for degranulation triggered by FcεRI-XL, however in both murine mast cell phenotypes IL-33 does not induce any discernible ß-hexosaminidase release regardless of cell type or priming by IgE (Figure 4.5 A-D).

In order to confirm these data, an alternative method of quantifying degranulation based on the Lamp1 expression method developed for CD8+ T-cells [162] was adapted for mast cells. The method is described previously (2.9.2). Graphic representation of GMFI analysis of the right peak, demonstrates that as expected, PMA/Ionomycin and XL induce increased surface Lamp1 expression, and thus degranulation.

As IL-33-induced degranulation could not be recorded using ß-hexosaminidase release (Figure 4.5 A-D), this more sensitive method was employed for all 3 subtypes of murine mast cells. PDMC (Figure 4.5 E), BMMC (Figure 4.5 F) and CTMC (Figure 4.5 G) all exhibit a strong response to PMA/Ionomycin and measureable responses to XL. Interestingly in the BMMC and CTMC there also is a low level of LPS-induced degranulation. Although this was not statistically significance, it could indicate LPS induces exocytosis in mast cells at a level not previously detected by the conventional method of ß-hexosaminidase release. Alternatively LPS could stimulate exocytosis of vesicles that do not contain ß-hexosaminidase but are labelled by Lamp1. However, even using this technique there is again no evidence for any exocytotic response induced by IL-33 with no change in % cells expressing Lamp1 (not shown) or the GMFI.

4.3.1.3 IgE sensitization increases ST2 expression

Interestingly, it was found that whilst IgE sensitization does not modulate expression of CD117 (Figure 4.6 A) or TLR4 (Figure 4.6 E), and decreases that of FcεRI (Figure 4.6 B), it slightly increases the surface expression of ST2 by PDMC (Figure 4.6 D). Using geometric mean fluorescence intensity (GMFI) there is an average increase in ST2 expression of 18% after sensitization and an apparent reduction of 35% of FcεRI expression (Figure 4.6 F). The effect on cytokine production in sensitized MC at the higher concentration of IL-33 (Figure 4.4) may reflect that IgE-mediated upregulation of ST2 allows a higher signal strength response at high concentrations of IL-33.

Such downregulation of FcεRI expression by IgE is controversial as some groups have reported that exposure to monomeric IgE may increase FcεRI expression [275] whilst others note that rather than upregulating FcεRI, IgE stabilises its expression on the surface [280]. It is therefore important to note that FACS analysis of FcεRI expression on sensitized cells should be approached with caution as the antibody used interacts with the α-subunit of the receptor, the IgE-binding subunit lacking signal-transducing ability, and hence the apparent downregulation observed here could be due to the reduced availability of binding sites for the anti-FcεRI antibody caused by the binding of IgE to FcεRI during sensitization. To further explore the effects of sensitization on these mast cell receptors, their expression over a 42 h period of sensitisation was analysed. This revealed that by 42 h, the increase in ST2 appears to resolve and its surface expression returns to that of non-sensitized cells (Figure 4.7 A). By contrast, FcεRI expression is progressively reduced with sensitization (Figure 4.7 B) whereas expression of CD117 is increased after 42 h of sensitization (Figure 4.7 C).

As the upregulation of ST2 is rapid and reversible, this could potentially reflect recycling from internal stores, a suggestion supported by the finding that the total cellular pool of ST2 expression is greater than that on the surface (Figure 4.7 D). Thus, ST2 signaling may involve a cycling trafficking mechanism similar to that reported for TLR4 in macrophages and DCs [281, 282] to ensure sustained signaling. Perhaps surprisingly, the profiles of TLR4 expression did not mirror that of ST2 as the total expression of TLR4

appeared to be similar to that on the surface of mast cells (Figure 4.7 E). However, this may reflect reports that TLR4 appears to only signal via the MyD88 pathway in mast cells and so may not undergo internalisation of TLR4 after LPS signaling, as required during TRIF-dependent, MyD88-independent TLR4 signaling [28, 48, 51, 283].

4.3.2 *IL-33 signaling involves mobilisation of calcium in murine mast cells.*

4.3.2.1 IL-33 induced calcium mobilisation in murine mast cells requires IgE sensitization, extracellular calcium and ST2 expression.

Mast cells in tissues are primed by binding of IgE and this sensitization is essential for mast cell activation via cross-linking of FcεRI (XL). However, mast cells respond to a wide range of stimuli including LPS and IL-33 and the effect of sensitization on these other methods of mast cell activation is not well defined. It is widely established that Ca^{2+} mobilisation is crucial to normal mast cell responses to crosslinking of FcεRI (Figure 1.5) and indeed, the importance of sensitization in the activation of MC by XL, LPS and IL-33 is seen in Figure 4.8 A. IL-33, like LPS, induces a slow calcium mobilisation that is almost entirely dependent on prior IgE sensitisation of PDMC. As this low profile of calcium mobilisation was suggestive of calcium influx, the role of extracellular calcium on the responses induced by IL-33 was investigated by repeating the experiments using buffer depleted in Ca^{2+}. This resulted in an initial small peak in intracellular calcium mobilisation resulting from release from internal stores followed by a return to basal levels due to the lack of extracellular calcium (Figure 4.8 B). PDMC (Figure 4.8 C) and BMMC (Figure 4.8 D) derived from ST2 -/- mice exhibited significantly reduced calcium responses after IL-33 treatment. As with previous experiments with other stimuli, including LPS and FcεRI crosslinking (Figure 3.9), IL-33-stimulated PDMC again displayed reduced calcium responses when compared to those of BMMC.

4.3.3 *IL-33-mediated activation of NFκB and ERK requires IgE sensitization and ST2 expression.*

As sensitization is crucial for XL and IL-33-mediated activation of calcium signaling, it was important to determine if these effects were maintained downstream with the activation of ERK and NFκB, as determined by Western Blotting analysis of dual phosphorylation of ERK and degradation of IκBα respectively (2.10). After the addition of the cross-linking agent DNP to mast cells in the absence of prior IgE sensitization, NFκB is not activated as determined by the maintenance and even increase in IκBα expression (Figure 4.9 A). By contrast, when cells are sensitised with IgE over night prior to DNP addition, FcεRI is crosslinked (XL) and IκBα expression is decreased and maintained at lower levels. Interestingly, given the calcium data, in the absence of sensitization IL-33 stimulates a rapid degradation of IκBα at 20 min that returns almost to basal levels by 30 min (Figure 4.9 B). When the cells are sensitized prior to IL-33 stimulation expression of IκB does not return towards basal levels until about 60 min, an effect dependent on ST2 expression (Figure 4.9 C). This lengthened period of reduced IκBα expression allows a more prolonged NFκB signal from IL-33.

ERK activation is almost absent in non-sensitized cells after addition of Ag (Figure 4.10 A), the slight increase in activity seen at 60 minutes is preserved in repeat experiments, presumably reflecting some basal cycling activity or alternatively that the Ag (DNP) induced some signaling in the cells independently of IgE-sensitisation of FcεRI. However, there is a bi-phasic profile of DNP-mediated ERK activation seen in sensitized cells representing cyclic activation of ERK in mast cells at 10 and 30 mins. By contrast, but reflecting the NFκB data, without IgE sensitization, IL-33 is able to induce ERK phosphorylation that peaks at 30 min after which it returns to basal levels (Figure 4.10 B). Sensitized mast cell also display a similar peak of ERK activation at 30 min, although this is exhibited at a higher signal strength and reflects a higher basal ERK phosphorylation. This enhanced basal ERK activation suggests that MAPK may be involved in the “priming” process occurring during sensitization of mast cells. ERK activation in ST2 -/- MC is essentially absent after IL-33 stimulation in BMMC (Figure 4.11 B). The PDMC results (Figure 4.11 A) are puzzling and suggest possible stimulation

contamination, indicted by the high ERK phosphorylation at 0 min in ST2 -/-. A further IL-33-induced signal is not apparent above this increased basal ERK signal, an effect preserved in two experiments.

4.3.4 *Role of phospholipase D in IL-33 signaling*

PLD-mediated hydrolysis of phosphatidylcholine (PtdCho) produces phosphatidic acid (PA) which can be further converted to DAG or lysophosphatidic acid (LPA), all products being second messengers important in both GPCR and RTK signaling, secretion, trafficking, cytoskeletal reorganisation and cell survival [284]. There are two mammalian PtdCho-specific PLD isoforms identified, which can occur as multiple splice variants and require PIP2 for activation [285].

Phospholipase D signaling has been reported to be crucial for mast cell responses to cross-linking of FcεRI [286-288] and has been suggested to play an important role in IL-33 signaling [126] potentially upstream of the IL-33-induced calcium mobilisation reported above (Figure 4.12).

Traditionally, roles for PLD signaling have been investigated using primary alcohols like Butan-1-ol that by trapping PLD-products, block functional responses, but this approach does not allow for analysis of individual PLD isoforms. However, several biochemical PLD inhibitors have been recently developed, including pan PLD, PLD 1 and 2 inhibitors that potentially act by an allosteric mechanism rather than by direct interaction with the catalytic domain [289, 290]. The pan-PLD inhibitor, 5WO, inhibits both PtdCho-PLD isoforms, and whilst the PLD 1 inhibitor, 809, exhibits > 100 fold selectivity for this isoform, the PLD 2 inhibitor, APV, is less selective, losing its specificity for PLD 2 at higher concentrations (>20 μM) [284]. Since performing the experiments in this thesis a more specific PLD 2 has been developed [291].

4.3.4.1 PLD inhibition affects murine mast cell viability.

As PLD is important in many mast cell processes it was important to check their toxicity before employing the inhibitors in any experiments. With both PDMC (Figure 4.13 A) and BMMC (Figure 4.13 B) the inhibitors showed little induction of cell death, as evidenced by the change in 7AAD staining, at lower concentrations (1 μM) but by 5 μM, there were obvious increases in cell death. Although administration of IL-33 helped to increase the basal level of cell survival, it did not rescue cell viability after treatment with any of the inhibitors, especially after APV treatment.

4.3.4.2 PLD signaling is involved in mast cell degranulation responses

Previous studies had reported that PLD activity is essential for the full degranulation response to FcεRI crosslinking in mast cells [286-288]. IL-33 does not induce degranulation in PDMC (Figure 4.5) but the role of PLD in FcεRI- and PMA/Ionomycin-stimulated degranulation was investigated. Analysis of the dose dependent effects of the inhibitors revealed that in the presence of the individual isoform inhibitors FcεRI-mediated degranulation (XL) was not significantly different from basal levels up to an inhibitor concentration of 5 μM (Figure 4.14 A). The pan-isoform inhibitor was more potent, with significant inhibition seen at 1 μM, suggesting that both PLD isoforms played a role in such degranulation.

PMA/Ionomycin potently induces degranulation in mast cells by directly activating PKC, an activator of PLD [292, 293]. When pre-treated with the PLD 2 Inhibitor and the PLD 1/2 inhibitor at 5μM, degranulation is significantly reduced compared to that of control cells but this effect is not seen in the presence of PLD 1 inhibition (Figure 4.14 B). As the inhibitors appeared to slightly increase basal levels of degranulation, the data are also presented as the "stimulated" degranulation values corrected for their respective basals (Figure 4.14 C). These data suggest that PLD 1 does not play a role in the regulation of degranulation in PMA/Ionomycin treated mast cells, an interesting finding as PLD 1 has been reported to be activated by PMA in fibroblast-like cell lines [292, 293].

4.3.4.3 PLD signaling is involved in mast cell cytokine responses

The cytokine response induced by FcεRI-XL in IgE sensitized mast cells is strongly inhibited by both of the individual PLD isoform inhibitors, again indicating a role for both PLD 1 and PLD 2 signaling in XL responses (Figure 4.16 A). In non-sensitized cells (PBS) PLD inhibition also reduced basal cytokine levels. Although IL-33 does not induce degranulation, it stimulates cytokine production that is augmented by prior IgE sensitisation of mast cells. The role of PLD in such enhancement was thus analysed (Figure 4.16 B). IL-13 production from both IgE sensitized and non-sensitized MC treated with IL-33 and potently inhibited by pan-PLD and PLD 2-specific inhibitors whereas PLD 1 inhibition produces a plateau response that preserves some IL-13 production.

Further investigation of the role of PLD in IL-33-stimulated cytokine production by IgE-sensitised mast cells was then performed to determine whether it applied to both PDMC MCP-1 and IL-13 production (Figure 4.17 A) and BMMC MCP-1 and IL-6 production (Figure 4.17 B). Low levels of MCP-1 are produced constitutively by sensitised mast cells and inhibition of PLD 1 or PLD 2 reduces such basal cytokine production by both PDMC and BMMC. Whilst inhibition of PLD 1 (809) only partially reduces IL-33-induced MCP-1 production by PDMC and BMMC, release of this chemokine is essentially completely abrogated by the inhibitor of the PLD 2 isoform (APV) in both cell types and by the pan-PLD inhibitor in BMMC. A similar pattern is observed with IL-13 production by PDMC and IL-6 release by BMMC and suggests that PLD 2 is indispensible for IL-33-induced cytokine production in mast cells whereas PLD 1 plays a role but may be redundant to PLD 2.

4.3.4.4 PLD inhibition suppresses calcium mobilisation in murine mast cells.

FcεRI-XL-mediated degranulation and FcεRI-XL- and IL-33-induced cytokine production are both modulated by inhibition of PLD (Figure 2.12). It was therefore investigated whether this reflected suppression of calcium signaling as this has been shown to be key to both functional responses. Indeed, PLD-

mediated activation of degranulation has been proposed to involve calcium signaling, either via PA- and DAG-mediated PKC or PA-mediated SphK activation [286-288].

All three PLD inhibitors were found to be effective in reducing calcium mobilisation resulting from FcεRI crosslinking (Figure 4.18 A) and IL-33 stimulation (Figure 4.18 B), when used at 5 μM to ensure inhibition of PLD activity. Rather surprisingly, given the above cytokine responses, the PLD 2 inhibitor appeared to be least effective in modulating IL-33-mediated calcium mobilisation, albeit there was no significant difference between the three inhibitors. This modulation appeared to reflect suppression of intracellular calcium release rather than influx of extracellular calcium as the second slower phase of calcium movement appeared still to be present. This was further investigated by performing the experiments with FcεR1-XL in the absence of extracellular calcium and EGTA to prevent the calcium influx phase and this revealed that indeed the PLD inhibitors were targeting the intracellular mobilisation phase (Figure 4.18 C).

As mentioned before, PLD 2 inhibition by APV needs to be interpreted with caution especially at higher concentrations where it loses selectivity. Moreover, calcium movement is very sensitive to environmental influences such as inhibitors [161] thus to further explore the role of PLD 2-specific responses, inhibitor dose response experiments were performed. Using decreasing doses of APV (Figure 4.18 A), there is still inhibition of calcium mobilisation in response to FcεRI-XL evident even at a dose of 0.5 μM (Figure 4.18 D), 10X less than the initial dose and at concentrations where no effect is detected in terms of degranulation. These dose effects were similarly reflected in the analysis of FcεRI-mediated calcium mobilisation in the absence of extracellular calcium (Figure 4.18 E). Collectively, therefore, these data suggest that APV is targeting PLD 2 and hence PLD 1 and PLD 2 both appear to play important but, as no further inhibition is obtained with the pan-PLD inhibitor, possibly redundant roles in FcεRI- and IL-33-stimulated calcium mobilisation.

4.3.4.5 PLD inhibition modulates FcεRI- and IL-33-stimulated NFκB and ERK activation.

As the PLD 1 and PLD 2 inhibitors inhibited calcium mobilisation, cytokine production and degranulation, their effect on signals potentially downstream of calcium mobilisation, namely ERK and NFκB activation were analysed by Western blotting.

The activation of NFκB, as evidenced by a decrease in IκB expression, was determined after FcεRI-cross-linking (XL) and as expected, this revealed a time-dependent decrease in IκB expression over 60 min to a level lower than that in unstimulated cells (Figure 4.19). However, in cells treated with the pan-PLD (1/2) inhibitor, IκBα expression was not decreased but rather, since exposure to the inhibitor alone appeared to visually slightly decrease IκB expression relative to that observed in control cells, was if anything slightly increased, suggesting that blocking PLD activation not only prevented NF-κB activation but that the feedback mechanisms that regulate such responses resulted in an increase of IκBα levels to above basal levels. Interestingly therefore, inhibition of PLD 1 had no apparent effect on such NF-KB activation whilst inhibition of PLD 2 resulted in similar modulation to that observed with the pan-PLD inhibitor, suggesting that PLD 2 may be important for efficient NF-κB activation.

By contrast, when similar experiments were performed using IL-33 as a stimulus, the overall results were quite different (Figure 4.20). Thus, IL-33 stimulates the transient degradation of IκB within the first 20 min, before expression of IκB recovers towards basal levels by 60 min. Whilst the pan-PLD inhibitor appeared to have no effect, when the cells are stimulated with IL-33 in the presence of the PLD 1 inhibitor, there appears to a reproducible faster/stronger recovery of IκB levels whilst inhibition of PLD 2 appeared to antagonise the recovery of IκB levels. Collectively, these data suggested that PLD 1 and PLD 2 could counter-regulate IL-33 mediated NF-κB activation, with PLD 2 acting to terminate signaling. Moreover, whereas for FcεRI-signaling the pan-PLD and PLD 2 inhibitors appeared to target the activation phase with both inhibitors preventing degradation of IκB, for IL-33, PLD activation was associated with regulation of maintenance of NF-κB activation

as whilst neither of the PLD 1 or PLD 2 inhibitors modulated the initial degradation phase, they promoted or antagonised the subsequent up-regulatory, negative feedback phase, respectively.

FcεRI-mediated activation of p42 and p44 ERK follows a biphasic pattern with peaks at 10 and 30 min post stimulation, with both isoforms being activated to a similar degree. In the presence of the pan-PLD inhibitor such activation, although still biphasic, was reduced after the initial peak at 10 min. Rather surprisingly therefore, exposure to the selective PLD 1 and PLD 2 Inhibitors essentially suppressed all ERK activation from 20 min post stimulation (Figure 4.21 A). Interestingly pre-treatment with the selective inhibitors, particularly that targeting PLD 2, increased basal levels of pERK and this may impact on the cell in terms of a "stimulation index"-type of sensing of responses and is also reflected by an increase in the first peak of ERK activation, particularly by the PLD 1 inhibitor. As inhibition of either of the individual PLD isoforms completely ablates the second peak of phosphorylation, it implies not only that PLD activity is required for this signal but also that a distinct, PLD-independent pathway is required for the initial phase of ERK activation.

ERK activation by IL-33 differs considerably from that induced by FcεRI-XL, with a slower, weaker monophasic response peaking at 30 min and activation predominantly representing the p42 isoform. Inhibition of the individual PLD isoforms again increases the basal levels of dually phosphorylated ERK, again indicating a role for PLD in regulating basal ERK activity. However, in the case of IL-33 inhibition of either PLD 1 or PLD 2 results in enhancement of both the duration and extent of ERK activation; in particular, especially following inhibition of PLD 2, an early phase of ERK activation is revealed and this could perhaps reflect relief of suppression of early PLD-independent peak observed following stimulation via FcεRI, which was also observed to be increased in the presence of PLD inhibitors (Figure 4.21 B).

4.3.4.6 Analysis of PLD activity in murine mast cells.

To validate the role of PLD in the above mast cell processes, PLD activity was directly quantified by the transphosphatidylation assay. Such analysis showed

that whilst no PMA or FcεRI-mediated accumulation of PtdEtOH could be detected within 10 min of stimulation, significant levels were detected for both stimuli within 60 min relative to the basal levels in IgE-sensitised PDMC (Figure 4.22 A). As expected, IgE sensitization was required for FcεRI-stimulated activity and interestingly, IL-33-stimulated activity (Figure 4.22 B). Moreover, although the selective PLD inhibitors tended to suppress both FcεRI- and PMA-stimulated PLD activities, these could only be blocked significantly by the pan PLD inhibitor suggesting that both PLD 1 and PLD 2 signaling contribute to these responses (Figure 4.22 C). By contrast, and rather surprisingly given the effects of PLD inhibitors on IL-33 signaling and functional responses, no IL-33-coupled PLD activity could be detected within 60 min. Indeed, no IL-33-stimulated PLD activation could be detected over a period of 18h stimulation (Figure 4.22 D). However, what this analysis clearly showed was that there is significantly increased "basal" PLD activity in IgE-sensitised mast cells over this 18 h period. To determine the source of this constitutive activity and to investigate whether this reflected sensitization, the ability of IgE-sensitisation to induce PLD activation was determined; this revealed that although there was a significant constitutive basal PLD activity, this was increased by exposure to IgE with significant effects observed within the 18 h sensitisation period. As IL-33 only induced calcium mobilisation in IgE-sensitised cells, this would explain the observed PLD-dependence of IL-33-associated calcium signaling.

In order to determine which isoforms are involved in the basal and IgE-sensitised responses, the PLD inhibitors were employed at a low concentration of 1 μM to prevent any potential loss of specificity by the PLD 2 inhibitor. Whilst, and consistent with data in the literature, basal PLD activity was significantly inhibited by the PLD 2, but not PLD 1, inhibitor, IgE-sensitised PLD activation was only significantly inhibited by the pan-PLD inhibitor suggesting that both PLD 1 and PLD 2 are involved (Figure 4.22 F).

These data strongly imply that sensitization does increase the basal homeostatic PLD activity in mast cells and confers the PLD-sensitivity of IL-33 responses in mast cells. By contrast, although sensitisation contributes to the overall FcεRI-mediated PLD response, comparison of the "sensitised" and "sensitised and XL" FcεRI-mediated responses with those occurring in the

absence of IgE show a substantial additional component resulting from XL that is more sensitive to PLD inhibition, particularly that of PLD 1, than IL-33 signaling, perhaps reflecting the faster, stronger calcium responses as well perhaps as the differential degranulation responses via FcεRI and IL-33 signaling in murine mast cells.

