INVESTIGATING THE IMPACTS OF MAST CELLS AND INTERLEUKIN-33 IN RESOLUTION OF INFLAMMATION AND CARDIAC FIBROSIS

by

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ABSTRACT

Cardiac fibrosis is characterized by excessive extracellular matrix deposition and tissue remodeling that leads to heart failure. Chronic inflammation promotes cardiac fibrosis and occurs after initial inflammatory responses to damage that are not effectively resolved. Mast cells are resident innate immune cells present in virtually all body tissues that act as sentinels to damage. Mast cells robustly respond to interleukin (IL)-33, a damage associated molecular pattern released upon stromal cell death. IL-33 exerts cytokine effects upon release via signaling through its cognate receptor ST2 to promote type 2 immune responses. We hypothesized that mast cells prevent excessive remodelling in the heart that leads to fibrosis via an IL-33-dependent mechanism. This hypothesis has been examined using human and mouse models. Atrial tissues from human cardiac surgery patients were assessed for mast cells, in relation to fibrosis, and clinical outcomes. We observed that patients with high mast cell density had lower atrial collagen content and improved functional outcomes post-operative. IL-33 did not have a role at this later stage in remodelling. In response to IL-33 *in vitro*, human mast cells produced a number of mediators of relevance to angiogenesis, such as vascular endothelial growth factor-A and urokinase plasminogen activator, but not those classically associated with fibrosis or inflammation. Finally, a mouse model of efferocytosis was employed, to determine impacts of IL-33 on inflammation resolution. IL-33 administration to the peritoneal cavity led to increased populations of macrophages bearing the efferocytosis receptor MerTK. Additionally, IL-33 promoted efferocytosis when apoptotic cells were introduced. This was not a mast cell dependent process. Through studies of human atrial tissues and related model systems, we have identified a novel association between mast cells, IL-33 and the resolution of cardiac fibrosis. Mast cell-derived angiogenic mediators identified *in vitro* may be relevant in this system. Our *in vivo* efferocytosis model can inform future work on inflammation resolution with relevance to the heart and other inflammatory sites. This work demonstrates the complexity of the cell types, mediators and interactions involved in a disease process such as cardiac fibrosis and has provided mechanisms to inform the development of more effective therapies.

LIST OF ABBREVIATIONS USED

- ACE – Angiotensin Converting Enzyme
- ACKR – Atypical Chemokine Receptor
- AF – Atrial Fibrillation
- Ang – Angiotensin
- AnnA1 – Annexin A1
- ATP – Adenosine Triphosphate
- BAI1 – Brain-specific Angiogenesis Inhibitor 1
- Bcl-2 – B cell lymphoma-2
- BMI – Body Mass Index
- BMMC – Bone Marrow-derived Mast Cell
- CABG – Coronary Artery Bypass Grafting
- CBMC – Cord Blood-derived Mast cell
- CFSE – Carboxyfluorescein Succinimidyl Ester
- CHF – Chronic Heart Failure
- CLR – C-type Lectin Receptors
- COPD – Chronic Obstructive Pulmonary Disease
- CTL – Cytotoxic T Lymphocytes
- CTMC – Connective Tissue Mast Cells
- cTRM – cardiac Tissue Resident Macrophage
- CVD – Cardiovascular Disease
- DAMP – Damage-Associated Molecular Pattern
- DC – Dendritic Cell
- DCM – Dilated Cardiomyopathy
- ddPCR – droplet digital Polymerase Chain Reaction
- ECM – Extracellular Matrix
- EF – Ejection Fraction
- ELMO1 – Engulfment and Cell Motility Protein 1
- ERK – Extracellular Signal-Related Kinase
- FBS – Fetal Bovine Serum
- FcεRI – Fc Epsilon Receptor 1
- FGF-2 – Fibroblast Growth Factor-2
- FM – Free Mitochondria
- FM+33 – Free Mitochondria + IL-33
- FRC – Fibroblastic Reticular Cell
- FSC – Forward Scatter
- GDF-15 – Growth/Differentiation Factor-15
- GEO – Gene Expression Omnibus

- GIT – Gastrointestinal Tract
- GM-CSF – Granulocyte Monocyte-Colony Stimulating Factor
- H&E – Hematoxylin & Eosin
- Hello Kitty – *Cpa3-Cre; Mcl-1*$^{fl/fl}$
- HMGB1 – High Mobility Group Box 1
- hs-TnT – High Sensitivity Cardiac Troponin T
- HSC – Hematopoietic Stem Cell
- HSP – Heat Shock Protein
- HTB – Hepes Tyrode's Buffer
- HTN - Hypertension
- HUVEC – Human Umbilical Vein Endothelial Cell
- i.p. – intraperitoneal injection
- ICAM – Intercellular Adhesion Molecule
- IFN – Interferon
- IHC – Immunohistochemistry
- IL – Interleukin
- IL-1RAcP – IL-1 Receptor Accessory Protein
- IL-33FL – IL-33 Full Length
- ILC2 – Type 2 Innate Lymphoid Cell
- IRAK – IL-1 Receptor-Associated Kinase
- IRI – Ischemia/Reperfusion Injury
- JAK – Janus Kinase
- JNK – Jun N-terminal Kinase
- LC3 - Microtubule-associated protein 1A/1B-light chain 3
- LOS – Length of Stay
- LPC – Lysophosphatidylcholine
- LPM – Large Peritoneal Macrophage
- LPS – Lipopolysaccharide
- LRP – Lipoprotein Receptor-Related Protein
- LRRC3 – Leucine Rich Repeat Protein 3
- LT – Leukotriene
- LVEDP – Left Ventricular End Diastolic Pressure
- LXR – Liver X receptor
- MAPK – Mitogen Activated Protein Kinase
- MCG – Mast Cell Granule
- Mcl-1 – Myeloid Leukemia Cell differentiation protein -1
- MCp – Mast Cell Progenitor
- MC_T – Tryptase Positive Mast Cells
- MC_{TC} – Tryptase Chymase Positive Mast Cells

- MDA5 – Melanoma Differentiation Gene 5
- MDSC – Myeloid Derived Suppressor Cell
- MerTK – Myeloid Epithelial Reproductive Tyrosine Kinase
- MFGE-8 – Milk Fat Globule-Endothelial Growth Factor 8
- MI – Myocardial Infarction
- miR – Micro Ribonucleic Acid
- MMC – Mucosal Mast Cell
- MMP – Matrix Metalloproteinase
- MRGPRX2 – Mas-related G protein-coupled Receptor X2
- mRNA – messenger Ribonucleic Acid
- MSC – Mesenchymal Stem Cell
- MyD88 – Myeloid Differentiation Primary Response 88
- NF-κB – Nuclear Factor-κB
- NK – Natural Killer Cells
- NLR – NOD-like Receptor
- NOD – Nucleotide-Binding Oligomerization Domain
- Nr4a1 – Nuclear Receptor Subfamily 4 Group A Member 1
- NSA – Normalized Standard Amount
- NSAID – Non-Steroidal Anti-Inflammatory Drug
- NT-proBNP – N Terminal-pro Brain Natriuretic Peptide
- NYHA – New York Heart Association
- PAMP – Pathogen-Associated Molecular Pattern
- PBMC – Peripheral Blood Mononuclear Cell
- PDGF – Platelet-Derived Growth Factor
- PG – Prostaglandin
- PROS1 – Protein S1
- PRR – Pattern Recognition Receptor
- PtdSer – Phosphatidylserine
- PVD – Peripheral Vascular Disease
- qPCR – quantitative Polymerase Chain Reaction
- R10F – RPMI 1640 Medium + 10% Fetal Bovine Serum
- RAAS – Renin Angiotensin Aldosterone System
- Rac1 – Ras-related C3 botulinum toxin substrate 1
- RAGE – Receptors of Advanced Glycation End Products
- RIG-I – Retinoic Acid-Inducible Gene I
- RLR – RIG-I-like Receptors
- RNS – Reactive Nitrogen Species
- ROS – Reactive Oxygen Species
- RP105 – Radioprotective 105

- RvD1 – Resolvin D1
- RvE2 – Resolvin E2
- S1P – Sphingosine-1-Phoshpate
- scRNA seq – single cell RNA-sequencing
- SCF – Stem Cell Factor
- SLE – Systemic Lupus Erythematosus
- SOCS – Suppressor of Cytokine Signaling
- SPM – Small Peritoneal Macrophage
- SRFG – Sirius red fast green
- SSC – Side Scatter
- sST2 – Soluble ST2
- ST2 – IL1RL1
- ST2L – membrane bound ST2
- STAT – Signal Transducer and Activator of Transcription
- t-SNE – t-distributed Stochastic Neighbour Embedding
- TAM – TYRO3, AXL and MerTK receptor family
- T_{h2} – T helper 2 cells
- TIM – T-cell Immunoglobulin and Mucin Domain Containing
- TLR – Toll-like Receptors
- TNFR – Tumour Necrosis Factor Receptor
- TRAF6 – Tumour Necrosis Factor Receptor Associated Factor 6
- T_{reg} – T regulatory cell
- TTP – Tristetrapolin
- TYRO3 – Tyrosine Protein Kinase Receptor 3
- uPA – Urokinase Plasminogen Activator
- UTP – Uridine Triphosphate
- VEGF – Vascular Endothelial Growth Factor
- VR – Valve Replacement
- WSH – $Kit^{Wsh/Wsh}$

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CHAPTER 1: INTRODUCTION

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1.0 – Overview

Cardiac fibrosis is the excessive deposition of scar tissue that occurs in the heart after damage and is driven by chronic inflammation [1,2]. Research in this field has historically focused on initial causes of chronic inflammation and fibrosis, rather than their resolution. Resolution of inflammation is an active process that transitions tissues to adaptive homeostasis [3,4]. Understanding how resolution occurs naturally and how it can be activated provides opportunities to improve patient outcomes. The inflammatory process is regulated by tissue resident immune cells at sites of damage. This includes mast cells and macrophages, which act as sentinels to co-ordinate immune responses to infection and tissue damage [5,6]. Resident tissue immune cells respond to various stimuli to mediate these responses. One unique dual-function cytokine that acts on mast cells is interleukin (IL)-33. IL-33 is a nuclear damage-associated molecular pattern (DAMP) released upon death or damage in stromal cell populations. It exerts cytokine effects via signaling through its cognate receptor ST2 to promote type 2 immune responses. To introduce the experimental work in this thesis, the following chapter will present relevant literature on mast cells, IL-33, cardiac fibrosis, inflammation resolution, and macrophage-mediated efferocytosis to provide relevant context for the work described herein.

1.1 – Mast cells

The immune system in mammals has evolved to serve a plethora of purposes, from defense against pathogens, to restoration of tissue function after injury, and maintenance of homeostasis. The mast cell is an innate immune cell that has an impact on all these functions. Mast cells are highly evolutionarily conserved and have homologues in invertebrates, which function to regulate many processes within and outside of the

immune response through a diverse array of actions [6]. Mast cells were initially identified and characterized by the high concentrations of metachromatic granules present in their cytoplasm. These granules contain a variety of mediators, such as histamine, serine proteases, and cytokines [5,7]. Mast cell degranulation occurs in response to numerous stimuli and is classically associated with Fc Epsilon Receptor I (FcεRI) crosslinking in allergy. However, mast cells also synthesize and secrete mediators *de novo* without degranulation to enact more discrete functions. Finally, a combination of degranulation and *de novo* mediator synthesis can occur upon mast cell activation, allowing for these cells to greatly impact the tissue environments in which they reside (Fig. 1.1) [5,8]. The mast cell proteomic profile is distinct amongst hematopoietic cells, leukocytes, and granulocytes, but conserved between humans and mice [9]. Mast cells are very long lived in tissues and unlike other granulocytes such as neutrophils, they can re-granulate and persist in tissues following degranulation [10]. Therefore, mast cells are poised to orchestrate inflammatory processes that follow infection and injury, allowing them to act as sentinels within the body.

1.1.2 – Mast cell origins

Mast cells are not observed in mature forms in circulation. Mast cell progenitors (MCp) are derived from bone marrow hematopoietic stem cells (HSCs) and circulate in the periphery until they enter tissues and undergo maturation to become mast cells [10,11]. Certain mast cell populations are derived from yolk-sac progenitors in young mice, particularly in the peritoneal cavity and skin, but in mature animals most populations are seeded from bone marrow MCps [12]. MCps are rare in bone marrow and circulation due to the rapidity with which they are recruited to tissues, and low rates of tissue mast cell turnover [11,13]. This makes the study of MCps particularly challenging. In mice, MCps can be identified in circulation as $Lin^{-}CD117^{+}ST2^{+}$Integrin $\beta7^{+}CD16/23^{hi}$ with variable expression of FcεRI between strains ($Fc\varepsilon RI^{int}$ in BABL/c and $Fc\varepsilon RI^{-}$ in C57BL/6) [14]. Upon entry into tissues, MCps upregulate CD45 and FcεRI on their cell surface as they mature into tissue resident mast cells. The identification of MCps in humans is more difficult, as a definitive progenitor population has yet to be identified. $CD34^{+}CD117^{+}CD13^{+}$ cells in circulation have the potential to be mast cell-forming in culture, but only a small percentage give rise to mast cells [10] and are therefore likely not

definitive MCps. Once within tissue, MCps require IL-3 [15] and stem cell factor (SCF) [16] signaling to differentiate into mature mast cell forms *in vivo* [17]. Although MCp identity and biology is poorly understood, it is well accepted that mast cells mature within the tissue niches they occupy and as such their function is derived from both cell identity and micro-environmental influences [10,11].

1.1.3 – Mast cell properties

Mast cells are distributed in tissues and organs throughout the body, at particularly high density at sites in contact with the external environment [18,19]. Mature mast cells can be identified morphologically by their high content of electron-dense secretory granules [20]. These granules can be visualized with cationic dyes to produce classical metachromatic staining of mast cells (Fig. 3.1A). The contents of mast cell granules differ between subsets, and mature mast cells have historically been divided into two phenotypes largely based around granule-associated proteases. This classification scheme differs between humans and mice. Murine mast cells have historically been divided based on heparin and chondroitin sulphate content in their granules into mucosal (MMC) or connective tissue mast cells (CTMC), the latter of which has more heparin content [6,20]. In humans, mast cells are divided based on chymase expression in granules into tryptase positive (MC_T) or tryptase and chymase positive mast cells (MC_{TC}) that generally have mucosal and connective tissue distribution respectively [21,22].

The structure of this schema is being challenged, as recent transcriptomic studies of 14 lymphoid and myeloid populations in mice identified mast cells as a transcriptionally diverse population depending on tissue residence[23]. While mast cells contained a "transcriptomic signature" consistent across tissues, each population contained a separate and distinct "tissue-specific" signature that regulated their activity [23]. Similar data has begun to emerge on human mast cells [9]. Therefore, mast cell function is likely regulated by the tissues these cells reside in, similar to tissue resident macrophages [24]. It is likely that mast cells residing in tissues with common needs, such as those with predominantly mucosal vs. connective tissue morphology, appear similar from histological perspectives. However, with the advent of transcriptomic and proteomic sequencing more diverse functions will likely be identified than previously appreciated.

Mast cells respond to a wide variety of stimuli via expression of numerous receptors on cell and inner membrane surfaces (Table 1.1). Mast cells can respond to activation in several ways: (1) degranulation and release of pre-formed mediators, (2) secretion of *de novo* synthesized mediators, or (3) a combination of degranulation and secretion of *de novo* mediators (Fig. 1.1). Degranulation results in largely pro-inflammatory actions through the release of mast cell granule products (Table 1.2). Mast cells trap a diverse array of mediators in anionic gel matrices of chondroitin sulfate and heparin to avoid the large and thermodynamically intensive task of storage [8,25]. Several of these mediators (e..g SCF, VEGF) can be generated by *de novo* synthesis pathways as well[5]. Examples of stimuli that exclusively induce degranulation in mast cells includes UV radiation [26], complement components C3a and C5a [27], and insect venoms [28], among others [8]. The magnitude of granule release differs based on the given receptor that is stimulated. In response to FcεRI cross-linking, mast cells rapidly degranulate and release most, if not all, of their granules into the extracellular environment which can result in anaphylaxis. However, in response to activation of Mas-related G protein-coupled Receptor X2 (MRGPRX2) by various stimuli, mast cells can limit granule release, with some evidence suggesting preferential release of certain types of granules [29].

Mast cells also synthesize and secrete mediators *de novo* in response to various stimuli. This includes cytokines, chemokines, lipids, and growth factors with actions ranging from pro-inflammatory, to pro-fibrotic, to angiogenic (Table 1.3). The activating stimuli and tissue microenvironment influence the subsequent mast cell secretion pattern. Mast cells generate lipid mediators *de novo* to exert physiological and inflammatory roles [30]. Lipid mediators can be synthesized with or without the generation of protein mediators, as well as in the context or independent of mast cell degranulation [31]. Mast cells have been historically regarded as pro-inflammatory cells, but the consequences of mast cell secretion are numerous. Mast cells can interact with neurons to regulate functions of the central and peripheral nervous system [32]. Mast cell serotonin secretion regulates intestinal homeostasis through direct nerve interactions [33] and distal impacts on hippocampal function [34]. Production of IL-10 by mast cells promotes allograft tolerance after transplantation through the promotion and maintenance of T regulatory cell (T_{reg}) populations [35–37]. In contrast, mast cell secretion of vascular endothelial growth factor

(VEGF)-A can promote tumour progression via neovascularization for tissue invasion [38–40]. Furthermore, mast cell IL-1β production is a key contributor to rheumatoid arthritis progression [41,42]. Stimuli associated with exclusive mast cell secretion include TLR2 activators [43,44], TLR4 activators [45,46], cytokines (IL-4 [43], IL-6 [47], IL-13 [48], IL-33 [49,50], interferon(IFN)α, IFNγ [51]), chemokines (CXCL12 [52]), and growth factors (SCF [53]), among others. Of note, pattern recognition receptor (PRR) signaling in mast cells has largely been examined in the context of pathogen-associated molecular patterns (PAMPs) [54], with less data available on the impact of DAMPs on mast cell secretory actions.

Finally, mast cells can combine both degranulation and *de novo* secretion, which is most classically observed in mast cell activation via IgE cross-linking in allergic disease. FcεR1 on the mast cell surface binds antigen-specific IgE, [55] which sensitizes the cell upon first exposure to pathogens, such as helminths, or allergens [56]. Subsequent activation of FcεR1-bound IgE by target antigen crosslinks these receptors [57], leading to immediate degranulation. FcεR1 mediated signaling induces synthesis and secretion of a number of mediators, including arachidonic acid metabolites (prostaglandins and cysteinyl leukotrienes), cytokines (TNF, CXCL8), chemokines (CCL2), and growth factors (VEGF-A) [55,56,58]. Combinations of degranulation and secretion have also been observed in response to activation of MRGPRX2 [29], neurokinin receptors (NKR) [59], and opioid receptors [60,61], among others [8].

Mast cells have been implicated as key cellular mediators in a number of pathological processes including defense against pathogens [62,63], allergic inflammation [64–66], cancer [67,68], vascular disease [69], autoimmune disease [32,70], cardiovascular disease [71,72], and fibrosis [73–77]. Mast cell actions in these disease settings have been frequently reported to be influenced by the dual-function cytokine IL-33.

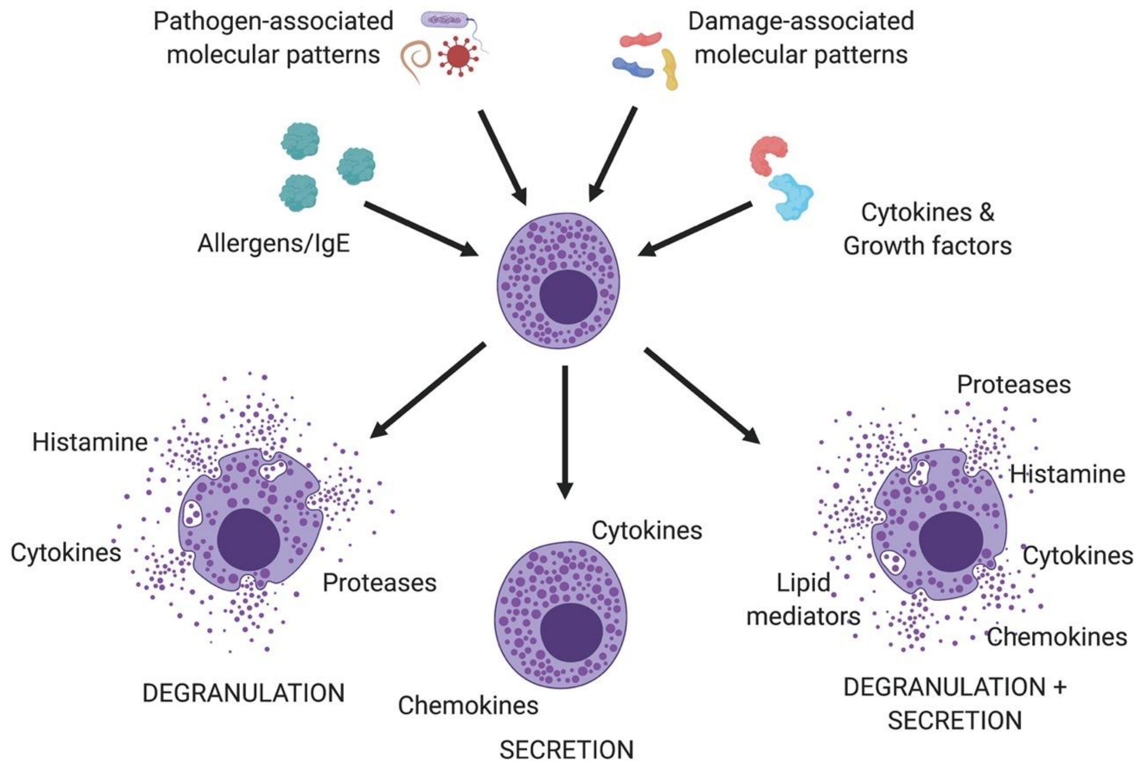


Figure 1.1 – Mast cells responses to activation. Mast cells can degranulate in response to activation by a variety of stimuli, characterized most typically by release of histamine, proteases and cytokines. Mast cells can also be activated to secrete mediators *de novo* without degranulation. Finally, a combination of degranulation and secretion can occur, in which tailored *de novo* mediator synthesis follows mast cell degranulation. Figure generated with BioRender.

Table 1.1 – Summary of known mast cell receptors [7,78,79]

Immunoglobulin receptors	
Surface Ig receptors	FcεRI
	FcγRI
	FcγRIIA
	FcγRIIB
	FcγRIIIA
	Surface Ig- free light chain receptor (FLCR)
Pattern recognition receptors (PRRs)	
Surface	TLR2/TLR1 or TLR2/TLR6
	TLR4
	TLR5
Endosomal	TLR3
	TLR7
	TLR8
	TLR9
	TLR10
C- type lectin receptor (CLRs)	
	Dectin- 1
	CD48
Retinoic acid- Inducible Gene- I (RIG- I)- like Receptors (RLRs)	
Cytoplasmic	RIG- I (Retinoic acid- Inducible Gene- 1)
	MDA- 5 (melanoma differentiation gene 5)
	LGP2 (laboratory of genetics and physiology 2)
Nucleotide Oligomerization Domain (NOD)-like receptors (NLRs)	
Cytoplasmic	NOD1
	NOD2
	NLRP3
Nuclear receptors	
	Glucocorticoid receptor (GR)
Hormone receptors	
	Estrogen
	Progesterone
	Testosterone
	Aryl hydrocarbon receptor (AhR)

Hormone receptors	
	Vitamin D receptor (VDR)
	Peroxisome proliferator- activated
	Receptors
G- protein-coupled receptors (GPCRs)	
MAS- related GPCRs	MRGPRX2
	MRGPRB2
	MRGPRB3
Complement receptors	C3aR
	C5aR
Endothelin receptors	ET_A
	ET_B
Neuropeptide and neurotransmitter receptors	NTSR1 (Neurotensin receptor 1)
	CRHR- 1 (corticotropin- releasing hormone receptor)
	NK- 1R (neurokinin- 1 receptor)
	CLR (calcitonin- like receptor
	VPAC2 (Vasointestinal peptide and pituitary adenylate cyclase-activating peptide receptors 2)
	VPAC1 (Vasointestinal peptide and pituitary adenylate cyclase-activating peptide receptor 1)
	ADRB2 (beta2 adrenergic receptor)
Lipid mediator receptors	
Sphingosine 1 phosphate (S1P) receptors	S1P1
	S1P2
Prostaglandin D2 receptors	PTGDR-1 (DP, D prostanoid receptor)
	PTGDR-2 (DP2 or CRTH2)
Prostaglandin E2 receptors	EP1 (E prostanoid receptor)
	EP2
	EP3
	EP4
Leukotrienes (LTs) receptors	CYSLTR1 (Cysteinyl leukotriene D4 receptor)
	CYSLTR2
	GPR99 (G-protein-coupled receptor 99)
	GPR17 (G-protein-coupled receptor 17)
Leukotrienes (LTs) receptors	
	BLT1R (B4 leukotriene receptor 1)

	BLT2R
	PAFR (PAF receptor)
Purinergic receptors	A2aR (Adenosine 2a receptor)
	A2bR
	A3R
	P2Y (P2 purinoceptor subtype Y)
Chemokine receptors	Not listed
Other GPCRs	ADGRE2 (Adhesion G-protein-coupled receptor E2) or
	EMR2 (EGF-like module-containing mucin-like hormone receptor-like 2)
	5HTR (Serotonin receptor)
Endocannabinoid receptors	CB1
	CB2
Corticotropin-releasing hormone receptor	CRHR1
	CRHR2
Alarmin receptors	
	IL-1 receptor (IL-1R)
	IL-33 receptor (ST2)
Purinergic receptors	P2Y receptors
	P2X receptors (P2X7)
RAGE/AGER	
Other receptors	
Integrins	CD29
	CD49d
	CD49e
	CD61
	CD51
	$\alpha_1\beta_1$
	$\alpha_2\beta_1$
	α4β7
Tetraspanins	
	CD9

	CD37
	CD53
	CD63
	CD81
	CD82
	CD151
Complement receptors	C1q-R
	C3AR1
	C5AR
Cytokine Receptors	Not listed
Stem cell factor receptor	c-Kit
Growth factor receptors	Not listed
Antigen presenting receptors	MHCII

Table 1.2 – Summary of known mast cell granule contents [20]

Granule constituent	Key features
Lysosomal enzymes	
β-hexosaminidase	Enzyme involved in turnover of carbohydrates
	Routinely used as a marker for mast cell degranulation responses
β-glucuronidase, N-acetyl-β-glucosaminidase and β-D-galactosidase	Enzymes involved in turnover of carbohydrates
Arylsulphatase A	Enzyme involved in turnover of glycosphingolipids
Cathepsin B	Cysteine protease
Cathepsin C	Cysteine protease
Cathepsin L	Cysteine protease
Cathepsin D	Aspartic acid protease
Cathepsin E	Aspartic acid protease
Biogenic amines	
Histamine	Present in all subtypes of mast cells in all species
Serotonin	High levels in rodent mast cells
	Low levels in human mast cells
Dopamine	Low levels
	Not found in human mast cells
Polyamines (for example, spermidine and spermine)	Ubiquitous components of mammalian cells
Cytokines and growth factors	
TNF	First cytokine shown to be stored in mast cell granules
IL-4	Released by IgE receptor crosslinking
bFGF	Seems to be associated with heparin
VEGF	Released by IgE receptor crosslinking
TGFβ	Released during degranulation
Nerve growth factor	Released by IgE receptor crosslinking
IL-5	Present in the cytoplasm
	Presence within granules unclear
IL-6	Present in the cytoplasm
	Presence within granules unclear
IL-15	Associated with granules but not released upon mast cell activation
Stem cell factor	Present in the cytoplasm
	Not released upon IgE receptor crosslinking

Proteoglycans, Proteases and Others	
Serglycin	High expression in mast cells
	Also expressed by other haematopoietic cells and by endothelial cells
Tryptases	Serine proteases
	Highly expressed by mast cells
	Low expression in basophils
Chymases	Serine proteases
	Highly expressed by mast cells
CPA3	Metalloproteinase
	Highly expressed by mast cells
	Low expression in basophils
Cathepsin G	Serine protease
	Major product of neutrophils
MMP9	Metalloproteinase
	Activated by chymase
Active caspase 3	Cysteine protease
	Present in granules of viable mast cells
ADAMTS5	Metalloproteinase
Granzyme B	Serine protease
	Major product of cytotoxic lymphocytes
Renin	Aspartic acid protease
VAMP2, VAMP3, VAMP7 and VAMP8	v-SNAREs
Syntaxin 3	t-SNARE
Synaptotagmin II and synaptotagmin III	Accessory protein in SNARE-mediated granule fusion events
MUNC18-2	Accessory protein in SNARE-mediated granule fusion events
MUNC13-4	Accessory protein in SNARE-mediated granule fusion events
SCAMP1 and SCAMP2	Accessory proteins in SNARE-mediated granule fusion events
CD63	Tetraspanin
RAB3D, RAB5, RAB7, RAB9A, RAB19, RAB27A, RAB27B, RAB42 and RAB43	Belong to a family of GTPases that regulates membrane trafficking
LC3-II	Autophagy-related protein
MHC class II	Presents antigens mainly derived from extracellular sources
Heparanase	Endoglycosidic enzyme that degrades heparin and heparan sulphate
LL37	Antimicrobial peptide
Eosinophil granule major basic protein	Appears in granules after uptake from environment
Secretogranin III and chromogranin A	Members of the granin protein family
	Present in granules of neuroendocrine cells

Table 1.3 – Summary of known mast cell *de novo* synthesized mediators[1]

Factor	Mast cell type(s)	References
IL-1β	Mouse in vivo, BMMCs[2], cell lines, Human CBMCs[3]	41, 44,80–85
IL-2	Mouse BMMCs, lung MCs	86–88
IL-3	Mouse BMMCs, Human gastroduodenal	89,90
IL-4	Mouse BMMCs, peritoneal MCs, cell lines, Human nasal, bronchial, skin	85,91–98
IL-5	Human PBMCs, CBMCs, airway, gastroduodenal, bone marrow-derived, Mouse BMMCs	46, 50, 85, 90, 94,95,99,100
IL-6	Mouse in vivo, BMMCs, cell lines, Rat PMCs[4], Human bronchial, nasal, PBMCs[5], CBMCs	50, 80, 92, 94,101–105
IL-9	Mouse BMMCs	106–108
IL-10	Mouse BMMCs, Human PBMCs, CBMCs	100 50
IL-12	Mouse BMMCs, Human PBMCs	45
IL-13	Human PBMCs, CBMCs, Mouse in vivo, BMMCs	46, 50, 114, 85, 87, 100,109–113
IL-16	Human BM-derived and lung	115
IL-33	Mouse BMMCs	116
Amphiregulin	Human skin, CBMCs	117,118
IFNα2	Human CBMCs	119
IFNγ	Mouse in vivo, Rat PMCs	103,104,120
IFNλ1	Human CBMCs	119
EGF	Human thyroid	121
Endothelin-1	Human skin	117
bFGF/FGF-2	Human dermal, cutaneous, lung, skin, thyroid, PBMCs, Rat PMCs, Mouse BMMCs	121–128
GM-CSF	Human skin, gastroduodenal, BM-derived, PBMCs, CBMCs, Mouse BMMCs	44, 50, 89,90, 99, 117,129,130
MMP-1	Rat liver	131
MMP-3	Rat liver	131
MMP-8	Human skin	117

[1] Some mediators have traditionally been considered as either primary or secondary mast cell mediators, but an increased understanding of mast cell biology has limited the usefulness of that classification in the context of chronic disease.
[2] BMMCs – Bone Marrow-derived Mast Cells
[3] CBMCs – Cord Blood-derived Mast Cells
[4] PMCs – Peritoneal-derived Mast cells
[5] PBMCs – Peripheral Blood-derived Mast Cells

MMP-9	Human CBMCs, HMC-1 cell line, Mouse BMMCs	[132–134]
PAI-1	Human skin	[117]
PDGF	Human thyroid, Mouse BMMCs	[121,135]
Factor	**Mast cell type(s)**	**References**
Pentraxin 3	Human skin	[117]
Factor	**Mast cell type(s)**	**References**
SCF	Human lung, skin, cardiac, mastocytosis in bone marrow, CBMCs, PBMCs	[136–140]
TGF-β1	Mouse lung, BMMCs, cell lines, mouse PMCs, Dog mastocytoma, Rat PMCs, Human CBMCs	[87,88,140–143]
TIMP-1	Human skin, HMC-1 cell line	[117,144]
TNF	Mouse BMMCs, peritoneal MCs, cell lines, Rat peritoneal MCs, Human skin, CBMCs	[44, 46, 150–154, 85, 97, 141,145–149]
VEGF	Human thyroid, skin, layryngeal squamous cell carcinoma, malignant melanoma, CBMCs, PBMCs, Mouse in vivo, BMMCs, peritoneal MCs, Rat small intestine, peritoneal MCs	[39,40, 117, 121, 125,126,155–161]
CCL1	Mouse liver-derived, Human skin, PBMCs, CBMCs	[50,162,163]
CCL2	Mouse BMMCs, Human skin, PBMCs, CBMCs	[49,50, 110, 112,113, 117,164]
CCL3	Mouse liver derived, BMMCs, Human CBMCs	[110, 113,163,164]
CCL4	Mouse skin, Human CBMCs	[165,166]
CCL5	Human CBMCs	[49]
CCL11	Human skin	[167]
CCL17	Human PBMCs, CBMCs	[50]
CCL20	Human CBMCs	[130]
CCL22	Human PBMCs, CBMCs	[50]
CXCL2	Human skin, synovium, PBMCs	[45, 151,168,169]
CXCL8	Human skin, PBMCs, CBMCs	[50, 117,166]
CXCL10	Human CBMCs	[166,170]
CXCL16	Human skin	[117]
LTB_4	Murine CTMCs, MMCs	[30,171]
LTC_4	Murine CTMCs, MMCs	[30,171]
LTD_4	Murine CTMCs	[30,171]
LTE_4	Murine CTMCs, MMCs	[30,171]

PGD_2	Murine CTMCs, MMCs	[30,171]
PAF	Murine BMMCs, Rat CTMCs	[172]
S1P	Human PBMCs, Murine BMMCs	[173–175]
TXB_2	Murine MMCs	[30,171]

1.2 – IL-33: a dual function cytokine

IL-33 is regarded as a dual-function cytokine, in that it is a DAMP co-localized with the nucleus of structural cells as well as an IL-1 family cytokine that signals through its cognate receptor IL-1RL1 (ST2) [176,177]. In humans, IL-33 is abundant in endothelial cells throughout the vascular tree, epithelial cells in tissues in contact with the external environment, and fibroblastic reticular cells (FRCs) of lymphoid organs [176] at rest. Several subsets of activated fibroblasts in a myriad of tissues can increase expression of *IL33* [178]. In mice, IL-33 can be found in the brain, eye, barrier tissue epithelium, FRCs of lymphoid organs, and stromal cells of cavity linings, but is notably absent from endothelial cells at rest [179–181]. The endothelium in several tissues can upregulate *Il33* expression under inflammatory conditions, such as in the heart and lungs, but lacks basal expression [179]. While some evidence would suggest IL-33 can be secreted by certain leukocyte populations [178], there is skepticism as to the validity of these claims. This is primarily because IL-33 does not contain a signal sequence for trafficking through the endoplasmic reticulum-Golgi network, and therefore it is unknown if IL-33 can be secreted as a traditional cytokine [182]. Evidence for release of IL-33 from live endothelial cells and fibroblasts does suggest alternative mechanisms [183,184] such as inclusion in extracellular vesicles, but these remain poorly defined [183,185].

IL-33 is a unique DAMP in that it signals through a cytokine receptor [177], allowing it to influence local leukocyte populations upon its release in a different manner than traditional DAMP signaling via PRRs. In this way IL-33 functions as a cytokine with emerging roles across a broad spectrum of disease, including cancer [186], autoimmune disease [187], allergy [188–190], inflammatory disease [191], fibrotic disorders [192], and cardiovascular disease (CVD) [193,194]. Therefore, understanding how its release is regulated, and its subsequent effects on target leukocytes could help to identify novel therapeutic targets.

1.2.1 – Regulation of IL-33 activity

IL-33 was initially thought of as a transcription factor capable of dampening nuclear factor-κB (NF-κB) signaling [195]. However, IL-33 was found to have no impact on transcription on further assessment, and as such nuclear localization of IL-33 is likely a mechanism by which to restrict release to conditions of cell death or damage [196]. Further

mechanisms exist to regulate IL-33 activity in the extracellular space (Fig. 1.2). The IL-33 receptor protein exists in both membrane-bound (ST2L) and soluble (sST2) forms as a result of alternative splicing [197]. sST2 acts as a decoy receptor to sequester IL-33 in the extracellular environment and prevent signal transduction [178]. IL-33 is thought to have a short half-life in the extracellular environment. Once released from the nucleus, IL-33 is highly susceptible to oxidation of its cysteine residues [198] (Fig. 1.2) that results in an inactive disulfide-bonded form of the cytokine. Additionally, IL-33 is cleaved and inactivated by apoptotic caspases 3 and 7 to restrict release of bioactive IL-33 to the extracellular environment only upon inflammatory cell death [199,200]. Mechanisms to restrict release are important, as full length IL-33 is bioactive, unlike other IL-1 family cytokines [182]. IL-33 can have its activity significantly increased through proteolytic processing by neutrophil cathepsin G and mast cell chymase (Fig. 1.2). The processed form of IL-33 has been shown to have increased potency on type 2 innate lymphoid cells (ILC2s) [201–203]. The impacts on IL-33 activity by proteolytic processing coupled with its susceptibility to oxidation in the extracellular space likely limits the presence of IL-33 outside of the cell to a matter of hours [178]. Therefore, the actions of IL-33 are thought to be particularly potent on ST2L-bearing cells within damaged tissues.

1.2.2 – ST2L signaling for IL-33 cytokine function

ST2L signals via the IL-1 receptor accessory protein (IL-1RAcP) upon IL-33 binding. After the assembly of the IL-33/ST2L/IL-1RAcP complex, recruitment of myeloid differentiation primary response 88 (MyD88)/IL-1 receptor-associated kinase-1 (IRAK-1)/IRAK-4/tumour necrosis factor receptor(TNFR)-associated factor 6 (TRAF6) mediates NF-κB and mitogen activated protein kinase (MAPK) activation, typical of IL-1 family cytokines [204] (Fig. 1.2). Downstream effector protein signaling induced by ST2L is quite complex, and can result in activation of numerous pathways and effector molecules depending on the cell type [205]. While notable, the complex cell-cell variability in ST2L signaling [205] is beyond the scope of this thesis.

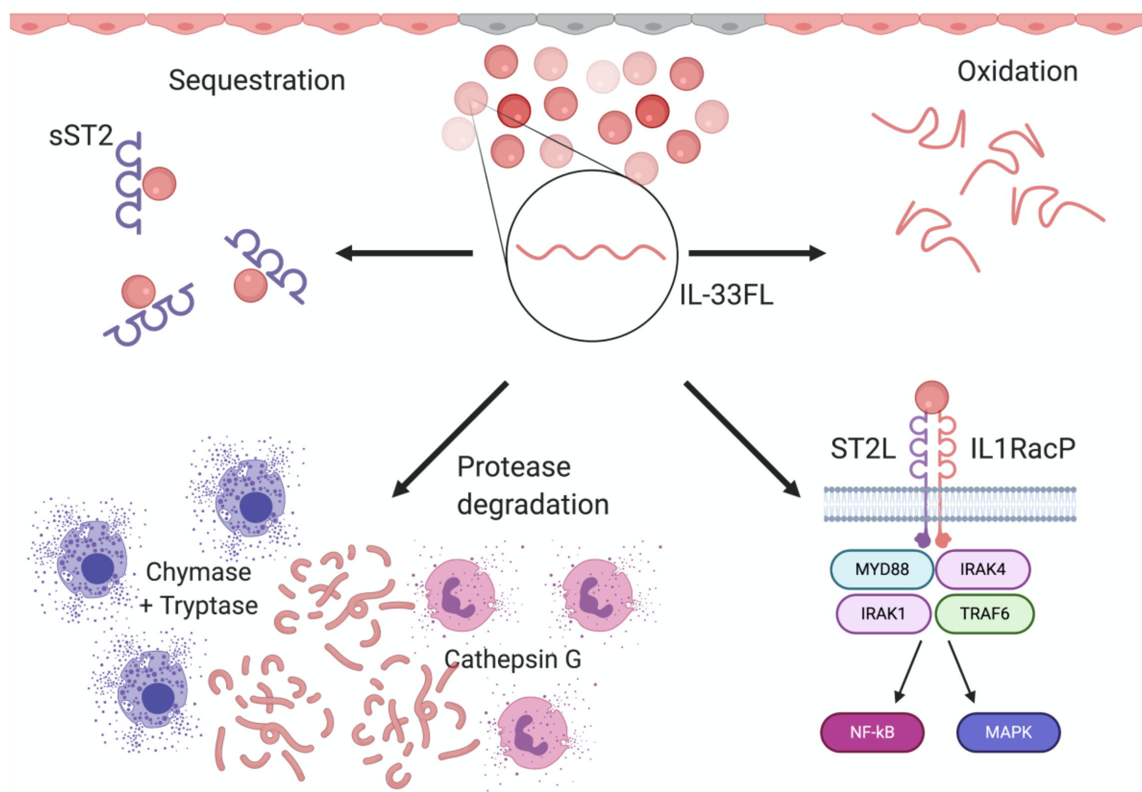


Figure 1.2 – Regulation of the bioactivity of IL-33. IL-33 is released as a full length (IL-33FL) protein from dead or damaged structural cells. It is quickly oxidized at cysteine residues once in the extracellular environment, as well as proteolytically processed by neutrophil Cathepsin G and mast cell chymase and tryptase. The membrane-bound receptor ST2L signals via IL1RacP to induce downstream activation of NF-κB and MAPK, while the soluble receptor sST2 acts to sequester IL-33 in the extracellular environment to prevent ST2L signaling. Figure generated with BioRender.

Unlike IL-33, ST2L is concentrated primarily on leukocytes, with expression also noted on some fibroblasts, endothelial, and epithelial cells [182,206]. Constitutive surface expression of ST2L is abundant in resident innate immune cells including ILC2s [207], mast cells [50], T_{regs} [208], and T_{H2} cells [209], while expression can be induced in basophils [210], eosinophils [211,212], macrophages [213,214], natural killer (NK) cells, NKT cells [215,216], $CD8^+$ cytotoxic T cells [217], dendritic cells [218], and neutrophils [219,220]. IL-33 activation of ST2L culminates in induction of type 2 immune responses, characterized by production of IL-5, IL-9, IL-13 and granulocyte monocyte-colony stimulating factor (GM-CSF) from numerous sources [178,206].

1.2.3 – sST2 as decoy receptor and disease biomarker

The splice variant sST2 was first identified as a soluble mediator produced by murine fibroblast cell lines in response to serum prior to the identification of ST2L [197]. The major sources of sST2 have not been fully elucidated, but human mast cells produce sST2 upon *in vitro* activation with IL-33 [221], while T_{H2} cells [222] produce sST2 in response to activation by anti-CD3 and anti-CD28 monoclonal antibody stimulation. Analysis of pooled human tissue mRNA has shown robust expression of sST2 mRNA in the lung, kidney, and heart, with lung epithelial cells and cardiac myocytes showing the highest individual cell population expression [223]. However, given that sST2 and ST2L differ by a truncation of 3 exons, the specificity of chosen primers in this study does not preclude ST2L detection.

In addition to its role as a decoy receptor to regulate IL-33 signaling, sST2 is emerging as a potent biomarker in several disease settings. In asthma, elevated serum sST2 levels predict severe exacerbation [224], while in the serum of pregnant women sST2 has shown prognostic efficacy in predicting pre-eclampsia [225]. However the exact mechanisms behind the detrimental impacts of sST2 in this context are not well understood. The most prominent and promising use of sST2 as a biomarker has been in CVD, in which sST2 serves as an accurate prognostic marker for fibrotic remodelling and progression to heart failure in patients with pre-existing CVD [226–228].

1.3 – Cardiovascular disease and cardiac fibrosis

CVDs are the leading cause of mortality worldwide, accounting for 31% of all annual deaths. CVDs are a collection of diseases that impact the physiology of the heart, such as myocardial infarction (MI), hypertensive heart disease, atherosclerosis, valvular heart diseases, myocarditis, and viral/bacterial cardiomyopathies [229]. A common characteristic across CVD regardless of etiology is myocardial damage. This can be hypoxic damage, such as in MI and ischemia reperfusion injury (IRI) due to atherosclerosis, stretch injuries in hypertension, or infection and cell death of cardiomyocytes. Hypoxic damage in the heart results in local inflammation due to DAMP release from necrotic cardiomyocytes and cardiac fibroblasts. Scar tissue deposition is crucial to restoration of tissue function, but excessive scarring leads to cardiac fibrosis, a key contributor to development of chronic heart failure (CHF) [230,231]. Therefore,

understanding the mechanisms that drive cardiac fibrosis is crucial to limiting CHF and improving outcomes for patients living with CVD.

1.3.1 – Kinetics of cardiac repair

Fibrosis is the deposition of excessive extracellular matrix (ECM) components in response to tissue damage that can lead to permanent scarring, organ malfunction and death [232]. The inflammatory response to damage is crucial in the initiation of wound healing and repair, but when it becomes chronic, inflammation is a key driver of fibrosis [233]. In most tissues, the goal of wound healing is to restore homeostasis with as little extracellular matrix deposition as possible to retain organ function. This is primarily mediated by proliferation of endothelial and epithelial cells to replace dead and damaged tissues over induction of ECM depositing pathways [234]. However, the cells of the cardiac parenchyma are primarily muscle, and their regenerative capacity is very limited [235,236]. As such, to restore function to tissue, some ECM deposition is required. When excessive scar tissue is deposited, it can interfere with excitation-contraction coupling in neighbouring cardiomyocytes, resulting in tissue stiffening and decreased contractility that ultimately leads to CHF [230,231]. It has become increasingly recognized that controlling inflammation may be the key to limiting adverse remodelling in the heart [237]. Proper cardiac wound healing progresses through three overlapping phases: inflammatory, reparative, and maturation (Fig. 1.3). These steps have been most clearly elucidated in the context of ischemic heart disease, which will be discussed below. That being said, there are still gaps in the knowledge landscape around repair in the heart. Both mast cells and IL-33 have been implicated in the pathogenesis of cardiac fibrosis, and how they are thought to contribute will be discussed in subsequent sections.

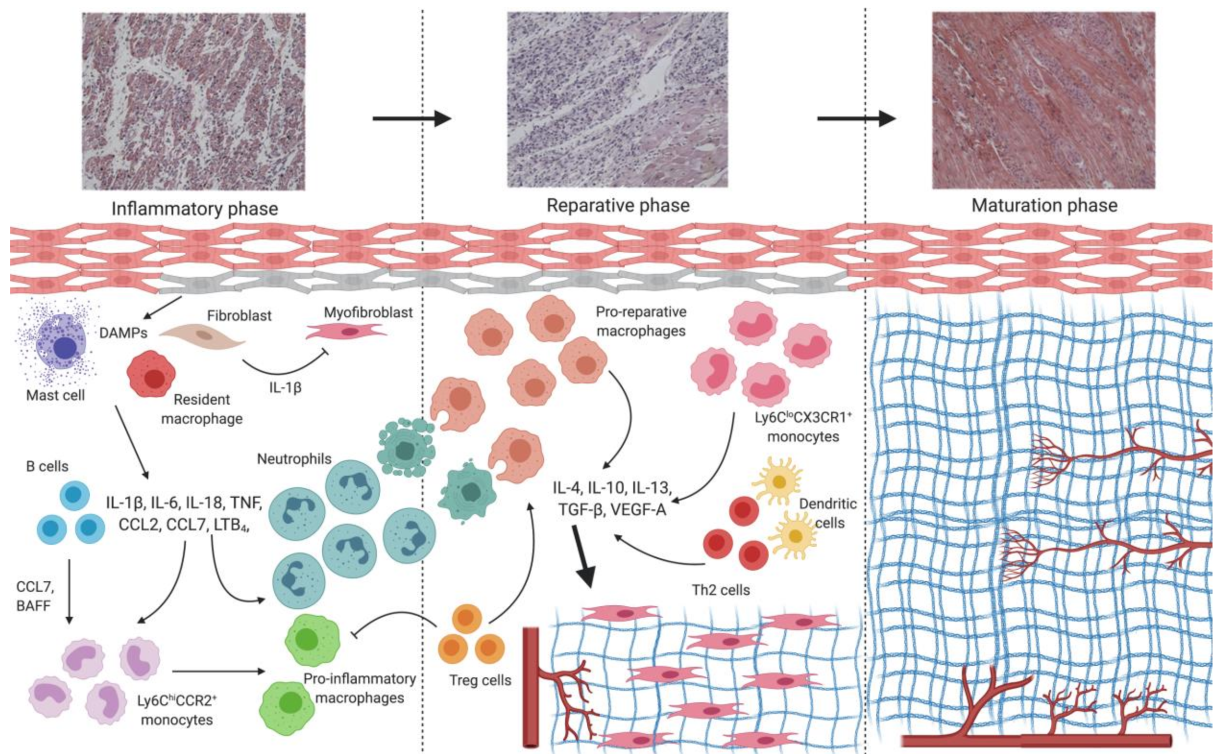


Figure 1.3 – The three phases of cardiac wound repair. In the inflammatory phase, initial DAMP release activates local resident leukocyte populations to clear debris, and initiate neutrophil and monocyte recruitment. The generation of pro-inflammatory mediators by leukocytes at the site promotes debris clearance to allow for transition to the reparative phase. During repair, fibroblasts are activated to the myofibroblast phenotype to deposit ECM components as a scaffold for tissue support and angiogenesis. Once sufficient ECM has been deposited and neovascularization has occurred, the wound transitions to the maturation phase. During maturation, the scar and blood vessels are matured to both withstand contractile forces of the heart and properly oxygenate remaining cells. Histology figures reproduced with permission from Dobaczewski et al., 2010, *JMCC*, 48 (3). Figure generated with BioRender.

1.3.1.1 – Inflammatory phase of cardiac wound healing

When a coronary vessel is occluded during MI, the local surrounding tissue becomes ischemic. This hypoxic environment increases vascular permeability in local blood vessels to facilitate leukocyte extravasation. Additionally, hypoxia causes localized cell death of cardiomyocytes, cardiac fibroblasts, and other resident cardiac cells. These necrotic cells release DAMPs, including HMGB1, ATP, and IL-33, into the local microenvironment to initiate the inflammatory phase [231]. The release of DAMPs activates TLRs, NLRs and receptor of advanced glycation end products (RAGE) on parenchymal cells, tissue resident leukocytes, and infiltrating leukocytes to induce NF-κB and MAPK

pathways [238]. Tissue resident mast cells degranulate in response to numerous stimuli in the infarcted microenvironment, such as hypoxia [239], and their situation in close proximity to blood vessels [240,241] allows them to assist in initiating the inflammatory cascade. The cumulative results of these activities is a dramatic increase in several pro-inflammatory cytokines, chemokines and other mediators including IL-1, IL-6, IL-18, TNF, leukotrienes, histamine, CCL2, and CCL7 [238,242]. The contribution and importance of individual immune or parenchymal subsets to this inflammatory response is unclear, and there may be redundancy to ensure inflammation is induced. It is important to note that the inflammatory response is a required event for wound repair (Fig. 1.3), as tissue debris and degraded ECM components must be cleared before effective repair can take place [243,244]. Previous efforts to oppose these signals with biologics led to human clinical trials with anti-TNF and IL-1R antagonists. These therapies were not effective in improving outcomes, and in some cases increased incidence of heart failure and death [245]

Neutrophils are the first cells recruited in response to CXC-chemokine gradients, lipid mediators, histamine, and complement, and emigrate into the infarct to contribute to clearance of debris [246,247]. The recruitment of neutrophils is rapid and is followed closely by monocytes. The first wave of monocyte recruitment to the cardiac microenvironment consists of $Ly6C^{hi}CCR2^{+}$ cells mobilized from the bone marrow [248,249] and splenic reservoirs upon induction of systemic inflammation mediated by IL-1 [250]. Locally, IL-1 signaling inhibits cardiac fibroblast activation to delay myofibroblast differentiation until the infarct is clear of debris [251]. The resident tissue macrophage population in the heart is relatively small at steady state [252] and how they contribute to the inflammatory phase of wound repair is not fully understood. It has been suggested that $Ly6C^{hi}CCR2^{+}$ monocytes differentiate into macrophages upon entry into tissues and promote pro-inflammatory actions at this stage [253], while resident macrophages may have more important roles in repair [252]. Regardless, monocyte and macrophage populations largely promote debris clearance in the infarct zone, while their populations rapidly expand towards the end of the inflammatory phase [238]. Lymphocyte recruitment to the heart also occurs following MI and serves important purposes in promoting initial inflammation. For example, recruited B cells are important in recruiting $Ly6C^{hi}CCR2^{+}$ monocytes to cardiac tissue via chemokine production [254].

How the inflammatory phase progresses sets the overall tone for cardiac wound repair; too robust or prolonged of a response, or ineffective resolution of inflammation results in sustained tissue damage that will impair transition to the reparative phase [238]. The initiation of the reparative phase begins during the inflammatory phase (Fig. 1.3) with neutrophil apoptosis and macrophage efferocytosis. Neutrophils are short lived cells that begin to undergo apoptosis within 24-48 hours of extravasation into tissues [255]. Efficient removal of apoptotic neutrophils is key to prevent them from becoming necrotic and releasing their highly caustic intracellular components that would prolong inflammation and perpetuate tissue damage. This includes proteolytic enzymes, cytokines, and reactive oxygen and nitrogen species (ROS and RNS) typically sequestered in granules [256]. Therefore, macrophages must phagocytose apoptotic neutrophils, stromal, and parenchymal cells, to clear them from the cardiac microenvironment. This process is known as efferocytosis, from Latin *effere* meaning carry to the grave [257], and is a crucial component in promoting proper cardiac wound healing. The relevance of efferocytosis was demonstrated effectively in mouse models lacking the efferocytosis receptor MerTK. Cardiac fibrosis was significantly increased, in association with impaired cardiac function after experimental MI and in hypertension models [258–260]. Once macrophages phagocytose apoptotic neutrophils, they are further activated to pro-reparative phenotypes by neutrophil gelatinase-associated lipocalin to induce IL-10 and TGF-β production [247]. Additionally, apoptotic neutrophils release specialized pro-resolving mediators, annexin A2 and lactoferrin, to negatively regulate neutrophil transmigration and promote their own apoptosis [261]. Finally, Tregs recruited in the inflammatory phase via CCR5 signaling contribute to dampening of inflammatory signals [262]. This allows the cardiac microenvironment to transition to the reparative phase (Fig. 1.3).

1.3.1.2 – Reparative phase of cardiac wound healing

The reparative phase is characterized by extracellular matrix deposition and angiogenesis. These programs are mediated by several immune cells in the cardiac microenvironment. $Ly6C^{hi}CCR2^{+}$ monocytes switch to $Ly6C^{lo}CX3CR1^{+}$ monocytes via nuclear receptor subfamily 4 group A member 1 (Nr4a1) activation to promote wound healing [263,264]. Resident tissue macrophages [252,265] and monocyte-derived

macrophages[249,266] act to promote wound repair programs characterized by IL-10 and TGF-β production. It is important to note that classical, dichotomous M1/M2 phenotyping is not useful in the myocardium, and macrophages have been identified within a wide spectrum of nuanced phenotypes at this site [252,267]. How exactly these macrophages contribute to the reparative phase is not well understood but involves the secretion of mediators to promote remodelling and angiogenesis.

Dendritic cells (DCs) infiltrate the myocardium in the reparative phase, and have been shown to reduce the accumulation of $Ly6C^{hi}$ monocytes and promote endothelial cell proliferation [268]. DCs in the myocardium can then present autoantigen to $CD4^+$ Th2 cells [269], which have been implicated in promoting pro-fibrotic signaling programs through as yet undefined mechanisms. $CD4^+$ $FoxP3^+$ Tregs have also been implicated in the reparative process through modulation of macrophage differentiation to "M2-like" phenotypes [270]. Mast cells have been shown to increase in density during cardiac fibrosis, but as will be discussed herein, how they contribute to remodelling in the heart is controversial and poorly understood. This is due in part to fundamental differences in murine cardiac mast cell density in comparison to other mammals [271].

The pro-reparative pathways activated here work in co-ordination to induce fibroblast differentiation into the myofibroblast phenotype (Fig. 1.3). This is another instance of a redundant system in which several cell types contribute to a common outcome. Myofibroblasts are the major contributors to deposition of ECM components in the heart, where they deposit collagens and elastin to make up the ECM [230]. Myofibroblasts originate primarily from activated resident tissue fibroblasts [272], and are characterized by increased expression of α-smooth muscle actin [273]. The ECM network functions as a scaffold for proliferating endothelial cells in the myocardium that rapidly form new blood vessels (Fig. 1.3). VEGF-A is a crucial signal for cardiac angiogenesis. VEGF-A promotes initial neovascularization within the remodelling and maturing tissues to supply oxygen to local cells [274–276]. Additionally, VEGF-A prevents excessive myofibroblast activation from endothelial sources by preventing endothelial-to-mesenchymal transition [277,278]. VEGF-A also activates further angiogenic pathways, such as urokinase plasminogen activator (uPA) [279]. The new vessels formed in the reparative phase are immature, enlarged, and extend out into the infarct zone. They lack smooth

muscle cell and pericyte mural coatings, and are hyper-permeable to allow continued leukocyte extravasation [238]. Once sufficient ECM deposition and angiogenesis occurs, the scar is then matured (Fig. 1.3).

1.3.1.3 – Maturation phase of cardiac wound healing

The maturation phase is characterized by further development of blood vessels and crosslinking of ECM to promote a strong scar that withstands the contractile forces in the heart (Fig. 1.3). Fibrotic growth factors are reduced as cellular content in the scar recedes, leading to quiescence and subsequent apoptosis of fibroblasts [280,281]. Immature blood vessels develop mural coatings and reduce permeability to provide a stable blood supply to tissues in a platelet-derived growth factor(PDGF)-β dependent manner [282,283]. Without proper vessel maturation, inflammation persists in tissues as leukocytes will continue to enter into the infarct via the leaky barrier of immature blood vessels [231]. Inflammation persistence, throughout wound repair, directly contributes to cardiac fibrosis, as constant damage restarts the entire process. Indeed, targeting of inflammation in animal models of cardiac fibrosis with the specialized pro-resolving mediator resolvin D1 (RvD1) improved outcomes and reduced uncontrolled remodelling [284].

1.3.2 – The controversial role of mast cells in cardiac fibrosis

How mast cells influence fibrosis throughout the body is controversial. A plethora of clinical and animal studies show contradictory roles for mast cells in fibrotic disease, reviewed here [74]. These discrepancies extend into cardiac fibrosis. Some incongruities could be accounted for by documented issues with blood pressure regulation in some mast cell deficient mouse strains [285,286], and differences in distribution of murine cardiac mast cell populations [271,287]. It is also likely that at different temporal and physical locations during cardiac fibrosis, mast cells exert differing effects.

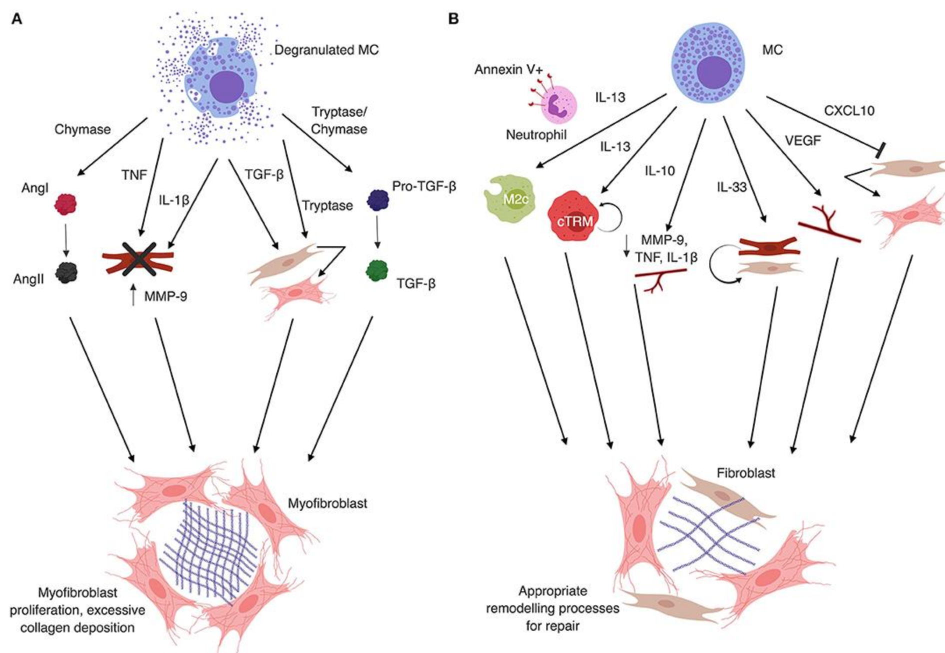


Figure 1.4 – Divergent mast cell roles in cardiac fibrosis based on degranulation or secretion activity. (A) Mast cell granule products are typically associated with fibrosis. Mast cell chymase converts Angiotensin I (AngI) to AngII independently of ACE. AngII generation directly contributes to fibrosis by inducing differentiation of fibroblasts to myofibroblasts. Mast cell degranulation-derived TNF and IL-1β induce cardiomyocyte apoptosis, MMP-9 production and inflammatory cell recruitment that enhances tissue remodeling. Mast cell tryptase can act directly on fibroblasts to induce proliferation and differentiation to the myofibroblast phenotype. Tryptase and chymase both act on latent TGF-β to convert it to the active form, which also induces fibroblast differentiation to the myofibroblast phenotype and collagen deposition. Additionally, mast cells release TGF-β upon degranulation, further contributing to the activation and differentiation of fibroblasts. (B) Mast cell secretion products can protect against fibrosis. Mast cells can produce IL-13, which in the presence of apoptotic neutrophils can induce M2c phenotypes of macrophages. M2c macrophages are associated with decreased fibrosis. IL-13 can also induce proliferation of local cTRM via IL-4Rα signalling, which are known to be anti-fibrotic. Mast cells can also produce IL-10, which acts in the heart to decrease IL-1β and TNF levels, reduce MMP-9 expression and activity, and increase capillary density to reduce fibrotic remodelling. IL-33, which is released by stressed cardiomyocytes and fibroblasts, but can also be produced by mast cells, has been shown to protect cardiomyocytes and fibroblasts from death under hypoxic conditions. This results in decreased inflammation and reduction in fibrosis. VEGF, which promotes angiogenesis and recapillarization of the cardiac tissue, is associated with reduced fibrosis and is another mast cell product. Finally, CXCL10 has been shown to inhibit fibroblast migration into the myocardium and delay differentiation to the pro-fibrotic myofibroblast phenotype. Reproduced with permission from Legere et al., 2019 *Frontiers in Immunology*, 10:580. Figure generated with BioRender.

1.3.2.1 – Mast cells as promoters of cardiac fibrosis

Mast cell degranulation products have important impacts on fibrosis (Fig. 1.4A), though degranulation stimuli within the cardiac microenvironment are not well defined. Mast cell chymase and tryptase generate the active pro-fibrotic form of TGF-β1 from latent forms present in the microenvironment and released by mast cells during degranulation [142,288–294]. TGF-β1 then promotes fibroblast activation, myofibroblast differentiation and collagen synthesis [230,238]. Mast cell tryptase can also directly activate fibroblasts independently of TGF-β1 [295–300]. *In vitro* mast cell chymase induces TGF-β1 production by rat cardiac fibroblasts [301]. Angiotensin II (AngII) is a major mediator of fibrosis that activates fibroblasts to the myofibroblast phenotype for proliferation and collagen deposition [230,238]. Mast cell chymase is an Angiotensin Converting Enzyme (ACE)-independent generator of AngII in humans, dogs and mice [74, 290,302–304]. Studies employing ACE inhibition, and reduction in AngII, show decreased cardiac fibrosis [305–308], suggesting further pro-fibrotic actions for chymase.

In addition to tryptase and chymase, mast cells store fibroblast growth factor-2 (FGF-2) in their granules [7, 74, 289,309], which, as its name suggests, is pro-fibrotic in the heart. Mast cells serve as sources of TNF, which is released during degranulation [20] and promotes cardiac fibrosis via induction of cardiomyocyte apoptosis, inflammation and MMP-9 production [103,310–312]. Finally, mast cells produce IL-1β following degranulation [5], which promotes fibrotic remodelling of the heart in a similar manner to TNF [312–316]. Although mechanisms of action are not well elucidated, Wang et al. found that blocking TNF and IL-1β with neutralizing antibodies reduced cardiac remodelling and cardiomyocyte apoptosis during AngII-induced fibrosis [312].

Numerous studies have attempted to understand mast cell roles in cardiac fibrosis *in vivo* (Table 1.4). Studies in rats, dogs and mice have shown that inhibition of mast cell degranulation or chymase activity reduces expression of fibrosis-associated genes and collagen deposition in models of dilated cardiomyopathy (DCM), ovariectomy-induced left ventricular diastolic dysfunction and MI [317–321]. These studies are limited in their assessment of mast cell function exclusively through degranulation capacity, and not mast cell involvement in fibrosis through *de novo* mediator production. In a spontaneously hypertensive rat (SHR) model, degranulation inhibition increased

myocardial IL-10 and IL-6 content in co-ordination with increased mast cell numbers, leading to improved outcomes and reduced fibrosis compared to untreated SHR [322]. Mast cells are well established sources of both IL-6 and IL-10 [5,323], therefore these results could suggest a potential role for mast cells independent of degranulation.

Studies assessing mast cells in cardiac fibrosis often analyze mast cell density changes that occur during remodeling, concluding a pro-fibrotic role. In canine MI and murine pulmonary artery bypass models, increases in mast cell density occurred alongside increases in inflammatory cell infiltration [324], fibrosis and cardiomyocyte hypertrophy [325], but no mechanistic relationships were found. These studies often only identify granulated mast cell populations. Common immunohistochemical (IHC) techniques for mast cell identification target granule-associated contents, ignoring populations of mast cells that are not granulated, either due to immaturity or recent granule release. Additionally, mast cell degranulation releases SCF, a potent growth and chemotactic factor for mast cells [20,326], resulting in local proliferation [327] and recruitment [328]. Therefore, increases in mast cell density may be due to activation of mast cells from degranulation and not tissue damage.