4.3.5 *The role of sphingosine kinase in IL-33 signaling.*

Many immunoregulatory receptors including FcR, couple to sphingosine kinase (SphK) activation downstream of PLD [126, 294, 295]. Mast cells express both sphingosine kinase (SphK) 1 and SphK 2 isoforms, although the relative role of each in FcεRI signaling is still under debate as anti-sense studies with RBL cells suggested that FcεRI was coupled to SphK1 for degranulation [296] whereas analysis of Sphk1, SphK 2 and double knockout models have indicated that whilst FcεRI is coupled to both SphK1 and 2, SphK2 is key and non-redundant in FcεRI signaling [195, 297]. However recent evidence also suggests a crucial role for SphK1 in mast cell-dependent allergic inflammation [298]. To determine if SphK activity plays a role in IL-33 signaling in murine mast cells, a series of experiments were performed using the SphK inhibitors DMS and 5c. DMS is isoform non-selective inhibitor of SphK that has also been reported to inhibit PKC activation [299], while 5c has been reported to selectively target SphK1 [300]. Consistent with a role for one or more SphK activities in IL-33 functional responses, in IgE-sensitised PDMC (Figure 4.23 C) and BMMC (Figure 4.23 D), increasing concentrations of DMS inhibit cytokine production. However, at 25 μM DMS, where there is essentially no cytokine production observed, this most likely reflects the loss of cell viability in both PDMC (Figure 4.23 A) and BMMC (Figure 4.23 B). Nevertheless, in PDMC there is substantial inhibition of cytokine production at 10 μM DMS, a concentration that is not toxic to the cells.

As SphK activity has been reported to regulate calcium signaling (Figure 3.8), it was important to determine if inhibition would affect calcium mobilisation and hence place SphK upstream of this important signal. Consistent with previous published data, DMS (10 μM) was able to significantly inhibit calcium

mobilisation induced by FcεRI cross-linking (XL), (Figure 4.23 E), apparently by modulating both mobilisation of intracellular stores and influx of extracellular calcium. Similarly, calcium mobilisation following stimulation with LPS is reduced by inhibition of SphK (Figure 4.23 F) but analysis in calcium-free buffer suggests that for this stimulus, this predominantly reflects inhibition of influx, consistent with the results reported above suggesting LPS stimulates little or no mobilisation of intracellular calcium (Chapter 3). Interestingly therefore, DMS-induced inhibition of IL-33-induced calcium mobilisation (Figure 4.23 G) mimics the IL-33 signal observed when extracellular calcium is absent and only intracellular calcium movement occurs. This suggests an alternative mechanism of mobilising intracellular calcium during IL-33 signaling compared to FcεRI-XL whereas, and as observed for LPS, DMS prevents influx of extracellular calcium influx.

To explore this further, the effect of the selective SphK1 isoform inhibitor, 5c was examined. This inhibitor does not substantially increase cell death of either PDMC (Figure 4.24 A) or BMMC (Figure 4.24 B), allowing use of a high concentration (30 μM) to ensure inhibition of SphK1. Consistent with what has previously been reported, the calcium response induced by crosslinking FcεRI (XL) is weakly but significantly reduced by 5c (Figure 4.24 C) and it appears that this effect is due to a reduction in intracellular store mobilisation. By contrast, the SphK1 inhibitor has no effect on the calcium profile after IL-33 stimulation (Figure 4.24 D) perhaps indicating that SphK2 is the more dominant isoform in IL-33 signaling and consistent with previous reports that this isoform is important for extracellular influx [195].

Consistent with the above findings, 5c generally only weakly inhibits IL-33-stimulated cytokine production by PDMC (Figure 4.24 E) and BMMC (Figure 4.24 F), although MCP-1 production in PDMC is reduced almost to the (high) basal levels observed in these cells, perhaps reflecting the poor ability of these cells to release this chemokine in response to receptor stimulation and also suggesting that the distinct cytokine responses to IL-33 may be differentially regulated. Basal cytokine production appears mainly unaffected. Collectively, these findings indicate that Sphk1 activation is unlikely to be critical for IL-33-stimulated cytokine production, although the DMS data may implicate an important role for SphK 2 in these responses.

To validate these studies, the activity of SphK was directly determined in cell lysates from FcεRI- or IL-33-stimulated cells. Following FcεRI-XL, there is an initial slight increase in SphK activity at 30 seconds followed by a sharp decrease to levels below that of basal (Figure 4.25 A). By contrast, in response to IL-33 stimulation, SphK activity steadily increases over time (Figure 4.25 B), again reflecting the slow and steady signaling induced by IL-33 compared to XL. As with previous signaling studies the mast cells were sensitized and although this may have modulated basal levels of SphK, as seen with PLD, this was not quantified. SphK produces S1P, an important second messenger in cell signaling. S1P may act both intracellularly and extracellularly in mast cells either directly as a second messenger in signal transduction and calcium mobilisation or by interaction with cell surface receptors S1P1 and S1P2. [301-303]. To corroborate the SphK data, it was important to determine the levels of S1P produced after IL-33 stimulation of mast cells. Using conditioned medium and cell lysates of IL-33-treated PDMC as sources of extracellular and intracellular pools of S1P respectively, the production of S1P was quantified by ELISA. Intracellular S1P expression in cell lysates (Figure 2.34 C) was found to increase, peaking by 30 min then diminishing towards basal levels with kinetics consistent with the SphK assay (Figure 4.25 B). Analysis of S1P released from the cell (supernatant) displayed faster kinetics, with release maintained throughout the timecourse and were reminiscent of those recorded previously after Ag-mediated crosslinking of FcεRI in BMMC [304].

Extracellular S1P can act via the receptors, S1P1 and S1P2 to induce migration, degranulation and trigger hyper-responsiveness in FcεRI-stimulated cells [296, 305] to amplify the responses of mast cells both *in vitro* (human cord blood) and *in vivo*, the latter in a model of mouse anaphylaxis [195, 306]. S1P activation of mast cells also plays an important role in an OVA model of allergic asthma [298]. To investigate this, the serum and broncheoalveolar lavage fluid (BALf) from individual mice treated with PBS or OVA plus Alum in a murine model of acute asthma were tested for the production of SIP and hence SphK activity during development of asthma (Figure 4.25 D) and this revealed that whilst there was an increase in the

levels of S1P in the serum, but not the BALf, after the induction of airway hyper-responsiveness by OVA and Alum in this model.

4.4 Conclusions

It is well established that sensitisation by IgE is required for signaling via FcεRI in mast cells but the results presented here confirm the suggestion [171, 277] that this also primes/licenses the cells for full signaling, in this case, via IL-33/ST2 and LPS/TLR4. As ST2 is the only one of these receptors that is directly upregulated on the surface of mast cells by IgE-sensitisation, potentially via trafficking of internal pools, it is likely that these priming effects reflect cooperative signaling occurring amongst downstream pathways.

Thus, although IL-33 can induce cytokine production by MC, this is enhanced by IgE-sensitization that reflects more prolonged NFκB activation and increased ERK signaling, potentially resulting from the IgE-mediated licensing of the resulting IL-33-stimulated extracellular calcium mobilisation, data consistent with previous studies reporting calcium mobilisation during sensitization in BMMC and RBL-2H3 cells [277, 278, 307, 308]. Calcium mobilisation in response to IgE-FceR1 ligation was not directly analysed, but as IgE sensitization appears to increase PLD activity and prime PLD-dependent calcium signaling in response to IL-33, PLD appears to be a key priming signal associated with IgE-sensitisation as previously reported for FcεRI responses in BMMC [308].

Consistent with a key role for PLD signaling in IgE-sensitisation of mast cell responses, basal cell viability, proliferation and cytokine production is suppressed by inhibitors of PLD. In terms of receptor-signaling, however, the role of PLD appears to depend on the stimulus and the cell type. Thus, for FcεRI-signaling, both PLD 1 and 2 isoforms are involved in calcium, ERK activation and degranulation responses, an effect confirmed by direct analysis of PLD activity. Previous reports, investigating the role of PLD in degranulation [309], found PLD 1 may be the more dominant isoform in FcεRI

signaling [288]. However, recent evidence suggests that PLD 2, previously thought to be restricted to basal constitutively active homeostatic role, can be stimulated by XL [310, 311]. PLD 2, similar to PLD 1, can be stimulated by PKCα and DAG, resulting from increased PIP_2 - PLCγ signaling following FcεRI crosslinking [285]. Furthermore, KO models and siRNA studies have suggested that PLD 2 functions to regulate cell morphology and compartmentalisation of the cytosol [312-315], ensuring the correct location and morphology of the Golgi complex, secretory granules and lysosomes. Of particular interest to this study, it appears that such signals and important for determining FcεRI localisation within the cell surface lipid microdomains, crucial for signaling [287, 316] and that fusion of mast cell granules with the plasma membrane may also be regulated by PLD 2 in a calcium-dependent manner [317].

The relative roles of the PLD isoforms in IL-33-ST2 signaling has been more difficult to determine but appears that in MC, PLD 2 is the more important activity. Thus, whilst inhibition of either PLD prevented IL-33-induced calcium mobilisation, downstream cytokine responses were more reliant on PLD 2 activity. This was initially surprising considering it is generally considered that PLD 1 is the important isoform in agonist responses, but presumably reflects that the PLD-dependence of IL-33 effects is dependent on the IgE-licensing of PLD 1 and PLD 2 activity. Furthermore, as it has been reported that PLD requires extracellular as well as intracellular calcium for full activation [318, 319], it may be that the calcium and PLD assays, performed over a shorter time-course *in vitro,* do not reflect the status of these signals elicited by IL-33 in IgE-sensitised mast cells over the 24 h period of the cytokine assay. Nevertheless, as PLD 1 activity is associated with vesicular membranes, regulation of translocation and secretion of granules, it is more dominant in driving exocytosis/degranulation following FcεRI crosslinking [314, 317, 320]. The predominant role of PLD 2 may go some way to explaining the absence of degranulation induced by IL-33 as reported here and previously [42, 60, 268, 321]. Similarly MC degranulation is dependent on intracellular calcium release [190, 202, 203, 322], a response not initiated by IL-33. These results suggest a link between PLD 1 activity and calcium release from the ER in murine mast cell degranulation. Interestingly as some IL-33-induced cytokine production was preserved despite PLD inhibition after IgE

sensitisation, there is a suggestion that another co-factor, other than IgE, may be promoting PLD activity.

Unfortunately due to the crucial role for PLD signaling in a variety of homeostatic and protective cell processes, inhibition of PLD is likely to be a difficult route for therapeutic intervention. However, PLD-dependent regulation of hyper-inflammatory IL-33-mediated mast cell induced by IgE-sensitization suggests this could be more safely targeted by therapeutics, such as omalizumab, which have been designed to prevent inhibit IgE ligation to FcεRI [323, 324] and could therefore be exploited to limit IL-33-stimulated pathology.

Previously, it was demonstrated that IL-33 failed to stimulate calcium mobilisation in BMMC [60, 321]. These data are confirmed here but are extended by the finding that IgE-sensitisation of mast cells licenses IL-33-mediated calcium mobilisation, predominantly via influx of extracellular calcium influx but with some evidence of a low level of mobilisation of intracellular stores in BMMC but not in PDMC. Both PLD and SphK signaling appear to be involved in such IL-33-mediated calcium mobilisation and whilst inhibitor studies suggested both PLD isoforms could mediate this response, they supported a role for SphK2 but not SphK1 in IL-33-induced calcium influx.

Activation of both SphK isoforms was reported to be involved in FcεRI and SCF responses in BMMC [304] and the data presented here suggested that both SphK1 and 2 were likely involved in FcεRI-mediated calcium mobilisation in PDMC. However, and consistent with the findings of functional plasticity of mast cell subtypes presented in this thesis, recent evidence suggests that SphK isoform usage depends on species of origin and culture method/maturation state of the cells [325]. Thus, in BMMC and PDMC, SphK 2 was found to be required for FcεRI-mediated calcium mobilisation, degranulation, cytokine and leukotriene production, whereas only SphK 1 was employed by human BMMC for FcεRI-induced cytokine production [325]. Thus, our studies using the SphK 1 inhibitor, suggesting that SphK 2 is the predominant isoform involved in IL-33 responses, is consistent with these findings although further studies with recently developed, better SphK

isoform inhibitors would allow more detailed analysis of isoform dependence in MC function [326].

As both PLD isoforms reduce intracellular calcium mobilisation, the rise in intracellular calcium concentration could be mediated by PA stimulated S1P-SphK release from ER, however as PLD inhibition does not completely ablate the intracellular calcium concentration there may also be a further mechanism of IL-33- induced calcium mobilisation. The recent association of PI3K p100δ in IL-33-ST2 signaling in Th2 cells [327], a molecule that can directly promote influx of calcium via membrane bound SOCs or promote PLCγ-mediated IP_3 production [328] may provide an alternative mechanism and an interesting focus for future studies.

Figure 4.1 IL-33 signaling

IL-33 signaling in mast cells requires the recruitment of the receptor ST2 and the co-receptor IL-1RAcP. TIR domains then recruit MyD88, to activate IRAK1 and IRAK4 using TRAF6 and TAK1. Further signal propagation leads to activation of ERK, JNK and p38 MAPK alongside the degradation of IκB resulting in activation of NFκB.

Figure 4.2 Murine mast cells express the IL-33 receptor, ST2 and respond to IL-33 stimulation.

IgE-sensitized PDMC, BMMC and CTMC were treated with the indicated dose of IL-33 for 24 h at 37ºC before analysis of IL-6 and IL-13 levels in culture supernatants by ELISA. Data are presented as the mean of duplicate samples from a single experiment with error bars representing SD.

Figure 4.3 IL-33-induced cytokine production requires ST2 expression by murine mast cells.

PDMC obtained from WT or ST2 $^{-/-}$ (BALB/c) mice were sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight then stimulated with IL-33 (100 ng/ml) for 24 h at 37^{o}C before release of IL-13 and IL-6 was measured by ELISA. Data are presented as the mean values of triplicate samples ± SD and are from a single experiment representative of 3 independent experiments. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. *** $p < 0.001$.

Figure 4.4 IgE sensitization modulates IL-33-induced cytokine production from PDMC.

PDMC sensitized or not with murine anti-DNP IgE (0.5 µg/ml) overnight were stimulated with IL-33 at 10 ng/ml (A) or 100 ng/ml (B) for 24 h at 37^{o}C before release of IL-13, IL-6 and MCP-1 was measured by ELISA. Data are presented as the mean values of triplicate samples ± SD and are from a single experiment representative of 2 independent experiments. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. *** $p < 0.001$.

Figure 4.5 IL-33 does not induce degranulation of murine mast cells.

PDMC (A, C, E,), BMMC (B, D, F) and CTMC (G), were sensitized with murine anti-DNP IgE (0.5 µg/ml) then stimulated with DNP (0.5 µg/ml) to induce cross-linking (XL), IL-33 (10 ng/ml), LPS (0.5 µg/ml) or PMA (1 µM) plus Ionomycin (1 µM) for 30 mins at 37^{o}C. Degranulation was determined as the % ß-hexosaminidase released relative to the total activity of the cells (A-D) Data are presented as single samples from a single experiment (A, B) or the combined results of 5 (C) or 2 (D) independent experiments where error bars represent SEM. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. Alternatively (E-G), degranulation was determined by flow cytometric analysis of cell surface Lamp1 expression by 7AAD^{-}FcεRI^{+}CD117^{+} mast cells. Geometric mean fluorescence (GMFI) of positive gate of Lamp1 expression was determined relative to the isotype control as described (2.9.2). Data are presented as triplicate samples ± SD and are from a single experiment representative of a single (F, G) or 2 (E) independent experiments. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

Figure 4.6 Effect of IgE sensitization on expression of mast cell surface phenotypic markers.

Flow cytometric analysis of PDMC sensitized, or not, with murine anti-DNP IgE overnight were gated for live 7AAD- cells then analysed for CD117 (A), FcεRI (B), ST2 (D) and TLR4 (E) expression. The single stain for FcεRI is shown in (C) to demonstrate that the reduction in staining is not a result of interference by other stains. The geometric mean fluorescence (GMFI) of cells positively expressing each marker was determined relative to gating against the relevant isotype control obtained with non-sensitised cells (F). CD117 data is represented as a ratio of non-sensitized CD117 expression. Results are combined data of at least 3 independent experiments where error bars represent SEM and statistical analysis is by unpaired, two-tailed t-test. * $p < 0.05$, *** $p < 0.001$

Figure 4.7 Effect of IgE sensitization on mast cell surface markers at 18 h and 42 h and the comparison of surface and total expression of ST2 and TLR4.

Flow cytometric analysis of PDMC sensitized, or not, with murine anti-DNP IgE for 18 h or 48 h were gated for live 7AAD- cells then analysed for CD117 (A), FcεRI (B), ST2 (D) and TLR4 (E) expression. Non-sensitized, 7AAD-, FcεRI+, CD117+ cells were analysed for surface and total (surface and intracellular) ST2 (D) and TLR4 (E) expression. The geometric mean fluorescence (GMFI) of cells positively expressing each marker was determined relative to gating against the relevant isotype control obtained with non-sensitised cells. Results are representative of a single experiments performed in triplicate where error bars represent SD and statistical analysis is by unpaired, two-tailed t-test. **** p< 0.0001.

Figure 4.8 IL-33 stimulated calcium mobilisation is dependent on IgE sensitization, extracellular calcium and ST2 expression.

WT or ST2 $^{-/-}$ PDMC (A, B, C) and BMMC (D) sensitised (A-D) or not (A) with murine anti-DNP IgE (0.5 µg/ml) and loaded with Fura-2/AM were stimulated at 50s in serum-free HBSS with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (A), 0.5 µg/ml LPS (A) or 100 ng/ml IL-33 (A-D). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (B). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Statistical analysis using a two-tailed, paired t-test. **** $p < 0.0001$.

Figure 4.9 Effect of sensitization and ST2 expression on NFκB activation by FcεRI and IL-33.

WT (A-C) or ST2 -/- (C) PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight (IgE) or not (PBS) were cultured with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI (A) or IL-33 (10 ng/ml) (B, C) for the indicated times and expression of IκBα analysed by Western blotting. "0 min" represents zero timepoint control. ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκBα compared to ß-actin for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.10 Effect of sensitization on ERK activation by FcεRI and IL-33.

PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight (IgE) or non sensitized (PBS) were cultured with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI (A) or IL-33 (10 ng/ml) (B) for the indicated times and expression of Pp44/p42 (pERK) analysed by Western blotting. "0 min" represents zero timepoint control. p44/42 (ERK) was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of Pp44/42 compared to p44/42 for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.11 IL-33 mediated ERK activation is dependent on ST2 expression.

WT or ST2 -/- PDMC (A) or BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight (IgE) were cultured with IL-33 (10 ng/ml) for the indicated times and expression of Pp44/p42 (pERK) analysed by Western blotting. "0 min" represents zero timepoint control. p44/42 (ERK) was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of Pp44/42 compared to p44/42 for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.12 Proposed involvement of PLD and SphK in IL-33 signaling.

Phospholipase D signaling has been reported to be crucial for mast cell responses to cross-linking of FcεRI [286-288] and has been suggested to play an important role in IL-33 signaling [126]. Although the mechanism of PLD coupling to ST2 is unknown, PLD-derived PA potentially induces calcium mobilisation via SphK-produced S1P production.

Figure 4.13 Effect of PLD inhibition on murine mast cell viability.

PDMC (A) or BMMC (B) were pre-treated with the indicated concentrations of PC-PLD inhibitors 5WO (pan PLD 1/2), 809 (PLD 1) or APV (PLD 2) for 30 min at 37^{o}C before stimulation with IL-33 (10 ng/ml) for 24 h before cell death was assessed by 7AAD uptake by flow cytometry and is presented as % change in 7AAD+ cells compared to untreated cells. Data represents are from a single experiment.

Figure 4.14 FcεRI-induced mast cell degranulation requires PLD signaling.

PDMC sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were pre-treated with the indicated dose (A) or 1 µM (B, C) of the inhibitors 5WO (pan PLD 1/2), 809 (PLD 1) or APV (PLD 2) for 30 mins prior to stimulation with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (A) or PMA (1 µM) plus Ionomycin (1 µM) (B, C) for a further 30 mins at 37^{o}C. Degranulation was determined as the % ß-hexosaminidase released relative to the total activity of the cells and was then adjusted to represent increase in ß-hexosaminidase release over unstimulated cells (basal) (C). Data represents the combined results of 3 independent experiments where error bars show SEM. Statistical analysis using two-way ANOVA with Bonferroni multiple comparison test. * $p<0.05$ and ** $p< 0.01$.

Figure 4.15 PLD signaling plays a role in IL-33-induced IL-13 production.

PDMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight (IgE) or not (PBS) then pre-treated with the indicated dose of PC-PLD inhibitors 5WO (pan PLD 1/2), 809 (PLD 1) or APV (PLD 2) for 30 mins at 37°C before stimulation with DNP (0.5 μg/ml) to induce FcεRI cross-linking (XL) (A) or IL-33 (10 ng/ml) (B) for 24 h at 37°C. Release of IL-13 was measured by ELISA from supernatants. Results from a single experiment performed in duplicate where error bars show SD.

Figure 4.16 IL-33-induced cytokine production is dependent on PLD 2 but not PLD 1.

PDMC (A) and BMMC (B) were sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight then pre-treated with the described dose of PC-PLD inhibitors, 5WO (pan PLD 1/2), 809 (PLD 1) or APV (PLD 2) for 30 mins at 37^{o}C before stimulation with IL-33 (10 ng/ml) for 24 h at 37^{o}C. Release of IL-13 and MCP-1 was measured by ELISA from supernatants. Results from a single experiment, where error bars represent SD, and are representative of at least 2 independent experiments.

Figure 4.17 Calcium mobilisation in murine mast cells requires PLD signaling.

WT PDMC sensitised with murine anti-DNP IgE (0.5 µg/ml) were simultaneously loaded with the described dose (D, E) or 5 µM (A- C) of PC–PLD inhibitors and Fura-2/AM before stimulation in serum-free HBSS at 50s with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (A, C-E)) or IL-33 (100 ng/ml) (B). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (C, E). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Statistical analysis using a two-tailed, paired t-test. *** $p < 0.001$.

Figure 4.18 FcεRI-induced NFκB activation is downstream of PLD signaling.

PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight then pre-treated with 1 μM of PC–PLD inhibitors for 30 mins at 37^{o}C were treated with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI for the indicated times or left unstimulated for 60 mins as a control and expression of IκBα analysed by Western blotting (A). ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκB to ß-actin for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.19 IL-33-induced NFκB activation is downstream of PLD signaling.

PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight then pre-treated with 1 μM of PC–PLD inhibitors for 30 mins at 37^{o}C were treated with IL-33 (10 ng/ml) for the indicated times and expression of IκBα analysed by Western blotting (A). "0 min" represents zero timepoint control. ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκB to ß-actin for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.20 Basal, FcεRI- and IL-33- induced ERK phosphorylation in murine mast cells is downstream of PLD signaling.

PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight then pre-treated with 1 μM of PC–PLD inhibitors for 30 mins at 37^{o}C were treated with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI (A) or IL-33 (10 ng/ml) (B) for the indicated times or left unstimulated for 60 mins as a control (A). For IL-33 treatment "0 min" represents zero timepoint control (B). Expression of P-p44/42 was analysed by Western blotting with p44/42 used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of P-p44/42 to p44/42 for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 4.21 Determining the direct PLD activity in mast cells induced by IgE sensitization, FcεRI, IL-33 and PMA.

PDMC were cultured in the presence of 5 μCi [^{3}H]Palmitate and murine anti-DNP IgE (0.5 μg/ml) overnight (A-E). Cells were then washed and resuspended in serum-free HBSS before being loaded with 1% ethanol and, where appropriate, pre-treated with 1 μM of the indicated PC–PLD inhibitor for 20 mins at 37^{o}C. Where indicated, a sample was taken and the reaction terminated (Control (E). Alternatively, cells were then stimulated with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI (A-C), IL-33 (100 ng/ml) (B, E), PMA (1 μM) (A, D) or murine anti-DNP IgE (0.5 μg/ml) (F, G) for 60 mins (B, C, D, G) or the indicated time course. Data are presented as mean value of [3H]PtdEtOH (cpm) expressed as a % of the total incorporation of [3H]palmitate ± SD of triplicate cultures from a single experiment representative of at least 2 independent experiments. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test (A), two-way ANOVA with Bonferroni multiple comparison test (B-D) or unpaired, one-tailed t-test (E- H). * $p < 0.05$ and ** $p < 0.01$.

Figure 4.22 IL-33 signaling in murine mast cells involves SphK.

PDMC (A, C, E-G) or BMMC (B, D) sensitized overnight with murine anti-DNP IgE (0.5 µg/ml) and pre-treated with the indicated concentrations (A-D) or 10 µM (E-G) of the SphK inhibitor, DMS for 30 min were then stimulated with IL-33 (10 ng/ml) for 24 h at 37^{o}C. Cell death was assessed by 7AAD uptake using flow cytometry as described (2.6) (A, B) and is presented as % change in 7AAD+ cells compared to untreated cells. Release of IL-6 and MCP-1 was measured by ELISA from supernatants. Viability and ELISA results are matched and are representative of 2 independent experiments (A, C) or a single experiment (B, D) where error bars show SD.

For calcium results PDMC were simultaneously loaded with 10 µM DMS and Fura-2/AM. Cells were stimulated at 50s in serum-free HBSS with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (E), LPS (0.5 µg/ml) (F) or IL-33 (100 ng/ml) (G). The intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (E-G). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 2 independent experiments. Statistical analysis using a two-tailed, paired t-test. **** $p < 0.0001$.

Figure 4.23 IL-33 signaling in murine mast cells involves the SphK 2 isoform.

PDMC (A, C-E) or BMMC (B, F) sensitized overnight with murine anti-DNP IgE (0.5 µg/ml) and pre-treated with the indicated concentrations or 30 µM (E, F) of the SphK 1 isoform specific inhibitor, 5c for 30 min were then stimulated with IL-33 (10 ng/ml) for 24 h at 37^{o}C. Cell death was assessed by 7AAD uptake using flow cytometry as described (2.6) (A, B) and is presented as % change in 7AAD+ cells compared to untreated cells. Release of IL-6 and MCP-1 was measured by ELISA from supernatants. Viability and ELISA results are matched and are representative of 2 independent experiments (A, E) or a single experiment (B, F) where error bars show SD.

For calcium results PDMC were simultaneously loaded with 30 µM 5c and Fura-2/AM. Cells were stimulated at 50s in serum-free HBSS with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (E) or IL-33 (100 ng/ml) (F). The intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment. Statistical analysis using a two-tailed, paired t-test. **** $p < 0.0001$.

Figure 4.24 FcεRI and IL-33 treatment directly stimulates SphK activity in mast cells to produce S1P found in the serum of a murine model of asthma

IgE-sensitised PDMC were treated with DNP (0.5 μg/ml) (A) or IL-33 (100 ng/ml) (B) for the indicated times before measurement of SphK as described (2.12). The results were analysed to determine fold difference to 0 min baseline, assigned the value "1" Data is combined from 2 (A) or 3 (B) independent experiments. Values are represented as mean ± SEM.

S1P concentration from IgE-sensitised PDMC were treated with IL-33 (10 ng/ml) for the indicated times and then S1P levels in culture supernatants or cell lysates were determined by ELISA (C). Data are presented as a single value. Serum and BALf samples obtained from mice treated with PBS (control) or OVA/ALUM in a model of OVA-induced airway hyper-responsiveness as described (2.14) was analysed for S1P expression by ELISA (D). Data presented as a single value from a single experiment.

Figure 4.25 Proposed revision of IL-33 signaling.

IL-33-ST2 can signal independently of IgE sensitization however, IgE ligation of FcεRI, without the need for crosslinking, can augment IL-33 signaling potentially via increased PLD 1 and PLD 2 activity. This increased PLD platform, especially PLD 2, allows further signal propagation by IL-33-ST2 and Sphk2 activation to allow calcium mobilisation and increase NFκB activation.

5 FcεRI, ST2 and TLR4 crosstalk in murine mast cells; potential for modulation by ES-62.

5.1 Introduction

5.1.1 *ST2 and FcεRI crosstalk*

Data presented in the previous chapter highlighted the cooperative interactions between IgE sensitization and IL-33 signaling in murine mast cells. To further explore these interactions and their potential role *in vivo*, the potential crosstalk occurring during antigen-mediated activation of IgE sensitized mast cells in the presence of IL-33 was investigated, as both of these signals are likely to be present in abundance under pathological conditions such as those pertaining in asthma. Indeed, previous data in human primary mast cells have demonstrated increased degranulation [274] and cytokine production [62] following co-stimulation via IL-33/ST2 and FcεRI-XL. Likewise, FcεRI-mediated induction of cytokines, including MCP-1, TNFα and IL-13 from murine BMMC is increased by simultaneous treatment with IL-33- treatment [268] although the degranulation responses of BMMC and RBL-2H3 were largely unaffected [60]. Consistent with a physiological role for such crosstalk, in a model of passive cutaneous anaphylaxis, prevention of IL-33-ST2 ligation limits the response to IgE administration [329], whilst in a model of antibody-induced arthritis, exposure to IL-33 increases mast cell activation to exacerbate disease.

Although the signaling mechanisms involved have not been defined, such cooperation leading to IL-33-mediated augmentation of FcεRI responses was lost in MyD88$^{-/-}$ BMMC, reflecting loss of IL-33, but not FcεRI, signaling. Neither IRAK1 nor ERK activation appeared to be increased during crosstalk whereas TAK1, p38, JNK and NFκB activation was augmented in studies using RBL-2H3 mast cells and to a lesser extent in BMMC. Such increases in signaling activity were only apparent during simultaneous administration of IL-33 with the FcεRI crosslinking agent, and not following IL-33 pre-treatment [60]. IL-33

has been similarly shown to promote mast cell activation in response to signaling via other Fc receptors including the IgG receptor, FcγRIII resulting in increased IL-1 production in early inflammation [330]. Interestingly, long-term exposure (>72 h) to IL-33 has been reported to suppress FcεRI-mediated calcium signaling in mast cells potentially via downregulation of PLCγ [321], indicating alternative roles for IL-33 in acute and chronic antibody-mediated inflammatory conditions.

5.1.2 *ST2 and TLR4 crosstalk.*

The ability of LPS, a lipopolysaccharide on the surface of Gram-negative bacteria, to augment immune responses is well documented. Consistent with this, LPS has been reported to increase FcεRI-mediated mast cell responses in terms of cytokine production [32, 49, 58, 59] and, when co-administered with OVA, to increase AHR, eosinophil infiltration, cytokine production and degranulation of histamine in a murine model of asthma [49].

The potential interaction between LPS and IL-33 signaling is therefore of great interest due to their combined presence during infection [253] as well as the close relationship between their receptors, TLR4 and ST2, and consequent similarity in their signaling pathways (Figure 5.1) Not only does the physical presence of bacteria simulate IL-33 release after tissue damage but LPS signals, via TLR4, induce IL-33 production from macrophages [223], human mucosal epithelial cells [331], murine monocytes [332] and DC [333, 334]. Moreover, this LPS-induced IL-33 release from macrophages further augments anti-bacterial responses via TNFα production [335, 336].

Although crosstalk mechanisms between the receptors for LPS (TLR4) and IL-33 (ST2) have not been defined, there are clear opportunities for this to occur as ST2 exhibits some structural homology with TLRs [337] with both types of receptors possessing TIR domains that utilise MyD88-dependent signaling machinery. Rather surprisingly therefore, despite the seemingly positive relationship between IL-33 and LPS, it has generally been reported

that ST2 negatively regulates MyD88-dependent TLR responses by sequestering MyD88 and Mal using its TIR domain [178, 179]. More recently, structural and protein-protein docking analysis has supported this negative regulatory role by demonstrating that membrane bound ST2-TIR domains can prevent Mal and MyD88 from associating with TLR4 [338]. Furthermore and consistent with this, mutations in its TIR domain resulted in a failure of ST2 to inhibit LPS-induced NFκB activation [179]. As LPS upregulates ST2 on macrophages and monocytes, this suggested that ST2 acts to signal in a feedback mechanism to limit TLR4 signaling [179, 339]. Moreover, it has also been suggested, that in macrophages, ST2 signaling inhibits LPS responses by downregulating TLR4 expression [340]. Conversely, recent evidence suggests that IL-33 signaling has no effect on TLR4 or MyD88 expression but rather instead, ST2 inhibits LPS-TLR4 signaling by inducing degradation of IRAK1 that is essential for TLR4 signaling in mast cells [341].

These molecular studies provided evidence that the ST2 receptor can negatively modulate TLR4 signaling however only one of these studies were performed under conditions of IL-33-ST2 signaling [341], perhaps going some way to explaining the contradictory reports in the literature where IL-33 acts to increase LPS responses [335, 336]. In these studies, IL-33 upregulates expression of TLR4, MD-2, CD14 and MyD88, findings corroborated by their reduced expression in cells from $ST2^{-/-}$ mice. Moreover, IL-33-deficient mice have been reported to exhibit reduced responses to LPS [235] and additionally, soluble ST2, an inhibitor of IL-33 signaling, has been found to inhibit cytokine production in response to LPS in a murine model of LPS-induced acute lung injury [342]. Although it is not clear what switches ST2 between a positive and negative regulator of TLR4 signaling, it is well established that TLRs are able to associate with other innate receptors, for example C-type lectins [343].

5.1.3 ***Potential modulation of IL-33 responses by ES-62.***

ES-62 can inhibit FcεRI-induced responses in human mast cells[126]) where this inhibition is due to TLR4-mediated PKCα degradation, preventing

subsequent coupling of FcεRI-mediated coupling to PLD/SphK-driven calcium mobilisation and consequent NFκB activation. Data presented in this thesis (Chapter 3) has shown that ES-62 can similarly target FcεRI responses in murine PDMC, BMMC and CTMC and in addition to suppressing calcium mobilisation and inducing degradation of PKCα, in all three subtypes. ES-62 has also been reported to inhibit FcεRI and TLR4 signaling in murine BMMC via targeted degradation of PKCα, PKCδ and MyD88 [134] and perhaps consistent with the latter finding identifying a commonality of action, ES-62 has also been shown to downregulate MyD88 in Th17 cells [135], macrophages and DC (Eason, McGrath, Rodgers and Harnett unpublished). Interestingly, therefore IL-33 signals via MyD88 and is associated with promoting pathology in several diseases in which ES-62 has demonstrated therapeutic potential, including rheumatoid arthritis and asthma. Moreover, mast cells have been reported to play an increasingly dominant role in the pathology of Th17-mediated disease, especially in those associated with chronic infection and/or inflammation where a Th17 response prolongs pathology. In particular, mast cells stimulated with IL-33 have been found to drive Th17 development in a model of allergic asthma [244] and chronic intestinal inflammation [344]. As ES-62 exhibits protection against autoimmune and allergic inflammatory disease by suppressing IL-17-mediated inflammation [135, 345], it was possible that ES-62 was acting, at least in part, by suppressing IL-33-mediated induction of such responses.

5.1.4 *ST2 deficient mast cells*

ST2-deficient mice were created to study the role of ST2 in immune responses [158] and since the identification of IL-33 as its ligand, the role of ST2 in IL-33 signaling. Although ST2 is expressed early in mast cell development, $ST2^{-/-}$ mice have equivalent mast cell numbers in the skin as WT mice and these mast cells appear to degranulate normally. Moreover, BMMC can be derived from $ST2^{-/-}$ bone marrow, confirming that ST2 is not essential for mast cell development [346].

5.2 Aims

The availability of $ST2^{-/-}$ mice allowed the validation of the dependence of IL-33 signaling on ST2 expression in various mast cell responses (Chapter 4). However, as there are contradictory reports that whilst IL-33 augments LPS responses, ST2 signaling can inhibit LPS responses [179, 340]**, it** was planned to exploit this resource to further examine the relationship between ST2 and TLR4 in terms of crosstalk between IL-33 signaling and the classical pro-inflammatory ligand, LPS. Moreover, in earlier studies showing ES-62-mediated desensitisation of pro-inflammatory TLR4-signaling it was suggested that ES-62 could perhaps signal directly via ST2, and although this did not seem to be the case in macrophages [132], it was also decided to test the effect of ST2 deficiency on the anti-inflammatory effects of the non-canonical TLR4 ligand, ES-62 in mast cells.

5.3 Results and Discussion

5.3.1.1 IL-33 signaling modulates FcεRI-mediated cytokine production in murine mast cells.

Whilst FcεRI-XL induces little or no production of IL-6 or IL-13 by PDMC or BMMC from WT or ST2-/- BALB/c mice, IL-33 generally induces low levels of these cytokines by WT mast cells, although there is a relatively strong production of IL-6 by BMMC. All of these IL-33 responses, as were the negligible FcεRI, were completely abrogated in cells from ST2-/- mice. In terms of co-stimulation, with PDMC there was no reproducible effect on production of IL-6 however that of IL-13 was increased, but not significantly, in 2/3 experiments (Figure 5.2 A). By contrast, following such co-stimulation of BMMC, IL-13 is consistently increased (Figure 5.2 B) whilst IL-6 production is significantly reduced (2/3 experiments), potentially reflecting the different signaling responses to FcεRI-XL seen amongst PDMC, BMMC and CTMC (Chapter 3).

5.3.1.2 The availability of ST2 affects FcεRI-induced calcium mobilisation and downstream signaling in murine mast cells.

To address whether ST2 expression modulated FcεRI-signaling in an IL-33-independent manner analogous to that reported for LPS/TLR4 signaling the effects of ST2 deficiency on FcεRI-induced calcium mobilisation, NF-κB and ERK activation were assessed. Although FcεRI-XL induced little or no cytokine production from either WT PDMC or BMMC, any such production appeared to be lost in ST2-deficient cells (Figure 5.2) and perhaps consistent with this, PDMC (Figure 5.3 A) and BMMC (Figure 5.3 B) generated from ST2 -/- mice exhibited significantly reduced calcium responses after XL which appeared predominantly to be a as a result of reduced influx of extracellular calcium (Figure 5.3 C, D).

Likewise, IκB expression is not reduced to the same extent and recovers faster in ST2-/- BMMC relative to that observed in WT cells, indicating weaker NFκB activation in response to FcεRI-XL (Figure 5.3 E). Moreover, FcεRI-mediated ERK activation is similarly reduced in both PDMC (Figure 5.4 A) and BMMC (Figure 5.4 B) from ST2-/- compared to WT mice. These data suggest that rather than the negative feedback role proposed for ST2 in LPS/TLR4 signaling, FcεRI requires ST2 expression for full signaling.

5.3.2 *ST2 negatively regulates LPS signaling in mast cells.*

As LPS responses are reportedly increased in mast cells deficient in ST2, the interaction between IL-33-ST2 and LPS-TLR4 signaling was further examined. When IL-33 and LPS are co-administered there is a significantly increased effect on IL-6 and IL-13 production in both PDMC (Figure 5.5 A) and BMMC (Figure 5.5 B) relative to either single stimuli alone, supportive of previous results [335, 336] and in conjunction with their cooperative role in infection. Although in this experiment the stimulation of IL-13 production in PDMC appears synergistic, over several experiments all of these enhanced responses are less than additive responses of the single stimuli perhaps suggesting they

are signaling via a shared pathway or both utilising core elements. In the absence of ST2 signaling, LPS-induced IL-6 and IL-13 cytokine production in PDMC and IL-6 in BMMC is consistently, significantly increased and to higher levels than that resulting from co-stimulation with LPS and IL-33. This could perhaps suggest that by functionally signaling via ST2, IL-33 is relieving its negative regulation on TLR4 and therefore these findings could some way to resolving the contradictory data relating to the regulation of TLR4 signaling by IL-33/ST2 in the literature. In contrast BMMC IL-13 is significantly reduced in ST2 -/- in 3/3 experiments. Interesting, although as expected the IL-33 response is lost in the ST2-deficient cells, ST2 -/- PDMC and BMMC co-stimulated with LPS and IL-33 exhibited reduced rather than equivalent cytokine responses compared to those treated with LPS (IL-6; 2/3, IL-13; 3/3). This surprising finding perhaps suggests that although ST2 is essential for its signaling, IL-33 can interact with another (undefined) receptor, possibly as part of the normal active IL-33-ST2/IL-1RAcP complex, that is not sufficient to transduce IL-33 functional responses in the absence of ST2 but can still sequester signaling elements shared with TLR4, or act otherwise, to negatively modulate TLR4 responses in the presence of IL-33. IL-1RAcP, bearing TIR domains, assists in MyD88 recruitment within the ST2 complex. It has been proposed that IL-1RAcP may also bind IL-33, although to a lesser extent than ST2 [347], so perhaps IL-1RAcP expression is maintained on ST2 -/- cells, allowing the observed effects.

These findings support previous data suggesting ST2 inhibits TLR4 signaling but perhaps instead of true inhibition, the interaction is more of regulatory signal, to tune the phenotype of immune response and/or prevent hyper-responses resulting from the high levels of LPS and/or IL-33 potentially observed under conditions of pathological inflammation. Thus, the absence of ST2 allows TLR4 to signal to its full potential. In the case of BMMC, this would suggest that TLR4 signaling would act to suppress IL-13 production, possibly reflecting the role for LPS in driving Th1-type responses and limiting Th2-promoting signals by IL-33 stimulated BMMC, cells expected to play roles in Th2-driven allergy in the mucosa. Although this latter proposal is somewhat at odds with reports that LPS augments airway-hyperresponsiveness [49, 348], evidence in the literature also demonstrated that LPS inhibits Th2 cytokine

responses in the BALF and AHR [349] as well as the perinatal exposure to LPS inducing protection against asthma [350].

5.3.2.1 Expression of ST2 and TLR4-MD2 on mast cells is linked

To begin to identify the mechanisms of crosstalk between ST2 and TLR4 signaling, their relationships in terms of their surface expression was investigated. LPS has been shown previously to upregulate ST2 expression on macrophages and monocyte [179, 339] and here flow cytometric analysis of CD117+ FcεRI+ cells demonstrated that whilst ST2 was strongly expressed on all WT PDMC (Figure 5.6 A, C, E, G) and BMMC (Figure 5.6 B, D, F, H) but not on ST2-/- mast cells, it was further up-regulated following exposure to LPS, indicating that LPS may induce either positive or negative feedback responses by upregulating the IL-33 receptor. To address the mechanisms involved, the effects of LPS on surface and total (permeabilised cells) ST2 expression were investigated and this revealed a general upregulation of ST2 expression indicating this this did not simply involve trafficking of intracellular pools of ST2 to the surface (Figure 5.7 G, H).

In these experiments on BALB/c mice, TLR4 staining was relatively weak and hence was only detected on about 60% of PDMC (Figure 5.7 A, C, E, G) and BMMC (Figure 5.7 B, D, F, H). Although not significant, and surprisingly given that LPS responses tended to be increased, there appeared to be a decrease in the proportion of cells (Figure 5.7 C, D) and expression levels (Figure 5.7 E, F) of TLR4 in ST2 -/- mast cells. Analysis of permeabilised cells showed a similar reduction in expression indicating that this was not as a result of a defect in trafficking TLR4 to the surface (Figure 5.7 G, H). These data suggest that ST2 and TLR4 expression may be linked and as we have found TLR4 to be expressed only relatively late (4 weeks) in their development *in vitro*, these findings may suggest that ST2 -/- cells may be slightly more immature than those derived from WT mice.

5.3.2.2 The availability of ST2 affects LPS-induced calcium mobilisation and downstream signaling murine mast cells.

Analysis of LPS-stimulated calcium mobilisation in WT and ST2-/- PDMC (Figure 5.8 A) and BMMC (Figure 5.8 B) revealed, consistent with the reduced levels of TLR4 expression, that in BMMC the calcium response was suppressed and that this reflected reduced intracellular calcium mobilisation (Figure 5.8 B, D). It should be noted however, that in these experiments, the LPS response in WT PDMC is very low (Figure 5.8 A). In BMMC although data reflects a lower TLR4 expression and IL-13 production by ST2 -/- cells, they are rather at odds with the increased levels of IL-6, suggesting that IL-13 and IL-6 production by such cells is differentially regulated, with IL-13 production perhaps being more dependent on calcium mobilisation signals.

To address this ST2-TLR4 crosstalk further, activation of NFκB was assessed and this showed that as with WT cells, IκB is downregulated in ST2 -/- PDMC treated with LPS (Figure 5.9 A), however, quantitation of the data indicated that ST2 -/- PDMC exhibited a delay in the degradation of IκB, although a slightly enhanced level of IκB (relative to ß-actin) was found in PDMC from ST2-deficient mice. The increase in this negative regulator was strongly pronounced in the equivalent BMMC but this was almost completely ablated in response to LPS, indicating a strong NF-KB activation. Moreover, in WT but not ST2-/- BMMC, IκB expression is beginning to recover by 60 min (Figure 5.9 B). Interestingly, LPS-induced ERK phosphorylation in increased in PDMC (Figure 5.10 A) and BMMC (Figure 5.10 B) from ST2 -/- mice both in terms of faster kinetics and also stronger activation.

Collectively, these data indicate that whilst ST2 expression acts to promote LPS/TLR4 coupling to calcium signaling in BMMC, it limits LPS-stimulated NF-KB and ERK responses in PDMC. This suggests that whilst the stronger production of IL-13 observed in BMMC may be dependent on the ST2-mediated enhancement of calcium mobilisation in WT cells, that of IL-6 is more dependent on NFκB and ERK signaling. Recent evidence suggests that alternate IL-13 production from IL-33 stimulated MC, may arise from differing requirements for co-stimulation with IL-3, provided in BMMC culture conditions, to activate STAT5 [351].

5.3.3 *ES-62 can modulate IL-33 signaling in murine mast cells.*

It has previously been shown that ES-62 can desensitise FcεRI and LPS-induced mast cell responses [126, 134] and consistent with this, suppress OVA-induced AHR by resetting Th17/Th2 polarised responses [345]. Thus, due to the emerging role for IL-33 and mast cells in these responses [220, 221, 243-246], the effects of ES-62 on IL-33-stimulated production of a range of pro-inflammatory cytokines (IL-6, IL-13, IL-17, IL-22 and IL-23) and chemokines (MCP-1), implicated in promoting the Th17/Th2 polarised responses observed in allergic inflammation, were investigated. This revealed that in IgE-sensitised PDMC, pre-treatment with ES-62 (2 μg/ml) for 18 h suppressed production of all these cytokines, albeit this did not reach significance in all experiments (Figure 5.11 A). Interestingly, pre-treatment with ES-62 for only 2 h was sufficient to inhibit IL-13, IL-17 and MCP-1 and under these conditions, also inhibited production of IL-17 and MCP-1 by non-sensitised cells, even at concentrations lower than the "standard" dose of 2 μg/ml (Figure 5.11 B).

That such ES-62 immunomodulation was not mediated by receptor downregulation was evidenced by the flow cytometric analysis of surface expression of FcεRI, CD117 and ST2 (Figure 3.7) that showed that pre-treatment with the parasite product had negligible effect on expression of any of these markers on either resting or IgE-sensitised mast cells. By contrast, as described for its effects on FcεRI- and LPS-stimulated pro-inflammatory responses (Figure 3.9), ES-62-mediated inhibition of cytokine responses was reflected by inhibition of IL-33-stimulated calcium mobilisation, with both the extent and kinetics of calcium mobilisation reduced (Figure 3.12 A). The mechanisms involved in such desensitisation of calcium mobilisation are not clear but it has been proposed ES-62 may signal abortively during its initial interaction with TLR4 on macrophages as evidenced by production of a low level of cytokines [184]. Thus, in order to determine if ES-62 could potentially induce signaling upon initial binding to TLR4 on mast cells its ability to activate calcium mobilisation was determined. This revealed that ES-62 induces a very slight immediate calcium response in mast cells (Figure 5.12 B) that may contribute to the, as yet

unknown, signals that promote degradation of PKCα, δ and/or MyD88 expression in order to uncouple TLR4, FcεRI and potentially, ST2 signaling in mast cells.

5.3.4 *ES-62 modulates FcεRI and LPS-induced cytokine responses from mast cells derived from ST2 -/- mice.*

As shown previously, ES-62 significantly inhibits IL-6 and IL-13 production induced by IL-33 from PDMC (Figure 5.13 A) and as expected, this is dependent on ST2 expression, although in this experiment there was residual IL-33-stimulated IL-13 production that was also inhibited by ES-62 in ST2 -/- PDMC, a finding that was not observed in repeat experiments. Such ES-62-mediated inhibition of IL-33-mediated, ST2-dependent cytokine responses were also observed in BMMC (Figure 5.13 B).

As shown in Chapter 3, ES-62 inhibits LPS-induced IL-6 and IL-13 production from both PDMC and BMMC derived from WT mice (Figure 5.14 A). Such ES-62 activity in BMMC does not require ST2 expression as demonstrated by the maintenance of inhibition of LPS-induced cytokines from ST2 -/- BMMC (Figure 5.14 B). Interestingly in ST2 -/- PDMC, LPS-induced cytokines are not significantly inhibited by ES-62 (3/3 experiments for IL-13 and IL-6). Consistent with this, the ES-62-mediated suppression of IL-6 produced by PDMC (Figure 5.15 A) but not BMMC (Figure 5.15 B) in response to co-stimulation with IL-33 and LPS is also lost in ST2-deficient cells. IL-13 responses mirrored this trend, although for this cytokine these effects were not statistically significant.

IL-33 stimulation generally enhanced FcεRI-mediated cytokine responses although the precise effect depended on the MC subtype and the cytokine analysed (Figure 5.2). ES-62 is able to significantly inhibit this increased cytokine production in both PDMC (Figure 5.16 A) and BMMC (Figure 5.16 B). In ST2 -/- cells, cytokine production is significantly and dramatically decreased, coinciding with the loss of IL-33 signaling but is still significantly modulated by ES-62 in the case of BMMC IL-13 production.

Collectively, these data again highlight signaling differences between the PDMC and BMMC subtypes and their subsequent response to ES-62 modulation, which may reflect differential negative regulatory mechanisms of action of ES-62 in PDMC and BMMC.

5.4 Conclusion

ST2 has been reported to be non-essential for the generation of mast cells [346] and our ability to generate expanded PDMC and *in vitro* differentiated BMMC corroborate this proposal. Although ST2-deficiency does not prevent MC generation it may, however, affect their maturation or activation capacity as the reduced calcium, NFκB and ERK activation and subsequent cytokine responses observed in response to FcεRI-XL in PDMC and BMMC from ST2 -/- mice, indicates an important role for ST2 in the activation of MC by antigen-mediated IgE crosslinking (Table 5.1). This may suggest that IL-33 signaling is important for the maturation of murine mast cells and indeed their lower expression of TLR4 suggests ST2 -/- MC may not reach full maturity, or alternatively take longer to mature, with their altered responses to stimuli reflecting this. As IgE sensitization of FcεRI appears to be required for optimal IL-33-ST2 responses, these new findings further support crosstalk between FcεRI and ST2 receptors. The requirement of ST2 (independently of IL-33 ligation) for full FcεRI function suggests an involvement of ST2 in formation or stabilization of signaling clusters during the early stages of FcεRI-mediated activation, perhaps to prevent abortive signaling.

Similarly, our data provide further support to the proposal that ST2 may interact with TLR4 to modulate mast cell responses. There is conflicting evidence in the literature relating to the role of ST2 in regulating LPS/TLR4 signaling with IL-33 reported to increase LPS responses whilst ST2 expression has been shown to inhibit LPS signaling. The differential effects of ST2 expression on IL-13 production observed between the PDMC and BMMC may therefore go part of the way to resolve such discrepancies reported, as given

their differential signaling, the precise subtype of mast cell and/or receptor targeted may influence the observed response. For example, although ST2-deficiency results in reduced FcεRI- and LPS-mediated calcium mobilisation (the latter only significant in BMMC), whilst FcεR1-coupling to NF-κB and ERK activation is also suppressed, that of TLR4 is enhanced. Moreover, whilst ST2 expression is important for ES-62-mediated inhibition of PDMC, it is dispensable in BMMC.

Regulation of TLR4 signaling is important to prevent unnecessary pathology due to hyper-inflammation during immune responses and reflecting this, failure to regulate TLR4-mediated responses has been implicated in several diseases [352]. Although exposure to LPS can directly downregulate TLR4 surface expression resulting in "LPS tolerance" [353], regulation of LPS-responses is commonly controlled by heterologous receptor signaling, which may be constitutive or upregulated following TLR4 stimulation and functions to downregulate surface expression and promote degradation of TLR4 as well as suppressing TLR4 gene induction. TLR4 expression on macrophages has been reported to be decreased by ST2-deficiency [340] and consistent with this, it was found here that there is also decreased TLR4 expression in ST2-/-MC. TLR4 expression is constantly cycled in macrophages allowing environmental sampling, and it has been suggested that ST2, the expression of which appears to be linked to that of TLR4, behaves in a similar manner by cycling though early endosomes alongside TLR4 [281, 282].

LPS upregulates ST2 levels in PDMC and BMMC indicating a negative feedback response perhaps analogous to that described for macrophages where MyD88 sequestration by ST2 via TIR domains has previously been reported [178, 179]. Perhaps consistent with this, a recent study suggested IL-33-ST2 signaling induced proteasome-dependent degradation of IRAK1, a signaling element that is recruited to activated MyD88 and non-redundant for TLR4 signaling in murine mast cells [341], In addition, it has also been reported that TLR4 signaling can be inhibited by sustained interaction of the p85 regulatory subunit of PI3K with MyD88 [57, 354] and ES-62 targets MyD88 in mast cells and T cells to prevent IL-1R/TLR signaling suggesting this is a key intersecting point in ST2 and TLR4 signaling (Figure 5.17), the latter both for canonical pro-inflammatory signaling (eg LPS) and also subversion to an anti-

inflammatory phenotype (ES-62). Regardless of the mechanism, however, IL-33 modulation of TLR4-induced mast cell activation is important in both inflammation and homeostasis as aberrant responses to commensal bacteria may be limited by low level, constitutive exposure to IL-33 at epithelial surfaces [341].

Although LPS is well established to drive Th1 immune responses, ST2-modulation of LPS responses in MC provides a mechanism whereby IL-33 may drive the development of a strong Th2 phenotype or alternatively, a mechanism whereby LPS can limit Th2 responses. IL-33 has been shown here to have the capacity to induce IL-17, IL-22 and IL-23 generation by MC, cytokines associated with a Th17 phenotype. The ability of Th17 cells to master regulate polarisation towards a Th1 or Th2 phenotype in autoimmune (role in type 1 diabetes reviewed in [355]) or allergic (role in asthma reviewed in [356, 357]) disease respectively, conditions in which IL-33 has been proposed to play a pathogenic role [221, 358, 359], may therefore suggest an important role for ST2-TLR4 crosstalk in mast cells in regulating such responses. Indeed, MC have previously been demonstrated to promote Th17 T cell differentiation via their effect on DC [93] and abundant IL-6 production [360]. The IL-33 stimulated production of IL-17, IL-22 and IL-23 by MC shown here now supports previous work reporting direct stimulation of Th17 differentiation by IL-33-stimulated mast cells *in vitro* and IL-33-mediated exacerbation of Th17-pathogenesis in OVA-induced allergic asthma in mice [244]. This regulatory mechanism may also provide a means whereby IL-33 and MC can exhibit both protective and pathological roles in disease, depending on the tissue involved and the surrounding inflammatory situation.

The ES-62-mediated inhibition of Th17-related cytokines from MC reflects the ability of the parasite product to ameliorate Th17/Th1 [135] and Th17/Th2 [345] biased inflammatory situations and may suggest that mast cells may be targeted under these conditions. Indeed, ES-62 is able to significantly reduce mast cell cytokine production in response to all of the key immunoregulatory stimuli tested as evidenced by its inhibition of LPS/TLR4, IL-33/ST2 and FcεRI signaling, either independently or in combination. The mechanisms involved have not been fully defined but with respect to FcεRI and LPS/TLR4 (Chapter 3), this appears to involve targeted degradation of PKCα, PKCδ and MyD88

[126, 134]. The mechanism underpinning its action against IL-33 signaling is much less understood but preliminary studies presented here again indicate suppression of calcium mobilisation.

Interestingly, it was proposed previously [132] that ES-62 might exploit the ability of ST2 to sequester MyD88 [179, 340] to modulate TLR signaling in monocytes, however, this did not appear to be the case as ES-62 was still able to inhibit IL-12 production in response to LPS stimulation in cells from ST2-deficient mice [132]. The finding that FcεRI and LPS responses in BMMC can still be inhibited by ES-62 in the absence of ST2 expression, supports and extends these previous data. Interestingly, therefore, the preliminary data presented here also suggest that ST2 is required for inhibition of PDMC responses by ES-62. It was previously reported that whilst ES-62 downregulated expression of PKCα in each of PDMC, BMMC and CTMC it potentially mediated it's suppressive effects on the strong, TLR-biased cytokine responses of BMMC, but not PDMC, at least in part by downregulating MyD88 [134]. These new data may suggest that as PDMC only make weak cytokine responses, even in response to LPS/TLR4, that exploiting the ability of ST2 to sequester MyD88, rather than directly inducing its degradation is sufficient to enable ES-62-mediated suppression of such responses (5.18).

Figure 5.1 IL-33/ST2 and LPS/TLR4 share common signaling molecules.

Members of the IL-1R family and TLR family of receptors such as ST2 and TLR4 have similarities in signaling pathways. Using TIR domains, ST2 and TLR4 recruit MyD88 to initiate signaling using IRAK1, IRAK4 and TRAF6, resulting in canonical NFκB, ERK, JNK and p38 activation.

Figure 5.2 IL-33 increases cytokine production induced by FcεRI, an effect dependent on ST2 expression.

IgE-sensitized PDMC (A) and BMMC (B) obtained from WT or ST2 -/- BALB/c mice were treated simultaneously with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI and IL-33 (10 ng/ml) for 24 h at 37^{o}C before analysis of IL-6 and IL-13 levels in culture supernatants by ELISA. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test. *** $p < 0.001$.

Figure 5.3 FcεRI-stimulated calcium mobilisation and NFκB activation is dependent on ST2 expression.

WT or ST2 $^{-/-}$ PDMC (A, C) and BMMC (B, D) sensitised with murine anti-DNP IgE (0.5 µg/ml) and loaded with Fura-2/AM were stimulated at 50s in serum-free HBSS with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI. Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (C, D). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Statistical analysis using a two-tailed, paired t-test. *** $p < 0.001$.

WT or ST2 -/- BMMC (E) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were cultured with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI for the indicated times and expression of IκBα analysed by Western blotting. “0 min” represents zero timepoint control. ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκBα compared to ß-actin for each timepoint. Data are from a single experiment.

Figure 5.4 FcεRI induced ERK phosphorylation is decreased in ST2 -/- murine mast cells.

WT or ST2 -/- PDMC (A) or BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were cultured with DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI for the indicated times and expression of Pp44/p42 (pERK) analysed by Western blotting. "0 min" represents zero timepoint control. p44/42 (ERK) was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of Pp44/42 compared to p44/42 for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 5.5 Co-administration of IL-33 and LPS increases cytokine production from murine mast cells and is increased further in the absence of ST2 expression.

IgE-sensitized PDMC (A) and BMMC (B) obtained from WT or ST2 -/- BALB/c mice were treated simultaneously with LPS (0.5 μg/ml) and IL-33 (10 ng/ml) for 24 h at 37^{o}C before analysis of IL-6 and IL-13 levels in culture supernatants by ELISA. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test. ** $p < 0.01$ and *** $p < 0.001$.

Figure 5.6 LPS upregulates frequency and expression of ST2 on murine mast cells.

Flow cytometric analysis of PDMC (A, C, E, G) or BMMC (B, D, F, H) sensitized with murine anti-DNP IgE and treated with LPS (0.5 µg/ml) overnight. Cells were gated for live 7AAD- , CD117+ and FcεRI+ cells then analysed for surface (A-H) and total (surface and intracellular) (G-H) ST2 expression. The gating plots relative to WT isotype (A, B) were used to determine % frequency (C, D) and the geometric mean fluorescence (GMFI) (E-H) of ST2+ cells. Data represents the combined results of two independent experiments performed in duplicate where error bars represent SEM. Statistical analysis is by unpaired, one-tailed t-test. * $p < 0.05$ and *** $p < 0.001$.

Figure 5.7 Absence of ST2 reduces the TLR4 expression of murine mast cells.

Flow cytometric analysis of PDMC (A, C, E, G) or BMMC (B, D, F, H) sensitized with murine anti-DNP IgE and treated with LPS (0.5 μg/ml) overnight. Cells were gated for live 7AAD-, CD117+ and FcεRI+ cells then analysed for surface (A-F) and total (surface and intracellular) (G-H) TLR4 expression. The % frequency (C, D) and the geometric mean fluorescence (GMFI) (E, H) was determined relative to gating against the relevant WT isotype control. Data represents the combined results of two independent experiments performed in duplicate where error bars represent SEM. Statistical analysis is by unpaired, one-tailed t-test.

Figure 5.8 LPS-stimulated calcium mobilisation is dependent on ST2 expression.

WT or ST2 $^{-/-}$ PDMC (A, C) and BMMC (B, D) sensitised with murine anti-DNP IgE (0.5 µg/ml) and loaded with Fura-2/AM were stimulated at 50s in serum-free HBSS with LPS (0.5 µg/ml). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (C, D). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 3 independent experiments. Statistical analysis using a two-tailed, paired t-test.* $p<0.05$ and *** $p< 0.001$.

Figure 5.9 LPS-stimulated NFκB activation is dependent on ST2 expression.

WT or ST2 -/- PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight were cultured with LPS (0.5 μg/ml) for the indicated times and expression of IκBα analysed by Western blotting. "0 min" represents zero timepoint control. ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκBα compared to ß-actin for each timepoint. Data are from a single experiment representative of a single (A) or 2 independent experiments (B).

Figure 5.10 LPS- induced ERK phosphorylation is increased in ST2 -/- murine mast cells.

WT or ST2 -/- PDMC (A) or BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were cultured with LPS (0.5 µg/ml) for the indicated times and expression of Pp44/p42 (pERK) analysed by Western blotting. "0 min" represents zero timepoint control. p44/42 (ERK) was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of Pp44/42 compared to p44/42 for each timepoint. Data are from a single experiment representative of at least 2 independent experiments.

Figure 5.11 ES-62 modulates IL-33-induced cytokine responses.

PDMC were sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight (A) or non-sensitized (B) in the presence of 2 µg/ml or the indicated dose (B) of ES-62 or 2 h ES-62 (2 µg/ml) treatment prior to stimulation with IL-33 (10 ng/ml) for 24 h at 37^{o}C.Release of IL-13, IL-6, IL-17, IL-22, IL-23 and MCP-1 was measured by ELISA from culture supernatants (A). (B) presented as % change compared to uninhibited cytokine production. Results from a single experiment representing at least 2 independent experiments with the exception of the IL-17 ELISA. Error bars represent SD. Statistical analysis is by unpaired, one-tailed t-test. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 5.12 ES-62 modulates calcium mobilisation induced by IL-33 and directly stimulates calcium mobilisation in mast cells.

PDMC were sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence (A) or absence of (B) ES-62 (2 μg/ml) then loaded with Fura-2/AM before stimulation at 50s in serum-free HBSS with IL-33 (100 ng/ml) (A) or ES-62 (2 μg/ml) (B). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of at least 2 (B) or 3 (A) independent experiments. Statistical analysis using a two-tailed, paired t-test. **** $p < 0.0001$.

Figure 5.13 IL-33-induced cytokine production is modulated by ES-62.

WT or ST2 -/- PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) were stimulated with IL-33 (10ng/ml) for 24 h at 37^{o}C before release of IL-13 and IL-6 was measured by ELISA from culture supernatants. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test.* $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 5.14 LPS-induced cytokine production is increased in the absence of ST2 expression and is still modulated by ES-62.

WT or ST2 -/- PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight in the presence or absence of ES-62 (2 µg/ml) were stimulated with LPS (0.5 µg/ml) for 24 h at 37^{o}C before release of IL-13 and IL-6 was measured by ELISA from culture supernatants. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test.* $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 5.15 Cytokine production following co-administration of LPS and IL-33 is altered in the absence of ST2 expression and is modulated by ES-62.

WT or ST2 -/- PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight in the presence or absence of ES-62 (2 µg/ml) were stimulated with LPS (0.5 µg/ml) and IL-33 (10 ng/ml) for 24 h at 37^{o}C before release of IL-13 and IL-6 was measured by ELISA from culture supernatants. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test.* $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 5.16 Cytokine production following co-stimulation of FcεRI by IL-33 is reduced in the absence of ST2 expression and is modulated by ES-62.

WT or ST2 -/- PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 μg/ml) overnight in the presence or absence of ES-62 (2 μg/ml) were stimulated with DNP (0.5 μg/ml) to induce cross-linking (XL) of FcεRI and IL-33 (100 ng/ml) for 24 h at 37^{o}C before release of IL-13 and IL-6 was measured by ELISA from culture supernatants. Data are presented as the mean of triplicate samples ± SD from a single experiment representing 3 independent experiments. Statistical analysis using unpaired, two-tailed t-test.* $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Table 5.1 Summary of the role of ST2 in FcεRI-XL and LPS/TLR4 signaling and responses in BMMC and PDMC.

In the absence of ST2 expression, FcεRI-XL calcium signaling, NFκB and ERK activation and subsequent IL-6 and IL-13 cytokine responses are reduced in both BMMC and PDMC. LPS-induced responses in ST2-deficient MC vary based on murine MC subtype. In BMMC, LPS calcium mobilisation is inhibited but not in PDMC whereas NFκB and ERK activation is increased in both BMMC and PDMC. IL-6 production is increased in ST2-/- MC after LPS treatment but IL-13 is alternatively regulated in BMMC (decreased) and PDMC (increased). Co-stimulation with IL-33 decreases LPS-induced IL-6 and IL-13 even in the absence of the IL-33 receptor, ST2. ES-62 does not require ST2 expression to inhibit FcεRI-XL cytokines, in both BMMC and PDMC and LPS responses in BMMC, however fails to modulated LPS responses in ST2 -/- PDMC.

Figure 5.17 Proposed method for ST2-TLR4 interaction and signaling modulation by ES-62.

Previous data indicates that ST2 sequesters MyD88 from TLR4, limiting LPS signaling (ref) and reflects that MyD88 is shared between the receptors as it is required for both IL-33 and LPS to signal (A). With the absence of ST2, TLR4 has greater access to MyD88 and so signaling propagation increases (B). ES-62 does not need ST2 expression to signal (at least in BMMC) but is able to modulate ST2 signaling, most likely via TLR4-mediated MyD88 sequestration and degradation as seen previously in murine mast cells (C) [134].

Figure 5.18 Model for alternate ES-62 modulation of BMMC and PDMC

FcεRI, TLR4 and ST2 responses can be inhibited by ES-62 in both BMMC and PDMC, however the mechanism of suppression may vary in each mast cell type. Suggested methods of inhibition involve targeted degradation of PKCα and MyD88 to suppress calcium responses. In BMMC, but not PDMC, FcεRI and TLR4 modulation does not require ST2 expression suggesting that the proposed mechanism of ST2-mediated MyD88 sequestration is not used by ES-62 in BMMC. However, with their requirement for ST2 expression, ES-62 may hijack ST2 to inhibit TLR4 signals via sequestration of MyD88 in PDMC, an ability made possible by the reduced cytokine responses from PDMC compared to BMMC.