In a transverse aortic constriction model of hypertension induced fibrosis (TAC), reconstitution of irradiated WT mice with bone marrow from WBB6F1/J-Kit^{W}/Kit^{W-v}/J (W/W^{v}) mast cell-deficient mice led to decreased collagen content compared to WT bone marrow recipients [135] . Mast cells are unusually radioresistant [329], and efficiency of mast cell removal after irradiation was not assessed in this study. In a rat cardiac allograft model, fibrosis was positively correlated with certain subsets of "mucosal" mast cells (MMC) but not "connective tissue"(CTMC) as defined by expression of mouse MCP-1 and MCP-2, respectively [330]. This may reflect changes in the maturity of mast cell populations at this site and the presence of newly recruited cells. Mast cell activation in atherosclerosis was associated with plaque progression and destabilization [69], which implicate mast cells in promoting MI but does not directly link them to later fibrotic changes. Overall, mast cells have the potential to promote cardiac fibrosis and increased numbers of granulated mast cells are often associated with fibrosis in animal models, but mechanistic data is lacking.

1.3.2.2 – Mast cells as inhibitors of cardiac fibrosis

Mast cells can synthesize and secrete a wide array of proteins and lipids without degranulating, allowing them to modify the cardiac microenvironment after ischemic damage or reperfusion injury in the heart (Fig. 1.4B). Mast cells produce a wide array of pro-inflammatory cytokines and chemokines with proven roles in the recruitment of immune cells [5,20]. Conversely, mast cells produce mediators such as IL-10 [323], IL-13 and CXCL10 that can serve different functions. IL-10 is known to prevent excessive cardiac remodelling via signal transducer and activator of transcription 3 (STAT3) activation and NF-κB suppression [331–333]. CXCL10 acts in the damaged myocardium independently of CXCR3 to delay fibroblast migration and differentiation [334–337]. While not classically considered part of the anti-fibrotic response, mast cells can produce VEGF-A [5,20], among other important angiogenic mediators, which can increase capillary density in damaged tissues and promote proper repair in cardiac and hepatic fibrosis [338–340].

IL-13 is produced by mast cells in response to several stimuli [5], including IL-33 [50]. Mast cells express the IL-33 receptor ST2L abundantly on their cell surface [64, 221,341,342]. IL-33 is released by cardiomyocytes and fibroblasts after damage. IL-33 is known to promote cardiomyocyte survival and reduces fibrosis after MI [343–345]. Some of these actions may be via IL-13 induction. IL-13 acts on cardiac tissue resident macrophage (cTRM) populations, which are seeded embryonically in the heart and display M2-associated and anti-fibrotic phenotypes [265,346–348]. cTRM self-renew and expand their populations in response to sterile inflammation and IL-4Rα signaling [349]. Cardiac mast cell IL-13 production could expand the cTRM population locally. IL-13 also reduces expression of pro-inflammatory cytokines by infiltrating cells and may impact efferocytosis, the clearance of apoptotic cells from injured or inflamed tissues [350].

Anti-fibrotic roles of mast cells have also been analyzed *in vivo* (Table 1.4). Direct mast cell transplantation into the murine myocardium post-coronary artery ligation increased cardiac function, and capillary density and decreased scar size [351]. It is important to note that traditional mast cell-deficient models (rat and mouse) involve mutations in the gene for c-kit [352], which encodes the SCF receptor, a growth factor critical for mast cells. These genetic defects also reduce hematopoietic stem cells, germ

cells and melanocytes, among other effects [352]. Reconstitution of mast cell deficient mice with wild type mast cells would confirm if observations are truly mast cell dependent, though it is not practical in all models. Several studies have focused on mast cell granule (MCG) contents in fibrosis. Administration of MCGs isolated from rat peritoneal mast cells to the myocardium during acute MI decreased fibrosis and increased capillary density. *In vitro* MCG treatment of cardiomyocytes promoted survival under hypoxic conditions [353]. MCG treatment of mesenchymal stem cells (MSC) *in vitro* prevented TGF-β1 mediated transition of MSCs to myofibroblasts in an alternative fibrotic pathway [354], even though individual mast cell granule products chymase and tryptase are pro-fibrotic. While there is limited evidence showing that mast cells are protective during cardiac fibrosis, these studies indicate that mast cell can have an anti-fibrotic role and could potentially be targeted therapeutically.

1.3.2.3 – Mast cells as bystanders in cardiac fibrosis

Several studies suggest mast cells do not influence cardiac fibrosis (Table 1.4). In a norepinephrine model, rats treated with degranulation inhibitor disodium cromoglycate had comparable collagen and *Col1* mRNA content compared to untreated mice, and mast cells were thought to be irrelevant [355]. However, degranulation inhibition would have little impact on *de novo* mast cell production of many fibrosis regulating mediators. The transverse aortic constriction model of hypertension resulted in hypertrophy and impaired cardiac function but equivalent fibrosis in Wsh mast cell-deficient mice, another kit-dependent deficiency model, compared to WT mice [286]. Ngkelo et al. compared a newly developed mast cell-deficient mouse strain to a classical c-kit mutation-dependent model. In previous studies with older mast cell-deficient strain W/W^v mice and WT mice treated with disodium cromoglycate and subjected to experimental MI had increased fibrosis and infarct size. Upon utilization of mast cell-deficient $Cpa3^{Cre/+}$ mouse model, a more mast cell-specific deficiency, no difference in fibrosis was observed. Rather, mast cells were shown to be important in myofilament Ca^{2+} sensitization and cardiac contractility [356]. It remains problematic that animal models for cardiac fibrosis are limited in their ability to mimic chronic fibrotic changes seen clinically. Several potential factors in experimental design may also contribute to discrepancies between animal models that will be discussed herein.

Table 1.4. – The role of mast cells in animal models of cardiac fibrosis

Study	Findings	Confounder?
Pro-fibrotic		
Zweifel et al., 2010	Rat cardiac allograft model, fibrosis correlated to mucosal MC density	Formaldehyde fixed tissue
Palaniyandi et al., 2008	Rat dilated cardiomyopathy; degranulation inhibitor reduced fibrosis &MC density	Formaldehyde fixed tissue, fibrosis associated with granulated MC density
Kanemitsu et al., 2008	Rat MI &left ventricular repair, chymase inhibition reduced fibrosis-associated gene expression	None
Wang et al., 2016	OVX rats, degranulation inhibition reduced collagen content &MC density	Formaldehyde fixed; fibrosis associated with granulated MC density
Somasundaram et al., 2009	Canine MI, MC density elevated 7-28dpMI, associated with increased inflammatory infiltration	Fibrosis associated with granulated MC density
Matsumoto et al., 2003	Canine heart failure, chymase inhibition decreased type I &III collagen gene expression	None
Luitel et al., 2017	Murine pulmonary artery bypass, MC density, fibrosis, hypertrophy increased 21 days post overload	Formaldehyde fixed; fibrosis associated with granulated MC density
Liao et al., 2010	Murine transverse aortic constriction, DC reduced atrial fibrillation & associated fibrosis, reconstitution of WT mice with W/W^v bone marrow decreased collagen content	Use of kit-dependent MC deficient mice, formaldehyde fixed, fibrosis associated with granulated MC density, improper use of disodium cromoglycate
Wei et al., 2010	Rat MI, chymase inhibition reduced hypertrophy, fibrosis & infarct size	None
Levick et al., 2009	Spontaneously hypertensive rats, degranulation inhibition decreased collagen volume fraction &improved outcomes	Telly's fixative (contains formaldehyde & glacial acetic acid), degranulation inhibition increased MC density & improved outcome
Akgul et al., 2005	Human end stage cardiomyopathy, positive correlation between MC & collagen content pre-LVAD that did not persist post-LVAD	Formaldehyde fixed
Dilsizian et al., 2007	Human ischemic cardiomyopathy, MCs elevated in ischemic patients	Formaldehyde fixed; fibrosis associated with granulated MC density
Batlle et al., 2007	Human idiopathic dilated cardiomyopathy, positive correlation between MC density & collagen content	Formaldehyde fixed
Roldão et al., 2012	Human Chagas disease, MC chymase content positively correlated to collagen content	Autopsy samples, no indication of fixative used
Anti-fibrotic		
Joseph et al., 2005	Rat homocysteine-induced hypertrophy, Ws/Ws MC deficient rats have increased fibrosis & collagen content	Kit-dependent MC deficiency, formaldehyde fixed

Study	Findings	Confounder?
Shao et al., 2015	Murine ischemic injury, W/W^{v} MC deficient mice had impaired fractional shortening & increased scar size, MC transplantation into the myocardium increased cardiac function, capillary density &decreased scar size	Kit-dependent MC deficiency, no indication of fixative used
Kwon et al., 2011	Rat MI, administration of low doses of MC granule content increased capillary density &decreased fibrosis at infarct	No indication of fixative used
Nazari et al., 2016	Murine MI, MCs injected into hearts of mice promoted mesenchymal stem cell proliferation early after MI &reduced fibrosis	No indication of fixative used
Neutral		
Briest et al., 2003	Rat norepinephrine cardiac fibrosis, degranulation inhibition did not impact collagen content or gene expression	None
Buckley et al., 2011	Murine transverse aortic constriction, Wsh MC deficient mice had no difference in fibrosis compared to WT	Kit-dependent MC deficiency, formaldehyde fixed (but didn't assess MC density)
Ngkelo et al., 2016	Murine MI, $Cpa3^{cre+/-}$ mice had no difference in fibrosis compared to WT	No indication of fixative used
Frangogiannis et al., 2002	Human chronic ischemic LV dysfunction in LV samples from CABG patients, no relationship between MC density &fibrosis	Formaldehyde fixed
Milei et al., 1996	Human Chagas disease, no relationship between MC density &fibrosis	No indication of fixative used nor of disease stage; controls were autopsy samples

1.3.2.4 – Relevance of research in human cardiac tissue

Similar to animal models, data on mast cell involvement in human cardiac fibrosis is inconsistent (Table 1.4). Several human studies of cardiovascular disease have equated increases in mast cell density to a detrimental role in fibrotic remodeling without a clear functional relationship between the two variables [357–359]. Positive correlations were observed between mast cell density and collagen content in human idiopathic dilated cardiomyopathy [360], end stage cardiomyopathy [357], and Chagas disease [361]. It remains unclear whether this is a protective response, epiphenomenon or pathological process. Studies have also indicated that mast cells have no association with human cardiac fibrosis in data from patients with ischemic dysfunction [362] and Chagas disease [363]. Overall, data varies as to the role of mast cells in human cardiac fibrosis.

1.3.2.5 – Confounding factors in mast cell cardiac fibrosis research

The role of mast cells in cardiac fibrosis is controversial, although it is clear they have the potential to modify fibrotic responses and tissue repair. There are several potential reasons for observed discrepancies. First, mice are not an ideal model to study cardiac mast cells. Unlike rats and dogs, mice have low myocardial mast cell content. Dogs on average have 6.8 ± 1.6 cardiac mast cells/mm^2, while C57BL/6 mice have 0.6 ± 0.2 cardiac mast cells/mm^2 [287]. Data shows that mast cell density increases in murine hearts after damage [135, 317, 322, 326, 360,364,365], but it is unclear if statistically significant increases in mast cell content have physiological relevance, or that murine cardiac mast cell responses mirror those in other mammals.

Second, there is widespread improper use of mast cell stabilizing agents. Mast cell stabilization drugs prevent calcium-dependent mast cell degranulation, while mast cell secretion of mediators independently of degranulation is not impeded. Disodium cromoglycate is a stabilizing agent used to inhibit mast cell degranulation in mice and rats. However, while disodium cromoglycate can inhibit IgE-dependent mast cell degranulation in rats, it does not inhibit this response in mice at similar or higher doses [366], though may have efficacy on CTMCs [367]. This calls into question the validity of studies in which disodium cromoglycate has been used to treat mice.

Third, mouse models of mast cell deficiency involving mutations in c-kit result in a lack of hematopoietic stem cells, germ cells, and melanocytes, among others [352]. The

advent of several kit-independent models of mast cell deficiency have allowed researchers to determine if lack of mast cells impacts the pathogenesis of various diseases, or if differences are due to deficiencies in other areas. Preferable models include *Cpa3*$^{Cre/+}$ and *Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice, as the mast cell deficiencies are more specific in these animals and have less overlap with other genes, which is an issue in the traditional kit-based mast cell deficient mice [352]. Discrepancies are already starting to appear [356,368,369], suggesting that increases or decreases in density of numerous cell types in these models contributes more to disease than lacking mast cells. Reconstitution experiments help in this respect, but only if appropriate reconstitution is achieved and reported, which is rarely the case. This is a difficult task, as mast cells inefficiently reconstitute the heart [73]. Therefore, the use of multiple complementary models of mast cell deficiency would be helpful to validate conclusions.

Finally, tissue fixation for mast cell staining greatly impacts ability to visualize mast cells. Aldehyde based tissue fixation, such as routine formalin fixation, does not allow for proper visualization of mast cells but reduces detection of mast cells by 30-80% depending on the method and tissue being examined. Proper identification of mast cells via histochemical methods following paraffin embedding requires fixation with Carnoy's fixative or basic lead acetate to fully visualize mast cells in tissue [370]. Care needs to be taken in designing studies of mast cells in cardiac fibrosis, with consideration given to the variety of actions of these cells and the difficulty of their experimental manipulation.

These confounding factors in cardiac fibrosis research need to be kept in mind in the design of studies to assess mast cell roles in cardiac fibrosis. Future work should be aimed at assessing mast cells in human cardiac tissue wherever possible, due to discrepancies in mouse models. Efforts should be made to utilize atrial appendage samples from human cardiac surgery patients, as these tissues are often disposed of as medical waste but contain valuable information along with patient demographics and outcomes at follow up to study. Additionally, work should focus on the development of mouse models that better reflect human cardiac mast cell content to more accurately model the human cardiac microenvironment.

1.3.3 – IL-33 as a protective mediator in cardiac fibrosis

In contrast to the controversy surrounding mast cells, IL-33 has been consistently identified as a cardioprotective mediator. In the heart it is expressed in human cardiomyocytes, fibroblasts, endothelial cells, and coronary smooth muscle cells, with increased nuclear IL-33 detected under conditions of pro-inflammatory release [371]. Furthermore, biomechanical strain, such as myocardial pressure overload, can increase IL-33 mRNA and protein levels in the heart [372–375]. IL-33 promotes cardiomyocyte survival under hypoxic conditions by inhibiting Jun N-terminal Kinase (JNK) signaling to prevent apoptosis [344,375–378]. IL-33 has consistently been shown to reduce collagen deposition, hypertrophy [379], pro-inflammatory cytokine signaling [372, 374,380] and infarct size [343–345,378] while improving cardiac function and survival [376,381]. Furthermore, IL-33 increases pro-reparative cytokine production, including IL-10, IL-13, and TGF-β from cardiac resident leukocytes [372,382]. IL-33 is cardioprotective in chronic allograft rejection [382–384], atherosclerosis [385], and diabetes [345]. The cellular mechanisms responsible for these cardioprotective effects beyond cardiomyocyte survival have not been clearly elucidated. However, the immune system is an ideal target for IL-33 in the heart, due to the abundance of ST2L on leukocyte cell surfaces [178] and the extensive recruitment and activation of leukocytes in response to damage [231,238].

Therapeutic strategies to target the beneficial effects of IL-33 have been proposed. Due to its pleiotropic actions outside of the heart [182], systemic administration of IL-33 would likely have detrimental effects, and as it is short lived in the extracellular space [198,201–203] this would limit its efficacy. Furthermore, evidence from a mouse model of viral myocarditis showed that IL-33 can induce eosinophilic pericarditis when given systemically [386], as IL-33 is a known activator of eosinophils [211]. Therefore, cardiac targeted approaches are likely better suited. Statins have been shown to increase IL-33 expression in human cardiomyocytes and fibroblasts *in vitro* [387], but this has not been observed in human tissues. Mesenchymal stem cells transfected with IL-33 in animal models increased pro-reparative macrophage phenotypes while reducing IL-1β, IL-6 and TNF signaling in rats in a Janus kinase(JAK)-dependent manner [380]. However, more work is required to understand the mechanisms behind the cardioprotective effects of IL-33 before therapeutics can be designed.

1.3.4 – sST2 as a prognostic biomarker for cardiac fibrosis

Perhaps the best evidence of the cardioprotective actions of IL-33 is the efficacy of sST2 as a prognostic marker for fibrotic remodelling and progression to heart failure in CVD patients. The use of sST2 as a biomarker for progression to CHF and early mortality was first raised in the early 2000s [373,388,389] and has since gained utility. It was observed in a rat model of MI that a significant increase in serum sST2 was induced one day post infarct [388]. In initial human studies, serum sST2 was significantly higher in patients with severe heart failure, and elevated levels at baseline were predictive of mortality or transplant endpoint within the study [373]. This work was replicated concurrently by a separate group observing acute MI patients, where baseline sST2 levels were significantly higher in patients who developed CHF or died within 30 days of MI [389]. Subsequently, interest has increased in the utility of sST2 as a biomarker for CHF and mortality that have become the focus of ongoing clinical trials [390]. It is important to note that sST2 is not a viable diagnostic biomarker, as levels can be elevated in healthy individuals. Interestingly, sST2 is elevated in healthy males compared to females [391–393], but upon diagnosis of CVD, increases in sST2 are observed at similar magnitudes between sexes [227,394,395].

sST2 has emerged as an accurate prognostic marker for predicting development of CHF in CVD patients with various etiologies, including MI [396–398], congenital heart disease [227], acute heart failure[399], and myocarditis [395]. Its utility is increased in combination with other biomarkers, such as N terminal-pro Brain Natriuretic Peptide (NT-proBNP), high sensitivity cardiac Troponin T (hs-TnT) [228], and galectin-3 [400]. In patients diagnosed with CHF, elevated sST2 levels were accurate in predicting one year and five year all cause and cardiovascular mortality, as well as heart failure hospitalization [228, 394,401].

sST2 was identified as a fibrosis-associated biomarker upon the identification of its ligand IL-33's cardioprotective actions [376, 379,381]. *In vivo* sST2 administration directly opposed the benefits of IL-33 in preventing cardiomyocyte apoptosis [376], and was associated with increased mRNA for type I collagen, type III collagen, IL-6, TNF, and TGF-β [374] in acute mouse models of MI. These responses were also associated with increased cardiac fibrosis. Additionally, sST2 opposed the anti-hypertrophic and anti-

fibrotic benefits of IL-33 in a pressure overload model [381]. Further work in human subjects has cemented this role. Heart failure patients with baseline sST2 levels less than the study median had reduced left atrial volume, indicative of a reduction in remodelling [402]. In acute MI patients there was a positive association with sST2 level at diagnosis and benefit from anti-fibrotic therapies, where patients with elevated sST2 levels benefited from anti-fibrotic therapies over those with less than the median sST2 [403]. These patients also had a reduction in sST2 levels once administered anti-fibrotic drugs, with independent association with reduced risk of CHF and cardiovascular death [404,405]. However, further work is needed to directly relate sST2 in circulation of CVD patients with ECM deposition in human heart tissue.

1.3.5 – Cardiac fibrosis as a product of chronic inflammation

As detailed previously, inflammation is crucial to proper wound repair and the inflammatory response itself initiates this process. Overlapping steps of inflammation, repair and maturation allow cardiac tissue to regain function after damage, and proper initiation and termination of each phase results in appropriate repair [238,406]. As the first stage of remodelling, the inflammatory phase needs to be resolved to allow for the reparative phase to occur. If inflammation persists in the myocardium and becomes chronic, this induces a positive feedback loop in the myocardium characterized by a persistent state of ECM deposition that results in fibrosis [230,231,238]. Therefore, understanding chronic inflammation and the mechanisms behind its resolution are crucial to effectively targeting cardiac fibrosis. Initiation of an active program of resolution rather than opposition of inflammation is key to turn off chronic processes [407].

1.4 – Chronic inflammation drives pathology

Acute inflammation is crucial to elimination of pathogens and debris at sites of damage, but once chronic, inflammation drives pathology in a number of disease contexts including autoimmune disease [408], cancer [409], and fibrosis [232]. Previous work has focused on anti-inflammatory therapies to treat chronic inflammation, such as non-steroidal anti-inflammatory drugs (NSAIDs), anti-cytokine therapies like anti-TNF, and administration of "anti-inflammatory" cytokines like IL-10 [410]. Despite the utility of NSAIDs in controlling inflammation in acute settings, current anti-inflammatory therapies are insufficient at controlling chronic inflammation. In fact, several have had detrimental

results once introduced in the clinical setting. For example, anti-TNF therapies for heart failure patients were proposed due to efficacy in animal models, and observed TNF elevation in plasma of heart failure patients [411]. Upon use in clinical trials anti-TNF worsened prognosis for those patients [412,413]. In endotoxemia studies, IL-10 treatment enhanced pro-inflammatory mediator production after administration of LPS [414]. This pro-inflammatory action of IL-10 was recapitulated in several inflammatory disease conditions [415–417], with a suggested role for IFNα-induced STAT1 upregulation [418]. These cautionary tales suggest two issues: first, systemic anti-inflammatory treatment is too broad a stroke with which to control chronic inflammatory processes, and second, opposing inflammation does not turn it off. It has increasingly been recognized that anti-inflammatory methods of treating chronic inflammation dampen but do not lessen inflammatory processes [408]. Inflammation resolution is an active process distinct from anti-inflammatory signaling that is likely a more effective therapeutic target for CVD and cardiac fibrosis [419].

1.5 – Resolution of inflammation

Transition out of the inflammatory response into resolution is a co-ordinated program consisting of several steps [410,419]. Triggering of pro-resolution pathways begins in the inflammatory phase, which suggests a balance between induction vs. resolution [410]. Classically, the molecular and cellular events of inflammation are: increased blood flow, capillary dilation, neutrophil and leukocyte infiltration, and production of inflammatory mediators initiated by resident innate immune cells [420]. In a similar vein, the cardinal signs of resolution (Fig. 1.5) are: (1) removal of stimuli, (2) dampening of pro-inflammatory signals, (3) clearance and catabolism of pro-inflammatory mediators, (4) exit and removal of granulocytes and efferocytosis, (5) increase in pro-resolution phenotypes of macrophages, monocytes, DCs, and T_{reg} cells, and (6) adapted homeostasis [4,410]. Although these steps have been presented sequentially, they likely occur simultaneously or in varying order depending on the tissue site and degree of inflammation.

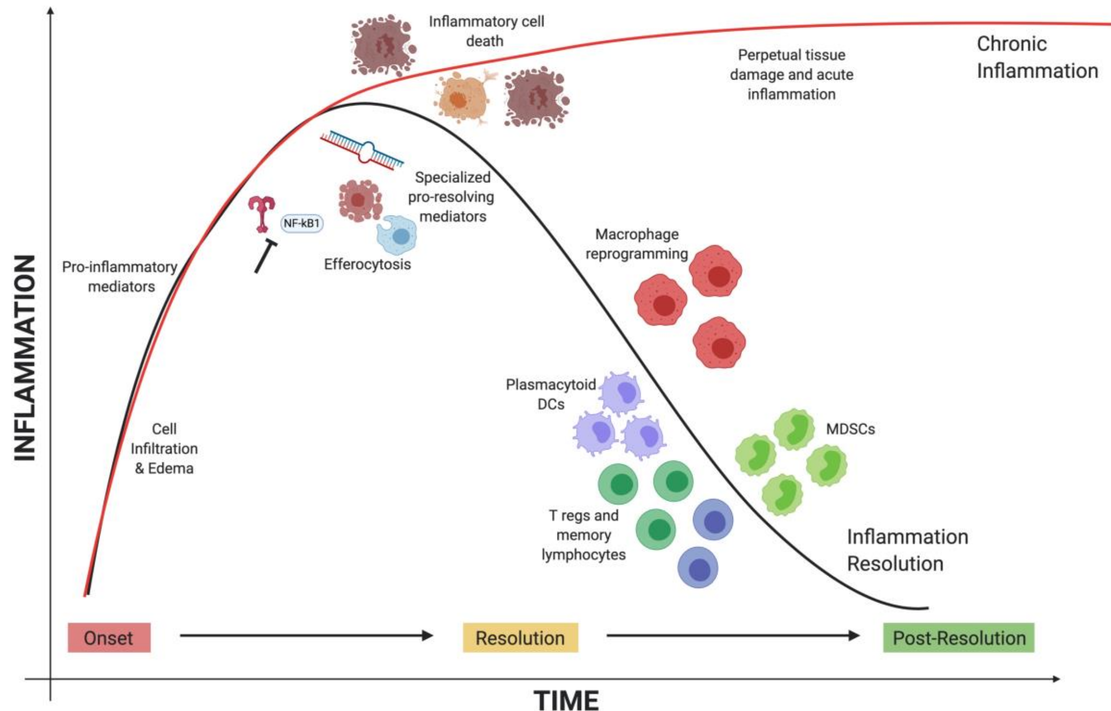


Figure 1.5 – Kinetics of inflammation resolution. After initial acute inflammatory processes, resolution occurs (1) removal of stimuli, (2) dampening of pro-inflammatory signals, (3) clearance and catabolism of pro-inflammatory mediators, (4) exit and removal of granulocytes and efferocytosis, (5) increase in pro-resolution phenotypes of macrophages, monocytes, DCs, and T_{reg} cells, and (6) adapted homeostasis. If resolution is frustrated, chronic inflammation can result. This is characterized by inflammatory cell death that further perpetuates tissue damage and reactivates acute inflammatory responses in a feedforward loop that leads to a chronic inflammatory state. Figure generated with BioRender.

1.5.1 – Debris removal

Dying cells release DAMPs upon injury, which are detected by PRR and non-PRR DAMP receptors on resident innate immune cells to induce pro-inflammatory protein and lipid mediator production. The initial goal is to disrupt barriers via endothelial cell activation to increase vascular permeability and allow for influx of plasma proteins, platelets, and, most importantly, leukocytes [421]. The serine protease system becomes rapidly activated after tissue damage by DAMPs and can be further bolstered in hypoxic, acidic microenvironments [422]. The complement cascade is a crucial part of the serine protease system [423]. Complement activation contributes to recruitment of neutrophils and monocytes via anaphylatoxins C3a and C5a, and activation of pro-

inflammatory pathways in resident leukocytes to perpetuate initial responses. Activation of the complement cascade also generates opsonins, C3b and C4b, that bind and target debris for phagocytosis [424]. Neutrophils, as in the heart, are the first leukocytes recruited to damaged tissues from the circulation, often within 30 minutes. They enter into tissues to assist in removal of debris [246,247] through release of ROS, serine proteases, and matrix metalloproteinases (MMPs), as well as direct phagocytosis [261]. Monocytes are the next subset of cells recruited to damaged tissue [420], where they can join tissue resident macrophages already activated by DAMPS [422] to mediate phagocytosis of debris and damaged cells [425].

1.5.2 – Dampening of pro-inflammatory signaling

In concert with neutrophil recruitment and debris clearance, pro-inflammatory signals are dampened. At the transcriptional level, NF-κB induction of pro-inflammatory gene expression after DAMP activation of leukocytes induces gene products associated with resolution. Leucine rich repeat protein 3 (LRRC3) [426] and radioprotective 105 (RP105) [427] are TLR homologues that lack cytosolic signaling domains and inhibit general TLR signaling [426], and TLR4 signaling [427]. At the translational level, pro-inflammatory genes can be suppressed by micro RNAs (miR) such as miR-146a [428] and miR-21 [429], which are induced by NF-κB and act to supress inflammatory NF-κB subunit signaling. NF-κB signaling itself can be switched to a pro-resolution phenotype. The p65/p50 dimer is the pro-inflammatory form of NF-κB that is first translocated to the nucleus in DAMP signaling [410]. The p65/p50 dimer induces production of the cRel subunit, which can form p65/cRel heterodimers and outcompete p65/p50 for κB binding sites on AP-1, STAT1, and STAT3 [430]. The p50/p50 subunit is induced later in the inflammatory cascade [431,432] and can also outcompete p65/p50 for κB binding sites to reduce TNF, IL-1β and IL-6 induction in macrophages in response to LPS [433].

IFN-γ and TLR4 activation of macrophages initially mediates pro-inflammatory actions, but also induces tristetrapolin (TTP) via p38 MAPK and STAT1 signaling [434,435]. TTP inhibits translation of mRNA encoding pro-inflammatory cytokines and chemokines, including IFN-γ [436], IL-1β [434], IL-6, CCL2, CCL3 [435], and TNF [437]. IFN activation can also result in protein 202a production that inhibits formation of the pro-inflammatory NF-κB subunits in favour of p50/p50 homodimers [438].

1.5.3 – Clearance and catabolism of pro-inflammatory mediators

Removal of mediators already released to the extracellular environment further promotes inflammation resolution. Catabolism of pro-inflammatory lipids such as prostaglandins is mediated by induction of 15-hydroxyprostaglandin dehydrogenase (PGDH) [439] and nicotinamide adenine dinucleotide(NADH)- and NAD phosphate(NADPH)-dependent reductases [4]. Neutrophils produce elastase, which can selectively degrade IL-1β and TNF [440]. Additionally, neutrophil-derived cathepsin G can degrade IL-3, IL-6, IL-15, IL-18, and IL-33 [202]. Neutrophils [441,442], eosinophils [443], mast cells [133,444], and macrophages [445] can also act as sources of MMPs, which degrade extracellular chemokines [446,447] and cytokines [448].

Scavenging of chemokines is mediated by atypical chemokine receptors (ACKRs) and apoptotic cells. ACKR2 is upregulated on numerous leukocytes and lymphatic endothelial cells under inflammatory conditions [447]. ACKR2 can bind chemokine ligands for CCR1, CCR2, CCR3, CCR4, and CCR5, and traffic them to the endo-lysosomal compartments for degradation [449,450]. ACKR2 is then recycled to the cell surface, allowing for rapid depletion of chemokines from the extracellular environment. Apoptotic neutrophils contribute to chemokine scavenging via upregulation of cell surface CCR5 upon activation of programmed cell death signaling to bind ligands from the extracellular environment [451]. By removing chemotactic and pro-inflammatory signals from the microenvironment, pathways promoting inflammation can be further dampened.

1.5.4 – Removal of infiltrating leukocytes

Infiltrated leukocytes, such as neutrophils and monocytes, must be removed to allow for adapted homeostasis to occur. Neutrophils can undergo reverse transendothelial migration to re-enter the circulation [452]. Recruited monocytes and dendritic cells exit tissues via the lymphatics [408]. Neutrophils are short lived cells under homeostatic conditions, and upon exit from the bone marrow their lifespan typically lasts a number of hours [255]. Inflammatory signaling pathways can enhance survival in neutrophils via B cell lymphoma-2 (Bcl-2) and Myeloid leukemia cell differentiation protein-1 (Mcl-1) activation [453]. Despite this, neutrophils will undergo apoptosis within 24-48h of entry into inflamed sites [255]. Apoptotic neutrophils promote resolution of inflammation in several ways. Release of Annexin A1 (AnnA1) and lactoferrin in microvesicles by apoptotic

neutrophils promotes their phagocytosis by macrophages [261,454,455]. Neutrophil-derived microparticles also act on endothelial cells to prevent further leukocyte attraction and trafficking across the endothelium via downregulation of adhesion molecule expression in an AnnA1-dependent manner [456,457]. Finally, apoptotic neutrophils produce RvD1, resolvin E2 (RvE2) and PGE2 [458], which are known to promote macrophage phagocytic activity.

Efficient removal of apoptotic neutrophils is crucial to prevent their progression to secondary necrotic cell death. In tissues throughout the body, including the heart [459,460], apoptotic neutrophils are phagocytosed by macrophages, termed efferocytosis [461]. Evidence suggests that tissue resident macrophages are specifically responsible for efferocytosis during inflammation to prevent Ly6C^{hi} monocytes from ingesting apoptotic cells and presenting self-antigen [462]. Upon induction of efferocytosis, macrophages will differentiate into a unique pro-resolution phenotype characterized by increased expression of *NOS* and *COX2* and production of specialized pro-resolving mediators (resolvin D1, protectin D1 and maresin 1), TGF-β, IL-10, and PGE2 [458,463–467]. Particularly, induction of specialized pro-resolving mediators in macrophages further promotes inflammation resolution as they dampen neutrophil recruitment, promote local neutrophil apoptosis, macrophage efferocytosis, and pro-resolution macrophage phenotypes [468].

1.5.5 – Post-resolution and adapted homeostasis

Recent work has shown that inflammation fundamentally alters a tissue setting even when it has resolved effectively, leading to the term adaptive homeostasis. This is characterized by an immunosuppressive environment [408]. After resolution of inflammation in a low dose zymosan model of peritonitis, the peritoneal cavity is populated by pro-resolving resident macrophages, monocytic myeloid derived suppressor cells (MDSCs), plasmacytoid DCs, and T_{reg} cells[469]. Changes to the leukocyte population in the peritoneal microenvironment were driven by macrophage-derived PGE2 production suppressing T cell proliferation and promoting MDSCs [470]. Furthermore, draining lymph node expansion following resolution increased presence of memory B and T cells [469]. Interestingly, in high dose zymosan models where resolution was delayed, this immunosuppressive environment was not observed [469]. The mechanisms and purpose

of this post-resolution phase are still being elucidated, but it has been suggested that induction of an immunosuppressive environment would aid in maintenance of tolerance to self-antigen and prevention of autoimmune diseases, at the risk of secondary infection [408]. While the mechanisms at work in adapted homeostasis are outside of the scope of this thesis, it has potential implications for therapeutic design.