6 ST2-cKIT crosstalk

6.1 Introduction

6.1.1 *IL-33/ ST2 and SCF/ cKIT signaling*

SCF signaling has been reported to enhance responses via TLR4 [57] and FcεRI [55, 56] in mast cells (MC). For example, it appears that prolonged low levels of SCF stimulation allows MCs to be activated and degranulate in response to concentrations of Ag that would otherwise fail to induce activation; SCF appears to do this by increasing the basal phosphorylation levels of ERK, p38 and LAT [55]. Recently, evidence has emerged to suggest that IL-33 and SCF signaling are similarly linked, with SCF priming of cKIT allowing IL-33 binding to ST2 to induce a physical association between the receptors, facilitating a broader and more enhanced signaling profile [273].

Accordingly, in HMC-1, a human leukemic mast cell line, co-administration of IL-33 and SCF increases IL-6 and IL-13 production compared to that induced by IL-33 alone [361] and reflecting this, SCF increases ST2-mediated JNK and ERK phosphorylation in HMC-1 [361]. Indeed, in HMC-1 cells whilst ST2 signaling was found to cross-activate cKIT, cKIT signaling was required for full IL-33 responses in mast cells [273] as evidenced by the finding that Imatinib, an inhibitor of cKIT signaling, suppressed IL-33-induced STAT3, ERK, PKB and JNK1 activation in HMC-1 cells, resulting in reduced IL-4, IL-6 and IL-8 production. Although these data suggest that cKIT signaling is important for optimal IL-33 responses, the residual IL-33-mediated signaling observed in the presence of Imatinib indicates the activation of both cKIT-dependent and -independent signaling processes [273]. Consistent with this proposal, whilst cKIT $^{-/-}$ BMMC exhibit reduced PKB and JNK1 activation and no ERK activation in response to IL-33, the kinetics and extent of JNK2, NFκB and p38 signaling are similar to those observed in WT BMMC. The mechanisms for such crosstalk are not clear but there is a physical association between cKIT, IL-1RAcP and ST2 and this association only occurs if cKIT has been activated, either constitutively in HMC-1 or following SCF pretreatment in BMMC. Nevertheless, in addition to the physical association between their receptors, crosstalk

between IL-33 and SCF signaling appears to be dependent on PI3K in murine MC [57]. It has therefore been proposed that SCF priming of cKIT is required to allow cells to respond to IL-33/ST2 signaling.

6.1.2 *SCF and cKIT signaling*

Many different cell types express SCF; for example in the lung, it is expressed by epithelial cells, fibroblasts, smooth muscle cells, endothelial cells, eosinophils and mast cells and in humans, SCF is produced in a range of tissues including thymus, spleen, testes and placenta [56]. SCF can be expressed in membrane-bound or soluble forms, with particular cell types exhibiting differential patterns of expression [362]. Similarly, the high affinity receptor for SCF, cKIT, is a transmembrane, type III receptor tyrosine kinase (RTK) [56] that is expressed on a variety of cells including haematopoietic progenitor cells (HPC), where it plays roles in self-renewal and differentiation of HPC into cells of the myeloid and lymphoid lineages. During haematopoiesis, cKIT is generally downregulated as cells mature, with the exception of mast cells where it remains essential for survival, differentiation, chemotaxis and activation [363, 364].

Injection of recombinant SCF into patients induces skin hyperpigmentation and MC hyperplasia and hyperactivation, the latter as evidenced by degranulation and consequent increased levels of MC tryptase and histamine. SCF signaling creates chemotatic gradients for mast cell localization within tissues [56] and consistent with this, local SCF concentrations increase rapidly during early stages of wound healing then decline, mirroring the kinetics of mast cell recruitment and activation [362]. However, although in acute responses, SCF increases mast cell activation [41, 365], chronic exposure to SCF makes MC hyporesponsive to signals received by receptors coupled to tyrosine kinases, due to the induction of defective cytoskeletal reorganization [41].

6.1.3 *IL-33 and SCF in Disease*

6.1.3.1 IL-33 in cancer immunology

Crosstalk between IL-33/ST2 and SCF/cKIT broadens the network of signaling components recruited, promoting greater functional responses from mast cells. Such cooperative signaling therefore has a potentially important role in pathologies where these two molecules are found in abundance, not only in cutaneous inflammation but also in cancer pathology, where they contribute to angiogenesis, cell growth and metastasis. Elevated serum IL-33 levels have been associated with several cancers including gastric cancer [366] and hepatocellular carcinoma [367, 368]. Indeed, as higher serum levels of IL-33 correlate with increased metastasis, it has been proposed that they may be helpful in determining disease prognosis.

Dissection of the role (s) of IL-33 in cancer, however, has been complicated by differing homeostatic roles played by the cytokine, depending on the local microenvironment. For example, although IL-33 is barely detectable in the vasculature around tumors in human, as its expression is most likely downregulated by the general inflammatory milieu present at the tumor site [369], it can drive angiogenesis as characterized by increased proliferation, migration and differentiation of human endothelial cells *in vivo* by inducing NO from endothelial cells [370]. Likewise, the ability of IL-33 to drive development of fibrosis and aberrant tissue growth, such as that seen in psoriasis [371], may also contribute to pathogenesis in cancer. Moreover, the ability of this cytokine to produce pro-inflammatory cytokines is also important as, for example, the IL-33 produced by myofibroblasts in the pancreas induces release of IL-6 and IL-8 from cells in the tumor microenvironment that are partly responsible for the progression of pancreatic cancer [372]. Consistent with this role for pro-inflammatory cytokines, prolonged NFκB activation by IL-33 has also been implicated in tumorogenesis [371].

Relating to the role of inflammation in cancer, IL-33 may also increase tumour growth and metastasis by its ability to influence DC phenotype and hence, the Th phenotype [373] of the immune response to tissue damage.

Thus, IL-33 release is an early danger signal for skin damage with UVB exposure upregulating IL-33 mRNA and protein in the epidermis of both mice and humans. Interestingly, therefore, UVB–induced squamous cell carcinomas that are not destroyed by immune responses express high levels of IL-33. Although IL-33 does not appear to promote tumour growth directly, its ability to reduce Th1 responses protects the tumour by limiting antigen presentation and cytotoxic cell activation [374]. This effect is also seen in a model of metastatic breast carcinoma where inhibition of ST2 signaling can increase anti-tumor responses characterized by reduced metastasis, slower tumor growth and increased levels of $CD4^+$, $CD8^+$ T cells and NK cells which have greater cytotoxic activity and IFNγ production [373]. These responses are proposed to be due to the inability of IL-33 to drive Th2 development under these conditions allowing a Th1/Th17 phenotype, that can combat tumor development, to dominate.

It should be noted however, that IL-33-ST2 signaling in mast cell responses may also drive proinflammatory, anti-tumour responses as it has been proposed that mast cells responding to IL-33 released after cell injury and necrosis may invade necrotizing tumours [63]. The IL-33 released may not necessarily be produced by the tumor itself but could be generated by damaged cells surrounding the tumor [366, 372].

6.1.3.2 cKIT in cancer immunology

Dysregulation of cKIT signaling appears to play two major roles in the development of cancers; initially, during the initial transformation of neoplastic cells, including mast cells, usually via gain-of function mutations or during progression and expansion of tumours, via increased angiogenesis and metastasis [375], with cKIT contributing to tumor growth via autocrine and paracrine stimulation in response to SCF produced by transformed cells [376], including mast cells [377]. In malignant cancers, cKIT plays multiple (contradictory) roles; whereas in melanomas decreased cKIT expression prevents apoptotic signaling and increases cell survival, upregulation of cKIT expression increases survival, proliferation and oncogenic transformation in myeloid and lymphoid proliferative diseases. Similarly, in mastocytosis, high

cKIT expression can lead to poor prognosis and resistance to chemotherapy [376]. A number of potential oncogenic mutations of the cKIT receptor have been identified and the phenotype of disease can be determined by the type of mutation, the genetic background and the cell type affected, for example, mutations in the tyrosine kinase domain have been found to be particularly associated with mastocytosis [364] [376].

6.1.3.3 Mast cells in cancer immunology

Although their function in disease development and progression is not clear, mast cells have been implicated in playing a pathogenic role in a range of cancers including, skin [378], breast [379] [380], lung [381, 382] kidney [383], melanoma [384] and multiple myeloma [385]. Consistent with this, MC-derived mediators, such as TNFα, VEGF, IL-8 and serine proteases promote angiogenesis, allowing increased tumor growth in the early stages and later, promotion of metastasis. Moreover, the levels and activity of mast cell chymase, tryptase and carboxypeptidase A are increased during progression of skin tumors and correlate with the induction and development of blood vessels within the skin tumor [386] whilst the ability of mast cells to drive inflammation towards a Th2 phenotype, promotes a more tumor-protective environment. Indeed, the precise function of mast cells in cancers may be determined by the differing tumour size, type and location, [11] (reviewed in [364]) as recruited immature mast cells can become “tuned” by the tumor to provide pro-metastatic signals [386].

6.1.3.4 HMC-1; an *in vitro* model of neoplastic mast cells

Neoplastic mast cell diseases, termed mastocytosis can be divided into two categories, systemic and cutaneous with clinical symptoms depending on the location of mast cells and the release of specific mediators [387]. Although rare, systemic mastocytosis, is very aggressive and the pathology induced by the infiltrating mast cells generally results in a poor prognosis. Current treatments are limited to reducing total mast cell numbers, hence a more

neoplastic-specific therapy would allow the preservation of homeostatic and protective mast cell responses, reducing deleterious side-effects. Consistent with the role of SCF in mast cell survival and development, mastocytosis is frequently characterised by mutations in the cKIT receptor.

HMC-1 cells are a human neoplastic mast cell line derived from a patient with mast cell leukemia [388]. HMC-1 cells actually comprise two distinct lines; HMC-1.1 identified by their V560G mutation on cKIT and HMC 1.2, bearing the D816V mutation. In humans, D816V is the most common mutation in systemic mastocytosis [364] and this allows ligand-independent autophosphorylation [56]. Although the mutation is found in haematopoietic progenitors and hence, subsequently in multiple cell lineages, pathogenesis is limited to mast cells [364]. Interestingly, therefore, mice with an equivalent mutation, D814V exhibit various acute leukemias and lymphomas, but not mast cell disease, suggesting that in mice this cKIT mutation is sufficient to trigger proliferative disease. By contrast, cKIT mutations are rarely found in human leukemias or lymphomas, suggesting the existence of differential pathogenic pathways for these diseases in humans and mice.

HMC-1 represent immature mast cells that are independent of SCF for growth and survival and reflecting this maturation status, are characterised by their lack of well-formed granules, low tryptase and chymase expression as well as their lack of functional IgE receptors [389]. Reflecting their independence of SCF, HMC-1 exhibit constitutive cKIT signaling [390] and thus provide a good *in vitro* cell model to investigate SCF-cKIT signaling and its crosstalk with other signal transducers in cancer in order to identify potential novel therapeutic targets.

6.2 Aims

To investigate potential crosstalk between IL-33/ST2 and SCF/cKIT signaling in both primary (murine PDMC and BMMC) and transformed (HMC-1) mast cells, in particular to determine whether this involves PLD and SphK and can be modulated by ES-62

To investigate the role of mTOR signaling in such crosstalk in neoplastic human mast cells

6.3 Results and Discussion

6.3.1 *Cooperation between SCF and IL-33 signaling in mast cells*

6.3.1.1 The effect of SCF on IL-33-induced responses in murine mast cells.

Previous studies have reported increased cytokine responses following co-stimulation of mast cells with SCF and IL-33 [273, 274]. Whilst IL-33 can stimulate release of MCP-1, IL-6 and IL-13, SCF can induce significant production of MCP-1, but not IL-6 or IL-13 from IgE-sensitized PDMC (Figure 6.1 A). However, co-administration of IL-33 and SCF produced significantly increased responses of all of these cytokines, with synergistic responses being observed for both IL-6 and IL-13, where the resulting cytokine levels are greater than the sum of individual stimulation. MCP-1 production is less than additive, but as both IL-33 and SCF induce strong responses, this may reflect that a peak response has been reached.

SCF, unlike IL-33, induces proliferation of both PDMC (Figure 6.1 B) and BMMC (Figure 6.1 C). Interestingly therefore, this SCF response is significantly enhanced by co-stimulation with IL-33, such crosstalk perhaps providing a rationale for the ability of IL-33 to stimulate human MC proliferation [62], but not PDMC or BMMC, as these data may suggest that murine MC may require greater levels of SCF than that found endogenously in serum during culture.

The increased responses to IL-33 in the presence of SCF has potential implications for the role of mast cells in the development and progression of cancers, as for example mastocytosis, in which mutations generating constitutively active cKIT are observed, is characterised by MC hyperresponsiveness both in terms of cytokine production and proliferation (hyperplasia) [56, 391].

6.3.2 *SCF enhances IL-33-induced calcium mobilisation.*

In IgE-sensitized PDMC, SCF was found to stimulate calcium mobilisation in a dose-dependent manner (Figure 6.2 A), with intracellular and extracellular mobilisation being detected at the highest dosage tested (100 ng/ml; Figure 6.2 B). By contrast, at the lowest dose (1 ng/ml) the calcium profile has lost the rapid peak characteristic of intracellular mobilisation and appears to predominantly reflect influx of extracellular calcium (Figure 6.2 A), unfortunately this was not confirmed, perhaps suggesting that the intensity and phenotype of SCF calcium signaling may be regulated by levels of receptor ligation, in a similar manner to that described for FcεRI [392]. Interestingly, unlike IL-33, SCF does not require IgE-sensitization to mobilise calcium (Figure 6.2 C).

Co-administration of IL-33 induces stronger calcium mobilisation than that observed with either IL-33 or SCF alone, with these effects appearing to be additive at both the lowest dose (1 ng/ml Figure 6.2 D) or highest dose (100 ng/ml Figure 6.2 E) of SCF tested. Analysis of the responses observed in the absence of extracellular calcium (Figure 6.2 F) suggests that the additive effects in response to IL-33 at 100 ng/ml predominantly reflect enhancement of the influx of extracellular calcium as the mobilisation of intracellular calcium is not significantly modulated. These data indicate that IL-33- and SCF cooperate to increase calcium signaling although the additive nature of the response suggests that the enhanced influx of extracellular calcium appears to be autonomously controlled by each stimulus. This is similar to that reported for SCF amplification of FcεRI responses in BMMC, where SCF both induces calcium mobilisation and enhances FcεRI-mediated-calcium signaling. However, in this case it was concluded following baseline adjustment analysis that SCF acted to build a platform for further Ag-stimulation of calcium mobilisation [55].

After observing these amplifying effects of SCF on IL-33-mediated calcium mobilisation and cytokine production, it was hypothesized that when used in conjunction, IL-33 and SCF could overcome a threshold not reached by IL-33 alone in PDMC to initiate the degranulation responses observed with human mast cells that may have been exposed to SCF during their culture conditions

[62]. However, stimulation with IL-33 plus SCF did not induce degranulation of PDMC (Figure 6.3 A). This failure of IL-33 and/or SCF to induce degranulation by PDMC, despite inducing strong calcium mobilisation might perhaps reflect an inability of these stimuli to downregulate an inhibitor of degranulation. A precedent for this has been provided by the finding that PDGF fails to induce degranulation despite stimulating a strong calcium response because it does not downregulate cAMP signaling that acts to inhibit degranulation [393].

6.3.2.1 The effect of SCF on IL-33-induced NFκB activation.

As SCF augmented IL-33-ST2-mediated calcium signaling, it was investigated whether these effects were reflected in enhanced activation of NFκB. Interestingly, despite inducing strong calcium signaling, SCF was unable to activate NFκB signaling, at least of the canonical variety, as indicated by IκB degradation. However, when administered in combination with IL-33, IκB degradation appeared to occur slightly faster than observed following treatment with IL-33 alone (Figure 6.3 B). This inability of SCF to activate NFκB suggests that Ca^{2+} activation and NFκB are not directly associated in SCF signaling, although these experiments do not rule out the possibility that cKIT couples to NFκB activation via the non-canonical pathway, which does not require IκB degradation [394, 395].

6.3.2.2 IL-33 responses are modulated by serum factors in addition to SCF

As SCF can enhance IL-33 responses, it is possible that differential levels of SCF present in FCS sources [396, 397], may contribute to the observed discrepancies in the functional responses of IL-33 presented in this thesis with those in the literature. To investigate this, the responses to IL-33 in the presence and absence of exogeneous were assessed under conditions of conventional (10%) and reduced (1%) serum. In addition, as prolonged (>48 h)

culture with SCF has been reported to inhibit FcεRI-mediated MC activation [41], similar effects on IL-33-mediated MC responses were also determined.

Interestingly, the SCF-mediated amplification of IL-33-stimulated cytokine production is more pronounced in the presence of high (10%) levels of FCS, in PDMC (Figure 6.4 A) and, to a lesser extent, BMMC (Figure 6.4 B). Indeed, in 1% FCS, SCF can only restore IL-33 responses to the levels observed in 10% FCS with IL-33 alone, suggesting that as exogeneous SCF cannot compensate for the reduced level of serum, some additional serum factor(s) may be required for the optimal cooperative signaling between IL-33/ST2 and SCF/cKIT.

6.3.2.3 HMC-1 cells, a model of human mastocytosis, respond to IL-33.

To model the interaction of these cytokines in diseases such as mastocytosis the human leukemic mast cell line, HMC-1, was exploited to investigate IL-33 and/or SCF signaling although unfortunately, in these experiments, the HMC-1 subtype, HMC-1.1 or HMC-1.2 was not determined. Nevertheless, release of IL-6, MCP-1 and TNFα by HMC-1 cells was found to be significantly stimulated in response to an increasing dose of IL-33, despite the high levels of MCP-1 produced constitutively by these cells (Figure 6.5 A).

Interestingly, although, IL-33 induces cytokine responses by HMC-1 cells, it fails to induce calcium mobilisation, even in the presence of IgE (Figure 6.5 B). These data provide further support that mast cells require to be sensitized in order to license IL-33-induced calcium signaling as, in combination with data obtained in the presence of DNP to mimic Ag-mediated crosslinking of FcεRI, they confirm that HMC-1 cells do not express functional FcεRI receptors and hence, cannot be sensitized by IgE. Moreover, and perhaps surprisingly given the important role of calcium signaling in promoting cytokine generation in primary mast cells [190, 193], these data indicate that calcium mobilisation is not required for IL-33-stimulated cytokine production by HMC-1 cells. However, as the calcium mobilisation experiments are performed in the absence of serum, they do not rule out the possibility that SCF or some other serum factor(s) either induce calcium

mobilisation or license IL-33 to do so, under the culture conditions pertaining in the cytokine assays. Given that cKIT is constitutively active in HMC-1 cells, and SCF induces strong calcium signals in PDMC, it is also rather surprising that no calcium responses are observed. However the rapid, eccentric signal observed may be a reflection of a continuous, dysregulated calcium signal as a result of endless cKIT stimulation.

Perhaps consistent with the latter suggestion, IL-33 does not stimulate proliferation (as indicated by DNA synthesis) of HMC-1 cells either when administered alone or in the presence of exogenous SCF (Figure 6.5 C), findings conflicting with those in primary human MC [62]. However, the lack of effect of SCF may reflect the constitutive signaling induced by the cKIT mutation, although perhaps arguing against this, in reduced serum conditions, HMC-1 proliferation is reduced (Figure 6.5 D). Interestingly, therefore, there is evidence that although HMC-1 constitutively produce MCP-1, exposure to SCF is able to further increase this production [398, 399] via increased ERK activation [400]. MAPK signaling does not appear to be (fully) activated by the mutated cKIT [401] suggesting that cKIT activity can be further stimulated above that induced by the mutation or else, in addition to the kinase activity of cKIT, ligation by SCF is required to allow the "activated" receptor to interact with co-receptors and/or signal transducers in order to recruit the full repertoire of SCF-coupled signaling pathways.

To further investigate the role of SCF, and other serum factors, in IL-33-induced responses in HMC-1 cells, these cells were cultured in the presence of 1%, rather than 10%, FCS overnight prior to stimulation with IL-33. Basal MCP-1 production was found to be reduced under conditions of 1% FCS, and IL-33 failed to further stimulate MCP-1 (Figure 6.5 E). To address whether it is the SCF present in the serum that is required for IL-33-induced MCP-1 production by these cells, rSCF was introduced in the presence of both low (1%) and high (10%) serum conditions but this did not affect the response, further suggesting that there is another component of serum besides SCF required for IL-33 signaling in MC. Indeed, the levels of SCF in FCS are reported to be less than 100 pg/ml which the investigators claim would be “insufficient to promote additive biologic effects”, although this was based on treatment of haematopoietic progenitor cells [396].

6.3.2.4 The effect of inhibition of cKIT signaling on IL-33 responses.

To investigate SCF-independent IL-33 responses and probe for any other serum dependent effects, analysis was performed in the presence of inhibitors that have been reported to potently inhibit SCF-cKIT signaling. Imatinib is a tyrosine kinase inhibitor developed to block bcr-Abl signaling but that is known to also act on cKIT. It functions by binding to an inactive confirmation of the kinase at the site that binds ATP and perhaps reflecting this, cannot inhibit cKIT bearing the D816V mutation although it can inhibit the V560G variant [402], although this latter form is found in very few systemic mastocytosis cases. It has been proposed that resistance to Imatinib is most likely due to mutations preventing its binding [364] and to overcome this resistance a further cKIT inhibitor was developed. Dasatinib is a small molecule, ATP-competitive inhibitor of cKIT that can inhibit both of the D816V or V560G cKIT variants as well as Abl and the Src-related tyrosine kinases, Btk and Tec [56]. Dasatinib is structurally unrelated to Imatinib and can be used at much lower dose [403-405]. However, both inhibitors can also target PDGF-R and as this receptor is expressed by mast cells and PDGF is a potential candidate for the serum factor(s) [397] that can enhance IL-33 signaling independently of SCF, it should be borne in mind that Imatinib and Dasatinib will also block such signaling. To perform these studies, the dosage of each cKIT inhibitor chosen was based on the range used therapeutically and although cell viability was reduced following overnight treatment at the higher doses of Imatinib, a working window (≤ 10 μM) to assess effects independent of toxicity was obtained for both PDMC and BMMC (Figure 6.6 A). As SCF-cKIT signaling is essential for mast cell survival this high dose toxicity is not surprising and interestingly, the addition of IL-33, a promoter of cell survival [62, 268], did not generally overcome the toxic effects of the inhibitors, although it tended to protect against cell death in BMMC.