1.5.6 – The peritoneal cavity as a site for modeling inflammation resolution

It is evident that understanding tissue inflammation resolution kinetics is vital to inform therapeutics for targeting chronic inflammation. However, understanding how inflammation resolution can progress in tissues is more difficult. To isolate cell populations, mechanical and proteolytic digestion of tissues is required which can impact phenotype [471,472] and cell surface molecule expression of leukocytes. Valuable information such as chemokine and cytokine changes are also difficult to evaluate in solid tissues. To understand the kinetics and mechanisms of inflammation resolution, researchers have turned to the peritoneal cavity. The peritoneal cavity is a sterile serous cavity and is sealed off from the external environment, making it amenable to experimental manipulation. Peritoneal lavage allows for easy isolation of leukocytes, and inflammatory mediators [473]. Furthermore, inflammatory processes in the peritoneal cavity are largely self-limiting, thus kinetics of resolution can be observed [474]. The peritoneal cavity contains diverse populations of resident leukocytes, including mast cells, macrophages, lymphocytes, DCs, and NK cells and retains recruitment kinetics observed in tissues [475]. Extensive work to understand inflammation resolution, and the cells and mediators that enact it has been conducted in the peritoneal cavity, reviewed here [3, 408,476]. It is an especially useful model for studying efferocytosis, as this quick process [461] can be visualized in the peritoneal cavity via the administration of fluorescently labeled apoptotic cells generated *ex vivo* and assessed by flow cytometry [477–479].

1.6 – Macrophages and efferocytosis as key effectors of inflammation resolution

Efferocytosis is a crucial homeostatic function occurring throughout the body [461] that is increased during inflammation [4]. Of the 37.2 trillion cells in the human body [480], approximately 0.4% die on a daily basis [481]. Yet detection of apoptotic cells in tissues is quite difficult, even in those where apoptotic activity is high such as the thymus and bone

marrow. This would suggest phagocytes have enormous capacity to engulf apoptotic cells, even under homeostatic conditions [482].

Macrophages have crucial roles in inflammation resolution as mediators of efferocytosis. Upon phagocytosis of apoptotic cells, macrophages undergo phenotypic changes associated with increased repair and regulatory functions [483–490]. This includes suppression of NF-κB and NLRP3 inflammasome signaling [491–493], decreased pro-inflammatory cytokine production [489], and induction of surface MerTK and secretion of adaptor molecules [490]. As previously mentioned, efferocytosis also activates production of specialized pro-resolving mediators [485,486] which directly promote inflammation resolution [468,494]. Thus, proper induction, control, and balance of efferocytosis is crucial. Efferocytosis exists as a continuum that can be divided into four overlapping phases of recruitment, tethering, engulfment, and response [461].

1.6.1 – The efferocytosis continuum

In the first phase of efferocytosis, recruitment, apoptotic cells release a class of DAMPs after caspase activation that have chemoattractant effects on macrophages. These DAMPs are known as find-me signals and include lysophosphatidylcholine (LPC) [495,496], sphingosine-1-phoshpate (S1P) [497,498], nucleotides (adenosine triphosphate (ATP) and uridine triphosphate (UTP)) [499–501], and fractalkine or CX3CL1 [502]. Stressed cells can also release find-me signals to recruit macrophages as a precautionary measure [482]. The intensity of the chemoattractant gradient produced by apoptotic cells is unclear, but it is likely that tissue resident macrophages preferentially respond to find-me signals rather than a large scale induction of general phagocyte recruitment [461].

Next is the second phase of efferocytosis, tethering. Macrophages interact with apoptotic cells and become tethered prior to phagocytosis. This is mediated by exposure of molecules referred to as eat-me signals on the surface of apoptotic cells [503]. These molecules are either exposed on the cell surface after caspase activation, such as phosphatidylserine (PtdSer) [504] and AnnA1 [505], or are already present on the cell surface but modified after caspase activation, such as intercellular adhesion molecule (ICAM)-3 [506]. Of these eat-me signals, PtdSer is the most widely recognized and studied, and thus will be discussed herein. PtdSer is typically localized to the inner leaflet of the plasma membrane under basal conditions, mediated by the actions of several flippase enzymes

[507]. After caspase activation, flippases are inactivated [507], while scramblase enzymes are induced via caspase cleavage and changes in cytosolic Ca^{2+} concentrations [508–510]. Together, these two actions promote cell surface PtdSer exposure.

PtdSer on the surface of apoptotic cells functions as a ligand for tethering receptors on macrophages (Fig. 1.6). This includes T-cell immunoglobulin and mucin domain containing (TIM)-1 and TIM-4 [511], brain specific angiogenesis inhibitor 1 (BAI1) [512], and low-density lipoprotein receptor-related protein 1 (LRP1) [481], among others. Tethering receptors bind apoptotic cells to macrophages to allow for engulfment receptors to commit to the process of efferocytosis (Fig. 1.6). Tyrosine protein kinase receptor 3 (TYRO3), AXL, and Myeloid epithelial reproductive tyrosine kinase (MerTK) receptors make up the TAM family of receptors that largely mediate engulfment [513]. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [505,514,515] have also been shown to contribute. Binding of TAM receptors and integrins to PtdSer is mediated by bridging molecules Gas6, Protein S1 (PROS1), and milk fat globule-endothelial growth factor 8 (MFGE-8), respectively. Gas6 acts as a bridge for all three TAM receptors, while PROS1 is specific for MerTK and TYRO3 interaction with PtdSer [516,517]. MFGE-8 interacts with integrins to bridge PtdSer for engulfment [515]. Importantly, engulfment receptors require previous tethering receptor engagement of “eat-me” signals on apoptotic cells for phagocytosis to occur (Fig. 1.6). Tethering receptors alone cannot induce phagocytosis [518,519].

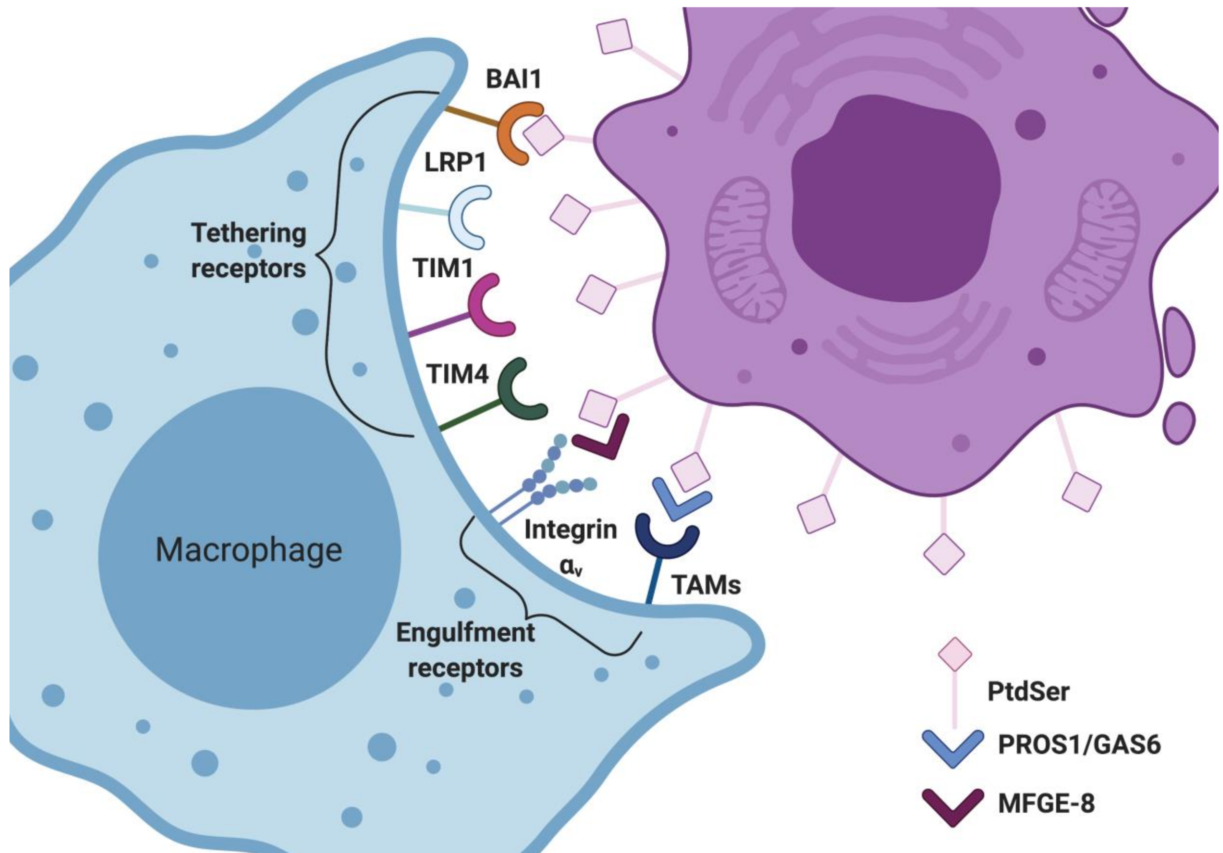


Figure 1.6 – Tethering and engulfment receptor interaction with apoptotic cells. Apoptotic cells expose 'eat-me' signals on their cell surface in response to caspase and Ca^{2+} flux inactivation of flippase enzymes and activation of scramblase enzymes. The best studied 'eat-me' signal is PtdSer, which interacts with a wide variety of receptors. To initiate efferocytosis, tethering receptors must first bind PtdSer on apoptotic cells to tether the cell to a macrophage. These include, but are not limited to, BAI1, LRP1, TIM1, and TIM4. Once the apoptotic cell is tethered to the macrophage, engulfment receptors can bind. Importantly, engulfment receptors require bridging molecules, and cannot interact with PtdSer directly. The TAM family of receptors utilizes GAS6 (all TAMs), and PROS1 (MerTK and TYRO3) to interact with PtdSer, while α_v Integrins utilize MFGE-8. Once tethering and engulfment receptors have bound their targets, the apoptotic cell is phagocytosed by the macrophage. Figure generated with BioRender.

Upon binding of tethering and engulfment receptors, the third phase of efferocytosis, phagocytosis, occurs. Through involvement of multiple receptors, signaling converges on Ras-related C3 botulinum toxin substrate 1(Rac1) activation [512, 514,515,520–523] that induces actin filament reorganization, membrane ruffling and phagosome formation [524,525]. Microtubule-associated protein 1A/1B-light chain 3 (LC3) assists in phagosome

formation and transfer of engulfed apoptotic cells to phagolysosomes for acidification and degradation [489,526]. Often, multiple apoptotic cells are present at the same location requiring timely removal. Macrophages have the capacity to engulf multiple apoptotic cells in quick succession via induction of mitochondrial fission [527].

Once the apoptotic cell has been phagocytosed, the final phase of efferocytosis, response, can occur. Macrophages take on distinct pro-resolution and anti-inflammatory phenotypes following efferocytosis. MerTK signaling has been shown to be crucial for inhibition of NF-κB [483,528] and STAT1 [484] signaling, and induction of specialized pro-resolving mediators [485,486] and TGF-β [487] via Liver X receptor (LXR)α , LXRβ [488], suppressor of cytokine signaling (SOCS)1 and SOCS3 [484]. Induction of specialized pro-resolving mediators following efferocytosis can act in a positive feedback loop on macrophages in an autocrine or paracrine manner to promote efferocytosis and further suppress NF-κB signaling [485]. Tethering receptor signaling has also been shown to have important impacts on macrophages to activate anti-inflammatory phenotypes, including via BAI1 [491], and TIM-4 [492,493]. Macrophage phenotype is also impacted by pathways induced after apoptotic cell phagocytosis. Induction of LC3-associated phagosomes acts to suppress pro-inflammatory cytokine signaling [489], while peroxisome proliferator activated receptor(PPAR)-δ increases production of opsonins, MFGE-8, IL-10 and cell surface MerTK expression [490]. All of these responses combine to generate a co-ordinated pro-resolution and anti-inflammatory program and can feed forward to promote further efferocytosis.

1.6.2 – The consequences of defective efferocytosis

Defects in the phagocytosis of apoptotic cells can have disastrous effects. Efferocytosis can be thought of as the biggest push towards resolution, as macrophages undergo drastic phenotypic changes that influence downstream outcomes [458, 463, 487–490, 464–467,483–486]. Without the signals provided by apoptotic cell uptake, macrophages maintain their pro-inflammatory phenotypes and likely contribute to chronic inflammation. Furthermore, progression of apoptotic cells to secondary necrotic cells and inflammatory cell death perpetuates tissue injury and feeds into pro-inflammatory pathways [461,529]. Of note, the complement cascade can act as a stopgap here, as opsonins C3b and C1q have been shown to specifically target late apoptotic and secondary necrotic

cells for efferocytosis [530,531]. Therefore, efferocytosis is required to prevent immunogenic cell death and activation of autoimmune programming via antigen presentation to adaptive immune cells [532]. This has been shown in animal models such as *Mertk-/-* mice which develop spontaneous autoimmune disease with age [461,533,534]. Furthermore, inefficient efferocytosis has been identified as a crucial factor in the development of systemic lupus erythematosus (SLE) [535,536], atherosclerosis [537,538], and chronic obstructive pulmonary disease (COPD) [539,540].

As efferocytosis operates within a continuum of feed-forward and feed-back regulation, failed initial clearance of apoptotic cells promotes local inflammation, and further recruitment of leukocytes that themselves increase apoptotic cell burden [419,482]. As such, initial defects become further accentuated as the inflammatory process progresses, ultimately resulting in chronic inflammation. Understanding the mechanisms of efferocytosis and ways in which to target its activity is a crucial contribution to understanding resolution of chronic inflammation.

1.7 – DAMPs regulate inflammation and promote repair

DAMPs typically have physiological roles within healthy cells, but under necrotic cell death conditions are released into sterile tissue environments and initiate or exacerbate pro-inflammatory signaling [541]. The importance of DAMPs to initiate inflammatory responses to injury is well understood and has been extensively reviewed [421,541–545]. DAMP signaling can be mediated by PRR (e.g. mitochondrial DAMPs, Heat shock proteins (HSPs)), non-PRR DAMP receptors (e.g. IL-33, LPC), or a combination of the two (e.g. High mobility group box 1 (HMGB1), S100 proteins) [544,546]. Canonical NF-κB signaling occurs downstream of the majority of DAMP receptor activation [432]. Regulation of NF-κB activity is mediated by which subunits are active, with important inflammatory responses, as detailed previously. Notably, the p50/p50 homodimer NF-κB subunit has been shown to be induced downstream of DAMP signaling to compete with p65/p50 for κB binding sites and block access to reduce inflammatory signaling [433,438]. TLR activation induces expression of TAM receptors which activates SOCS1/3 signaling to suppress IFNα receptor (IFNAR)-STAT1 signaling and reduce inflammatory cytokine and chemokine production [484]. Thus, while initial DAMP activation is inflammatory, pathways are activated from the outset to try and control the ensuing response.

There are emerging roles for DAMPs within instruction of adaptive immune responses and orchestration of tissue repair. Most of this work centres around HMGB1 and ATP. HMGB1 promotes stem cell migration [547–549], endothelial cell proliferation [550,551], and angiogenesis [552,553]. ATP is an important signal for endothelial cell proliferation [554–557] and angiogenesis [558]. Both of these DAMPs contribute to cardiac wound repair. HMGB1 increases angiogenesis and decreases leukocyte infiltration to the heart [559,560]. The role for ATP is less clear and likely temporally dependent [561–564]. ATP functions as both a DAMP and a find me signal for efferocytosis [481], while HMGB1 functions to exert DAMP and angiogenic effects via signaling through TLR4 and RAGE respectively [542,545]. Another dual function DAMP is IL-33.

1.7.1 – IL-33 as a tissue repair DAMP

The identification of IL-33 as a DAMP was first based on its processing by apoptotic caspases to prevent extracellular release upon programmed cell death [199,200,565,566]. The idea was further cemented when abrogation of the N-terminal nuclear localization sequence of IL-33 lead to lethal inflammation in mice [567]. Nuclear localization of IL-33 has no transcriptional function and is thought to be a mechanism to sequester IL-33 and prevent its release outside of a necrotic cell death setting [196]. IL-33 activates type 2 immune pathways via ST2L signaling in leukocytes [178,206], but its role in promoting cardiac repair has been largely attributed to induction of pro-survival signaling in cardiomyocytes [344,375–378]. However, the broad impacts of IL-33 on collagen deposition, hypertrophy [379], pro-inflammatory cytokine signaling [372, 374,380], infarct size [343–345,378] and improved cardiac function [376,381] seem too large to be exclusively due to cardiomyocyte survival.

1.8 – Rationale & Hypothesis

After injury in the heart, wound healing restores function to damaged tissue. The initial inflammation that occurs following damage is crucial for initiation of wound healing and is instigated by DAMP release from necrotic cardiomyocytes and cardiac fibroblasts [231,238]. Once local immune cells and first responder cells have cleared debris, the reparative phase occurs. This is characterized by ECM deposition for scar tissue formation by fibroblasts. Finally, remodeling of said ECM completes the process to

mature the scar for stability. These steps occur sequentially, and proper transition from one to the next is crucial to prevent detrimental outcomes, such as cardiac fibrosis [230].

Cardiac fibrosis is characterized by excessive scar tissue deposition that interferes with signal transduction and impairs cardiac function. A key driver of fibrosis is chronic inflammation [231,237,238], which occurs when the inflammatory stimuli are not removed due to persistent injury or ineffective clearance. Chronic inflammation is characterized by excessive production of inflammatory mediators and activation of inflammatory cell death pathways such as pyroptosis and necroptosis [541, 546,568]. These mechanisms of cell death damage tissues in a feedforward loop that perpetuates inflammation and prevents transition to wound healing. As a result, excessive ECM is deposited beyond the needs of the initial injury, decreasing contractility and cardiac function, and ultimately leading to CHF [230]. To prevent or reduce fibrosis, chronic inflammation needs to be resolved. Proper inflammation resolution hinges on efferocytosis, with extensive evidence identifying this process as the crux for adapted homeostasis to be achieved [461,529].

Mast cells are innate immune sentinel cells that reside in tissues and act to orchestrate immune responses to damage [5, 71,74]. IL-33 is a dual-function cytokine released from damaged structural cells that acts on $ST2L^+$ leukocytes to exert type 2 immunity-associated functions [178,182]. IL-33 is released by damaged cardiac parenchymal and stromal cells, including cardiomyocytes, and acts to promote protective responses [344,375–378]. Mast cells readily respond to IL-33 via ST2L signaling, characterized most ubiquitously as increased IL-13 production [64,569]. Mast cells are resident in cardiac tissue, abundant in human hearts, and have been shown via animal models to have controversial roles in cardiac fibrosis [75]. The impact of IL-33 on mast cell activity is well described in several disease contexts but has not been examined in the context of cardiac fibrosis.

We hypothesize that mast cells prevent excessive remodelling in the heart that leads to fibrosis via an IL-33-dependent mechanism. This hypothesis has been examined using both human tissue and mouse models in the context of three research objectives: (1) assess the influence of mast cells on fibrosis and post-operative outcomes in human atrial tissue from cardiac surgical patients, (2) determine the impact of mast cells on early angiogenic events after IL-33 activation, and (3) assess the impact of IL-33 on resolution of inflammation and efferocytosis at distal sites, and how mast cells may contribute.

CHAPTER 2: MATERIALS AND METHODS

2.1 – Mice

All animal experiments were approved by the Dalhousie University Committee on Laboratory Animals (Protocols 16-085, and 19-031). C57BL/6 mice were obtained from Charles River Laboratories (Quebec, Canada), or bred on site in the Carleton Animal Care Facility. Kit$^{W\text{-}sh}$/HNihrJaeBsmJ (Kit$^{W\text{-}sh/W\text{-}sh}$) were obtained originally from Jackson Laboratories (Bar Harbor, ME) and then bred on site in the CACF. *Cpa3-Cre; Mcl-1*$^{fl/fl}$ mice were obtained from collaborators at Queen's University and bred on site in the CACF. For Chapter 5, in IL-33 peritoneal experiments, mice were between 6-8 weeks of age. For Appendix A, in AngII mini-osmotic pump experiments, male mice were used between 6-8 weeks of age, except where mast cell reconstitution was conducted in mast cell deficient strains prior to Ang II administration, in which case mice were 14-16 weeks of age and compared to age-matched control mice.

2.2 – Murine surgical procedure: osmotic mini pump insertions

For Appendix A, mini-osmotic pumps were inserted into mice subcutaneously as previously described[265,570] (Alzet, Palo Alto, CA, USA). Animals received pumps filled with saline, or AngII (0.7/1.0/1.44mg/kg/day, Millipore Sigma, Oakville, ON, Canada) for 7 or 28 days. For the first 48h post operative, all animals were given 1mL subcutaneous saline to promote survival. Weights were monitored daily (endpoint >20% weight loss), as well as grooming, behaviour and feeding to assess morbidity. At the endpoint animals were sacrificed with isoflurane and CO_2 asphyxiation. To determine hypertrophy, hearts were extracted, flushed with saline and weighed to compare to both body weight at time of death and tibia length. Hearts were then dissected for histology, protein, and mRNA analyses, with apex of ventricles and atria split for protein and mRNA, while cross section of both ventricles was taken for histology.

2.3 – Human surgical procedures

Blood samples were collected from all patients prior to skin incision and atrial tissue samples were taken at the time of cannulation. A portion of right atrial appendage was excised during surgery and processed for RNA, protein, and histological analyses. In all patients, cardiac surgery was performed with cardiopulmonary bypass and anticoagulation was achieved using intravenous heparin (400 IU/kg) with a target

activated clotting time greater than 450 seconds. Antifibrinolytic agents were given to all patients and consisted mainly of tranexamic acid. Intermittent cold blood cardioplegia was delivered in an antegrade or retrograde fashion based on surgeons' preference. Protamine sulfate was given for reversal of heparin in all patients. Patients also received routine baseline 12-lead electrocardiograms upon admission to the surgical intensive care unit (SICU). Resumption of routine postoperative medications occurred as indicated and included anti-platelet agents within 24 hours, statins and β-blockers. Patients underwent coronary artery bypass grafting (CABG), atrial valve replacement (VR), mitral VR, or a combination of CABG and VR. All patients were monitored during their stay in hospital.

2.4 – Human Atrial Tissue Samples Acquisition

Patients were enrolled prospectively and limited to those receiving elective open-heart surgery at the New Brunswick Heart Centre in Saint John, New Brunswick, Canada or the QEII Health Science Centre in Halifax, Nova Scotia, Canada. All subjects gave informed consent. Approval from the Research Ethics Board (REB) was obtained from each individual institution (NSHA #: 1019404, NSHA #: 1017160, Horizon Health Network #2014-2006). All consented participants received the standard care in terms of treatment. Exclusion criteria included the need for urgent/emergent surgery, advanced age (>75 years) and patients with BMI < 18.5 kg/m^2.

2.5 – Variable Selection

Patients were assigned random ID numbers at enrolment. Pre-operative clinical characteristics of interest included age, sex, New York Heart Association (NYHA) functional class, urgency of surgery (in-hospital urgent if hospitalization required until time of surgery, elective or outpatient) and diabetes, among others (Table 3.1). Intraoperative variables included pump time and clamp time. Standard blood analysis included neutrophil-to-lymphocyte ratio and peak troponin (collected within 24hr after surgery). Outcome variables included: in-hospital mortality, atrial fibrillation, cerebrovascular complication (transient ischemic attack or stroke), length of hospitalization (hospitalization, LOS) and NYHA functional class at follow-up after discharge. All patient information, procedural details and outcomes were collected and maintained in the respective cardiac surgery registries of each institution.

2.6 – Primary Human Cord Blood-derived Mast Cell Culture

Umbilical cord blood samples were obtained following approval from the Research Ethics Board of the Izaak Walton Killam (IWK) Health Centre in Halifax, Canada (IWK HE 1005110). Adult donors provided written informed consent and Cord Blood-derived Mast Cells (CBMCs) were cultured according to an adaptation of Radinger et al[571] described as follows. Mononuclear cord blood cells were isolated by Ficoll-Hypaque (GE, Mississauga, Canada) density gradient centrifugation and cultured in maturation media for three weeks consisting of StemSpan media (Stem Cell Technologies, Vancouver, Canada) with 100 ng/mL stem cell factor (SCF, R&D Systems, Minneapolis, MN), and 10 ng/mL IL-6 (Biolegend, San Diego, CA), with 10 ng/mL IL-3 (Peprotech, Rocky Hill, NJ, USA) for the first week. CBMCs were then transferred to RPMI (ThermoFisher, Waltham, MA) and 10% Fetal Bovine Serum (FBS, ThermoFisher) supplemented with 100 ng/mL SCF (R&D Systems) and 10 ng/mL IL-6 (Biolegend) to further mature in culture for a total of six to eight weeks. CBMC maturity was determined via CD117 (c-Kit) staining, with a minimum of 95% CD117+ cells prior to use in experiments. CBMCs produced in this way were on average 96.5% positive for CD117. Prior to any activation (degranulation or long term), CBMCs were cultured overnight in RPMI (ThermoFisher) and 10% Fetal Bovine Serum (FBS, ThermoFisher) supplemented with 10 ng/mL SCF (R&D Systems) and 10 ng/mL IL-6 (Biolegend) to rest the cells prior to activation.

2.7 – CBMC degranulation assays

Mast cells were washed with cold Hepes Tyrode's buffer (HTB, pH 7.2-7.4) prior to combining with the following pre-warmed activators in HTB at 37^{0}C : HMGB1 at 100 ng/mL (Biolegend), HSP60 at 100 ng/mL(Biolegend), HSP70 at 100 ng/mL (Biolegend), ATP at 20 μM (MilliporeSigma, Oakville, Canada), S100A8 at 10 ng/mL (Biolegend), human IL-33 at 30 ng/mL (Peprotech), calcium ionophore A23187 at 5 μM (MilliporeSigma), or mock control. Cells were incubated at 37°C for 30 minutes prior to being placed immediately on ice for 10 minutes to stop degranulation. Cells were then pelleted in a pre-cooled centrifuge and aliquots of supernatant were made prior to storing cells and supernatant at -80°C.

2.8 – B-hexosaminidase assay

To detect degranulation of mast cells, the concentration of β-hexosaminidase contained in mast cell granules was assessed in supernatant compared to cell content. Cells were lysed by three cycles of freeze-thaw and vortex. Supernatant and cell pellets were plated on a 96 well plate, and 0.1M p-nitrophenyl-N-acetyl-β-D-glucosaminide (MilliporeSigma) was added to each well. The plate was then incubated at 37°C for 1 hour, prior to stopping the reaction with 0.1M carbonate buffer. Absorbance was read at 405nm and % degranulation was calculated with the following formula:

$$\% \text{ degranulation} = \frac{(\text{OD supernatant} - \text{OD HTB ctrl})}{(\text{OD supernatant} - \text{OD HTB ctrl}) + (\text{OD pellet} - \text{OD HTB ctrl})} \times 100$$

2.9 – Long term CBMC activations

After resting overnight, CBMCs were resuspended in activation medium (R10F with 10 ng/mL SCF and 100 μg/mL soybean trypsin inhibitor, MilliporeSigma) without supplementation to act as a mock control, recombinant human IL-33 at 30 ng/mL, 10 ng/mL, or 3 ng/mL (Peprotech), human recombinant $VEGF_{165}A$ at 10ng/mL (Peprotech) or 0.1μM calcium ionophore A23187 (MilliporeSigma) for 3 hours, 6 hours or 24 hours at 37°C. Cells were pelleted and lysed using Qiazol tissue lysis reagent (Qiagen, Toronto, Canada) for RNA isolation and reverse transcription, while supernatants were saved for further downstream Immunoassays.

2.10 – PBMC Isolation

Peripheral blood was obtained with informed consent from adult volunteers (REB IWK HE 1005110) and was subjected to density gradient centrifugation over Ficoll-Hypaque (GE) to isolate PBMCs. PBMCs were then lysed using Qiazol tissue lysis reagent (Qiagen) for RNA isolation and reverse transcription.

2.11 – Human Umbilical Vein Endothelial Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from collaborators (Thomas Issekutz lab, IWK Health Centre, Halifax, NS). HUVECs were cultured in complete media containing RPMI, 20% FBS (ThermoFisher), 50 μM 2-ME (Sigma), 1x Penicillin/Streptomycin (Wisent Inc.), 50 μg/mL endothelial cell growth supplement (Sigma), and 50 μg/mL Heparin (Sandoz Inc., Boucherville, Quebec,

Canada) at 37ºC and 5%CO_2. HUVECs were activated with IL-33 (BioLegend) at 30 ng/mL or $VEGF_{165}A$ (R&D Systems) at 10ng/mL

2.12 – HUVEC Angiogenesis Assay

A commercial *in vitro* angiogenesis assay (Abcam, Toronto, Canada) was used to assay capillary tube formation in HUVECs according to manufacturer's instruction. Briefly, HUVECs were grown to approximately 85-90% confluency and serum starved overnight prior to use in experimental assays with 1% FBS. Cells were resuspended in RPMI with 1% FBS (ThermoFisher), 50 µM 2-ME (Sigma), 1x Penicillin/Streptomycin (Wisent), 5 µg/mL endothelial cell growth supplement (MilliporeSigma), and 50 µg/mL Heparin (Sandoz) and 2 x 10^5 cells/well were plated in a 96 well plate pre-coated with extracellular matrix. Supernatants from CBMCs activated with IL-33 (BioLegend) at 30 ng/mL and Calcium Ionophore at 0.1µM were added to cells and incubated for 4h at 37ºC. $VEGF_{165}A$ (R&D Systems) in HUVEC media was used as a positive control for angiogenesis, and Suramin (Abcam) in HUVEC media was used as a negative control to inhibit angiogenesis. Cells imaged on EVoS microscope system (ThermoFisher). ImageJ (NIH) Angiogenesis Analyzer was used to assess extent of capillary tube formation [572].

2.13 RNA Isolation from Tissue

Up to 50 mg of flash frozen human or mouse tissue was placed in Qiazol tissue lysis reagent and lysed using the Qiagen TissueRuptor homogenizer on ice. RNA isolation was conducted according to an adaptation of protocols from the Qiagen miRNeasy mini and RNeasy Plus system. Briefly, chloroform was used to separate the aqueous and organic phases of the homogenized tissue samples. The aqueous phase containing RNA was then first bound to RNeasy filter matrix and treated with DNaseI to eliminate genomic DNA prior to RNA isolation. Subsequently, samples were treated according to the manufacturer's instructions for the RNeasy Plus kit RNA extraction procedure (Qiagen). RNA was deemed to be of good quality when the following criteria were met: A_{260}:A_{280} ratios between 1.8-2.1, and a 2:1 ratio of intensity of 28S:18S RNA band on an agarose RNA integrity gel. Samples that did not meet both of these criteria were excluded from analyses.

2.14 – RNA isolation from cells and reverse transcription

Cells were resuspended in Qiazol (Qiagen, Toronto, Canada) and chloroform separation was used to remove protein components from aqueous phase containing nucleic acids. RNA was then isolated form this aqueous phase according to manufacturer's instruction (RNA isolation kit, Qiagen). cDNA was generated from RNA according to manufacturer's instructions (reverse transcription kit, Qiagen). Briefly, 150-300 ng of RNA was used to generate cDNA in each reverse transcription reaction. Mixtures of oligo-dTs and random primers were used in the reaction mix.

2.15 – Angiogenesis microarray

cDNA generated from three separate donor CBMC cultures untreated or activated for 24 hours with 30 ng/mL human recombinant IL-33 (Peprotech) were pooled for an angiogenesis microarray (BioRad). BioRad synthetic control RNA and master mix were generated and added to the 384 well plate to warm to RT prior to addition of cDNA. Samples were then run on a Bio-Rad C1000 Touch Thermal Cycler qPCR machine in 384 well format under the following conditions: 95°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds.

2.16 – Quantitative PCR (qPCR)

Pooled samples of cDNA for each cell or tissue type were used to determine whether to use qPCR or droplet digital PCR (ddPCR) for an assay. When C_q values were <30, qPCR was used, while C_q values of 30-40 were taken to ddPCR. qPCR mixes consisting of 0.125 µM of the gene primer, and 1X SYBR green master mix (Bio-Rad, Mississauga, Canada) per well of the plate were prepared. A Bio-Rad C1000 Touch Thermal Cycler qPCR machine was used to carry out the procedure. All primer sequences used in these studies are proprietary to BioRad and Qiagen. The reference genes used in this study were *GUSB, HPRT,* and *TBP* for CBMCs, PBMCs, and HUVECs (Qiagen), *YWHAZ, GUSB* and *PPIA* for human atrial tissue (Qiagen) and *Tbp* and *Gusb* (Qiagen) for murine experiments. The human genes of interest were *SIGLEC6, MS4A2, VEGFA, PLAU, IL1RL1* and *IL33* (Bio-Rad, and Qiagen), while the murine genes of interest were *Acta2, Ccl2 , Col1a1, Col3a1, Il13, Mmp9, Tgfb,* and *Vegfa*. Gene expression values were determined using the formula $\Delta C_q = C_{q\text{ Geomean of reference genes}} - C_{q\text{ gene of interest}}$, and normalized expression was then calculated as $2^{\Delta Cq}$.

2.17 –ddPCR

Each ddPCR reaction consisted of 1X Eva Green (Bio-Rad), 0.2 µM primers (Qiagen), cDNA, and ddH_2O. Droplet generation oil (Bio-Rad) was combined with reaction mixes in the droplet generator prior to sealing the plate and running on a Bio-Rad C1000 Touch Thermocycler. PCR amplification went as follows: 95°C for 5:00, 95°C for 0:30, 58°C for 1:00, 72°C for 1:00, 58°C for 1:00, 72°C for 1:00 (repeating alternation from 58°C to 72°C 49x), 4°C for 5:00, 90°C for 5:00 with a ramp of 2°C/second between cycles. The QX200 droplet reader (Bio-Rad) was then used to assess gene expression with QuantaSoft software. The reference genes used in this study were *YWHAZ* and *GUSB* (supplementary Fig. 3) for human atrial tissue, and *Tbp* and *Gusb* (Qiagen) for murine peritoneal cells. The human genes of interest (GOI) were *MS4A2, SIGLEC6, MERTK* and *IL1RL1* (Qiagen), while the murine gene of interest (GOI) was *Il13*. Normalized standard amount (NSA) was determined by the following formula:

$$\text{NSA} = \frac{\text{Concentration of GOI} \times \text{Dilution factor}}{(\{\text{Reference Gene A} \times \text{Dilution factor}\} + \{\text{Reference Gene B} \times \text{Dilution factor}\})^{1/2}}$$

2.18 – Gene Expression Omnibus (GEO) profile retrieval from National Center for Biotechnology Information (NCBI) database

GEO datasets were accessed from https://www.ncbi.nlm.nih.gov/geoprofiles/ via searches for studies related to human fibrosis or heart samples. Datasets of interest were identified and probed for *MS4A2* (gene ID NM_00139) and *SIGLEC6* (gene ID NM_001245) relative mRNA expression.

2.19 – Tissue Sectioning and Histology

Human tissues were fixed in formalin while mouse tissues were fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid) each for 24 hours prior to paraffin embedding. Serial sections were cut at 5µm for staining. For analysis of tissue fibrosis, Sirius red/fast green (SRFG) staining was employed, and collagen content was assessed using Adobe Photoshop as previously described[265, 570,573,574]. For analysis of gross pathology, hematoxylin and eosin (H&E) staining was used[575]. To account for issues in mast cell detection due to formalin fixation, toluidine

blue staining of human tissues was conducted at pH 1 for 5 days prior to assessment of mast cell density. Mouse Toluidine blue staining was conducted at pH 2.5 overnight.

2.20 – Thymocyte isolation and induction of apoptosis

For each *in vivo* efferocytosis experiment, 2-3 female C57BL/6 mice aged 4-6 weeks were euthanized and dissected to remove the thymus. Thymocytes were isolated and stained with 1µM CFSE (MilliporeSigma) prior to induction of apoptosis by 1µM dexamethasone incubation for 3 hours at 37ºC and 5% CO_2. Thymocytes that were not previously CFSE labeled were used to assess efficiency of apoptosis induction. These cells were stained with viability dye (ef506, ThermoFisher, Waltham, MA) and Annexin V (ThermoFisher) to confirm apoptosis (30-40% Annexin V+, Fig. 5.2E) by flow cytometry.

2.21 – Free mitochondria isolation

Free mitochondria were isolated from mouse liver using mitochondria isolation kits according to manufacturer's instruction for option A, protocol 2 (ThermoFisher) and previously published methods [576]. Isolated mitochondrial pellets were suspended in HBSS and treated with Halt Protease Inhibitor Cocktail (ThermoFisher). Protein concentrations of free mitochondria were determined by Bradford Protein Assay (Bio-Rad, Hercules, CA).

2.22 – Intraperitoneal injection experiments

Mice were injected intraperitoneally (i.p.) with 0.5 µg IL-33 (BioLegend) in 100 µL saline, 50 µg Free mitochondria in 100 µL saline, 50 µg Free mitochondria and 0.5 µg IL-33 in 100 µL saline or with 100µL saline alone. Animals were housed for 48h prior to (A) sacrifice with isoflurane and CO_2 asphyxiation or (B) receipt of 2.5×10^7 CFSE-labeled thymocytes (35-40% AnnexinV+ apoptotic cells) i.p. Animals that received apoptotic thymocytes were housed for a further 30-minute incubation period prior to sacrifice. All necropsies were performed as follows. Peritoneal lavage was conducted with an initial 750 µL volume for protein analysis and subsequent 3 mL volume for cell collection in sterile 0.5% Bovine Serum Albumin (ThermoFisher), and 5mM EDTA (Invitrogen) in 1x PBS (Wisent Inc.). Cells were washed and counted on a hematocytometer. Single cell suspensions were then processed for FACS analysis.

2.23 – FACS staining and analysis

Single cell suspensions were stained with antibodies (Table 2.1). Where biotinylated antibodies were used, streptavidin-BV785 was subsequently conjugated for visualization (BioLegend). Isotype control antibodies and fluorescence minus one staining were used to establish gating. Stained peritoneal cells were acquired for analysis using a BD LSRFortessa cytometer. Data was analyzed using FlowJo software (Ashland, OR).

2.24 – Immunoassays

Cell culture supernatants, tissue lysates, and peritoneal lavage fluids were assayed for cytokines, chemokines and growth factors of interest by Luminex assay and ELISA. For Luminex of human patient plasma samples, fluorescence-coded magnetic microparticles coated with antibodies specific for NT-proBNP, Galectin-3, ST2, CCL2, TNFR2, MMP-2, and MMP-9 were purchased from R&D Systems and eBioscience (now ThermoScientific). For Luminex of murine peritoneal lavage samples, fluorescence-coded magnetic microparticles coated with antibodies specific for IL-4, IL-10, IL-13, GM-CSF, M-CSF, CCL2, CCL5 and VEGF were purchased from R&D Systems. The Luminex assay was conducted according to manufacturer's instructions. The samples were read using a Bio-Rad Bio-Plex 2 (Bio-Rad, Mississauga, Canada). ELISA was used to probe for PGD_2 (Cayman Chemical, Ann Arbor, Michigan), PGE_2 (Cayman Chemical), IL-6 (Biolegend), IL-10 (Biolegend), IL-13 (R&D), MMP-9 (R&D), TGF-β (R&D), TNF (R&D), uPA (R&D) and VEGF-A (R&D) in human cell culture supernatants.

2.24.1 – Leukotriene C_4 ELISA

Leukotriene C_4 (LTC_4) ELISAs were performed according to a lab developed protocol. Plates were coated with 1 µg/mL anti-LTC_4 antibody (Enzo Life Sciences, Farmingdale, NY) in 2.5 µg/mL borate buffer and incubated at 4°C overnight. Plates were blocked with 2% BSA (ThermoFisher) in 1x PBS for 2 hours. Samples and recombinant LTC_4 standard were defrosted and kept on ice prior to addition, diluted in 1% BSA in 1x PBS. Samples were incubated for 1 hour at room temperature (RT) prior to washing and addition of 2.5% glutaraldehyde (Sigma) for 5 minutes at RT. Plates were washed and received 0.1M NaBH4 (Sigma) for 5 minutes at RT, followed by washing

and addition of 100% Methanol (Sigma) for 2 minutes at RT. Plates then received 0.5 μg/mL in-house biotinylated anti-LTC_4 (Enzo Lifesciences) for 1 hour at RT. Streptavidin-HRP was added to plate for 30 minutes, followed by TMB substrate solution (Invitrogen) to develop for up to 30 minutes. The reaction was stopped with 2N H2SO4 and absorbance measured at 450 nm.

2.25 – Statistical analyses

For Chapter 3, D'Agostino-Pearson normality tests were conducted to determine appropriate statistical comparisons. Mann-Whitney U tests for non-parametric distribution were used to compare high and low mast cell containing patient groups relative to a number of other parameters, Pearson linear regression was used to assess significance of correlations, and a combination of Mann-Whitney U, chi-square, and Fisher's exact tests were used to compare variables in tables 1 and 2. Kaplan-Meier curves were used to assess end outcomes of NYHA re-classification or mortality. Post-hoc ANOVA was used to assess gene expression differences in CBMCs vs. PBMCs. Correlation matrices were generated in R (R Foundation for Statistical Computing, Vienna, Austria), while all other tests were conducted with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

For Chapter 4, D'Agostino-Pearson normality tests were conducted to determine appropriate statistical approach. Unpaired students t-tests were used to assess significant differences between untreated (mock) and IL-33 activated CBMCs and HUVECs, ordinary one-way ANOVA was used to assess differences between groups in degranulation experiments and HUVEC qPCR experiments, and Pearson linear regression was used to assess significance of correlations. All tests were conducted with GraphPad Prism (GraphPad Software Inc.).

For Chapter 5, D'Agostino-Pearson normality tests were conducted to determine appropriate statistical approach. Unpaired students t-tests and ordinary one-way ANOVA (FM+IL-33) or two-way ANOVA (large and small peritoneal macrophages in saline and IL-33-treated groups) were conducted to examine statistical significance between groups. Outliers were removed based off of ROUT outlier tests of neutrophil and overall cell number within each experimental group. All tests were conducted with GraphPad Prism (GraphPad Software Inc.).

Table 2.1. – Antibodies for flow cytometry experiments

Target antigen	Species	Fluorochrome	Company	Clone
Panel 1				
CD163	Mouse	APC	Biolegend	S150491
CD80	Mouse	Biotin	Biolegend	16-10A1
CCR2	Mouse	FITC	Biolegend	SA203G11
MerTK	Mouse	PE	Biolegend	21310C42
Ly6C	Mouse	APC efluor780	Invitrogen (now ThermoFisher)	HK1.4
F4/80	Mouse	PE Cy7	Invitrogen (now ThermoFisher)	BMB
MHCII	Mouse	Alexa Fluor 700	Invitrogen (now ThermoFisher)	M51114.15.2
CX3CR1	Mouse	PerCP Cy5.5	Biolegend	SA1011F11
CD206	Mouse	BV421	Biolegend	C068C2
CD11b	Mouse	BV650	BD Biosciences	M1/70
Panel 2				
ST2	Mouse	APC	eBioscience (now ThermoFisher)	RMST2-2
Ly6G	Mouse	Biotin	Biolegend	1A8
NK1.1	Mouse	FITC	Invitrogen (now ThermoFisher)	PK136
CD3	Mouse	PE	eBioscience (now ThermoFisher)	145-2C11
SIGLECF	Mouse	PE CF594	BD Biosciences	E50-2440
CD4 (in efferocytosis and free mitochondria experiments)	Mouse	Alexa Fluor 700	Biolegend	GK1.5
FcεRI	Mouse	PerCP efluor710	eBioscience (now ThermoFisher)	Mar-1
CD117	Mouse	BV421	BD Biosciences	2B8
Ly6C	Mouse	APC efluor780	Invitrogen (now ThermoFisher)	HK1.4
F4/80	Mouse	PE Cy7	Invitrogen (now ThermoFisher)	BMB
ST2 (in later experiments)	Mouse	BV605	Biolegend	D1H9
CD11b	Mouse	BV650	BD Biosciences	M1/70

Panel 3				
CD19	Mouse	BV605	BD Biosciences	1D3
CD5	Mouse	APC	Biolegend	53-7.3
IgM	Mouse	BV711	BD Biosciences	R6-60.2
ST2	Mouse	BV605	Biolegend	D1H9
CD11b	Mouse	BV650	BD Biosciences	M1/70
Cell Sort Experiment				
F4/80	Mouse	PerCP	Biolegend	BM8
CD11b	Mouse	PE Cy7	Biolegend	M1/70
CD117	Mouse	FITC	eBioscience (now ThermoFisher)	2B8
SIGLECF	Mouse	PE CF594	BD Biosciences	E50-2440

CHAPTER 3: INCREASED MAST CELL DENSITY IS ASSOCIATED WITH DECREASED FIBROSIS IN HUMAN ATRIAL TISSUE

This work appears in part in the publication: Legere, SA, Haidl, ID, Castonguay, MC, Brunt, KB, Légaré, JF, Marshall, JS, IMPART investigator team Canada. Increased mast cell density is associated with decreased fibrosis in human atrial tissue, *Journal of Molecular and Cellular Cardiology*. 2020. doi:10.1016/j.yjmcc.2020.09.001. This publication is open access, see appendix C for permissions.

Author Contributions: SAL designed the study, performed the majority of experiments, analysed data and wrote the paper. IDH designed aspects of the research, performed experiments and helped write the paper. MCC analysed data and helped write the paper. KB contributed analytic tools and helped write the paper. JFL designed clinical aspects of the study, analysed data and helped write the paper. JSM designed laboratory aspects of the study, analysed data and helped write the manuscript. The IMPART team provided clinical information and surgical tissues as well as analytical tools essential for this study.

3.1 – Introduction

Myocardial fibrosis is the accumulation of extracellular matrix components, such as collagen, within the myocardium. This process accompanies many forms of heart disease and is associated with poor patient outcomes [230]. However, some fibrosis is necessary for proper wound healing after myocardial injury, such as myocardial infarction or hypertension-induced stretch injury. Fibrosis can occur in all chambers of the heart and is often associated with myocardial stiffening and reduced signal transduction that ultimately leads to heart failure [577]. Understanding myocardial fibrosis requires better characterization of the fibrotic microenvironment for the development of future therapeutic strategies.

Evidence suggests that myocardial fibrosis is driven by dysregulated inflammation [230], implicating the immune system is a contributor to its pathogenesis. Resident immune cells, such as mast cells and macrophages, are thought to contribute to fibrotic remodeling in response to damage to the myocardium indirectly [238] or directly [578,579]. Mast cells are innate immune sentinel cells that respond to a variety of stimuli, including damage-associated alarmins [580], through a wide array of pattern recognition and mediator receptors. Upon activation, mast cells can degranulate to release stores of preformed mediators, or secrete a variety of compounds *de novo* with or without degranulation [5]. After myocardial injury, mast cell density is increased in animal models [353,581]. However, the exact role of mast cells in the pathogenesis of myocardial fibrosis is not well understood. Both beneficial and detrimental roles have been suggested [73,75], as various mast cell mediators have been shown to have pro- and anti-fibrotic effects [75]. Previous studies have often only considered mast cell degranulation as evidence of

activity. Mast cell granule contents are known to contain pro-fibrotic compounds such as chymase, tryptase, and TGF-β. However, as previously mentioned, mast cells can respond to stimuli released in the heart after damage without degranulation. Mast cells have been shown to produce anti-fibrotic compounds, such as CXCL10 [166,170], VEGF-A [582], and IL-33 [5,583], in other disease settings, yet it is unknown if these mediators are up-regulated by mast cells after myocardial damage. Studies in mice have been controversial, partly due to fundamental differences in the distribution and phenotype of myocardial mast cell populations between mice and humans, and their ontogeny as resident or itinerant populations [271]. To date, few studies have examined mast cells in human chronic cardiovascular disease [137,357–360].

Therapeutic approaches to limiting myocardial fibrosis have largely focused on blocking canonical pro-fibrotic pathways, such as the Renin-Angiotensin-Aldosterone System (RAAS) [584], rather than inducing anti-fibrotic mediators. There is increased recognition of the importance of inflammation in the pathogenesis of cardiovascular disease as a whole [170] including myocardial fibrosis [230]. Mast cells can respond to a wealth of stimuli [5, 7,585] to maintain homeostasis, and quickly respond to infection or damage, shaping the inflammatory response that follows. Therefore, we hypothesize that mast cells could play a critical role in regulating cardiac remodeling and as such, they could provide an important cellular mechanism determining the nature of fibrosis and tissue restitution following cardiac damage [5,75].

The current study examined the relationship between mast cells and myocardial fibrosis using tissues from cardiac surgery patients with established cardiovascular disease. Human right atrial appendage tissue was excised during cardiac surgical

procedures and used to provide insight into fibrotic remodelling and resident mast cells. Detailed preoperative and postoperative variables were collected in all patients to determine how mast cell density from tissue samples could relate to patient outcomes. This study demonstrates an association between high levels of atrial tissue mast cells, reduced fibrosis, and improved functional outcomes. These data indicate that increased mast cell density provides a cellular mechanism for reducing myocardial fibrosis in human patient samples.

3.2 – Results

3.2.1 – Mast cells are present in varying numbers in human atria

Human atrial tissue samples were obtained while patients (n=112) underwent cardiac surgery at two centres in Atlantic Canada (Halifax, NS, and Saint John, NB). The procedures performed included: isolated CABG (54%), isolated valve (21%), and combined CABG/valve (25%). The average heart function (defined by ejection fraction, EF) for the entire cohort of patients was 58% ± 13%, with the majority (94%) having a normal EF (defined as ≥ 50%). A significant proportion of patients (40.2%) were diagnosed with clinical symptoms of heart failure (NYHA functional class III/IV) pre-operatively (Table 3.1). This indicates a large proportion of patients in this cohort experienced Heart Failure with Preserved Ejection Fraction. Mast cell density was assessed in atrial tissues by Toluidine blue staining (Fig. 3.1A). Mast cells were observed in 94% of samples and primarily found in the myocardium, some associated with blood vessels or others within the tissue proper (e.g. within the myocardium). The median mast cell density was 7.3 ± 2.67 mast cells/mm^2 and was similar between male and female patients (p=0.664, Fig. 3.1B) and was not normally distributed (Fig. 3.2A). To determine how atrial mast cell density was related to cardiac function and other clinically relevant variables, patients were divided into quartiles based on mast cell content. These groups did not differ on the basis of EF, Body Mass Index (BMI), hypertension, or other comorbidities, though patients with high mast cell density (above the median) had increased incidence of cerebrovascular disease (Table 3.2). Patients with low mast cell density (below the median) had a 17.8% longer length of stay in hospital (LOS, ≥9 days in hospital prior to discharge, p=0.03) compared to those with high mast cell density

(Table 3.2). When assessing patients in the upper (>75%) and lower (<25%) quartiles of mast cell density, it was observed that patients in the lower quartile had increased incidence of pre-operative atrial fibrillation (AF), elevated Left Ventricular End Diastolic Pressure (LVEDP), and greater BMI compared to those in the upper quartile (Table 3.3). There was mild to moderate pericellular myocardial fibrosis in the majority of the atrial tissue sections as determined by Sirius red fast green (SRFG) [573] (Fig. 3.1D, F). One patient exhibited mural thrombus. Patients in the upper and lower quartiles of collagen content were assessed for general pathophysiology by an experienced clinical pathologist [586]. There was limited myocyte hypertrophy (31.9% of patients with mild hypertrophy and 2.1% of patients with moderate hypertrophy), and no vacuolization, or interstitial inflammation observed in H&E stained atrial tissue sections (Fig. 3.1C, E).

Table 3.1. – Total patient demographics from Halifax, NS and St John, NB, Canada.

Patient demographics (N=112)		
Age (years)		63.5 +/- 8.4
Female		22.3%
Comorbidities		
	Diabetes	29.5%
	Hypertension	50.9%
	Dyslipidemia	67.9%
	Body Mass Index	30.8 +/-6.5
	Peripheral vascular disease	5.4%
	Cerebrovascular disease	6.3%
	Renal failure (Creatinine >179 mmol/L)	1.8%
	Atrial fibrillation	15.2%
Ejection Fraction		58 +/- 13
Left Ventricular End Diastolic Pressure		18 +/- 10
% Patients Ejection Fraction >50		93.8%
% NYHA III-IV		40.2%
Etiology of heart disease	ischemic	78.6%
	non-ischemic	21.4%
Type of procedure	Isolated Coronary Artery Bypass Graft (CABG)	52.7%
	Isolated Valve Replacement (VR)	21.4%
	(CABG+VR)	25.9%
Medications	Anti-platelet (including ASA)	83.9%
	Statins	75.9%
	ACE inhibitors	46.4%
	ARB	14.3%
Neutrophil:Lymphocyte Ratio (NLR)		3.4 +/- 3.6
% Collagen in atrial sample		39.6% +/- 20.4

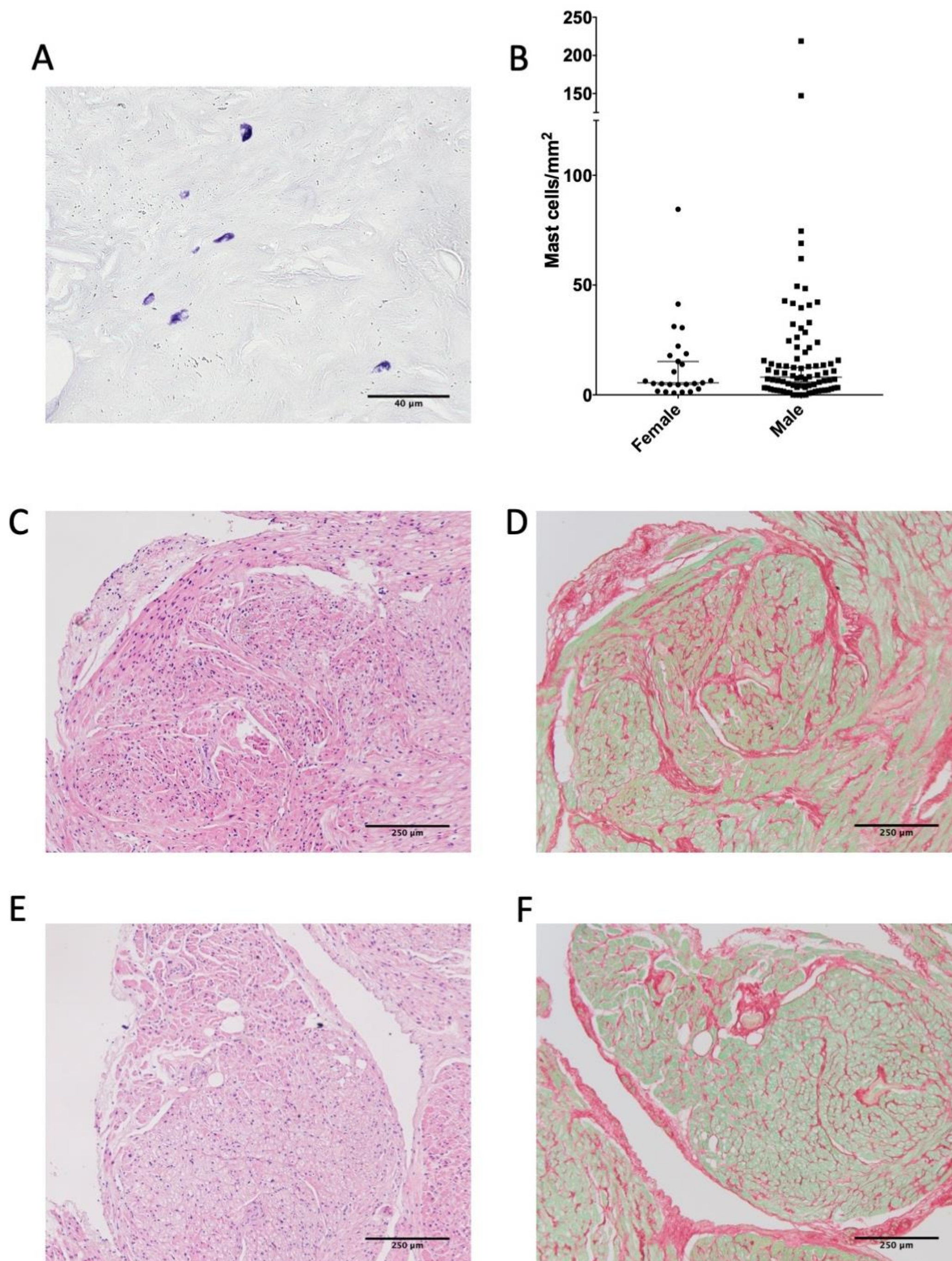


Figure 3.1. – Human atrial tissue histological assessment for mast cells, gross pathology and collagen content. Atrial tissues were stained for mast cells using toluidine blue (40x, A). Mast cells were counted, and surface area of tissue sections was assessed to reflect mast cell/mm^2. Male and female patients had equivalent mast cell content (B). H&E (C, E) and SRFG (D, F) stains were used to assess myocyte hypertrophy, inflammatory infiltrates, mural thrombus, and fibrosis (10x, C-F).

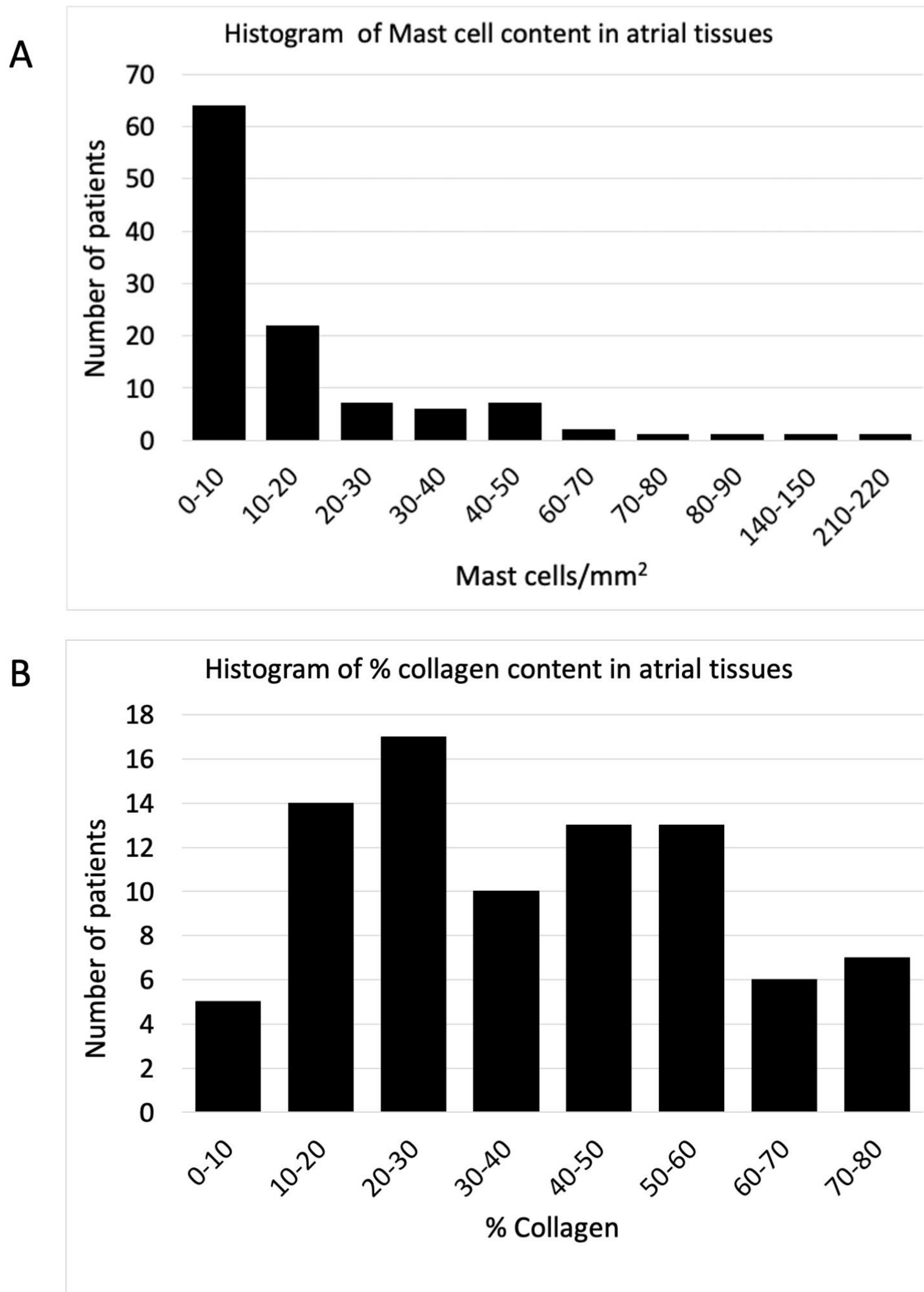


Figure 3.2. – Histogram distribution among patients from Halifax, NS, and St. John, NB. Patients (n=112) were stratified based on mast cell content at the time of surgery by toluidine blue staining (A) in comparison to total surface area in the tissue sections. Patients were also stratified by collagen content by Sirius red fast green staining (B) and photoshop-based quantification of scar tissue in atria at the time of surgery.

Table 3.2. – Patient demographics based on mast cell content in atria at the time of cardiac surgery

		Low MC (n=56)	High MC (n=56)	p value
Age		64.0	65.0	0.68
Female sex		30.4%	17.9%	0.12
Diabetes		28.6%	30.4%	0.84
Hypertension		44.6%	57.1%	0.19
Dyslipidemia		60.7%	75.0%	0.11
Peripheral Vascular Disease (PVD)		3.6%	7.1%	0.40
Cerebrovascular Disease		1.8%	10.7%	0.05
Renal failure		3.6%	0.0%	0.15
Ejection Fraction		59.0	60.0	0.82
LVEDP		18.0	15.0	0.39
Pre-op atrial fibrillation		21.4%	8.9%	0.07
% NYHA III-IV at surgery		39.3%	41.1%	0.85
% Patients EF >50		66.1%	66.1%	0.99
Urgency of procedure		23.2%	8.9%	0.04
BMI		31.1	29.6	0.20
	Normal	17.9%	23.2%	0.48
	Pre-obese	23.2%	32.1%	0.29
	Class I	25.0%	26.8%	0.83
	Class II	16.1%	12.5%	0.59
	Class III	17.9%	5.4%	0.04
Etiology of heart disease	Ischemic	76.8%	80.4%	0.65
	Non-ischemic	23.2%	19.6%	0.65
In-hospital				
	Atrial fibrillation	42.9%	41.1%	0.85
	In-hospital mortality	5.4%	1.8%	0.31
	LOS ≥ 9 days	35.7%	17.9%	0.03
Long-term outcomes				
% NYHA III-IV at follow up		16.1%	7.1%	0.14
Loop diuretic (Y)		21.4%	3.6%	0.004
Readmission		12.5%	14.3%	0.78
Troponin		551.00	667.50	0.11
Procedure	CABG	42.9%	62.5%	0.04
	VR	23.2%	19.6%	0.65
	CABG+VR	33.9%	17.9%	0.05

Table 3.3. – Patient demographics based on upper and lower quartiles of mast cell content in atria at the time of cardiac surgery

		<25% MC	>75% MC	p value
Age		64.0	64.5	0.98
Female sex		25.0%	21.4%	0.75
Diabetes		39.3%	35.7%	0.78
Hypertension		53.6%	67.9%	0.27
Dyslipidemia		60.7%	78.6%	0.15
PVD		3.6%	7.1%	0.55
Cerebrovascular Disease		0.0%	14.3%	0.04
Renal failure		0.0%	0.0%	0.99
Ejection Fraction		57.0	59.0	0.99
LVEDP		18.0	13.5	0.03
Pre-op atrial fibrillation		28.6%	3.6%	0.01
% NYHA III-IV at surgery		46.4%	35.7%	0.42
% Patients EF >50		64.3%	67.9%	0.78
Urgency of procedure		10.7%	0.0%	0.08
BMI		32.2	28.5	0.04
	Normal	17.9%	28.6%	0.34
	Pre-obese	17.9%	32.1%	0.22
	Class I	21.4%	25.0%	0.75
	Class II	25.0%	10.7%	0.16
	Class III	17.9%	3.6%	0.08
Etiology of heart disease	Ischemic	78.6%	85.7%	0.49
	Non-ischemic	21.4%	14.3%	0.49
In-hospital outcomes				
	Atrial fibrillation	50.0%	39.3%	0.42
	In-hospital mortality	3.6%	0.0%	0.31
	LOS >/=9 days	28.6%	10.7%	0.09
Long-term outcomes				
% NYHA III-IV at follow up		17.9%	3.6%	0.08
Loop diuretic (Y)		21.4%	0.0%	0.010
Readmission		10.7%	14.3%	0.69
Troponin		578.00	634.00	0.86
Procedure	CABG	39.3%	67.9%	0.03
	VR	21.4%	14.3%	0.49
	CABG+VR	36.7%	17.9%	0.11

3.2.2 – Mast cell density is associated with expression of mast cell-specific genes

To confirm histological assessment of mast cell density in human atria independent of levels of cell granulation, an assay was developed to analyze mast cell content through ddPCR. Expression of two mast cell-specific genes was assessed in human atrial tissue samples. This included samples from high and low mast cell density patients. RNA was isolated from all available tissues, however only a portion of the RNA obtained was of appropriate quality and integrity for subsequent analysis. Such RNA degradation was likely the result of variation in time from surgical excision to storage at -80°C. *SIGLEC6* encodes a membrane embedded protein highly expressed in mast cells [342,587] and a subset of circulating dendritic cells [588] that have not been observed in heart tissue. *MS4A2* encodes the FcεR1 β-chain that is restricted to mast cell surfaces in cardiac tissue [342,587], but also expressed by basophils in circulation. *SIGLEC6* and *MS4A2* were highly expressed in unstimulated cord blood-derived mast cells (CBMCs) and under various activating conditions compared to peripheral blood mononuclear cells (PBMCs) at rest (Fig. 3.3A, *** $p<0.001$, **** $p <0.0001$,). These data indicate the utility of these genes for mast cell quantification because their expression is activation independent and would not be expected to be influenced by mast cell-activating stimuli in the myocardium. Histologically determined mast cell density was positively correlated to both *MS4A2* (Fig. 3.3B, $r^2 = 0.6056$, $p < 0.0001$) and *SIGLEC6* (Fig. 3.3C, $r^2 = 0.494$, $p = 0.0001$) expression in atrial tissue as represented by normalized standard amount (NSA) through ddPCR (see methods). The range of mast cell densities observed via histology was wide, possibly the result of the limited amount of tissue sampled in individual sections. In contrast, RNA signals convey a sum of the entire tissue sample. The observed

correlation indicated that mast cell density, determined via histology, was not substantially biased by lack of detection of degranulated, undetected mast cells in our histological samples.