Analysis of cytokine release by PDMC and BMMC demonstrated a clear dose-dependent inhibition of IL-33-induced MCP-1 (Figure 6.6 B) and IL-6 (Figure 6.6 C) production following treatment with either Imatinib or Dasatinib. Unstimulated, “basal” MCP-1 production In the presence of 10% FCS was strongly inhibited by Imatinib and Dasatinib at non-toxic concentrations

suggesting that some of the MCP-1 generated was in response to SCF/cKIT and/or serum growth factor signaling. BMMC appear to be more dependent on SCF/PDGF signaling than PDMC, as when they are treated with Imatinib or Dasatinib, basal MCP-1 production is completely ablated. This may represent a greater requirement of SCF for survival by the more immature BMMC. IL-6 production is also sensitive to such inhibition with almost complete suppression of basal release. Although it is not possible to determine from this analysis how much of this reflects inhibition of cKIT signaling relative to that of PDGF, it suggests that the serum enhancer effects are predominantly Imatinib/Dasatinib-sensitive. Likewise, IL-33-stimulated MCP-1 and IL-6 production could be essentially ablated by the inhibitors, particularly by Imatinib perhaps reflecting the higher toxicity of this inhibitor at the concentrations tested and the protective effects of IL-33 observed in the presence of Dasatinib.

Dasatinib suppressed basal and SCF-induced MCP-1 production by PDMC and also reduced IL-33-induced MCP-1 (Figure 6.6 D) and IL-6 (Figure 6.6 E), but not as potently as it inhibited the SCF-MCP-1 response, perhaps suggesting that IL-33-induction of this cytokine can be independent of cKIT signaling. Moreover, the synergistic production of MCP-1 or IL-6 in response to the combination of IL-33 and SCF was reduced to about the level seen with IL-33 or SCF alone. It is not clear whether this reflects inhibition of SCF/PDGF-licensing of IL-33 signaling or direct targeting of downstream targets of ST2-cKIT crosstalk such as Src-kinases [406] and/or c-Abl, as described for cKIT and PDGF-R [407]. However, it was clear that irrespective of the culture conditions, inhibition of IL-33 responses by Dasatinib was not due to an effect on levels of surface ST2 (Figure 6.7 A) or cKIT (CD117) expression by PDMC (Figure 6.7 B).

As Imatinib and Dasatinib induced negligible toxicity over their therapeutic range in HMC-1 cells (Figure 6.7 C, D), these inhibitor studies were next extended to HMC-1 cells to determine the effects of blocking cKIT activity on the observed IL-33 responses in these neoplastic human mast cells. Perhaps consistent with the constitutive activity of cKIT in these cells, raising serum levels from 1-10% did not substantially increase basal levels of MCP-1 or IL-6 released by these cells. Interestingly, therefore, the IL-33 response was

completely ablated to the basal level by reduction of FCS to 1% suggesting that a non-SCF serum factor is also required for IL-33 responses in these cells (Figure 6.7 E). Nevertheless, in all cases, MCP-1 production was inhibited by Dasatinib, presumably via inhibition of cKIT. Interestingly, therefore Dasatinib (Figure 6.7 G) did not inhibit MC proliferation, although this was suppressed by Imatinib (Figure 6.7 F), suggesting that some factor other that the mutated cKIT activity was being targeted by Imatinib.

6.3.2.5 Role of PLD in crosstalk between IL-33/ST2 and SCF/cKIT signaling

PLD activation by IgE-sensitization was shown to license IL-33-ST2 signaling (Chapter 4). However, the cytokine responses to IL-33 in the absence of IgE-sensitization were also suppressed by PLD inhibitors perhaps suggesting that SCF or some other serum factor not present under the conditions of assaying either PLD or calcium signaling was responsible for licensing of such IL-33-functional responses. Thus, as cKIT signaling has been reported to activate PLD in a PI3K-dependent manner [408], the role of PLD in the cooperative signaling between IL-33 and SCF, and hence in the IL-33-stimulated cytokine responses observed in 10% FCS in the absence of IgE-sensitization, were investigated.

Firstly, SCF-induced MCP-1 production by PDMC was found to be inhibited by the pan-PLD inhibitor (5WO), regardless of the sensitization status of the mast cells, reflecting previous reports that cKIT was coupled to PLD activity, (Figure 6.8). Similarly, and as shown in Chapter 4, the IL-33-induced MCP-1 response in both resting and IgE-sensitized PDMC, was also inhibited by the pan-PLD inhibitor (Figure 6.8 A). It was therefore not surprising that the synergistic MCP-1 and IL-13 responses to IL-33 + SCF, which could be further enhanced by IgE-sensitization, were also ablated by the inhibitor, presumably reflecting an ability of SCF- (and/or other serum-containing growth factors) associated PLD and calcium signaling to license optimal IL-33 signaling as discussed earlier for IgE sensitization.

Interestingly, therefore, SCF cytokine responses appeared refractory to inhibition of PLD 1 (809; Figure 6.9). Perhaps consistent with this, although the weak release of MCP-1 observed in response to IL-33 appeared to be inhibited, the synergistic MCP-1 response to IL-33 and SCF was generally unaffected in non-sensitized PDMC. However, IL-13 production following such co-stimulation was partially blocked, perhaps again suggesting involvement of an additional serum factor in the synergistic responses of SCF and IL-33 as well as highlighting differential regulation of MCP-1 and IL-13 production. By contrast, the synergistic production of both MCP-1 and IL-13 in IgE-sensitized PDMC was almost completely ablated by inhibition of PLD 1, presumably reflecting recruitment of this signal resulting from IgE-ligation of FcεRI. By contrast to the PLD 1 results, inhibition of PLD 2 (APV) ablates cytokine production in response to SCF or IL-33, either alone or in combination and regardless of sensitization status of the PDMC (Figure 6.10). These data further support a role for PLD, particularly PLD 2, in licensing optimal IL-33 signaling, which in the absence of IgE-sensitization can be partially provided by SCF or perhaps some other serum factor.

To determine whether PLD signaling also plays a role in IL-33/ST2 and SCF/cKIT crosstalk responses in human MC, the effect of inhibition of PLD 1 and/or PLD 2 on basal and IL-33-stimulated HMC-1 responses was investigated. As HMC-1 cells express constitutive cKIT activation, this would allow analysis of ST2/cKIT crosstalk and performing the experiments in 1% and 10% serum would determine the need for SCF ligation of cKIT, or its interaction with other serum derived factors, to license the full signaling capacity of this receptor.

Again, it can be seen that the IL-33-stimulated MCP-1 production observed in the presence of 10 % serum is lost when FCS levels in culture are reduced to 1%, suggesting this serum enhancement is cKIT-independent and, perhaps consistent with this, this serum-licensing of IL-33 signaling is PLD 1-dependent. By contrast, inhibition of "basal" ie cKIT-mediated responses is only inhibited by the PLD 2 or pan-PLD inhibitors, and this is similar in 1% (Figure 6.11 A) and 10% (Figure 6.11 B) serum. Interestingly inhibition of proliferation by the PLD 1 inhibitor is overcome by high levels of FCS (Figure

6.11 C), suggesting involvement of a non-PLD 1-coupled serum factor, such as SCF, in promoting this rescue of cell proliferation.

6.3.2.6 Role of SphK in IL-33 plus SCF responses in mast cells.

SphK activity has been shown to be crucial to cKIT-mediated tumor survival, growth and metastasis [409-411], This appears particularly to be the case in leukemias and lymphomas [412] where, for example, in chronic myeloid leukemia, Imatinib inhibits SphK1 activation [413]. Thus, using PDMC and HMC-1 cells, as a model for the cKIT-driven development of mast cell leukemia, the role of SphK in such SCF and IL-33 crosstalk was investigated.

MCP-1 production induced by co-stimulation of PDMC with IL-33 and SCF was strongly inhibited by the SphK inhibitor, DMS (Figure 6.12 A) and the selective inhibitor of SphK1, 5C (Figure 6.12 B). Likewise, the proliferative response of HMC-1 induced by 10% serum was reduced to the level observed with 1% FCS by both DMS (Figure 6.12 C) and 5C (Figure 6.12 D). It was rather surprising, given the results presented for IL-33 in Chapter 4, that neither of the SphK-targeting reagents had much effect on the PDMC cytokine responses induced by either IL-33 or SCF alone (Figure 6.12 A, B). Nevertheless, as these data indicate that signaling induced by the co-administration of IL-33 and SCF is dependent on SphK1, and in the presence of 10% serum HMC-1 proliferation is also dependent on SphK1, these findings may suggest that SphK activation by some serum factor may be responsible for providing a platform for optimal SCF-IL-33 signaling in HMC-1 cells.

To further investigate the role of SphK in responses of HMC-1 cells to IL-33 and serum, the kinase activity of SphK was determined. Consistent with the proliferation data, culture of HMC-1 with 10 % FCS following serum starvation led to a significant increase in SphK activity within 30 min (Figure 6.13 A). Rather surprisingly given the cytokine data, treatment of HMC-1 cells with IL-33 in 1% serum also stimulates SphK activity (Figure 6.13 B) and a similar, but enhanced, profile of activation is observed in response to co-stimulation with IL-33 plus 10% serum (Figure 6.13 C) perhaps suggesting that IL-33 (or some serum factor) may be stimulating SphK activation in 1% serum that is not

essential for cytokine production or proliferation. Future experiments should include a control of HMC-1 in 1% serum alone to determine whether this is not just a constitutive SphK activity of these transformed cells. Western Blot analysis of SphK1 expression (Figure 6.13 D) revealed that long-term culture (in the presence of 10% serum) had a negligible effect on the protein levels of this signal transducer and this was not substantially modulated by either of the cKIT inhibitors, Imatinib or Dasatinib (Figure 6.13 E), ruling out the possibility that chronic activation of cKIT downregulated this signal under conditions of "basal" HMC-1 signaling.

6.3.2.7 Role of mTOR in IL-33 signaling.

mTOR is a serine/threonine kinase that plays a key role in cell proliferation, metabolism and differentiation in response to growth factors and nutrient conditions [414-416] as evidenced by loss of mTOR function leading to arrest in the G1 phase of the cell cycle and suppression of protein synthesis [416]. Association with a number of distinct signaling molecules, including the scaffolding proteins RAPTOR and RICTOR, allows mTOR to form the mTORC1 and mTORC2 complexes respectively (Figure 6.14). Based on studies with Rapamycin, which is a specific inhibitor of mTORC1 signaling, preventing mTOR and RAPTOR interaction, it is well established that a key function of the mTORC1 complex is to activate S6 kinase and and its downstream effector, 4E-BP1 to initiate RNA translation and protein synthesis [415, 416].

Although it has been proposed that long-term treatment with Rapamycin may also inhibit mTORC2 formation by sequestering mTOR, and hence reducing its phosphorylation [414, 417, 418], it is generally believed that Rapamycin is not an inhibitor of mTORC2 function as this complex contains the Rapamycin-insensitive transducer, RICTOR. Thus, due to the lack of mTORC2 inhibitors, function of this complex is less well-defined. Nevertheless, it is clear that mTORC2 phosphorylates and activates Akt at Ser473 [419, 420] allowing full activation of Akt by PI3K-dependent phosphorylation of Akt at Thr308 [419, 420]. This activation of Akt occurs upstream of coupling to mTORC1 and hence acts to perpetuate mTOR signaling by linking the mTORC2 and mTORC1 pathways and suppressing an inhibitor of mTORC1 formation [421]. The

signals required for activation of mTORC2 are unconfirmed but they may be initiated, either directly or indirectly, via growth factors [415].

In human and mouse mast cells, FcεRI and cKIT signaling activate mTORC1 via PI3K [414] and consequent PDK1-mediated phosphorylation of Akt Thr308 [415, 416]. Although Rapamycin inhibits cKIT and FcεRI-induced cytokine production and cKIT-mediated survival in mast cells, it has no effect on FcεRI-mediated degranulation, presumably reflecting the "pre-formed" nature of this response. Likewise, in Th2 cells and innate lymphoid cells (ILC), IL-33 requires PI3K-dependent activation of mTORC1 and S6K1 to produce IL-5 and IL-13 and consistent with this, *in vivo,* Rapamycin inhibited IL-33-induced airway inflammation [327]. Interestingly, as a consequence of the activating mutation of cKIT, HMC-1 cells express constitutively active mTOR and p70S6K [414] although such mTORC1 signaling remains sensitive to Rapamycin in both variants (D816V; HMC-1.2, V560G; HMC-1.1) of the cell line [422]. Thus, it was planned to investigate whether the synergism resulting from SCF and IL-33 crosstalk could perhaps also reflect interactions of cKIT/ST2 modulating mTOR signaling.

As mTOR signaling is important for mast cell growth and survival the potential toxic effects of Rapamycin on HMC-1 viability were first screened. As expected, the addition of serum or IL-33 reduces the cell death observed with serum starved (1% FCS; Control) HMC-1 cells (Figure 6.15 A). Perhaps counter-intuitively, treatment with Rapamycin also reduced the cell death triggered by nutrient withdrawal under conditions of serum starvation. However, this presumably reflects inhibition of mTORC1-driven apoptosis, resulting from S6K mediated inhibition of PI3K activity self-regulating the mTOR pathway to promote cell survival via phosphorylation of Akt at Thr308 [423] and activation of the ERK/MAPK signaling pathway [424]. Rapamycin does not completely prevent cell death, as other pathways involved in nutrient withdrawal-induced cell death are unaffected. Nevertheless, these data rule out the possibility that any observed effects are due to Rapamycin toxicity.

In the presence of 10% serum, MCP-1 is constitutively produced by HMC-1 cells, presumably due to the activating mutation of the expressed cKIT.

Rather surprisingly, if anything, at low concentration (≤10 nM) Rapamycin tended to stimulate such cytokine production but this may reflect the ability of Rapamycin to promote cell survival by inhibiting the apoptosis-inducing effects of mTORC1 as discussed above. However, at higher concentrations cytokine production was somewhat inhibited by Rapamycin in a dose-dependent manner (Figure 6.15 B), findings perhaps consistent with previous reports that cytokine production by HMC-1 cells requires mTORC1 signaling [414]. Similarly, Rapamycin also inhibits serum-stimulated proliferation of HMC-1, with significant reduction of DNA synthesis observed at the highest dose tested (Figure 6.15 C).

As SCF/cKIT and IL-33/ST2 couple to PLD/SphK signaling and these elements contribute to functional responses in murine mast cells and HMC-1 cells, their potential role in SCF/serum and IL-33-stimulated mTOR activation was investigated. A precedent for this has been provided by studies showing that PLD signaling can activate mTOR pathways where PLD-generated phosphatidic acid (PA) promotes the association between mTOR and RAPTOR or RICTOR [425-427]. Moreover, further support is provided by studies showing that FCS-stimulated S6K1 activation and phosphorylation of 4E-BP1 was prevented by using butan-1-ol to block PA formation [428, 429]. Thus, to determine whether serum- and/or IL-33-stimulated mTOR activation required PLD and/or SphK activation, the effects of Rapamycin and selective inhibitors of these lipid-directed signal transducers was assessed by Western blot analysis of the expression and phosphorylation/activation status of signaling components downstream of mTORC1, such as p70 S6K and RAPTOR whilst phosphorylation of AKT at Ser473 was exploited as a downstream readout of mTORC2 activation.

Firstly, p70 S6 kinase is key to the regulation of protein synthesis and cell growth downstream of mTORC1 in mast cells [414] and consistent with this, phosphorylation of p70 S6 Kinase at Thr389 is rapamycin-sensitive and correlates with p70 S6 kinase activation *in vivo* [430, 431]. Thus, following serum withdrawal for 18 h (Unstimulated 60 min), p70 S6 kinase is found in a dephosphorylated and inactive state in HMC-1 cells (Figure 6.16 A). On replacement of serum (10% FCS) the cells undergo a rapid phosphorylation at Thr389 and activation of p70 S6 kinase that peaks at 10 minutes before

returning towards basal levels of phosphorylation within 60 min (Figure 6.16 A), findings supporting previously reported data using SCF-treated HMC-1 cells [414]. IL-33 does not alter the kinetics of the response induced by serum but amplifies the response. As predicted, Rapamycin completely inhibits phosphorylation of p70 S6K induced by both stimuli due to its inhibition of mTORC1 formation (Figure 6.16 B).

Although IL-33 has been reported to activate mTOR using PI3K [327] and both IL-33 and SCF activate S6K, the role of PLD in the activation of this downstream signaling molecule by these stimuli has not been addressed. Interestingly, therefore, PA produced by PLD is crucial for mTORC1 and mTORC2 assembly [426], however the relative contributions of each PLD isoform in mTOR activation is controversial and may depend on subcellular localisation of the isoforms and/or the particular stimulus [427]. Thus, the relative contributions of PLD 1 and PLD 2 in SCF- and IL-33-mediated p70 S6K activation was addressed by the use of the selective inhibitors, 809 and APV respectively. This revealed that whilst neither inhibitor exhibited any substantial effect on the kinetics of S6K activation, inhibition of PLD 1 increases the extent of p70 S6K phosphorylation both in response to serum and IL-33 (Figure 6.16 C), whereas inhibition of PLD 2 only appeared to promote the serum response (Figure 6.16 D). Similarly inhibition of SphK1 (5C) increased the peak phosphorylation of p70 S6K induced by serum (Figure 6.16 E). These findings were rather unexpected given that S6K promotes protein synthesis and the Rapamycin data supported a role for mTORC1 in HMC-1 cytokine responses stimulated by serum. However, whilst the relative levels of Pp70 S6K were increased by the inhibitors, the finding that inhibition of PLD 2 appeared to dramatically suppress protein expression of p70 S6K, might suggest that it possibly acts in this way to reduce the overall activation of this mTORC1 effector and would be consistent with the observed PLD 2-dependence of serum-induced MCP-1 production in HMC-1 (Figure 6.11).

RAPTOR phosphorylation at Ser792 is shown to inhibit mTORC1 and prevent cell cycle progression during energy stress [432]. Corresponding to this we found that serum withdrawal induced RAPTOR phosphorylation (Unstimulated 60 min) but after serum reintroduction this phosphorylation was dramatically

reduced before recovering slightly (Figure 6.17 A). The addition of IL-33 appears to increase the reduction in RAPTOR phosphorylation when compared to unstimulated levels, suggesting an enhanced mTORC1 activation in response to this stimulation, a suggestion supported by its ability to increase the extent of Pp70 S6K (Figure 6.16 A). Association of RAPTOR with mTOR can be inhibited by Rapamycin [417] and this investigation suggests that the inhibitory phosphorylation of RAPTOR is also increased by Rapamycin (Figure 6.17 B). Pretreatment with PLD 1, PLD 2 and SphK1 inhibitors appeared to increase RAPTOR phosphorylation in unstimulated, serum and serum plus IL-33 stimulated cells, suggesting greater mTORC1 inhibition and reduction in cell cycling, confirming the role for PLD in cell cycle progression [433] and for PLD 2 in cytokine production by serum-stimulated HMC-1 cells (Figure 6.11).

Generally, serum withdrawal (Unstimulated) seems to reduce the levels of RAPTOR expression but this is rapidly upregulated by restoration of serum. This may indicate that during metabolic stress RAPTOR is downregulated as well as phosphorylated to regulate its activity and indeed, downregulation would amplify the relative level of phosphorylated element. Phosphorylation of RAPTOR targets the molecule to different subcellular compartments and it has been suggested that perhaps during nutrient stress, RAPTOR is located to the nucleus [434].

The inverse pattern of p70S6K and RAPTOR phosphorylation reflects mTORC1 activation with the increase in an activatory signal and a reduction in an inhibitory signal. Although perhaps contradictory with the PLD 1 and SphK1 p70S6K data (Figure 6.16), the Ser792 RAPTOR phosphorylation responses suggests that both PLD 1/2 and SphK1 signaling are involved in the activation of mTORC1 at least by serum.

Akt phosphorylation at Ser473 is induced by mTORC2 and allows further Akt activation by PDK1 at Thr308 [419, 435]. Total levels of Akt remain constant regardless of the serum status whereas in the absence of serum, Akt phosphorylation at this site is elevated indicating greater mTORC2 activity (Figure 6.18 A). Replenishment of serum dramatically and rapidly reduces this phospho-Akt signal, which then returns towards the levels observed under serum starvation (Unstimulated 60) within 60 min, although this appears

delayed in the presence of IL-33. As would have been predicted, Rapamycin had no effect on either response, reflecting its inability to inhibit mTORC2 activation (Figure 6.18 B). Although the data are more difficult to interpret because of an apparent downregulation of Akt expression, inhibition of PLD 1 (Figure 6.18 C) and PLD 2 (Figure 6.18 D) initially appears to further decrease the relative levels of p473 Akt induced by serum suggesting their inhibition also suppresses activation of mTORC2 as well as that of mTORC1 (Figure 6.18 A). Conversely, at later timepoints, the recovery of Akt phosphorylation in the presence of serum is increased, indicating a role for PLD in the suppression of mTORC2, which is reduced in the presence of IL-33, potentially indicating that IL-33 can inhibit mTORC2 by another means other than PLD signaling. Likewise, inhibition of SphK1 also increases Akt phosphorylation, promoting mTORC2 activation (Figure 6.18 E). That these PLD and SphK inhibitors all appear to promote mTORC2 activity may therefore support the p70S6K data indicating that they can all inhibit mTORC1 formation.

6.3.2.8 Effect of ES-62-mediated inhibition on IL-33 responses in HMC-1.

Under conditions of reduced serum (1%) IL-33 does not further stimulate the high spontaneous release of MCP-1 from HMC-1 cells, reflecting its requirement of a serum factor to license its functional signaling. Nevertheless, ES-62 significantly inhibits such basal MCP-1 production from HMC-1 cells (Figure 6.19 A) irrespective of the presence of IL-33. By contrast, when the cells have been cultured in 10% serum, ES-62 does not inhibit the spontaneous release of either MCP-1 or TNFα but there is a reproducible trend for the parasite product to slightly inhibit IL-33-induced MCP-1 release (Figure 6.19 B) (Figure 6.19 C).

6.4 Conclusion

6.4.1 *SCF and serum factors enhance IL-33 responses*

Although serum is used as a source of SCF [396] when exogenous rSCF is used to replace serum there is only partial restoration of the cytokine response in murine MC and HMC-1 cells. Moreover, the amplified response to IL-33/SCF co-stimulation only occurs in the presence of 10% serum, suggesting a role for a further unidentified serum component(s), such as PDGF or EGF, in ST2-cKIT signaling. This finding may be important *in vivo* as microenvironments can be highly variable and hence, depending on the location and context of the interactions involved can have an influential impact on MC responses. For example, the microenvironment of tumors can be tailored by the tumor itself as well as the surrounding stromal cells [436, 437]. Thus an increase in co-factors of IL-33 signaling could potentially modulate the immune response, particularly that of MC, to promote metastasis by increased angiogenesis, surrounding tissue destruction and release of growth factors [375].

IL-33 and SCF crosstalk resulting in amplified functional responses appears to occur by signaling via converging pathways. Thus, previous studies found that the augmented responses to IL-33 and SCF observed in both MC and CD34+ progenitor cells [273, 438] were the result of synergistic activation of NFκB, ERK, PKB and JNK [273]. Here the involvement of calcium, PLD, SphK and mTOR in such augmented responses in PDMC (Table 6.1 A) and HMC-1 (Table 6.1 B) were investigated and these studies revealed that, similarly to the IL-33 cytokine responses in IgE-sensitized PDMC, although dependent on PLD 2, SCF-stimulated responses were not sensitive to inhibition of PLD 1 unless the MC were sensitized. Presumably reflecting this, cytokine responses to stimulation with IL-33 plus SCF were only partially reduced by inhibition of PLD 1, while inhibition of PLD 2 induces more robust dose-dependent effects. PLD 2 has generally been reported to exhibit high basal activity and hence reflect homeostatic responses whereas PLD 1 is considered to be more often involved in agonist responses. However, whilst the data in this thesis support the idea that PLD 2 is involved in regulating basal responses, they suggest that in mast cells, agonists elicit PLD 2 as well as PLD 1 with both isoforms involved in convergent IL-33 and SCF signaling. This may account for the

rather unusual patterns of PLD activation observed in response to IL-33/ST2 signaling observed in mast cells. For example, it was reported in Chapter 4, and again above, that despite functional responses being susceptible to PLD inhibitors, IL-33 did not appear to directly stimulate PLD activity but rather that IgE sensitization licensed PLD activation and consequent IL-33-mediated calcium mobilisation. However, this did not explain the ability of IL-33 to stimulate cytokine release in the absence of IgE sensitization, a response also involving PLD signaling. The data presented here relating to SCF (and/or serum) enhancement of IL-33 responses, similar to that of IgE sensitization, may now explain this as SCF/cKIT signaling appears to be PLD- and calcium-dependent, as previously reported [408, 439] and these crosstalk effects would be masked in the assays measuring IL-33-mediated PLD activation and calcium mobilisation which are performed in the absence of serum. Likewise, SphK signaling may also contribute to the SCF-induced signaling platform that allows and/or enhances IL-33 responses (Figure 6.20).

The dual receptor (cKIT/ST2) and IgE-licensing requirement for full activation of IL-33-ST2 signaling potentially allows for both rigorous regulation and amplification of MC responses. Loss of (part of) this regulation, for example as exemplified by the constitutive cKIT signaling in HMC-1, could have important implications in the development of disease especially where both SCF and IL-33 are found in abundance such as asthma [440-442]. Interestingly, therefore, aberrant cKIT activation has been reported in non-IgE dependent anaphylaxis [443] and "idiophathic" anaphylaxis [444], conditions associated with elevated levels of IL-33 expression [329].

Thus, the ability of cKIT inhibitors such as Imatinib and Dasatinib to suppress IL-33/SCF cooperation and hence limit pro-inflammatory responses by MC could provide an attractive potential therapy in asthma. Indeed, Imatinib has been reported to prevent airway inflammation and remodeling in an OVA-induced model of asthma [445], a disease where IL-33 contributes to pathology. Similarly in cancer, even if MC are not themselves transformed, they appear to play an important role promoting angiogenesis and remodelling of the tumour environment and hence, cKIT inhibitors, although not targeting the tumour directly, may exhibit some efficacy by restricting MC responses. Moreover, in mastocytosis, they may also be directly effective

by targeting the aberrant cKIT/ST2 and serum-driven survival and proliferative responses of such cells.

6.4.2 *mTOR signaling in HMC-1 is regulated by serum and PLD activity.*

During nutrient starvation, such as that observed experimentally after serum withdrawal or cKIT inhibition, cells undergo a complex set of events to avoid death, including the slowing or ceasing of proliferation, increased recycling of proteins and organelles and reduced biosynthesis. mTOR signaling plays an important role in sensing and interpreting such changes in the environment and initiating mechanisms to protect the cell [415]. Not surprisingly, therefore, dysregulated mTOR is implicated in oncogenesis particularly with respect to the growth of cancer cells due to aberrant activity of Akt, which drives proliferation, survival and nutrient absorption. Moreover, excessively high mTORC1 activity has been suggested to be responsible for transformation in several cancers [433, 446]. Perhaps reflecting this, proliferation of HMC-1 cells, which are leukemic MC, involves mTORC1 signaling. Constitutive MCP-1 production also involves, in part, mTORC1 signaling, perhaps reflecting the role for MCP-1 in recruiting and activating MC and other immune cells such as monocytes to promote angiogenesis, promoting cancer metastasis [447].

In the absence of serum, mTORC1 activity (as evidenced by p70 S6K phosphorylation) is reduced but mTORC2 activity (as evidenced by AKT, Ser473 and RAPTOR, Ser792 phosphorylation) is increased in HMC-1 cells, responses reversed by the reintroduction of serum (Figure 6.21) and which appear to be regulated, at least in part by PLD and SphK signaling. Although both pathways can act simultaneously [415], this may not be the case in HMC-1 cells. Perhaps consistent with this, in human mast cells, there is evidence that each mTOR complex has a different role thus, whilst mTORC1 is crucial for survival, mTORC2 is important for proliferation, especially in neoplastic MC, with the relative predominance of particular mTOR complex varying depending on the maturation and nutrient status of the mast cells [448].

Using this model our results are surprising where in reduced nutrient conditions there is a reduction in survival-promoting mTORC1 but an increase in mTORC2, driving proliferation, a counterproductive activity.

As it has been reported that FcεRI and cKIT can activate mTORC1 in MC [414] and IL-33 stimulates mTORC1 and S6K1 in Th2 cells and innate lymphoid cells [327], it was proposed that ST2 could potentially activate mTOR signaling in mast cells. The data presented here for HMC-1 cells suggest this may be the case where IL-33 treatment increases p70 S6K phosphorylation and further decreases Akt Ser473 phosphorylation compared to serum alone (Figure 6.21). This effect is subtle and it would be interesting to determine the effect of IgE sensitization on mTOR activation by IL-33 in primary mast cells.

Figure 6.1 Co-administration of IL-33 and SCF increases cytokine production and proliferation.

PDMC (A, B) or BMMC (C) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were stimulated with IL-33 (10 ng/ml) ± SCF (100 ng/ml) for 24 h at 37^{o}C. Release of IL-13, IL-6 and MCP-1 was measured by ELISA from culture supernatants (A). Proliferation of was determined by the addition of [^{3}H] thymidine for 12 h (B, C). Results represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA. Results represent the combined data from 2 independent experiments ± SEM (A) or a single (B-C) experiment ± SD. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 6.2 Calcium mobilisation induced by IL-33 is augmented by increasing SCF dose.

PDMC sensitized (A-D) or not (C) with murine anti-DNP IgE (0.5 µg/ml) and loaded with Fura-2/AM were stimulated at 50s in serum-free HBSS with 100 ng/ml IL-33 (D, E, F) ± the indicated dose (A, D) or 100 ng/ml SCF (B, C, E, F). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. For the analysis of intracellular mobilisation alone, the cells were stimulated in calcium-free buffer supplemented with 100 µM EGTA to remove all extracellular calcium (EGTA) (B, F). Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment. Statistical analysis using a two-tailed, paired t-test. ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Figure 6.3 Co-administration of IL-33 and SCF increases NFκB activation.

PDMC sensitized with murine anti-DNP IgE (0.5 µg/ml) then stimulated with DNP (0.5 µg/ml) to induce cross-linking (XL), IL-33 (10 ng/ml), SCF (100 ng/ml) or PMA (1 µM) plus Ionomycin (1 µM) for 30 mins at 37^{o}C. Degranulation was determined as the % ß-hexosaminidase released relative to the total activity of the cells (A). Data are presented as the combined results of 4 independent experiments where error bars represent SEM. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. *** $p < 0.001$.

PDMC were sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight then cultured with IL-33 (10 ng/ml) ± SCF (100 ng/ml) for the indicated times and expression of IκBα analysed by Western blotting (B). "0 min" represents zero timepoint control. ß-actin was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of IκBα compared to ß-actin for each timepoint. Data are from a single experiment.

Figure 6.4 IL-33 requires serum or SCF to stimulate cytokine production.

PDMC (A) and BMMC (B) sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were stimulated with IL-33 (10 ng/ml) ± SCF (100 ng/ml) in 1% or 10% FCS supplemented RPMI for 24 h at 37°C. Release of IL-6 and MCP-1 was measured by ELISA from culture supernatant. Data is representative of a single experiment ± SD. Statistical analysis using two-way ANOVA with Bonferroni multiple comparison test. **** $p < 0.0001$.

Figure 6.5 IL-33 induces cytokine production but not proliferation or calcium mobilisation in HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were stimulated with IL-33 (10 ng/ml), SCF (100 ng/ml) or DNP (0.5 µg/ml) to induce cross-linking (XL) in 10% (A, C-E) or 1% FCS-supplemented RPMI (D, E) for 24 h (A, C-E) or the indicated time course at 37^{o}C. Release of IL-6, MCP-1 and TNFα was measured by ELISA from culture supernatants. Data were obtained in a single experiment (A) or a single experiment representative of 2 independent experiments where error bars show SD (E). Proliferation was determined by the addition of [^{3}H] thymidine for 12 h (C, D). Results represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA combined from 2 independent experiments ± SEM.

HMC-1 sensitized (IgE) or not (PBS) with murine anti-DNP IgE (0.5 µg/ml) in 1% FCS-supplemented RPMI and loaded with Fura-2/AM were stimulated at 50s in serum-free HBSS with 100 ng/ml IL-33 or DNP (0.5 µg/ml) to induce cross-linking (XL) of FcεRI (B). Intracellular calcium mobilisation and influx were recorded in real-time using excitation-emission ratios of 340/380 nm. Calcium levels were calculated from Rmax and Rmin values and the data presented as the mean calcium values of triplicate samples ± SD (baseline calcium values subtracted) from a single experiment representative of 3 independent experiments. Statistical analysis using a two-tailed, unpaired (D) or paired t-test (B), one-way ANOVA with Tukey's comparison test (A, C) or two-way ANOVA with a Bonferroni multiple comparison test (E). * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Figure 6.6 Inhibition of cKIT signaling reduces cell viability and cytokine production and prevents cooperative cytokine production induced by IL-33 and SCF in murine mast cells.

PDMC (C, D) or BMMC sensitized with murine anti-DNP IgE (0.5 µg/ml) overnight were pre-treated with indicated concentrations of cKIT (CD117) inhibitors, Imatinib and Dasatinib (A-C), or 100 nM Dasatinib (D, E) for 30 min at 37^{o}C then stimulated with IL-33 (10 ng/ml) (A-E) ± SCF (100 ng/ml) (D, E) for 24 h at 37^{o}C before cell death was assessed by 7AAD uptake by flow cytometry. Results are presented as % 7AAD+ cells in total population (A). Release of IL-6 (C, E) and MCP-1 (B, D) was measured by ELISA from culture supernatants. Data is representative of a single experiment performed in duplicate where error bars show SD.

Figure 6.7 cKIT inhibition does not affect receptor expression of HMC-1 but inhibits viability, cytokine production and proliferation.

HMC-1 incubated in 1% (Starved) (A-G) or 10 % FCS-supplemented RPMI overnight (Unstarved) were pre-treated with indicated concentrations of cKIT (CD117) inhibitors Imatinib (C, F) and Dasatinib (D, E, G) or 100 nM Dasatinib (A, B) for 30 min at 37^{o}C, followed by IL-33 (10 ng/ml) ± SCF (100 ng/ml) in 1% or 10% FCS supplemented RPMI (Serum) for 24 h at 37^{o}C. Cell death was assessed by 7AAD uptake (C, D) and release of IL-6 and MCP-1 was measured by ELISA from culture supernatants in duplicate (E). Proliferation of was determined by the addition of [^{3}H] thymidine for 12 h (F, G). Results, performed in duplicate, represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA. Surface CD117 (B) and ST2 (A) expression on HMC-1 was determined by FACs. Graphs represent geometric mean fluorescence (GMFI) of positive expressing cells as defined by isotype controls (not shown). Data is representative of a single independent where error bars show SD.

Figure 6.8 PLD signaling is involved in individual and combined SCF and IL-33-induced cytokine production in PDMC.

Release of IL-13, MCP-1 was measured by ELISA in duplicate from culture supernatants of PDMC sensitized with murine anti-DNP IgE (0.5 µg/ml) (B) or not (A) overnight, pre-incubated with the indicated concentrations of pan-PLD isoform inhibitor, 5WO, for 30 min at 37^{o}C, then stimulated with SCF (100 ng/ml) ± IL-33 (10 ng/ml) for 24 h at 37^{o}C. MCP-1 results represent combined data of 2 independent experiments ± SEM. IL-13 results represent a single experiment ± SD.

Figure 6.9 PLD 1 signaling is involved in SCF cytokine production in IgE sensitized PDMC.

Release of IL-13, MCP-1 was measured by ELISA in duplicate from culture supernatants of PDMC sensitized with murine anti-DNP IgE (0.5 µg/ml) (B) or not (A) overnight, pre-incubated with the indicated concentrations of PLD 1 isoform inhibitor, 809, for 30 min at 37^{o}C, then stimulated with SCF (100 ng/ml) ± IL-33 (10 ng/ml) for 24 h at 37^{o}C. MCP-1 results represent combined data of 2 independent experiments ± SEM. IL-13 results represent a single experiment ± SD.

Figure 6.10 PLD 2 signaling is involved in SCF and IL-33-induced cytokine production in PDMC and is enhanced by IgE sensitization.

Release of IL-13, MCP-1 was measured by ELISA in duplicate from culture supernatants of PDMC sensitized with murine anti-DNP IgE (0.5 µg/ml) (B) or not (A) overnight, pre-incubated with the indicated concentrations of PLD 2 isoform inhibitor, APV, for 30 min at 37^{o}C, then stimulated with SCF (100 ng/ml) ± IL-33 (10 ng/ml) for 24 h at 37^{o}C. MCP-1 results represent combined data of 2 independent experiments ± SEM. IL-13 results represent a single experiment ± SD.

Figure 6.11 PLD signaling is involved in IL-33-induced cytokine production and proliferation in HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were pre-incubated with the indicated concentrations of PLD inhibitors for 30 min at 37°C in 1% (A, C) or 10% FCS supplemented RPMI (B, C), then stimulated with IL-33 (10 ng/ml) (A, B) for 24 h at 37°C. Release of MCP-1 was measured by ELISA from culture supernatants (A, B). Proliferation of unstimulated HMC-1 was determined by the addition of [^{3}H] thymidine for 12 h (C). Results represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA. Results represent a single experiment ± SD (C) or the combined data from 2 independent experiments ± SEM (A, B).

Figure 6.12 IL-33 plus SCF responses in murine mast cells and serum-induced proliferation in HMC-1 involve sphingosine kinase signaling.

PDMC sensitized with murine anti-DNP IgE (0.5 μg/ml) (A, B) were pre-treated with the indicated concentrations of the non-isoform specific sphingosine kinase inhibitor, DMS (A) or the SphK1 specific inhibitor, 5C (B), for 30 min at 37^{o}C followed by IL-33 (10 ng/ml) ± SCF (100 ng/ml) for 24 h at 37^{o}C. Release of MCP-1 was measured by ELISA in duplicate (A, B). Data from a single experiment ± SD, representative of 2 independent experiments.

Unstimulated proliferation of HMC-1 incubated in 1% FCS-supplemented RPMI overnight (C, D) was determined by the addition of [^{3}H] thymidine for 12 h after 24 h incubation with the indicated concentration of inhibitors DMS (C) or 5C (D) in either 1% or 10% FCS-supplemented RPMI. Results represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA. Data obtained in triplicate from a single independent experiment where error bars show SD (C, D).

Figure 6.13 Sphingosine kinase activity is induced by serum and IL-33 in HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with 10% FCS (A, C) ± IL-33 (100 ng/ml) (B, C) for indicated time course. SphK activity was measured using a fluorimetric assay based on the increased fluorescence of the substrate JF02 following its phosphorylation by SphK as described (2.12). A representative experiment ± SD of at least 2 experiments is shown (left panel). The combined results were further analysed to more clearly display change in activity, where basal values "0 min" were subtracted from values at subsequent time points. Error bars show SEM. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p< 0.05$, ** $p< 0.01$ and *** $p< 0.001$.

Expression of SphK1 was analysed by Western blotting (D) of HMC-1 incubated in 1% FCS-supplemented RPMI overnight then pre-treated with Imatinib (10 μM) or Dasatinib (100 nM) before incubation in 10% FCS supplemented RPMI for 24 h or 48 h at 37°C. GAPDH was used as a loading control and SphK1 expression (42.5 kDa) was confirmed with a blocking peptide, pre-incubated with anti-SphK1 antibody at 37°C from 20 mins. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of SphK1 compared to GAPDH for each timepoint. Data are from a single experiment.

Figure 6.14 mTOR signaling.

mTOR is a serine/threonine kinase that plays a key role in cell proliferation, metabolism and differentiation in response to growth factors and nutrient conditions [414-416], with in particular, loss of mTOR function leading to arrest in the G1 phase of the cell cycle and loss of protein synthesis [416]. mTOR associates with multiple signaling molecules including the scaffolding proteins RAPTOR and RICTOR to form the mTORC1 and mTORC2 complexes respectively. mTORC1 complex activates S6 kinase and 4E-BP1 to initiate RNA translation and protein synthesis [415, 416]. Rapamycin is a specific inhibitor of mTORC1 activation that prevents mTOR and RAPTOR interaction, preventing mTORC1 formation. mTORC2 phosphorylates and activates Akt at Ser473 [419, 420] allowing full activation of Akt by PI3K-dependent phosphorylation of Akt at Thr308 [419, 420]. This activation of Akt is upstream of mTORC1 and perpetuates mTOR signaling by feeding into the mTORC1 pathway. The stimulus for activation of mTORC2 is unconfirmed but it may be directly or indirectly via growth factors [415].

Figure 6.15 Rapamycin inhibits cytokine production and proliferation of HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with the indicated concentrations of Rapamycin and microcystin LR (1 μM) for 30 min at 37^{o}C then IL-33 (10 ng/ml) or 10% FCS (Serum) for 24 h at 37^{o}C before cell death was assessed by 7AAD uptake by flow cytometry. Data is presented as % 7AAD+ cells in the total population from a single experiment (A). Release of MCP-1 was measured by ELISA from culture supernatants in triplicate where error bars show SD (B). Unstimulated proliferation was determined by the addition of [^{3}H] thymidine for 12 h after 24 h incubation with inhibitors. Results represent mean counts per minute (cpm) of [^{3}H] thymidine incorporation in newly synthesised DNA ± SD (C). Results represent a single experiment. Statistical analysis using one-way ANOVA with Tukey's multiple comparison test. * $p < 0.05$.

Figure 6.16 Serum and IL-33 stimulates p70 S6K Thr389 phosphorylation in HMC-1

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with Rapamycin (100nM) (B), the SphK1 inhibitor, 5C (30 μM) (C), the PLD 1 inhibitor, 809 (5μM) (D), or the PLD 2 inhibitor, APV (5μM) (E), for 30 min at 37^{o}C in 1% FCS supplemented RPMI then serum (10%) ± IL-33 (10 ng/ml) was added for the indicated time-course or left unstimulated for 60 mins as a control. Expression of phosphorylated p70S6K (Thr389) was analysed by Western blotting. Total p70S6K was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of Pp70S6K (Thr389) to p70S6K for each timepoint. Data are from a single experiment representative.

Figure 6.17 Serum and IL-33 inhibits RAPTOR Ser792 phosphorylation in HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with Rapamycin (100nM) (B), the SphK1 inhibitor, 5C (30 μM) (C), the PLD 1 inhibitor, 809 (5μM) (D), or the PLD 2 inhibitor, APV (5μM) (E), for 30 min at 37^{o}C in 1% FCS supplemented RPMI then serum (10%) ± IL-33 (10 ng/ml) was added for the indicated time-course or left unstimulated for 60 mins as a control. Expression of phosphorylated RAPTOR (Ser792) was analysed by Western blotting. Total RAPTOR was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of P-RAPTOR to RAPTOR for each timepoint. Data are from a single experiment representative.

Figure 6.18 Serum and IL-33 inhibits Akt Ser473 phosphorylation in HMC-1.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with Rapamycin (100nM) (B), the SphK1 inhibitor, 5C (30 μM) (C), the PLD 1 inhibitor, 809 (5μM) (D), or the PLD 2 inhibitor, APV (5μM) (E), for 30 min at 37^{o}C in 1% FCS supplemented RPMI then serum (10%) ± IL-33 (10 ng/ml) was added for the indicated time-course or left unstimulated for 60 mins as a control. Expression of phosphorylated Akt (Ser473) was analysed by Western blotting. Total Akt was used as a loading control. Graphical presentation of densitometric analysis was performed using Image J to determine the relative intensity of P-Akt to Akt for each timepoint. Data are from a single experiment representative.

Figure 6.19 ES-62 modulates HMC-1 cytokine production.

HMC-1 incubated in 1% FCS-supplemented RPMI overnight were treated with ES-62 (2 μg/ml) in 1% (A) or 10% (B, C) FCS-supplemented RPMI overnight at 37°C then stimulated with IL-33 (10 ng/ml) for 24 h at 37°C. Release of MCP-1 (A, B) and TNFα (C) was determined by ELISA from culture supernatants. Results represent the combined data from 2 (C) experiments ± SEM or a single (A, B) independent experiment ± SD. Statistical analysis using one-tailed, unpaired t-test. *** $p < 0.001$.