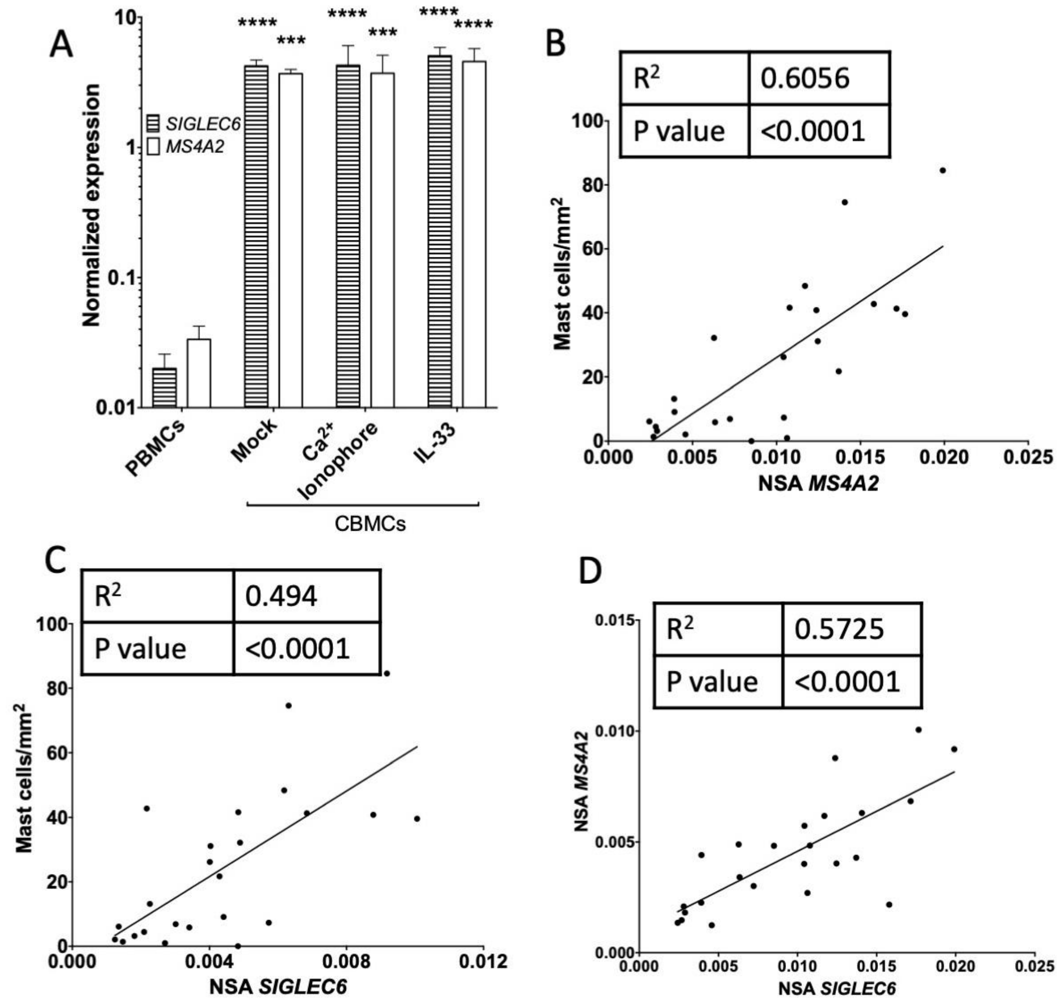


Figure 3.3. – Mast cell-specific genes reflect mast cell content as determined by histology. Cord blood-derived mast cells (CBMCs) had increased expression of mast cell-specific genes *SIGLEC6* and *MS4A2* under various activating conditions compared to peripheral blood mononuclear cells (PBMCs) (A). Mast cell counts in select patient samples (n=24) determined by histology were positively and significantly correlated to expression of *MS4A2* (B) and *SIGLEC6* (C). Both *MS4A2* and *SIGLEC6* expression were significantly and positively correlated to each other (D).

3.2.3 – Mast cell specific genes in other fibrotic disease settings

In human atrial tissue samples assayed in the current study, NSA of *MS4A2* and *SIGLEC6* were positively correlated to each other (Fig. 3.3D, r^2=0.5725, p<0.0001). To confirm that mast cell specific genes were relevant in other disease settings, public microarray datasets were accessed via NCBI through query for keywords "human fibrosis" and "human heart". Two separate and independent RNAseq studies were chosen where *MS4A2* and *SIGLEC6* gene expression was detected in human lung explants from pulmonary fibrosis lung patients (GDS4549) and human atrial tissue from CABG patients (GDS2772). *MS4A2* and *SIGLEC6* relative mRNA expression were positively correlated in both human lung (Fig. 3.4A, r^2=0.3878, p<0.0001) and human atrial tissue (Fig. 3.4B, r^2=0.6619, p<0.0001) from independent studies.

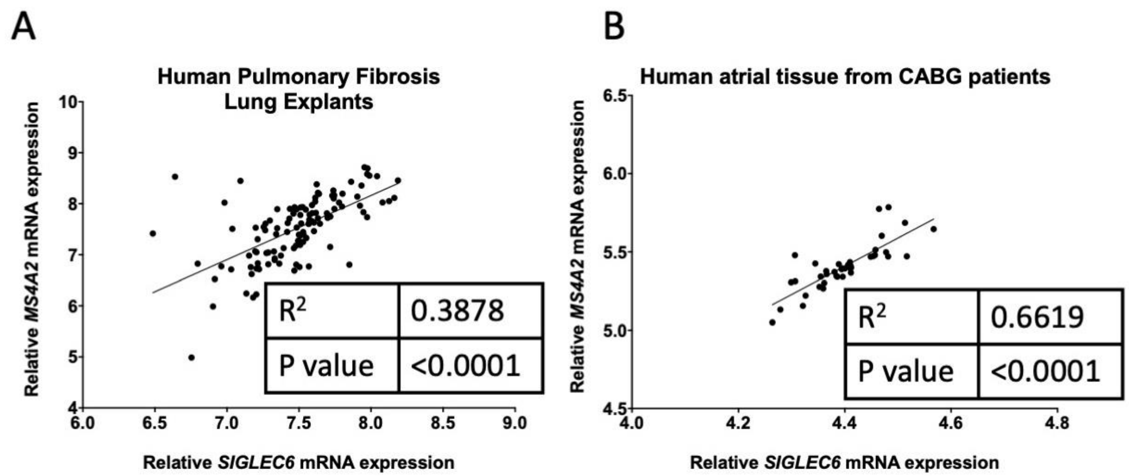


Figure 3.4. – Expression of mast cell-specific genes is consistent in the lungs as well as atria. GEO datasets were probed for relative mRNA expression of *MS4A2* and *SIGLEC6,* which were found to be positively correlated to each other in human pulmonary fibrosis lung explants (GDS4549, A) and human atrial tissue samples (GDS2772, B).

3.2.4 – Mast cell density is related to severity of atrial fibrosis and outcomes.

Collagen content was quantified on the basis of SRFG staining as previously described [574,589] (Fig. 3.1D, F). The median collagen content was 37.7 ± 20.2%. Fibrosis was commonly observed with variable severity (Fig. 3.2B). Patients with low collagen content in atria, below the median, had increased incidence of hypertension but decreased incidence of both pre-operative AF and prolonged LOS compared to those with high (above the median) atrial collagen content (Table 3.4). Varying collagen content was observed in relation to patients' mast cell density (Fig. 3.5A). Collagen content did not vary on the basis of sex (p=0.26, Fig. 3.5B). Patients defined as having high atrial mast cell density at the time of surgery, with mast cell densities above the 75th percentile, had a significantly lower atrial collagen level compared to both patients with low atrial mast cell content (p=0.036, Fig. 3.5C) and the remaining cohort as a whole (<75%, p=0.0106, Fig. 3.5D). The relationship between collagen content and mast cell density was independent of clinically defined metabolic or cardiovascular comorbidity (Table 3.2), or dysregulation of inflammatory markers in plasma (Fig. 3.6). A more than two-fold difference in pre-operative AF was observed between patients above and below the median mast cell density, but this failed to reach statistical significance (Table 3.2). No sex dependent differences in collagen content were observed (Fig. 3.5B, p=0.26). Mast cell degranulation was assessed in patients at the upper and lower quartiles of collagen content, as mast cell granule contents are known to be associated with fibrotic remodelling [75]. It was found that patients above the 75th percentile for collagen content had more degranulating mast cells in their atria than those below the 25th percentile (Fig. 3.5E, p=0.0020). This was not reflected in the larger low and high mast cell groups (Fig.

3.7, $p=0.877$). Within our cohort of human cardiac surgical patients, key biomarkers for fibrotic remodelling, inflammation, and heart failure were assessed in plasma by multiplex protein assays, where samples were available. Patients with high mast cell content (>75%) at the time of surgery had significantly less plasma MMP-9 content compared to low mast cell content (<25%, Fig. 3.5F, $p=0.0176$) and the remaining patients within the cohort (<75%, Fig. 3.5G, $p=0.0039$). Although only a small portion of atrial tissue explants were large enough to evaluate protein level, it was found that atrial MMP-9 was positively correlated to plasma MMP-9 (Fig. 3.6H, $r^2=0.3747$, $p=0.045$). Therefore, plasma MMP-9 level was, to some extent, reflective of tissue content. Levels of the heart failure biomarker NT-proBNP were similar between high and low mast cell patients at the time of surgery (Fig. 3.6A), as were several other mediators (Fig. 3.6C-G), while GDF-15 was significantly increased in low mast cell density patients (Fig. 3.6B). Of note, sST2 content in patient plasma was positively correlated to atrial collagen content at the time of surgery (Fig. 3.6J, $r^2=0.0834$, $p=0.0146$). Heart failure symptoms were assessed clinically using the New York Heart Association (NYHA) classification system in a subset of patients in this study during post-discharge follow-up. Post-operative outcomes analyzed using Kaplan Meier analysis (time) comparing mast cell content revealed that patients with low atrial mast cell content (<25%) have less freedom from NYHA III/IV heart failure symptoms or mortality than patients with high atrial mast cell content (>75%, Fig. 3.5H, $p=0.0155$). Additionally, patients with high atrial mast cell density had significantly increased freedom from NYHA III/IV heart failure symptoms or mortality than the rest of the patients in the cohort overall (<75%, Fig. 3.5I, $p=0.0227$).

Table 3.4. – Patient demographics based on collagen content in atria at the time of cardiac surgery

		Low Collagen (n=49)	High Collagen (n=49)	p value
Age		63.0	65.0	0.17
Female sex		22.5%	28.6%	0.64
Diabetes		32.7%	22.5%	0.37
HTN		59.2%	32.7%	0.02
Dyslipidemia		63.3%	69.4%	0.67
PVD		4.1%	2.0%	0.99
CVD		8.2%	4.1%	0.68
Renal failure		2.0%	2.0%	0.99
EF		62.0	56.5	0.36
LVEDP		16.0	15.5	0.95
Pre-op atrial fibrillation		8.2%	24.5%	0.05
% NYHA III-IV at surgery		36.7%	40.8%	0.84
% Patients EF >50		73.5%	59.2%	0.53
BMI		30.24	30.9	0.98
	Normal	18.4%	20.4%	0.99
	Pre-obese	30.6%	24.5%	0.65
	Class I	22.5%	28.6%	0.64
	Class II	14.3%	16.3%	0.99
	Class III	14.3%	10.2%	0.76
Etiology of heart disease	ischemic	71.4%	81.6%	0.34
	non-ischemic	28.6%	18.4%	0.34
In-hospital waiting		8.16%	28.57%	0.017
Procedure	Isolated CABG	42.6%	36.5%	0.58
	Isolated VR	28.6%	18.4%	0.34
	CABG+VR	18.4%	34.7%	0.11
Short-term outcomes	Mortality	4.1%	6.1%	0.99
	Troponin	636	443	0.12
	LOS > 9 days	14.3%	40.8%	0.006

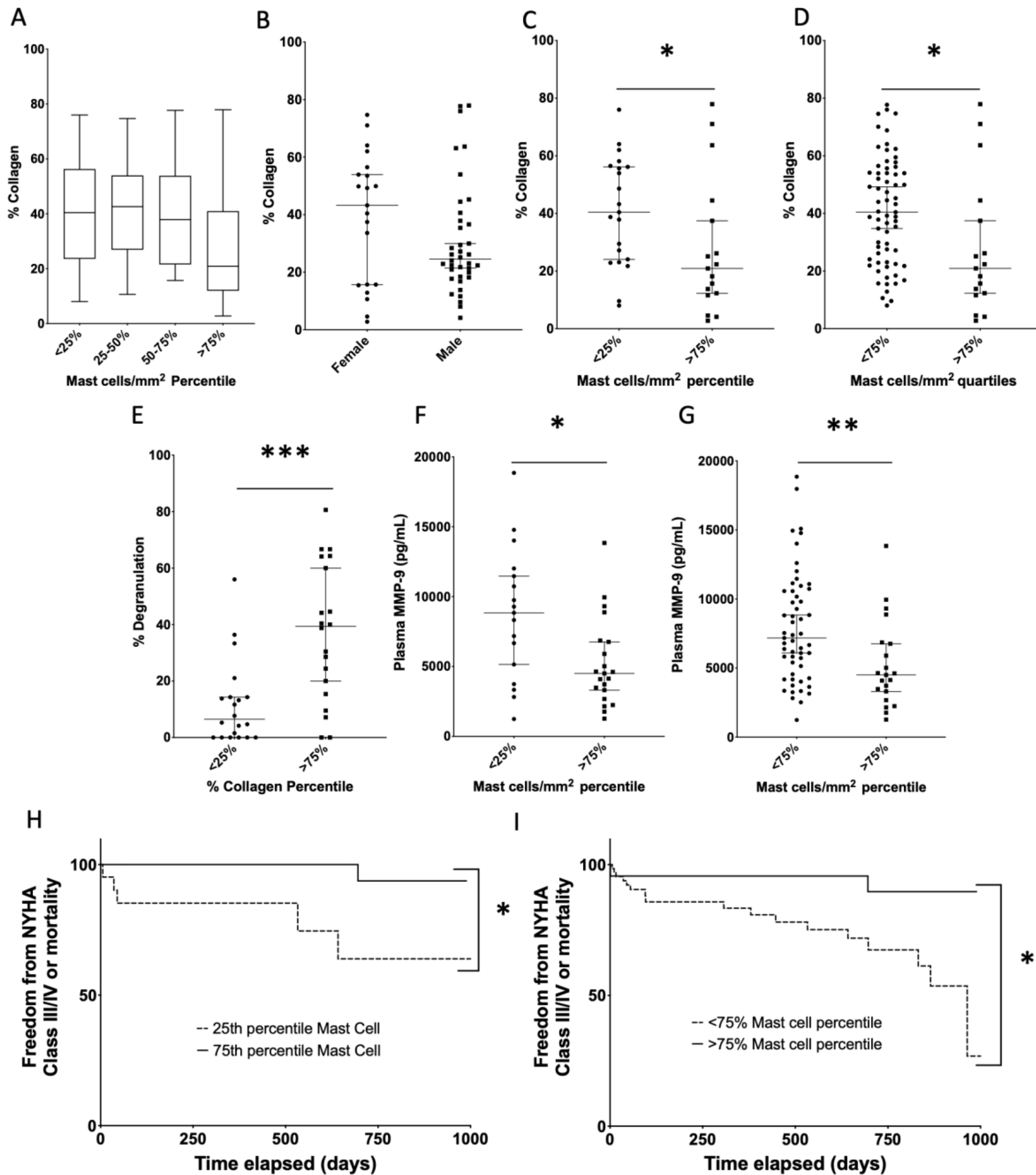


Figure 3.5. – Fibrosis in human atrial tissue is associated with mast cell content. Patients had varying degrees of collagen content in their atria at the time of surgery (n=84, A). Collagen content did not differ on the basis of sex (B). Patients in the 75th percentile of atrial mast cell density had significantly less collagen in their atria at the time of surgery compared to those in the 25th percentile (C) and those below the 75th percentile (D). Patients in the 75th percentile of collagen content had more degranulating mast cells in their atria than those in the 25th percentile (n=48, E). Patients with high atrial mast cell content had decreased MMP-9 plasma concentrations compared to in the 25th percentile (F) as well as those below the 75th percentile (G). Post-operative outcomes, as assessed by NYHA III/IV classification and mortality, were better in high mast cell containing patients compared to low (H) and compared to those below the 75th percentile (I).

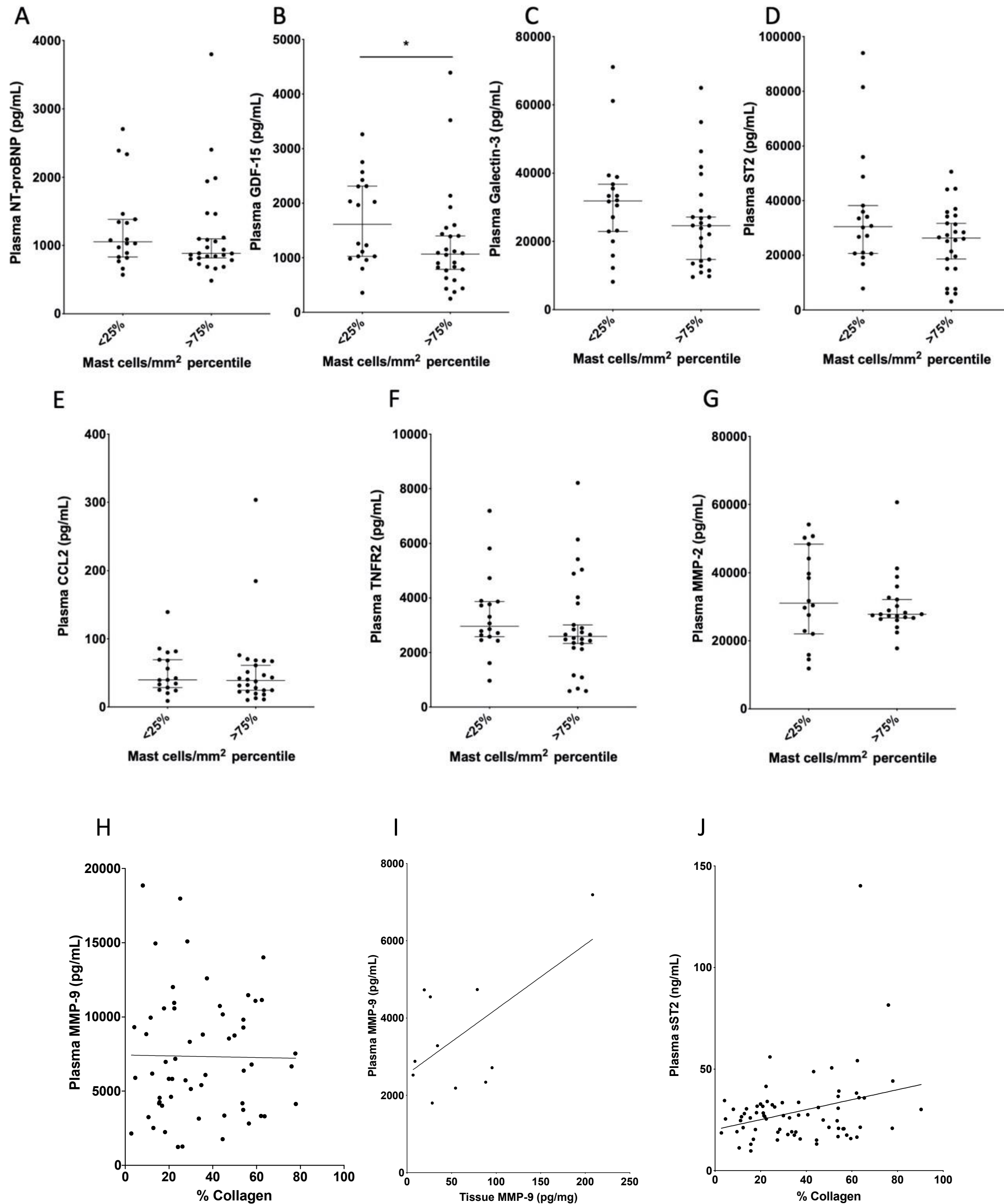


Figure 3.6. – Levels of plasma biomarkers are not related to mast cell content. When comparing patients in the upper and lower quartile of atrial mast cell density, NT-proBNP (A), GDF-15 (B), Galectin-3 (C), ST2 (D), CCL2 (E), TNFR2 (F), and MMP-2 (G) concentrations in plasma were assessed. MMP-9 was not correlated to collagen content in atria at the time of surgery (H). MMP-9 content in plasma was positively correlated to MMP-9 content measured in tissue (I). sST2 plasma content was positively correlated to atrial collagen content at the time of surgery (J).

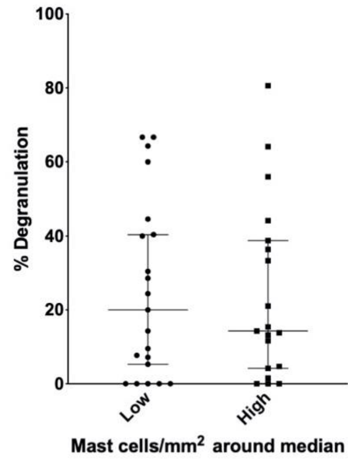


Figure 3.7. – Mast cell degranulation activity in human atrial tissue was equivalent between patients with high and low mast cell densities around the median. The proportion of degranulating mast cells in comparison to total mast cells in a subset of human atrial tissue were assessed by toluidine blue staining of human atrial tissue samples taken at time of surgery.

3.2.5 – Mast cell relevance to fibrosis and outcomes persists in a more homogeneous patient population

Patients within our cohort were broadly recruited while undergoing cardiac surgical procedures (Table 3.1). To limit multivariate impacts and gain a better understanding of how mast cells may relate to atrial fibrosis, we focused further on patients undergoing CABG surgery (n=88). Within the CABG cohort, patients below the median mast cell density had similar incidence of AF, diabetes and other co-morbidities compared to the cohort at large (Table 3.5, 3.6). CABG patients with high mast cell density, in the upper quartile, had significantly less atrial collagen content than patients below the lower quartile (Fig. 3.8A, p=0.0148) and the remaining patients in the cohort (<75% mast cell density, Fig. 3.8B, p=0.0028). High mast cell density patients also had significantly lower plasma MMP-9 content than the remaining patients in the cohort (<75%, Fig. 3.8C, p=0.0301). As was observed in the overall cohort, patients above the 75th percentile of collagen content within the CABG cohort had significantly more degranulating mast cells than those below the 25th percentile (Fig. 3.8D, p=0.0463). Finally, patients with high mast cell density had increased freedom from NYHA III/IV and mortality compared to the remaining patients in the cohort (Fig. 3.8E, p=0.0207). Overall, the majority of the relationships observed in high mast cell density patients in the broad cohort were still relevant within a more defined and homogeneous surgical subpopulation.

Table 3.5. – Patient demographics based on mast cell content in atria at the time of CABG surgery

		Low MC (n=44)	High MC (n=44)	p value
Age		63.5	65.0	0.24
Female sex		27.3%	11.4%	0.06
Diabetes		25.0%	36.4%	0.25
Hypertension		47.7%	65.9%	0.09
Dyslipidemia		68.2%	81.8%	0.14
Peripheral Vascular Disease		4.6%	9.1%	0.40
Cerebrovascular Disease		2.3%	9.1%	0.17
Renal failure		2.3%	0.0%	0.31
Ejection Fraction		61.0	59.0	0.75
Left Ventricular End Diastolic Pressure (LVEDP)		17.0	15.0	0.41
Pre-op atrial fibrillation		20.5%	9.1%	0.13
% NYHA III-IV at surgery		38.6%	47.7%	0.39
% Patients EF >50		70.5%	65.9%	0.46
Urgency of procedure (IHU status)		25.0%	6.8%	0.02
BMI		30.8	29.9	0.58
	Normal	18.2%	25.0%	0.44
	Pre-obese	27.3%	31.8%	0.64
	Class I	25.0%	22.7%	0.80
	Class II	18.2%	15.9%	0.78
	Class III	11.4%	4.6%	0.24
In-hospital				
	Atrial fibrillation	38.6%	47.7%	0.39
	In-hospital mortality	4.6%	2.3%	0.56
	LOS >/=9 days	31.8%	20.5%	0.23
Long-term				
% NYHA III-IV at follow up		13.6%	4.6%	0.14
Loop diuretic (Y)		25.0%	2.3%	0.002
Readmission				
Troponin		564.00	680.00	0.16
Procedure	CABG	56.8%	22.7%	0.04
	CABG+VR	43.2%	77.3%	0.04

Table 3.6. – Detailed surgical information on patients with high and low mast cell content in atria at the time of cardiac surgery

		<25% (n=22)	>75% (n=22)	p value
Age		64.0	65.0	0.28
Female sex		22.7%	9.1%	0.22
Diabetes		31.8%	45.5%	0.35
Hypertension		54.6%	77.3%	0.11
Dyslipidemia		68.2%	86.4%	0.15
Peripheral Vascular Disease		4.6%	9.1%	0.55
Cerebrovascular Disease		0.0%	9.1%	0.15
Renal failure		0.0%	0.0%	0.99
Ejection Fraction		61.0	59.0	0.81
Left Ventricular End Diastolic Pressure (LVEDP)		17.0	15.0	0.13
Pre-op atrial fibrillation		27.3%	4.6%	0.04
% NYHA III-IV at surgery		54.6%	54.6%	0.99
% Patients EF >50		63.6%	72.7%	0.52
Urgency of procedure (IHU status)		13.6%	0.0%	0.07
BMI		31.6	29.4	0.24
	Normal	22.7%	27.3%	0.73
	Pre-obese	18.2%	36.4%	0.18
	Class I	18.2%	18.2%	0.99
	Class II	31.8%	13.6%	0.15
	Class III	9.1%	4.6%	0.55
Outcomes at Follow Up				
In-hospital				
	Atrial fibrillation	50.0%	40.9%	0.54
	In-hospital mortality	4.6%	0.0%	0.31
	LOS >/=9 days	27.3%	13.6%	0.26
Long-term				
% NYHA III-IV at follow up		13.6%	4.6%	0.29
Loop diuretic (Y)		27.3%	0.0%	0.008
Troponin		578.00	668.50	0.73
Procedure	CABG	50.0%	81.8%	0.03
	CABG+VR	50.0%	18.2%	0.03

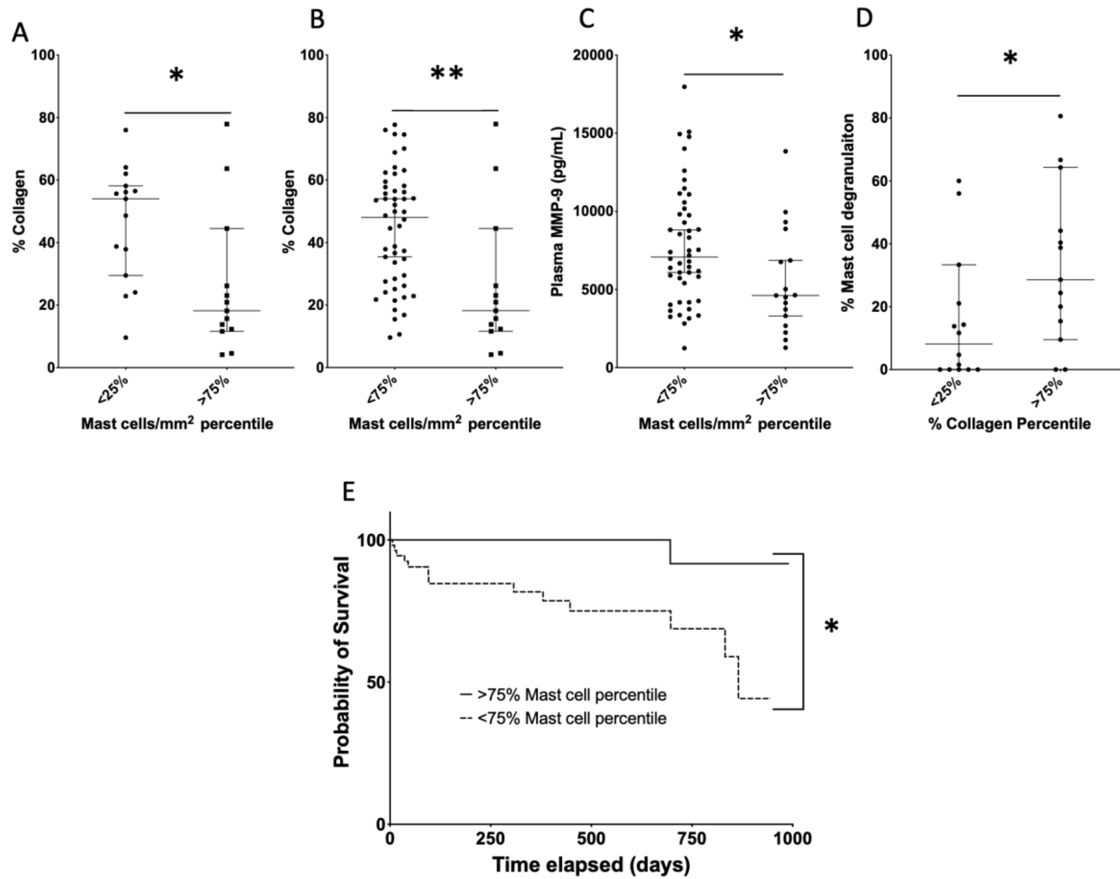


Figure 3.8. – CABG patients reflected observations seen in the heterogeneous cardiac surgical population. CABG patients with high atrial mast cell content (in the 75th percentile) at the time of surgery had significantly lower collagen content compared to their low mast cell density peers (in the 25th percentile A) as well as patients below the 75th percentile (B). Patients with high atrial mast cell content also had decreased MMP-9 plasma concentrations compared to those below the 75th percentile (C). Patients in the 75th percentile of collagen content had significantly higher degranulating mast cells in their atria than those in the 25th percentile (D). Post-operative outcomes in CABG patients, as assessed for the development of NYHA III/IV symptoms, were significantly better in high mast cell density patients compared to those below the 75th percentile (E).

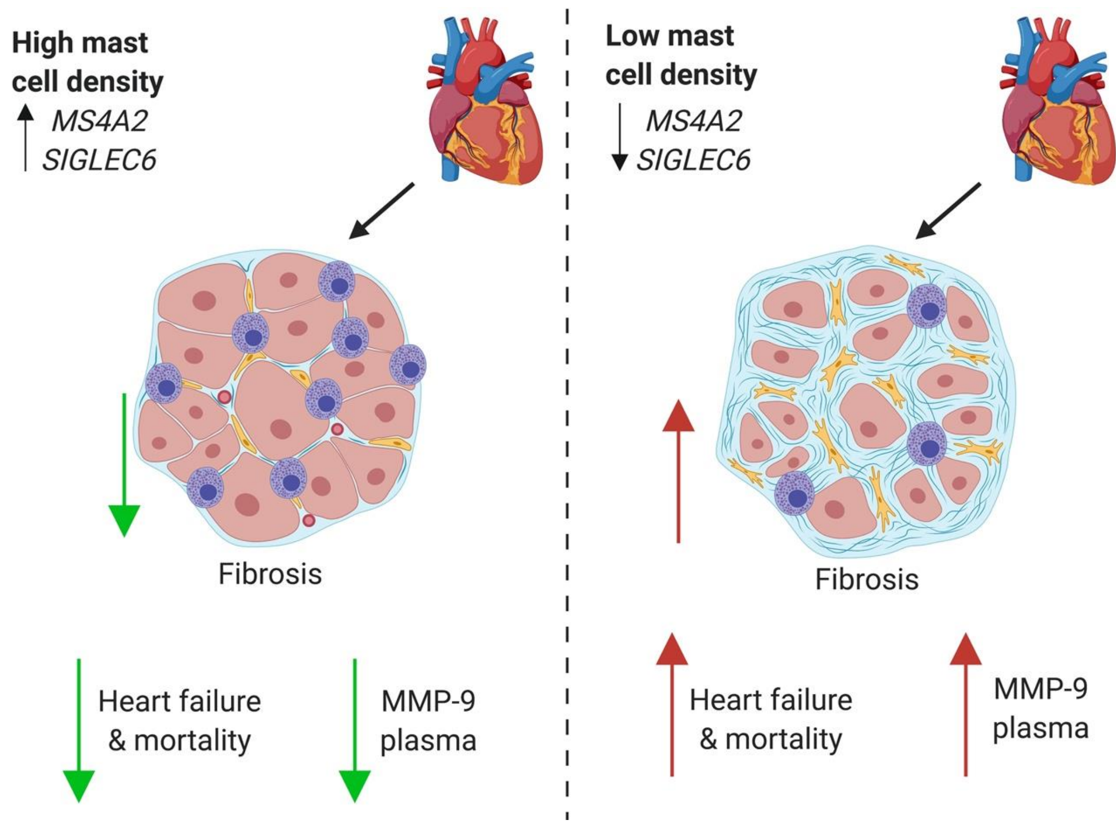


Figure 3.9. – Graphical summary of observations in the current study. Patients with high mast cell density as determined histologically had increased expression of the mast cell-specific genes *MS4A2* and *SIGLEC6*. Within their atrial tissue, these same patients had reduced atrial collagen and MMP-9 plasma content that coincided with a reduction in mortality or NYHA III/IV reclassification indicative of heart failure. In contrast, the opposite was observed in patients with low atrial mast cell density.

3.3 – Discussion

This study identifies an association between increased mast cell numbers and reduced cardiac fibrosis. This could indicate a potential role for mast cells as negative regulators of myocardial fibrosis or suggest that increased mast cells are functionally hallmarks of an anti-fibrotic process in which they do not directly participate. Patients identified to be within the upper quartile for atrial mast cell density at the time of cardiac surgery, had significantly less collagen in their atrial tissue than both the lowest quartile and the remaining 75% of patients in the cohort (Fig. 3.5C, D). When a more homogenous population of CABG patients were considered independently of the cohort at large, similar findings were observed (Fig. 3.8A,B). In addition, patients with high mast cell density had better long-term outcomes as characterized by a decreased incidence of NYHA III/IV heart failure symptoms or mortality (Fig. 3.5H,I, 3.8E). This study indicates that increased mast cell presence in atria is associated with decreased fibrosis and adverse functional outcomes, which may be related to alterations in MMP-9 content.