Table 6.1 Summary of SCF augmentation of IL-33 responses in PDMC and HMC-1.

Additive effect of co-stimulation with IL-33 and SCF in PDMC (A) and HMC-1 (B). No degranulation was detected in PDMC regardless of stimulation and IL-33 fails to induce proliferation independently however in combination with SCF, increases proliferation. MCP-1, IL-6 and IL-13 production in increased potentially as a result of PLD and SphK-mediated induction of cooperative calcium signaling and NFκB activation.

IL-33-induced MCP-1 production is increased by SCF in HMC-1 potentially as a result of PLD and SphK cooperative signaling but HMC-1 do not display calcium mobilisation or proliferation following IL-33 treatment. SCF fails to induce proliferation however experiments were performed in 10% FCS-supplemented serum, which increases basal HMC-1 proliferation.

Figure 6.20 Proposed PLD and SphK signaling changes during co-stimulation of ST2 and cKIT.

IL-33 and SCF signal predominantly using the PLD 2 isoform. Sphingosine kinase isoforms are differentially used by SCF (SphK1) and IL-33 (Sphk2), potentially cooperating to increase calcium and NFκB signaling, allowing an increase in cytokine responses.

Figure 6.21 Proposed involvement on mTORC1 and mTORC2 in HMC-1 under different serum and IL-33 conditions

In HMC-1 cells, mTORC1 and mTORC2 activation is opposing. In reduced serum conditions mTORC2 dominates with reduced mTORC1 activation, a situation reversed in the presence of serum. Whether this inverse relationship is due to negative feedback or an alternative method has not been determined in this investigation. When HMC-1 are treated with serum and IL-33 simultaneously mTORC1 activation is increased.

7 General Discussion

Mast cells have long been recognised as playing important roles in promoting immunity to infection and more recently in the resolution of inflammation and wound repair. Coupled with, their pathogenic roles in allergic and autoimmune inflammation, this has resulted in intense interest in their potential as therapeutic targets [14, 37, 75, 77, 142]. It is increasingly clear that such studies have been hampered by the gaps in our understanding of the multiple heterogeneous phenotypes of mast cells, their phenotypic and functional plasticity as well as the ability to respond to a diverse range of stimuli. The studies outlined in this thesis attempted to address these problems by characterising the pro-inflammatory responses of mucosal and connective tissue phenotypes of mast cells to a range of key immunomodulatory receptors. Moreover, and as a first step to identifying novel therapeutic targets, the signaling pathways involved in transducing such functional responses of mast cells were characterised, as well as their potential subversion by the helminth product, ES-62 as this has been used as a unique probe to identify targets of safe immunomodulation. Interestingly, the modulatory effects of ES-62 were found to vary with each MC subtype (Table 3.3) highlighting the need to fully understand the mast cell biology of distinct phenotypic and functional subtypes in order to develop effective therapeutics.

7.1 Mast cell phenotype can be modulated by *in vitro* culture conditions, resulting in diverse functional responses.

Initial investigations were directed at determining the phenotypic and functional differences between mucosal and connective tissue mast cell subtypes, derived *in vitro* (Table 3.2). Thus mast cells, or their precursors, were harvested from distinct locations (bone marrow and peritoneal cavity) and cultured in the presence of SCF, IL-3 and/or IL-4 to produce three phenotypes that may begin to reflect immature and mature versions of such

mast cells occurring *in vivo*. Consistent with this, mature PDMC (serosal) expanded with SCF *in vitro*, display greater degranulation responses to Ag-mediated FcεRI crosslinking than the immature BMMC (mucosal) and CTMC (connective tissue), derived from bone-marrow precursors. Interestingly, however, the bone marrow-derived mast cells exhibit greater prostaglandin and cytokine responses suggesting a functional bias towards degranulation by mature cells. Culture with IL-3, rather than IL-4, also defines a functional response bias, with IL-3 plus SCF generating the mucosal-like cells, BMMC, which are reported to be in an immature state [11, 159]. This latter proposal is supported by the data presented here, as when compared to PDMC or CTMC (IL-4 plus SCF treated cells), BMMC have limited degranulation capability and reduced granule content (Figure 3.2). However, BMMC display greater cytokine responses to LPS and Ag-mediated FcεRI crosslinking than CTMC, but equivalent prostaglandin production. As CTMC responses display similarities to those of PDMC, namely poor cytokine production, whilst exhibiting a greater degranulation capacity than BMMC, one hypothesis is that CTMC represent an intermediate state between immature BMMC and fully mature PDMC. This would suggest that as mast cells mature, they pass through different response biases, from a predominance of cytokine production to a downregulation of cytokines in favour of increased degranulation responses. This proposal may be supported by the differential cytokine responses resulting from co-stimulation via TLR4/ST2 (Figure 5.5) and ST2/FcεRI (Figure 5.2) in PDMC and BMMC.

As CTMC are not functionally equivalent to the PDMC, this suggests that additional maturational factors present within the peritoneal cavity are required, highlighting the subtlety of mast cell maturation *in vivo*. Of course, at this stage, it is not possible to rule out that the BMMC and CTMC populations characterised here represent branching pathways of mast cell differentiation arising from a common precursor or even from distinct precursors within the bone marrow population as limited dilution and progenitor isolation studies were not performed prior to culture.

Nevertheless, despite this functional plasticity, certain core signaling features appeared to be conserved. For example, despite the differential functional responses to FcεRI signaling in the three subtypes, the profiles of

calcium signaling were very similar across the mast cell subtypes, with involvement of both intracellular and extracellular calcium mobilisation (Figure 3.9). Interestingly, it was previously reported that LPS/TLR4 signaling did not involve calcium mobilisation [31, 188] in mast cells although it had been proposed that such signaling could promote FcεRI responses by enhancing calcium signaling via upregulation of SOC components [49]. Here, however, it was shown that following IgE-sensitization, LPS can indeed mobilize calcium, predominantly from extracellular sources, a finding in keeping with the ability of LPS to upregulate Orai1 in mast cells. More generally, BMMC were found to display the strongest calcium responses, typically utilizing calcium from both intracellular and extracellular sources, following stimulation with Ag, LPS or IL-33 whereas calcium signaling in CTMC and PDMC, appear to predominantly reflect influx of extracellular calcium. From this it is possible to create a tentative link between the strength of calcium mobilisation and volume of cytokine production. This link has previously been suggested for the rat RBL-2H3 "mast cell" line, where varying intracellular calcium availability was found to alter the levels of cytokine production [449]. This is perhaps not surprising as most functional responses of mast cells, including proliferation, gene expression, secretion, adhesion and migration [189-191] rely on calcium mobilisation to some extent. Regulation of these responses is likely to involve spatio-temporal regulation of calcium responses but, as yet, it is difficult to assign a type of calcium signal to a functional output as the data concerning the specific roles of influx versus ER release have been conflicting [190]. Nevertheless, calcium signaling is crucial for cytokine production [193] and likewise degranulation of mast cells is well established to be dependent on intracellular calcium release [190, 202, 203, 322].

7.2 Mast cell responses are defined by complex interplay between multiple stimuli and receptor interactions.

Mast cell responses are shaped by the interactions of a variety of stimuli and the studies in this thesis have focused on those implicated in driving allergic responses (FcεRI, IL-33/ST2 and LPS/TLR4) and cancer (SCF/cKIT) as a first step towards identifying key regulatory events in these pathways. Although there has been great interest in the emerging role of IL-33 in allergic inflammation, much of the data in the literature relating to mast cell responses to this cytokine are controversial. For example, there are conflicting reports as to whether IL-33 induces degranulation; however the data in this study show clearly that, even following IgE-sensitisation or SCF/serum co-stimulation, IL-33 does not stimulate degranulation by any of the subtypes of murine MC. The data presented here showing that IL-33 does not stimulate either PLD activation or calcium mobilisation in the absence of IgE –sensitization of the presence of SCF/serum may go some way to explaining this as degranulation of MC is dependent on intracellular calcium release [190, 202, 203, 322] and similarly, PLD 1, the predominant PLD isoform in FcεRI crosslinking [287, 288], is reported to be more dominant in driving exocytosis/degranulation [314]. The lack of degranulation even in responses to IL-33-stimulation of IgE-sensitised mast cells cultured in 10% FCS, may reflect the predominant PLD 2- and calcium influx-nature of this signaling.

IL-33 does, however, stimulate cytokine production and this is associated with calcium mobilisation (via influx from extracellular sources) (4.3.2), NFκB and ERK activation (4.3.3). Moreover, although there is evidence for a role(s) of both PLD and SphK involvement in such IL-33-ST2 signaling, the former appears to require licensing by IgE-sensitisation and/or perhaps co-stimulation with SCF or other serum-derived growth factors such as PDGF (Figure 7.1) and provides a rationale for the PLD-dependency of IL-33-stimulated calcium mobilisation in IgE-primed mast cells.

SCF is an important growth factor for MC [363, 364] and consistent with this, in addition to its amplification of IL-33-stimulated MCP-1, IL-13 and IL-6 responses shown here, it has been shown to increase FcεR1-mediated degranulation, cytokine and chemokine responses in MC [365, 440, 450]. Interestingly, therefore, long-term culture with SCF has been reported to inhibit FcεRI-mediated MC activation, suggesting "older" or chronically-activated MC are less sensitive to stimulation [41]. Acute SCF treatment has been shown to increase FcεRI responses through augmentation of calcium mobilisation using DAG and IP_3 [365] and although its ability to induce calcium mobilisation (6.3.2) would explain its ability to augment IL-33 signaling, there is also evidence for alternative mechanisms underpinning SCF-IL-33 interactions, including cooperative PLD and SphK signaling (6.3.2.5-6). As such cooperative signaling could be inhibited with pharmacological inhibitors of cKIT signaling, namely Imatinib and Dasatinib (6.3.2.4), these inhibitors could provide a mechanism to limit pathology under conditions where IL-33 and SCF are found in abundance, such as asthma [64, 242, 450] or cancer. Likewise, evidence of IL-33-mediated augmentation of serum (SCF)-induced mTORC1 activation (6.3.2.7), which is crucial for cell survival [448], was found in the human mast cell line, HMC-1, potentially providing a mechanism whereby IL-33 might increase (potentially aberrant) survival in human mast cells in tumour pathogenesis [62].

Interestingly there appears to be a cKIT-independent activity in serum that enhances IL-33 functional responses, as evidenced by the incomplete suppression of cytokine production by Dasatinib. Further support is provided by the failure of exogenous rSCF to completely reconstitute IL-33 –induced cytokine responses following serum withdrawal. Collectively, these data suggest the presence of an additional co-stimulator of IL-33 in serum. Although PDGF, also found in serum [397], would be a potential candidate to fit this model, it is also robustly inhibited by Dasatinib [56].

Similarly to the enhancement of IL-33-ST2 responses by SCF-cKIT signaling, IgE sensitization of FcεRI also increases IL-33-stimulated cytokine responses (4.3.1), possibly as a result of increased ST2 receptor levels and by a mechanism whereby IgE-stimulated PLD activity licenses IL-33-mediated calcium mobilisation and increases NFκB and ERK activation. Consistent with

this, data from both PDMC and BMMC demonstrate cooperative cytokine production following mast cell co-stimulation via IL-33/ST2 and IgE-FcεRI-signaling (Figure 5.2), mirroring previously reported studies [268] that also attributed such enhanced responses to increased calcium influx, NFκB and ERK activation. Consistent with these findings, TAK1, a downstream effector of TRAF6 has been proposed to be the point of convergence to increase NFκB activation following co-stimulation via IL-33/ST2 and FcεRI [60]. IL-33 and SCF crosstalk has been reported to be dependent on PI3K signaling [57]; however, although this mechanism was not investigated here, PLD activation, which is downstream of PI3K signaling by many immunoreceptors, including FcR, appears to be a common element of the crosstalk between IL-33 and either SCF or IgE sensitization.

In terms of PLD signaling, the data presented in this thesis suggest a rather more dominant role for PLD 2 in immunoreceptor signaling (Figure 7.1) than previously thought, as whilst generally PLD 2 has been proposed to be responsible for constitutive, basal signaling, PLD 1 has been defined as the dominant isoform in agonist activation of mast cells [288]. However, this apparent contradiction may be easily reconciled as the use of the isoform-selective inhibitors suggested that sensitization of mast cells resulting from FcεRI binding of monomeric IgE or by exposure to SCF, involves the contribution of PLD 2 signaling to bring about "priming" of murine MC to allow IL-33-signaling. IL-33 alone does not appear to stimulate PLD activity or calcium mobilisation presumably reflecting the fact that the assays used to determine these activities were performed in serum-free conditions, effectively removing the SCF-mediated starting platform for IL-33-mediated PLD activation. This “basal” PLD 2 signal, especially induced by IgE sensitization, allows for increased PLD 1 and PLD 2 signaling following stimulation with IL-33 or SCF and may be responsible for the IL-33-induced calcium response, only observed in IgE sensitized cells (Figure 4.8) and potentially identifies the role for PLD in maintaining NFκB, and subsequent cell activation. Interestingly this calcium signaling is not completely ablated by PLD inhibition, indicating an alternative method of calcium mobilisation used by IL-33 suggesting future experiments should investigate any role for PLCγ-dependent production of IP_3 in IL-33-induced MC signaling. In addition to its role in calcium signaling, the ability of IL-33 to rescue cell viability [62,

268] is lost following inhibition of PLD inhibition, suggesting a pro-survival role for PLD which may similarly provide a mechanism for IgE-induced MC survival [276, 279, 451]. The increased PLD response produced by cooperative IL-33 and SCF signal may also be reflected in serum-mediated activation of mTORC1, as in HMC-1, PLD 2 signaling appears to play a role in the regulation of both mTORC1 and mTORC2. Similarly to the role of PLD in mast cell priming, enhanced SphK signaling may also contribute to providing a signaling platform to allow and/or enhance IL-33-functional responses.

Although a subordinate role is attributed to PLD 2 in the literature, its location at the plasma membrane places it in an optimal position for immediate activation [318, 319] and perhaps consistent with this, PLD 2 appears to regulate the location, morphology and trafficking of signaling components [316]. Indeed, and of relevance to this study, siRNA studies revealed that downregulation of PLD 2 prevented localisation of FcεRI to signaling domains [287]. Interestingly, therefore, the reported dominance of PLD 1 signaling in FcεRI may be a reflection of its role in granule trafficking [314], crucial for degranulation but a process not stimulated by IL-33 [42, 60, 62, 155, 268].

7.3 Mast cell signaling and functional responses can be modulated by ES-62

The ability of ES-62 to modulate mast cell responses to Ag (FcεRI), LPS (TLR4) (Table 3.3) or IL-33 (ST2) (Figure 5.12) in both murine MC (at different stages of maturation) and the human mast cell line, HMC-1 (Figure 6.19), indicates that its modulation is highly adaptable and not restricted to a specific receptor in mast cells. This is also evidenced by its inhibition of PMA/Ionomycin responses, which indicates that it is likely targeting one or more downstream elements common to these signaling pathways. Similarly, as ES-62 as the ability to inhibit both degranulation and cytokine responses, which are reported to be controlled by alternative signaling pathways [25,

452], this is further support for the proposal that ES-62 can modulate recruitment of multiple pathways, presumably at a convergent upstream point. One option is calcium signaling as this is crucial for both of these MC functional responses [192] and indeed the data presented here reveal that ES-62 can inhibit influx of calcium from extracellular locations induced by Ag-mediated FcεRI crosslinking, LPS or IL-33 (Figure 7.2). Although this has not yet been investigated, this inhibition of calcium influx is interesting given that ES-62 subverts TLR4 signaling and classical LPS/TLR4 signaling acts to promote calcium influx in mast cells by upregulating Orai1, a component of SOCs [49]. Perhaps pertinent to this, evidence suggests that SphK2 may be involved in regulating calcium influx and hence, future studies, possibly using SphK1 or SphK2 KO mice could determine the potential role of individual SphK isoforms in ES-62 responses.

Another candidate of convergent signaling potentially targeted by ES-62 is PKCα (Figure 7.2) as this PKC isoform, which plays a key role in Ag receptor signaling [187, 212] as well as that of TLR4 [453, 454] and FcεRI signaling [126, 455], is downregulated by ES-62 not only in PDMC, BMMC and CTMC (Figure 3.11 and [134]) but also in B and T cells. Importantly for a model of ES-62 inhibition of IL-33, PKCα can be placed either downstream or upstream of PLD, reported here to be key to licensing IL-33 responses. Thus, PKCα, both via direct interaction and also combined with calcium, can activate both PLD isoforms [209, 210, 292, 293, 318, 319, 456, 457], whereas the PLD product, PA (in conjunction with DAG) can activate PKCα following FcεRI-crosslinking in RBL-2H3, an activity which has been reported to be responsible for calcium signaling and degranulation [285, 286]. Although ES-62 modulation of PLD activity has not been investigated here and previous studies [126] proposed that ES-62 targeted the former, the role of this PKC isoform in modulating calcium mobilisation suggests that by downregulating PKCα and hence suppressing both of these upstream and downstream roles, ES-62 may have developed an efficient strategy for inducing mast cell hyporesponsiveness. Alternatively, modulation of IL-33 responses may be mediated by downregulation of MyD88 as ES-62 has been shown to downregulate this key transducer of IL-1R/TLR family responses in BMMC [134], T cells [135] and macrophages (MA McGrath, DT Rodgers, RJ Eason, W Harnett & MM Harnett, unpublished). In the latter case, ES-62 has been found

to exploit this mechanism to inhibit TLR4-(LPS), TLR2- (BLP) and TLR9- (CpG) mediated pro-inflammatory signaling. Interestingly, therefore, evidence presented here suggests that ST2-signaling may also inhibits LPS-TLR4-responses in mast cells, and ST2 -signaling has previously been reported to inhibit TLR4 signaling by a mechanism also suggested to involve MyD88 sequestration and/or downregulation [179, 338]. If ST2 is restricting access to MyD88 in mast cells, under certain conditions ST2 may be functioning in a manner similar to ES-62, although the $ST2^{-/-}$ data presented here do not generally support a role for ST2 in transducing immunomodulation by ES-62. Interestingly, however, co-stimulation with IL-33 and LPS induced additive effects with respect to cytokine production by WT PDMC and BMMC (Figure 5.14); these apparently contradictory effects relating to ST2/TLR4 signaling are extremely interesting and reflect the conflicting data previously published [178, 179, 235, 335, 336, 338, 339]. Perhaps signaling by IL-33 and LPS convergently signals via MyD88 to increase cytokine production while, in the absence of IL-33, ST2 sequesters MyD88 to limit the LPS response. Moreover, as ES-62 can inhibit this enhanced cytokine response in BMMC, this supports the proposal of MyD88 as a common signaling element(s) in the interaction between ST2 and TLR4. Interestingly, therefore, especially given that ES-62 does not downregulate MyD88 in PDMC [134], in the absence of ST2 expression, ES-62-induced inhibition of LPS responses in these cells, but not BMMC, is lost. This result again highlights the functional plasticity of MC responses in response to their microenvironment. Thus, in BMMC, which produce a strong cytokine response upon TLR4 stimulation, ES-62 may use PKCα and/or MyD88 downregulation in order to mediate hyporesponsiveness, whereas in PDMC with their generally weak TLR4 responsiveness, ES-62 may only require to exploit, via its subversion of TLR4 signaling, ST2-mediated inhibition of TLR4 signaling to sequester, rather than degrade MyD88 (Figure 5.18).

The development of individual mast cell populations with the ability to interpret subtle signals of particular microenvironments to mould their response is crucial to the immune response as it increases efficiency and reduces unwanted or excessive responses, thereby limiting tissue damage and conserving energy. Thus, as inflammatory disease can be restricted to a particular organ such as the lungs in asthma, translational research on

clinically relevant mast cell subtypes is required to develop appropriate therapeutics. Collectively, these studies highlight potential mechanisms for therapeutically targeting MC whilst the further characterisation of the distinct MC subtypes that can be generated *in vitro* will potential allow design of future studies to target phenotypically-relevant signals and mast cells for particular inflammatory conditions. Furthermore, the complex and overlapping signaling interactions identified amongst such receptors involved in the homeostatic and inflammatory regulation of mast cells suggests potential novel targets for existing therapeutics such as Imatinib or Dasatinib, due to their ability to suppress "harmful" or "hyper-inflammatory" cooperative signaling arising from crosstalk between FcεRI, TLR4 or ST2 and their target, cKIT, whilst still preserving sufficient signaling and functional responses to allow protective mast cell function.

Figure 7.1 Summary of IL-33 signaling and its interactions with FcεRI, cKIT and TLR4

IgE-licensing of IL-33 (and SCF) signaling may be mediated by enhanced PLD 1, PLD 2 and SphK activity. Similarly SCF-licensing of IL-33 is most likely mediated by an increase in PLD 2 activity. This allows further PLD 1 and, predominantly, PLD 2 activation by IL-33 that may be required for downstream calcium mobilisation, potentially via PA-stimulated S1P-S1PR mediated release from the ER and the mobilisation of STIM1 to form a complex with CRAC1 allowing influx of calcium from the extracellular space. The increase in PLD signaling may also induce mTORC1 and mTORC2 activation via PA. In combination this cooperative signaling drives MC proliferation, increases survival and cytokine production. ST2 also has an inhibitory effect on TLR4 signaling, potentially via MyD88 sequestration.

Figure 7.2 Model of the mechanism of action of ES-62 in the induction of hyporesponsiveness to the key mast cell activators, FcεRI-XL, IL-33/ST2 and LPS/TLR4.

ES-62 may downregulate MyD88 expression in BMMC (A) to inhibit IL-33 signaling or alternatively/additionally by targeting PKCα, upstream of PLD, for degradation (B), the latter mechanism providing a common signaling element shared by FcεRI, TLR4 and potentially ST2 signaling in PDMC and BMMC (C). PKCα degradation would uncouple PLD activation, inhibiting calcium mobilisation and subsequent activation of NFκB. Similarly ES-62-mediated inhibition of influx of extracellular calcium induced by FcεRI crosslinking, LPS or IL-33 may potentially be via inhibition of SphK2 or prevention of TLR4-mediated upregulation of calcium channel components and assembly (D). In PDMC, rather than induce its degradation, ES-62 may exploit the ability of ST2 (E) to inhibit TLR4 signaling via MyD88 sequestration (F).

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