The impetus for changes in mast cell populations in human atria is unclear. The biology and tissue infiltration kinetics of mast cell precursors are poorly understood [590] and the extent of development of committed mast cell precursors in the human heart is unknown. Evidence from mouse models has shown mast cells were recruited from epicardial adipose tissue following myocardial damage [356], but this observation requires confirmation in human subjects. Additionally, it is possible that modified mast cell density in human myocardium could be due to failure of tissue resident mast cells to proliferate in response to stimuli after damage. Mast cell density may also be impacted by

signalling that results in apoptosis and a failure to renew those populations. Resolving these questions will require improved *in vivo* models of disease.

The observed increase in mast cell density concurrent with decreased fibrosis could indicate that having a higher number of mast cells in atrial tissue is related to improved tissue restitution. However, the stage of disease progression and time from the instigating event to tissue acquisition are unknown. The findings in this manuscript may reflect more prominent roles for mast cells at early stages of fibrosis, when patients with lower collagen levels experience evolving and active fibrotic processes. As fibrosis progresses and mast cell infiltrate resolves, higher collagen levels result where older established lesions and cross-linked matrices lead to lower cellular content. Although this is an important alternative hypothesis it should be noted that patients with high atrial mast cell content at the time of surgery had better long-term outcomes. Specifically, patients with high mast cell content had significantly lower incidence of re-classification to NYHA III/IV post-operative or mortality than the rest of the cohort as a whole, which was consistent in CABG patients (Fig. 3.5I,H, 3.8E). These observations indicate that patients with high mast cell content are experiencing less severe clinical disease leading to less heart failure symptoms or mortality.

Many studies relating to myocardial fibrosis are limited by their consideration of mast cell activity only in terms of degranulation, which represents only one of many complex mast cell responses [5,585]. Mast cells are capable of producing numerous anti-fibrotic mediators in response to stimuli present in the myocardium after damage, such as CXCL10, VEGF-A, and IL-33 [75]. Mast cell-fibroblast interactions have been studied extensively *in vitro,* however no direct impact of mast cells on fibroblast phenotype has

been consistently observed [74]. It is likely that the relationship between mast cells and fibrosis in the heart is more complex than direct cell-cell interactions and could involve multiple cells within the microenvironment. The current study of primary surgical tissue was limited in its ability to assess what mediators mast cells may be producing or cells they may influence. It was observed that patients in the upper quartile of atrial collagen content had significantly higher number of degranulating mast cells compared to those in the lower quartile (Fig. 3.5E), while no difference was observed in degranulation activity between patients in upper and lower quartiles of mast cell density (Fig. 3.7). Mast cell degranulation is thought to be pro-fibrotic [75], therefore it is possible that the activity of mast cells in atria rather than their relative abundance, may be a more important factor to consider in this context. However, mast cells are also known to degranulate in response to mechanical stimulation [471,472], and it is possible that stiffer tissues with high collagen content required more handling during excision than those with low collagen content. It is important to note that atrial appendage was collected in this study and should be distinguished from ventricular myocardium which could not be collected in our human subjects, including patients who experienced MI.

The present study is unique because it uses primary human tissues to assess their relationship to clinical disease. However, there are limitations when working with such tissues that can hinder the depth of understanding. Obtaining biopsy samples at the time of surgery provides a single point of data collection within a spectrum of disease that cannot be standardized. The instigating event that led to disease, the stage of disease progression, the variability of cell populations within tissues, and the reflection of the state of biopsy samples to the tissue as a whole cannot be controlled. As such, variability

is seen in data related to collagen content, mast cell density, and mast cell-associated gene expression. However, the impacts of mast cells on cardiac fibrosis, using human atrial biopsies can allow for the identification of clinically relevant questions for further investigation. This study is limited by a lack of standardized data on measures of cardiac function e.g. follow-up echocardiography. Surrogate measures of outcomes employed in this study allowed for the assessment of the importance of mast cells in atrial fibrosis. LOS after cardiac surgery is an important surrogate measure of functional outcomes early post-operative [591]. Patients with low mast cell content, in the bottom quartile, had increased incidence of prolonged LOS or hospitalization (Table 3.1, 3.6) and went on to have increased incidence of re-classification to NYHA III/IV functional class at follow-up or mortality in keeping with worse long-term functional outcome (Fig. 3.5H). Low mast cell density patients within the CABG cohort also had increased incidence of re-classification to NYHA III/IV at follow-up (Fig. 3.8B). Mast cell density progression was not followed with disease state as atrial tissue was only obtained intra-operatively, but data in the current study suggests that mast cell density at the time of surgery may be indicative of future outcomes. Atrial fibrosis is commonly described in association with AF and increased atrial size [592–594]. Atrial size was not recorded in this study, as size and function of atria measured by echocardiography remains operator dependent, lacks standardization and remains poorly captured [595]. The current study included patients with pre- and post-operative AF in the overall cohort. No significant relationship was observed between atrial fibrosis and pre- or post-operative AF in the overall cohort or within CABG patients specifically. Our findings are in keeping with previous work in which atrial fibrosis was independent of AF [589]. It is possible that atrial fibrosis is not an

exclusive driver of AF but rather a manifestation of aberrant myocardial processes at work in patients with cardiovascular disease [596].

To our knowledge, this is the first study to assess human atrial tissue mast cell density and its association with fibrosis in patients using a substantial cohort of fresh surgical tissue. Previous work has focused on smaller patient groups (n<30), with a mix of tissue acquired post-mortem [361,363] or intra-operatively [357,358, 360,362]. Post-mortem assessment of mast cells in tissue is confounded by hypoxia-induced degranulation after death, impeding detection [597]. An association between increased mast cell density and increased heart collagen content has only been described in relation to Chagas disease which results from infection [591], in two similar studies [361,363]. Two much smaller studies (n=13 [362], n=3 [358]) of uninfected heart tissue reported no significant relationship between mast cells and collagen content in human atria [358,362]. Recent work, using a group of 30 patients, showed a positive correlation between mast cell density as assessed by protease immunohistochemistry and collagen content in post mortem samples of the left ventricle of patients with hypertensive heart disease [598]. However, time from death to tissue excision is not noted, despite its importance to interpretation [597]. The use of anti-tryptase staining in this recent study can result in overestimates of mast cell numbers via identification of macrophages that have taken up apoptotic cells and granules [599] .

Collagen content was determined by SRFG staining of atrial appendage tissue removed from patients during cardiac surgery and quantified in a standardized manner as previously described [265, 570,574]. In the current study, there was a wide degree of fibrosis observed (Fig. 3.2B). Although this indicates extensive remodeling, similar levels of collagen have been observed in human atrial tissues [589,600,601]. In support of our findings,

40.2% of the patients included in this study were pre-operatively classified as NYHA III/IV heart failure symptoms, and 50.9% had a history of hypertension (Table 3.2). Therefore, the range and extent of remodelling seen in atrial biopsies is not entirely surprising given the level of disease severity within the cohort. The large sample size of the current study allows for assessment of fibrosis without being overly skewed towards high, and low extremes of collagen deposition.

This study also developed a novel method to determine mast cell density in human heart tissue by probing for mast cell specific genes via ddPCR. Accurate mast cell density quantification in tissues was critical for this study. Metachromatic staining with Toluidine blue, the usual standard to detect mast cells in paraffin-embedded tissues, can be confounded by several factors, including the small area of tissue samples, difficulty in detecting fully degranulated cells, and method of fixation. Assessing expression of mast cell specific genes is likely to give a better indication of true mast cell density in tissues. *MS4A2* and *SIGLEC6* expression in human atrial tissue were positively correlated to mast cell counts as observed by histology. *MS4A2* is also expressed on basophils not usually found in heart tissue, outside of allograft rejection [586]. *SIGLEC6* can be expressed in placental tissue as well as mast cells [342] but is considered mast cell-specific in heart tissue. The classical mast cell marker CD117 was not used as cardiac endothelial cells and cardiac progenitors have been identified as CD117+ [602,603]. Further work is needed to determine the utility of ddPCR assessment of mast cell content in atrial tissue for use clinically.

The current study of samples from 112 subjects strongly supports an association between increased mast cells numbers and decreased atrial fibrosis, which may reflect the

unusual regulation of fibrosis in the cardiac tissue setting. The novel findings reported in this study suggest greater consideration of mast cell functions in myocardial fibrosis and suggest caution in the use of drug treatments that reduce mast cell numbers or function in this setting.

CHAPTER 4: IL-33 PROMOTES ANGIOGENIC MEDIATOR PRODUCTION IN HUMAN MAST CELLS RELEVANT TO WOUND HEALING

4.1 – Introduction

Angiogenesis is the formation of new blood vessels and is a crucial process after tissue injury. It promotes effective wound healing by supplying oxygen, nutrients and immune effector cells to remodelling tissue sites [74]. However it can also be co-opted to support tumour growth and metastasis [38]. Mast cells are innate immune cells present in tissues and organs throughout the body where they act as sentinels and co-ordinators of immune responses to damage, infection, and any other tissue disturbances [32]. Mast cells are closely associated with blood vessels and can induce angiogenesis in tissues via degranulation and release of pre-formed pro-angiogenic compounds, and by synthesis and secretion of mediators *de novo* [5]. The strongest case for mast cell involvement in angiogenesis has been made in the context of solid tumour development, where mast cells have been shown to promote neovascularization in tumour microenvironments [604,605]. However, an underappreciated role exists for the promotion of blood vessel formation by mast cells in other contexts, such as wound healing [606,607]. Due to their broad distribution throughout the body, and long life spans in tissues [608,609] mast cells are ideal therapeutic targets.

IL-33 is a damage-associated molecular pattern (DAMP) released by structural cells [176,610] upon tissue injury that activates type 2 immune signaling in leukocytes [177,178,182]. Unlike many DAMPs, IL-33 does not signal through a pattern recognition receptor (PRR) but rather through the IL-1 family receptor IL-1RL1, or ST2L [177].

Multiple tissue-resident innate immune cells express ST2L , including ILC2s [207], T_{regs} [208], T_{H2} cells [209], and mast cells [50]. Furthermore, IL-33 is highly susceptible to post-translational processing by mast cell and neutrophil associated proteases that enhance its bioactivity [201–203] as well as oxidation that limits its systemic impact [198]. IL-33-ST2L signaling on leukocytes has been reported to induce many effects depending on co-stimuli and targeted cells. It is most commonly associated with the production of IL-5, IL-9, IL-13 and granulocyte monocyte-colony stimulating factor (GM-CSF) [206]. Several tissues have particularly high concentrations of IL-33, including the heart and the skin [178]. In the heart, IL-33 has been identified as an important signal to limit adverse remodelling and promote beneficial outcomes [194]. While these observations can be partially attributed to the pro-survival actions of IL-33 on cardiomyocytes [344,375–378], there are likely immune mediated actions that support.

The impacts of IL-33 on mast cells have been extensively studied, particularly in the context of allergic inflammation [64]. However, depending on the species and source of the mast cell, IL-33 can have different effects. Murine mast cell lines respond to IL-33 by producing IL-4, IL-5, and IL-6 [203], and increase *Il33* expression after degranulation [116], while primary bone marrow-derived mast cells (BMMCs) produce IL-1β, IL-6, IL-13 [110], TNF, CCL2 [611], CCL3, GM-CSF [221], but not IL-4. A similar relationship has been observed in human mast cells, where primary cell responses to IL-33 consist of increased production of IL-5, IL-6, CXCL8, IL-10, IL-13 [612], GM-CSF, CCL1, CCL2, CCL3 [221], CCL5 [49], CXCL8 [50], while human mast cell lines, such as LAD2 cells, typically do not respond as robustly to IL-33 [221] but have been shown to produce more classical pro-inflammatory cytokines such as IL-1β [613], and TNF [614]. Interspecies differences in

response to IL-33 have also been reported, for example murine mast cells do not produce IL-10 in response to IL-33 [611], but primary human mast cells do [50,615].

VEGF-A is an important angiogenic growth factor up-regulated in the heart after damage that promotes neovascularization of hypoxic tissues after damage and supports repair [274–276]. IL-33 has been shown to induce VEGF-A production in the human mast cell line HMC1 [616]. The extent to which IL-33 may, directly or indirectly, induce other regulators of angiogenesis by mast cells is unknown but could have relevance in several tissue settings. Importantly, VEGF-A can also act as an initiator of further angiogenic activity by induction of urokinase plasminogen activator (uPA), a serine protease initially characterized for its role in degradation of extracellular matrix [617]. It has been established that uPA serves important roles in promoting tissue angiogenesis, particularly in the context of cardiac damage repair [618–620]. The capacity for mast cells to produce uPA is not as well understood, though there is some evidence that unstimulated primary human skin mast cells may secrete it at low levels[117]. Therefore, we sought to determine how IL-33 may promote mast cell angiogenic activities. We looked *ex vivo* at human atrial tissue samples and *in vitro* at human CBMCs and human umbilical vein endothelial cells (HUVECs) to determine if mast cells have a pro-angiogenic repertoire of mediators produced in response to IL-33.

4.2 – Results

4.2.1 – IL-33 is present in human atrial tissue samples and relates to angiogenic pathways

We assessed the expression of *IL33*, *VEGFA*, *MERTK,* and *IL1RL1* (gene name of ST2) in human atrial appendage samples from a series of cardiac surgery patients (n=23). All genes examined were detected by qPCR (*IL33, VEGFA*) or ddPCR (*IL1RL1, MERTK*) with varying distribution (Fig. 4.1A-D). Expression of *IL33* and its receptor *IL1RL1* were negatively correlated to each other (Fig. 4.1E, r^2=0.2042, p=0.0304). In contrast, expression of *VEGFA* and *IL33* (Fig. 4.1F, r^2=0.5778, $p<0.0001$) were positively correlated to each other, as were *MERTK* and *IL33* (Fig. 4.1G, r^2=0.1774, p=0.0453). There was no significant relationship between *MERTK* and *VEGFA* (r^2=0.1652, p=0.0543), *IL1RL1* and *VEGFA* expression (r^2=0.0623, p=0.2396), or *IL1RL1* and *MERTK* expression (r^2=0.0176, p=0.5562).

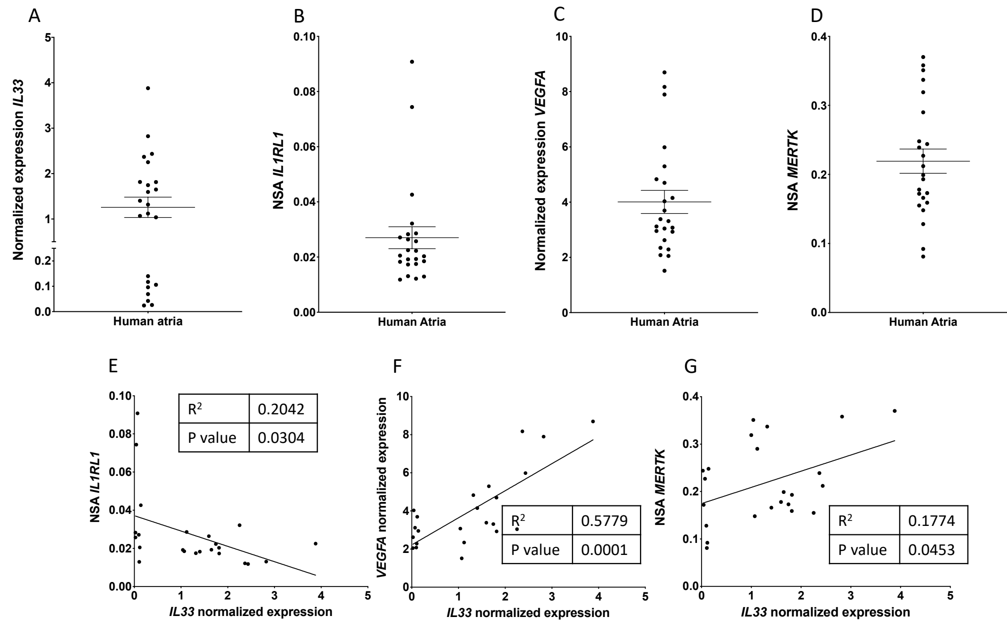


Figure 4.1. –Expression of *IL33*, *IL1RL1*, and *VEGFA* in human atria. Gene expression was assessed in human atrial tissues by qPCR (normalized expression) or ddPCR (normalized standard amount, NSA). *IL33* (A), *IL1RL1* (B), *VEGFA* (C), and *MERTK* (D) were detectable in all human atrial samples assessed. There was no association between expression of *IL1RL1* and *IL33* in human atria (E). An association was observed between *VEGFA* and *IL33* (F) and *MERTK* and *IL33* (G) in the same tissues.

4.2.2 – Human mast cells express angiogenesis-associated genes in response to IL-33

We next sought to assess the impact of IL-33 on angiogenesis-associated gene expression in human mast cells. CBMCs from three donors were activated for 24h with IL-33, or untreated (mock). mRNA from all three donors for each condition was then pooled such that samples could be run in duplicate within the microarray. In total, 85 genes associated with angiogenesis were assessed, of which 17 were not detected, and 48 were expressed at comparable levels between the two groups. We identified genes increased in expression 1.5-fold or decreased in expression 0.5-fold after IL-33 treatment. Eight genes had increased expression, including *FN1, PLAU, VEGFA* and *PDGFA*, while nine genes were decreased in expression within these criteria, including *IL6, IL8, CXCL1* and *CXCL5* (Fig. 4.2).

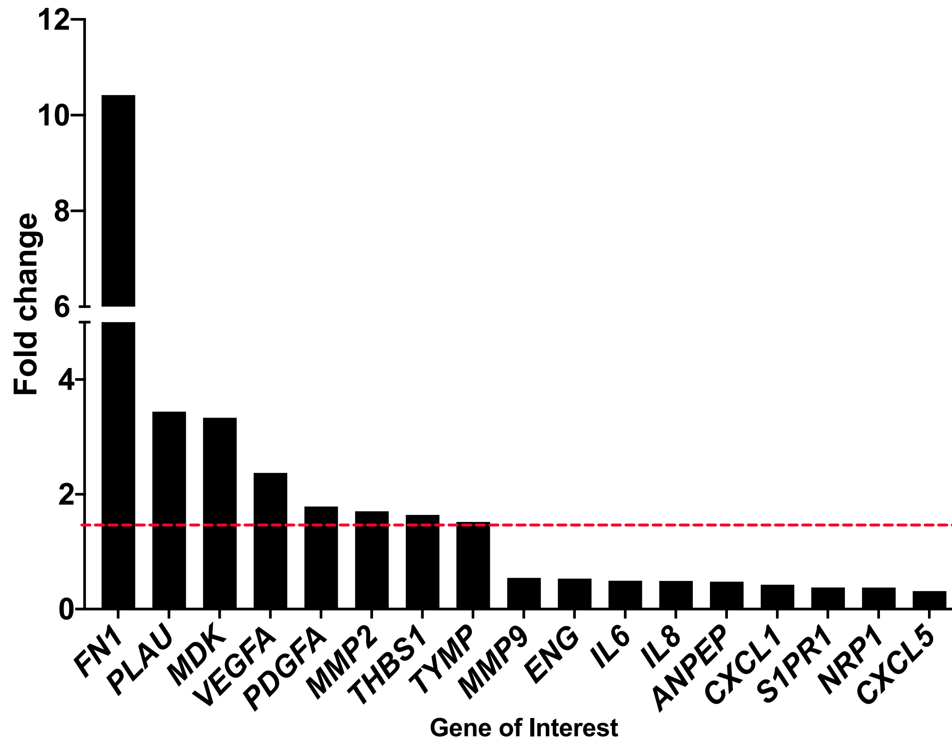


Figure 4.2. – IL-33 influences gene expression patterns in human mast cells. CBMCs (n=3/condition, pooled) were activated for 24h with IL-33 or mock and pooled for gene analysis. A BioRad microarray for angiogenesis-associated genes was used to assess gene expression changes

4.2.3 – IL-33 induces angiogenesis-associated mediators in human mast cells

Mast cells are well known for their degranulation capacity in response to various stimuli. Therefore, human CBMCs were activated with IL-33, HMGB1, HSP60, HSP70, S100A8, and ATP for 30 minutes to assess degranulation, with Ca^{2+} ionophore (A23187) treatment as a positive control. The 30-minute time point is appropriate when assessing mast cell degranulation, as this process is quick and can occur in as little as 15 minutes after initial stimulation [621]. Mast cells did not degranulate in response to any of the DAMPs assessed in this study (Fig. 4.3A). Furthermore, CBMCs did not produce PGD_2, PGE_2, or LTC_4 in response to IL-33 after 30 minutes of activation (Fig. 4.3B). Dose response and time course experiments were conducted to determine optimal activation conditions for CBMCs with IL-33 (Appendix B). Next, human CBMCs were activated with IL-33 for 24h to assess mediator production. CBMCs did not produce TGF-β, IL-6, MMP-9, or TNF in response to IL-33 (Fig. 4.3C). However, it was determined that CBMCs significantly increased production of VEGF-A, and uPA, as well as the type 2 associated cytokines IL-13 and IL-10 after IL-33 stimulation (Fig. 4.3D), compared to control diluent treated cells. HUVECs were treated with supernatants collected from control or IL-33 treated CBMCs. Similar to VEGF-A treated HUVECs, which were used as a positive control, capillary tubes were observed in cultures of HUVECs treated with both sets of CBMC supernatants (Fig. 4.3E). However, IL-33-treated CBMC supernatant did not appear to differentially impact capillary tube formation compared to control.

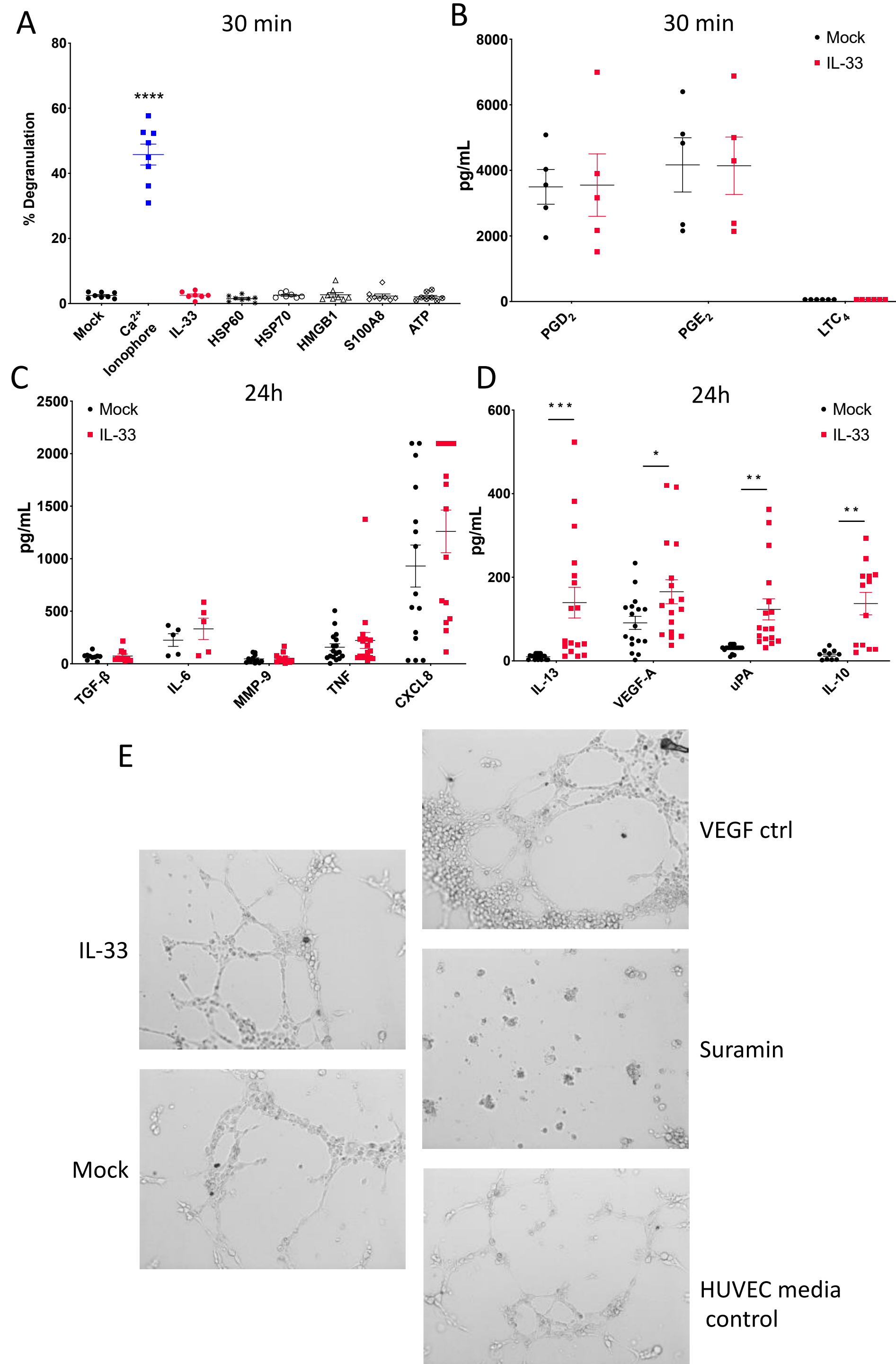


Figure 4.3. – Human mast cells produce angiogenic mediators but not pro-inflammatory or pro-fibrotic mediators in response to IL-33. CBMCs were activated for 30 minutes with DAMPs to assess degranulation (A, n=7/condition), and IL-33 to assess lipid mediator production (B, n=5/condition). CBMCs were activated for 24h with IL-33 or untreated were assessed for TGF-β (n=10/condition), IL-6 (n=5/condition), MMP-9 (n=12/condition) TNF (n=17/condition), CXCL8 (n=15/condition) (C), IL-13

(n=17/condition), VEGF-A (n=17/condition), uPA (n=17/condition), and IL-10 (n=10/condition) (D). HUVECs were treated for 4h with CBMC supernatants from IL-33, and mock CBMCs and monitored for capillary tube formation in comparison to positive (VEGF-A) and negative (Suramin) controls (E). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.2.4 – Human endothelial cells do not make VEGF or uPA in response to IL-33

To determine if endothelial cells may also respond to IL-33 by generating VEGF-A and/or uPA, we activated HUVECs *in vitro* with IL-33 for 24h before harvest. We did not see a significant change in VEGF-A or uPA protein production between untreated and IL-33 treated HUVECs (Fig. 4.4A). Gene expression of *IL33, IL1RL1* (ST2), *VEGFA* and *PLAU* (gene name for uPA) was also assessed in these samples, using VEGF-A treated HUVECs as a positive control. There was a significant decrease in *IL1RL1* expression in VEGF-A treated cells compared to mock untreated, and IL-33 treated cells, and a substantial increase in *PLAU* in IL-33 treated cells compared to VEGF-A (Fig. 4.4B). There was no change in *IL33* or *VEGFA* gene expression under any of the conditions assessed.

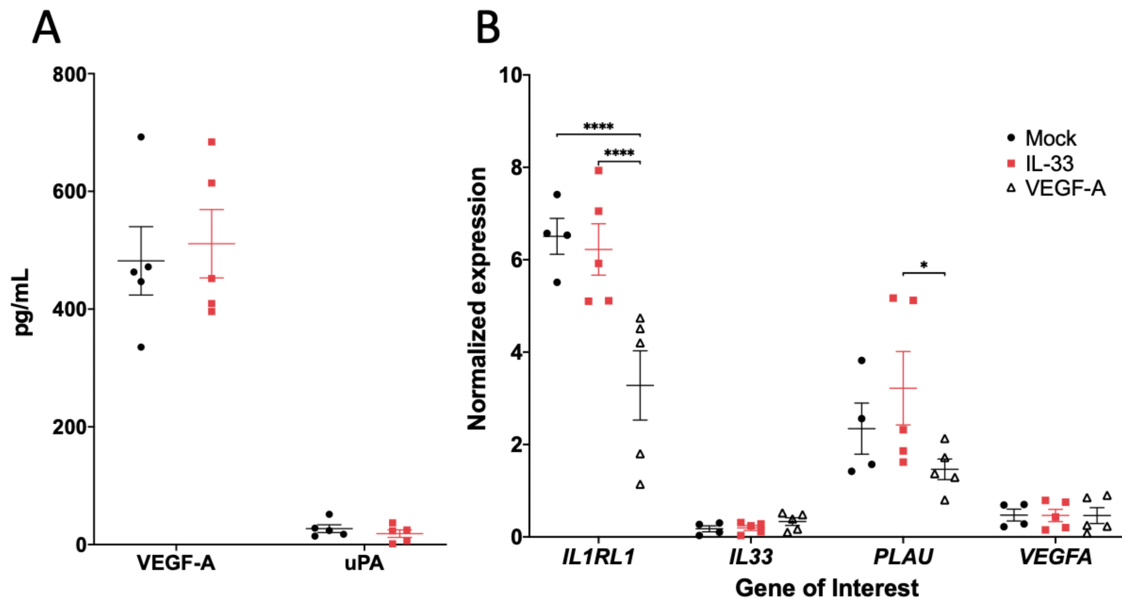


Figure 4.4. – HUVECs do not respond to IL-33 by producing angiogenesis associated mediators. HUVECs were activated for 24h with IL-33 at 30ng/mL or control media (n=5/condition). Production of VEGF-A and uPA was assessed in supernatants (A). Gene expression was assessed in these cells, as well as HUVECs activated with VEGF-A as a positive control, for *IL1RL1, IL33, PLAU,* and *VEGFA* (B). * p<0.05, **** p<0.0001.

4.3 – Discussion

In this study, we have demonstrated that IL-33 promotes VEGF-A and uPA pro-angiogenic mediator production in human mast cells (Fig. 4.2, 4.3D). IL-33 does not induce mast cell degranulation activity or pro-fibrotic mediator production (Fig. 4.3A-C). Mast cell products promoted capillary tube formation of HUVECs in culture (Fig. 4.3E) though this was not found to be IL-33 dependent. This observation is not a by-product of HUVEC production of VEGF-A or uPA in response to IL-33 (Fig. 4.4). Increased uPA production by mast cells in response to IL-33 is a novel observation, as mast cells have not been previously reported as significant sources of uPA. Human atrial tissues had a positive correlation between *IL33* and *VEGFA* gene expression *ex vivo*, suggesting that IL-33 induction of VEGF-A may be occurring in human atrial tissues (Fig. 4.1E). Future work should determine if mast cell responses to IL-33 are relevant in models of wound repair. Mast cells may be particularly important in tissues where IL-33 release from damaged cells is high and mast cells are found in large numbers, such as the human heart [194] and skin [178].

Mast cells are known to promote angiogenesis in several disease contexts [5,604,606,607]. While this activity promotes pathogenesis in tumour microenvironments [604], angiogenesis is critical for successful tissue repair [622,623]. Remodelling tissues have a high rate of cellular proliferation, migration, extracellular matrix deposition, and other metabolically intensive activities that require oxygen and nutrients. Therefore, the provision of proper blood supply as early as possible is crucial to the success of the repair process [622]. This highlights a potential benefit of activating angiogenic pathways subsequent to the local release of DAMPs, like IL-33. Production of VEGF-A is

particularly relevant in the cardiac microenvironment during wound healing, where it promotes new vessel formation [274–276], in addition to limiting excessive tissue remodelling at the site [277,278]. Additionally, VEGF-A activates other angiogenic pathways in local tissue microenvironments, such as uPA production [279]. By triggering cascading angiogenic pathways, initial VEGF-A induction likely promotes further vessel formation that would be beneficial during tissue remodelling.

Increased uPA production of mast cells in response to IL-33 activation (Fig. 4.3D) has not been previously described. Work in primary human skin mast cells has shown some uPA secretion at rest [117]. Mast cell tryptase has been shown to activate uPA to its proteolytic, bioactive, form [624], though no degranulation was observed in this setting (Fig. 4.3A) and as such tryptase activity may not be relevant here. This is the first instance to our knowledge in which a significant amount of uPA has been shown to be produced by mast cells. uPA promotes revascularization of damaged cardiac tissue after myocardial infarction for proper wound repair and reduced fibrosis [618–620]. However, caution should be exercised in considering the potential of mast cell uPA production in the heart, as uPA in its action as a serine protease has been shown to play pro-fibrotic roles in the cardiac setting [625–627]. It is likely that the temporal regulation of uPA in settings of tissue damage may dictate how it exerts pro-angiogenic versus pro-fibrotic effects, and that, like many systems in biology, regulation of its activity is important to prevent adverse effects. Mast cells are often localized around the vasculature in the heart [628], and as such are ideally located to activate angiogenesis early after damage and subsequent IL-33 activation via production of VEGF-A and uPA.

IL-33 is released upon stromal cell damage into local tissue microenvironments to activate immune responses [629]. We found that while HUVECs express the IL-33 receptor gene *IL1RL1*, they do not upregulate its expression in response to IL-33, nor do they respond by increasing gene expression or protein production of VEGF-A or uPA (Fig. 4.4). Choi et al. have previously shown that HUVECs can proliferate and form capillary tubes in response to IL-33 [624], but there is no data on HUVEC angiogenic mediator production in response to IL-33. Endothelial cells have been shown to respond to IL-33 in other ways. HUVECs increase surface expression of adhesion molecules such as VCAM-1, ICAM-1 and E-selectin, as well as the production of the chemokine CCL2 [630], in a response similar to IL-1β activation of HUVECs [631]. IL-33 also induces CXCL8 production in HUVECs for additional chemoattractant capacity [632]. VEGF-A is a well-known activator of HUVECs [624], and uPA has also been shown to exert pro-angiogenic effects on these cells [633,634]. Therefore, while HUVECs likely contribute to initiating inflammatory responses after IL-33 activation, our observations coupled with previous work suggest an important role for IL-33 activation of local mast cells in promoting angiogenesis in this population.

Mast cells also produce IL-10 and IL-13 in response to IL-33 (Fig. 4.3D). This observation has been previously described by several others [50, 216, 612,635]. However, it is relevant to note that IL-10 [332,333,636–639] and IL-13 [265,346–349] can contribute to proper wound healing via impacts on macrophage phenotypes. In this study expression of *MERTK* and *IL33* were also found to be positively correlated in human atrial tissues (Fig. 4.1G). *MERTK* encodes the gene for the efferocytosis receptor MerTK on macrophages. Both IL-10 [259,639,640] and IL-13 [350, 521,641] have been identified to contribute to

efferocytosis, the phagocytosis of apoptotic cells by macrophages by promoting $MerTK^+$ macrophage populations . Efferocytosis is a crucial step in the resolution of inflammation after tissue damage [461] that promotes macrophage differentiation to wound-repair phenotypes [642] implicated in fibrosis at various tissue sites [258,643–645]. Responding to IL-33 by producing IL-10 and IL-13 may be an additional mechanism by which mast cells can contribute to wound repair via IL-33 signaling.

Mast cells have been identified to contribute to fibrotic remodelling in several disease settings, though their role is controversial [74]. This controversy is likely due to the focus on mast cell degranulation activities and their pro-fibrotic actions, rather than assessing the *de novo* synthesis and secretory functions that mast cells can exert [75]. We have shown in this study that mast cells do not degranulate (Fig. 4.3A) in response to DAMPs or produce pro-inflammatory or pro-fibrotic mediators in response to IL-33 *in vitro* (Fig. 4.3B,C). Therefore, this narrow focus on mast cell degranulation activity in fibrosis is not accurately capturing impacts mast cells may have on damaged tissues through other routes. Future work should focus on better elucidating mast cell secretory functions in fibrotic settings, potentially through more advanced immunohistochemistry techniques such as MANTRA staining or focused studies comparing *in vitro* and *in vivo* observations.

In conclusion, we have shown that mast cells can selectively produce angiogenic mediators VEGF-A and uPA, as well as pro-repair cytokines IL-10 and IL-13 in response to IL-33 that could contribute to wound healing in the cardiac microenvironment and beyond. While IL-33 is typically associated with type 2 immune responses, such as allergic airway disease [178], this study shines light onto a growing body of evidence to

suggest IL-33 acts to promote angiogenesis [646–649]. It is important to note that these observations are limited to the *in vitro* setting. As such, future work should focus on elucidating how IL-33 signaling in mast cells could influence local tissue microenvironments to limit excessive inflammation and fibrotic remodelling. By better understanding how IL-33 impacts mast cells in tissues, we can identify better therapeutic targets for patients with cardiovascular disease.

CHAPTER 5: INTERLEUKIN-33 PROMOTES EFFEROCYTOSIS IN PERITONEAL MACROPHAGES

This work appears in part in the publication:
Legere, S.A., Hu, Q., Haidl, I.D., Légaré, J-F, Marshall, J.S. IL-33 promotes efferocytosis in peritoneal macrophages for inflammation resolution. Submitted December 2020.

This chapter is presented, as in the submitted manuscript, in the following sections: Introduction, Results & Discussion.

Author contributions: SAL designed the study, performed the majority of experiments, analyzed data, and wrote and edited the manuscript. QH performed experiments and edited the manuscript. IDH assisted in design of the study, analyzed data, and edited the manuscript. JFL assisted in design of the study and edited the manuscript. JSM assisted in design of the study, analyzed data, and edited the manuscript.

5.1 – Introduction

Inflammation is crucial for responding to infection and tissue trauma and, when properly regulated, is followed by restoration of tissue homeostasis [3,408]. The inflammatory process is often mediated by pattern recognition receptors (PRRs) on local leukocytes and stromal cells that detect damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) [421]. Resident immune cells generate multiple mediators such as cytokines, chemokines, and eicosanoids, to promote polymorphonuclear cell recruitment and elimination of pathogens or debris. DAMPs mediate sterile inflammation in response to tissue injury, which initiates a cascade of events ending in wound repair. Appropriate orchestration and induction of inflammation resolution is key, as aberrant mediator or resolution processes lead to chronic inflammation that can result in fibrosis, cancer and autoimmune disease [232, 409,650].

The resolution of inflammation is an active process. Debris removal from tissues must occur to close the positive feedback loop of inflammatory mediator production [408]. Neutrophils are recruited to sites of tissue damage [651]. These cells undergo apoptosis within 24-48h [255] and their removal is critical for resolution to occur, as apoptotic neutrophils can become secondary necrotic cells and perpetuate tissue damage [503]. Efferocytosis is the phagocytosis of apoptotic cells mediated by macrophages [459,461] and is a central linking step in the resolution process. However, the initiating steps for this process are poorly understood. Efferocytosis is extremely efficient, taking as little as 10 minutes to occur [461], and is difficult to observe in solid tissues *in vivo*. Clearance of apoptotic cells is continuous and non-inflammatory but can be upregulated during infection or tissue injury [459]. Macrophages are recruited to apoptotic cells in response to a

gradient of so-called “find me” signals [461]. Once within the apoptotic cell loci, macrophages bind and engulf apoptotic cells via recognition of “eat me” signals on the target cell surface, the most common being phosphatidylserine (PtdSer)[504]. PtdSer interacts with cell surface macrophage receptors including the efferocytosis receptor MerTK [652,653].

MerTK is a receptor tyrosine kinase of the TYRO3, AXL and Mer (TAM) receptor family [517] and is the most ubiquitously expressed TAM receptor on phagocytic macrophages [654]. Its induction can be mediated by a myriad of signals, including IL-13 stimulation in the presence of apoptotic cells [350, 521,641]. *Mertk-/-* mice have marked accumulation of apoptotic cells in their tissues [538] and develop spontaneous autoimmune disease [461,533,534]. MerTK has been implicated in resolution of inflammation processes in a number of sterile inflammatory and tissue injury settings [258, 260, 486,487,655,656].

IL-33 is known as a dual function IL-1 family cytokine. It is released from cells as a DAMP but signals through a cytokine receptor [178,657,658]. Upon cell death or nuclear damage, IL-33 is released from fibroblasts, endothelial cells, epithelial cells, and other stromal cells [182]. This cytokine acts to initiate type 2 inflammatory responses via signaling through its cognate receptor ST2, expressed on mast cells [50,612], type 2 innate lymphoid cells (ILC2s) [659,660], eosinophils [211,567], CD4+ T regulatory (Treg) [380,661] and T helper 2 cells (Th2) [662,663]. IL-33 signaling results in the generation of several mediators, including IL-13 [50, 211, 380, 567, 612, 635,659–665]. Once in the extracellular space, IL-33 is rapidly degraded by proteolysis [202,203] and oxidation [198]. This would suggest that initial local responses to IL-33 in the first minutes to hours after cell damage are most relevant.

In this study, we sought to determine if IL-33 may impact the extracellular environment by promoting efferocytosis in macrophage populations. We assessed changes in peritoneal leukocyte populations in response to IL-33, and how those changes impacted the efferocytosis ability of macrophages. We found that IL-33 increased overall populations of macrophages, as well as $MerTK^+$ macrophages, which allowed for increased efferocytosis in response to apoptotic thymocytes. IL-13 was increased in the peritoneal cavity as early as 3h post IL-33 administration, with elevated *IL13* mRNA signals from lymphocytes and eosinophils. Finally, increased $MerTK^+$ macrophages were also observed after IL-33 administration together with free mitochondria but not in response to free mitochondria alone, as an alternate source of DAMPs. Together, these data demonstrate a significant and rapid pro-efferocytic impact of IL-*33 in vivo*, associated with IL-13 induction, which has important implications for inflammation resolution processes.

5.2 – Results & Discussion

5.2.1 – IL-33 modifies populations of macrophages in the peritoneal cavity

Leukocyte populations were assessed in the peritoneal cavities of female and male mice at 48 hours post i.p. IL-33 injection via flow cytometry (Fig. 5.2A-C). IL-33 treated female mice had increased overall $F4/80^{+}CD11b^{+}$ macrophage populations (Fig. 5.1A, $p<0.0001$) compared to saline. There are two distinct populations of macrophages reported at this site [666], $F4/80^{hi}CD11b^{+}$ large peritoneal macrophages (LPM, Fig. 5.6B, $p<0.0001$), and $F4/80^{lo}CD11b^{+}$ small peritoneal macrophages (SPM, Fig. 5.6C^{+}, $p<0.0001$). Both were increased in response to IL-33. Total numbers of $MerTK^{+}$ macrophage in the peritoneal cavity ($F4/80^{+}CD11b^{+}MerTK^{+}$, Fig. 5.1D^{+}, $p<0.0001$) were increased at 48h post IL-33 injection, as were $MerTK^{+}$ LPMs (Fig. 5.6B), and SPMs (Fig. 5.6C).

Several leukocyte populations were increased in the peritoneal cavity after IL-33 treatment, including total lymphocytes ($FSC^{lo}SSC^{lo}$, Fig. 5.1E, $p=0.0003$), eosinophils ($SiglecF^{+}CD117^{-}$, Fig. 5.1F, $p=0.0329$), neutrophils ($Ly6C^{+}Ly6G^{+}$, Fig. 5.6G, $p=0.0077$), and total peritoneal lavage cells (Fig. 5.1I, $p<0.0001$). Inflammatory ($Ly6C^{+}CCR2^{+}$) monocytes were significantly increased in IL-33 treated mice compared to saline (Fig. 5.6H, $p=0.0009$), while patrolling monocytes ($Ly6C^{lo}CX3CR1^{+}$) were not (Fig. 5.6I). Interestingly, populations of mast cells (Fig. 5.1G, $p=0.2980$) and ILC2s (Fig. 5.1H, $p=0.2470$) were not significantly impacted. Male mice had less robust leukocyte population changes after IL-33 than female mice (Fig. 5.3) in keeping with previously reported literature in the peritoneal cavity [667,668]. Therefore, female mice were used for the remainder of the study.

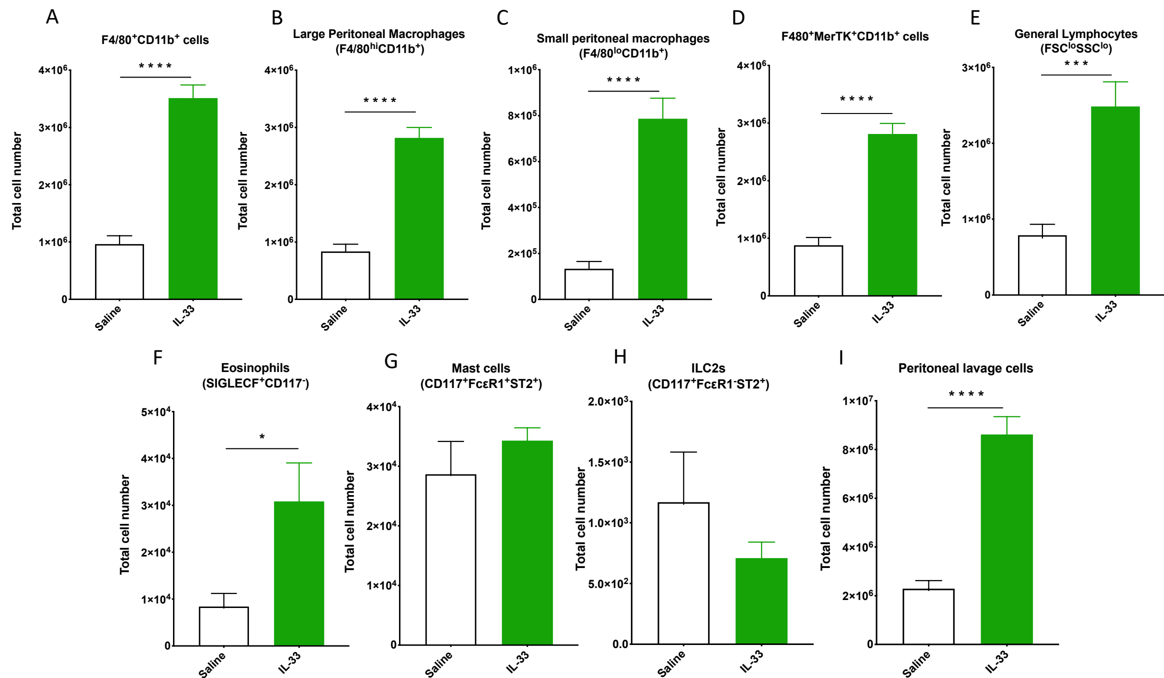
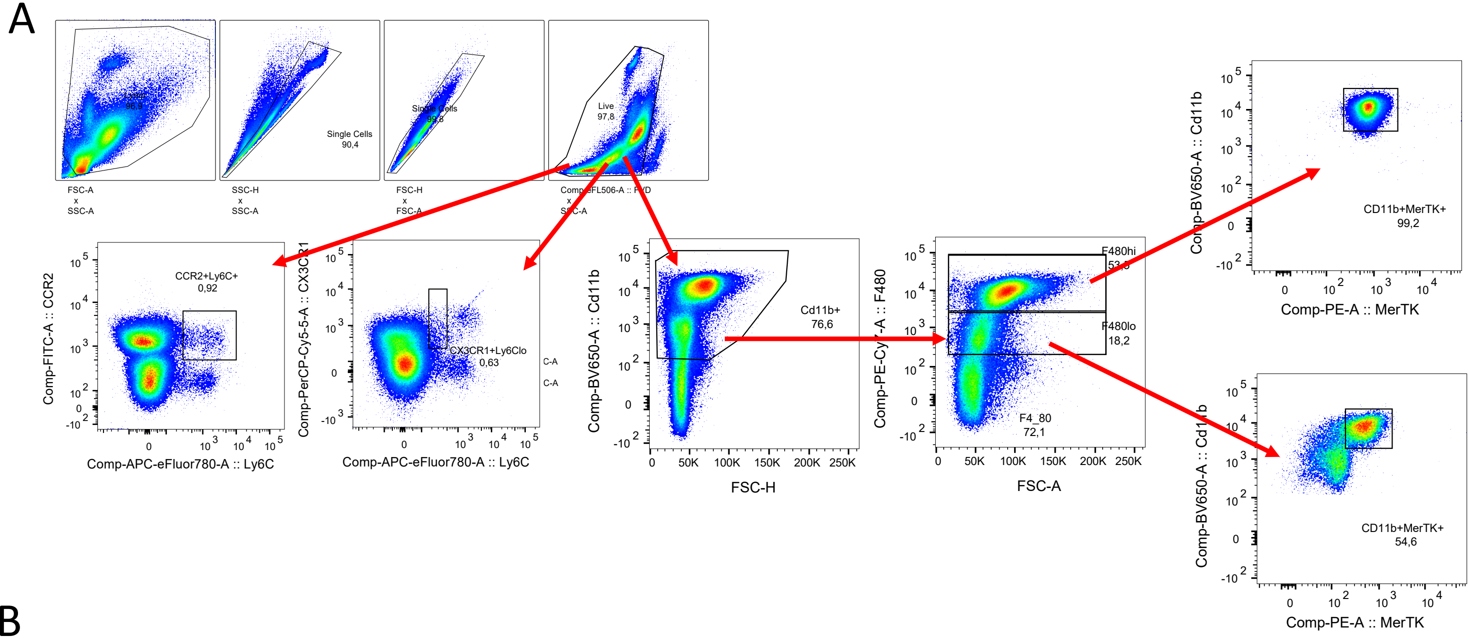
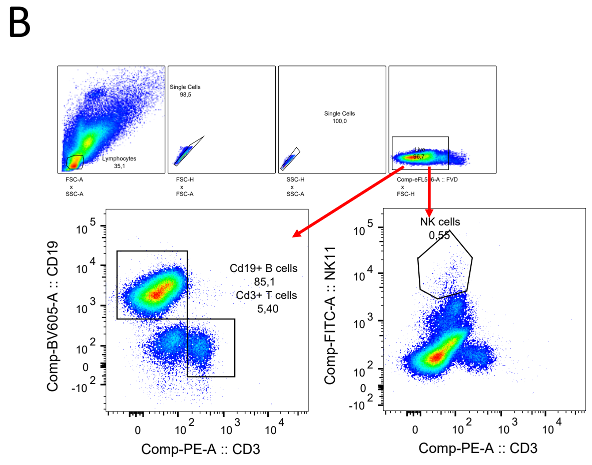


Figure 5.1. – IL-33 increases macrophage populations in the peritoneal cavity. Female C57BL/6 mice were injected i.p. with 0.5µg IL-33 (green, n=15) or saline (white, n=11) and sacrificed after 48h. Leukocyte populations were assessed by flow cytometry. Total numbers are represented as mean ± SEM. Overall macrophage populations were assessed as F4/80^{+}CD11b^{+} cells (A). LPM (F4/80^{hi}CD11b^{+}) and SPM (F4/80^{lo}CD11b^{+}) populations, as well as overall MerTK^{+} macrophage populations were identified (D). General lymphocytes (E, FSC^{lo}SSC^{lo}), eosinophils (F), mast cells (G), and ILC2s (H) were also assessed. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$.

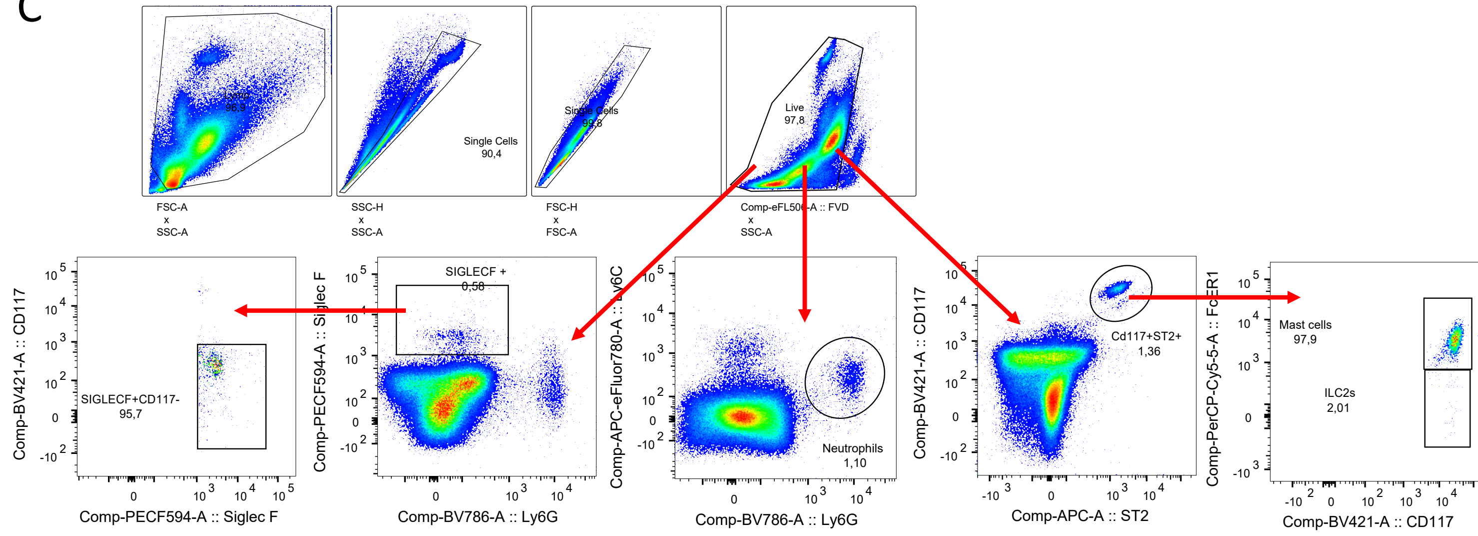


A
Single Cells
90,4
Live
97,8
CCR2+Ly6C+
0,92
Comp-FITC-A :: CCR2
Comp-APC-eFluor780-A :: Ly6C
Comp-PerCP-Cy5-5-A :: CX3CR1
CX3CR1+Ly6Clo
0,63
Cd11b+
76,6
Comp-BV650-A :: Cd11b
FSC-H
F480hi
F480lo
18,2
F4_80
72,1
FSC-A
CD11b+MerTK+
99,2
Comp-BV650-A :: Cd11b
Comp-PE-A :: MerTK
CD11b+MerTK+
54,6
Comp-PE-A :: MerTK

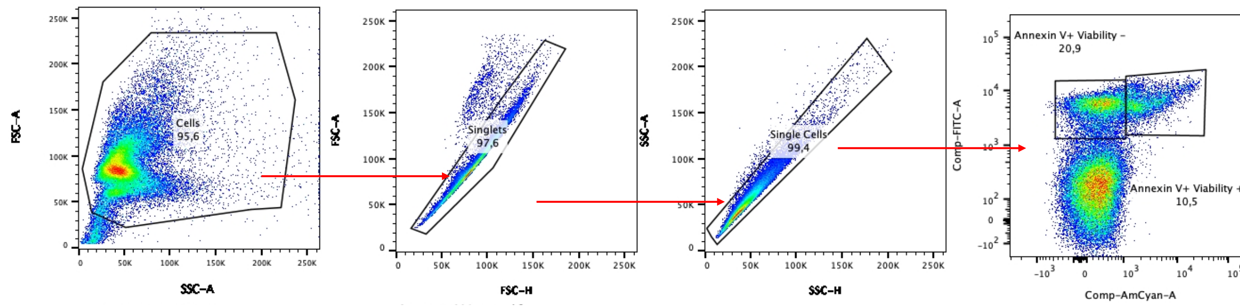


B
NK cells
0,55
Cd19+ B cells
85,1
Cd3+ T cells
5,40
Comp-BV605-A :: CD19
Comp-PE-A :: CD3
Comp-FITC-A :: NK11
Comp-PE-A :: CD3

C



D



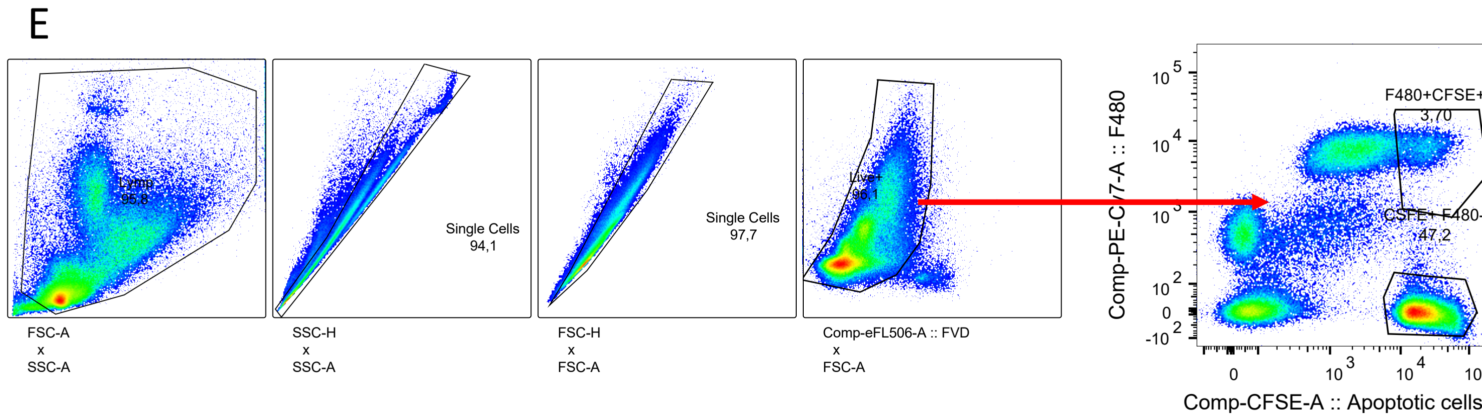


Figure 5.2. – Gating strategies to identify leukocyte populations. Macrophages were gated first on CD11b+ cells, then F4/80+ cells. Large peritoneal macrophages were identified as F4/80hiCD11b+ and small peritoneal macrophages were identified as F4/80loCD11b+. Within each macrophage gate, MerTK+CD11b+ macrophages were also identified. Monocytes were classified as inflammatory (CCR2+Ly6C+) or patrolling (CX3CR1+Ly6Clo) in the peritoneal cavity from overall live cells (A). Lymphocytes were identified as FSC lo SSC lo cells and fixable viability dye negative. T cells were identified as CD3+ CD19-, B cells were identified as CD19+CD3-, and NK cells were identified as NK1.1+CD3- (B) Granulocytes and ILC2s were identified as fixable viability dye negative cells. CD117+ST2+ cells were classified as mast cells or ILC2s based on FcεR1 gating. Eosinophils were identified as SiglecF+ Ly6G- and further CD117-. Neutrophils were identified as Ly6C+Ly6G+ cells. (C). Efficiency of apoptosis induction by dexamethasone treatment was determined by AnnexinV and Viability staining to identify primary apoptotic (AnnexinV+ Viability-) and secondary apoptotic (AnnexinV+ Viability+). If >10% of cells were necrotic (AnnexinV-Viability+), cells were not administered (D). Efferocytosis activity was assessed by gating on F4/80+CFSE+ cells compared to total CFSE+ signal (E).

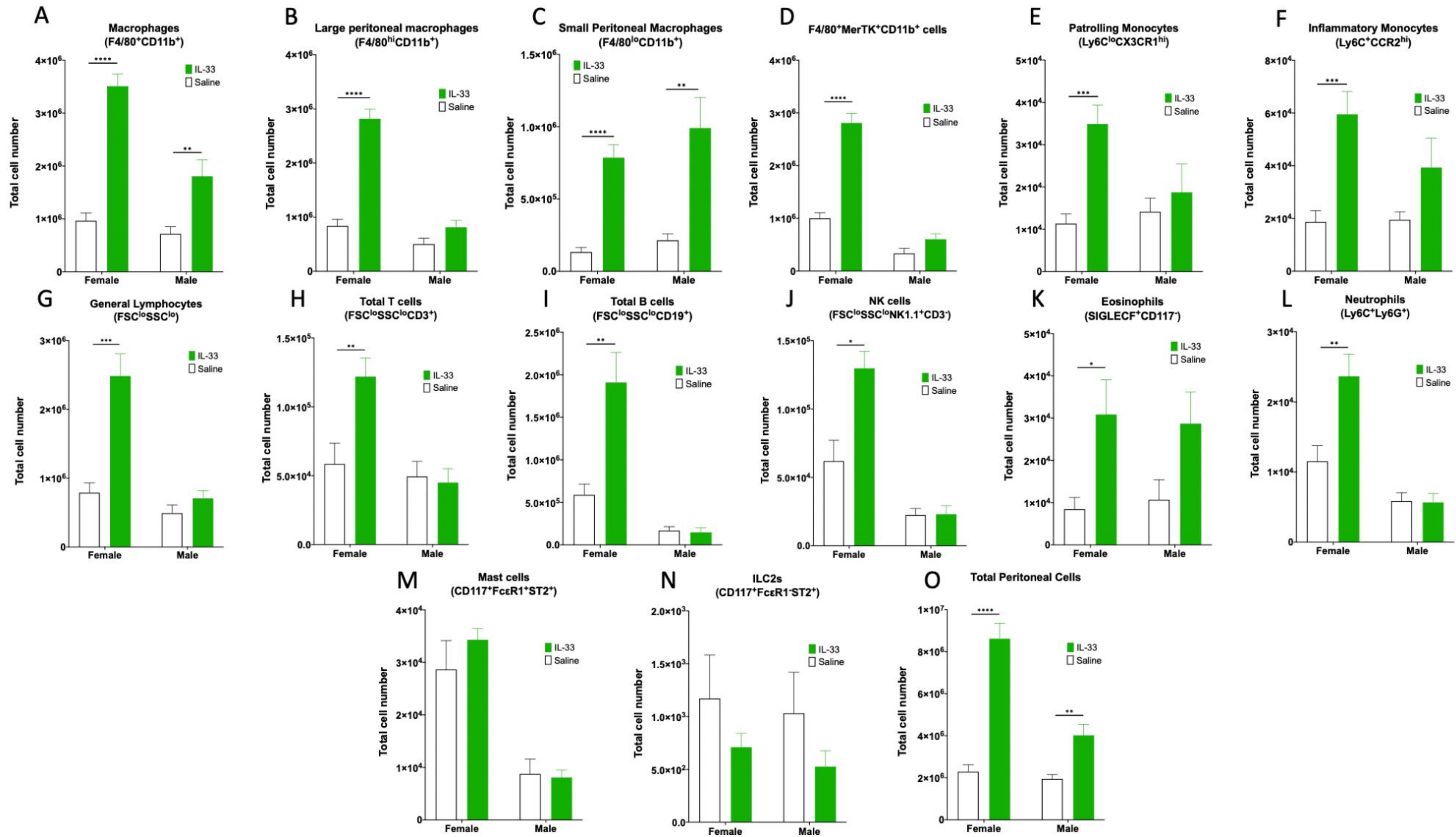

A
Macrophages (F4/80⁺CD11b⁺)
B
Large peritoneal macrophages (F4/80^hiCD11b⁺)
C
Small Peritoneal Macrophages (F4/80^loCD11b⁺)
D
F4/80⁺MerTK⁺CD11b⁺ cells
E
Patrolling Monocytes (Ly6C^loCX3CR1^hi)
F
Inflammatory Monocytes (Ly6C⁺CCR2^hi)
G
General Lymphocytes (FSC^loSSC^lo)
H
Total T cells (FSC^loSSC^loCD3⁺)
I
Total B cells (FSC^loSSC^loCD19⁺)
J
NK cells (FSC^loSSC^loNK1.1⁺CD3⁻)
K
Eosinophils (SIGLECF⁺CD117⁻)
L
Neutrophils (Ly6C⁺Ly6G⁺)
M
Mast cells (CD117⁺FcεR1⁺ST2⁺)
N
ILC2s (CD117⁺FcεR1⁻ST2⁺)
O
Total Peritoneal Cells
Total cell number
IL-33
Saline
Female
Male

Figure 5.3. – Leukocytes from female mice respond more robustly to IL-33 than males. Male mice were given IL-33 (green, n=8) or saline (white, n=8) i.p. for 48h prior to sacrifice and lavage, and compared to females. Total numbers are represented as mean ± SEM. Leukocyte populations were assessed by flow cytometry. Macrophages were assessed as F4/80^{+}CD11b^{+} cells (A), LPMs as F4/80^{hi}CD11b^{+} cells (B), SPMs as F4/80^{lo}CD11b^{+} cells (C), and MerTK+ macrophages as F4/80^{+}MerTK^{+}CD11b^{+} cells (D). Patrolling monocytes (E), and inflammatory monocytes (F) were also assessed in the myeloid compartment. In the lymphocyte compartment (G), total T cells (H), B cells (I), NK cells (J), and ILC2s (N). were examined. In the granulocyte/myeloid compartment, eosinophils (K), neutrophils (L), and mast cells (M) were assessed. Total peritoneal cells were counted from lavage fluid (O). ** $p<0.01$.

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5.2.2 – IL-33 enhances efferocytosis in the peritoneal cavity

To determine the influence of IL-33 administration on efferocytosis, mice were treated i.p. with IL-33 or saline for 48 hours followed by a second i.p. injection of 2.5 x 10^7 CFSE-labeled apoptotic thymocytes for 30 minutes, prior to sacrifice and flow cytometry analyses (30-35% apoptotic, Fig. 5.2D). Overall, F4/80$^+$CD11b$^+$ macrophage numbers were increased after administration of IL-33 and apoptotic cells compared to saline and apoptotic cells (Fig. 5.4A, p=0.0002). Efferocytosis was significantly increased in the peritoneal cavity after IL-33 administration, as indicated by an increased percentage of F4/80$^+$CFSE$^+$ cells as a proportion of total CFSE$^+$ cells (Fig. 5.4B, p=0.0031, Fig. 5.2E). The total numbers of CFSE$^+$ cells in the peritoneal cavity were consistent between groups (Fig. 5.4D, p=0.6518). LPMs performed significantly more efferocytosis in response to IL-33 and apoptotic cells (Fig. 5.4C, p=0.0271) compared to saline animals. SPMs did not significantly differ in their efferocytic abilities (Fig. 5.4C, p=0.6934). This observation is consistent with previous reports that LPMs have a higher capacity for efferocytosis than SPMs [669,670].

MerTK$^+$ macrophages (Fig. 5.4G, p=0.0007) were consistently increased following IL-33 treatment compared to saline. However, numbers of lymphocytes (Fig. 5.4H, p=0.4342), and eosinophils (Fig. 5.4I, p=0.1501) were not impacted by the presence of IL-33 and apoptotic cells in the peritoneal cavity. Mast cells (Fig. 5.4J, p=0.2628) and ILC2s (Fig. 5.4K, p=0.1548) remained unchanged after addition of apoptotic cells in IL-33 treated animals compared to saline. Additional studies on mast cells in Wsh mice showed that IL-33 and apoptotic cell administration had similar impacts on macrophages (Fig. 5.5A), and MerTK$^+$ macrophage populations (Fig. 5.5B), with little impact on

efferocytosis (Fig. 5.5C). Taken together with data showing no impact of IL-33 on mast cell numbers (Fig. 1G), it seems likely these cells are redundant for observed impacts on macrophages, and that their proteolytic activation of IL-33 [202,203] is not crucial to observations.

IL-33 has been previously associated with alternatively activated macrophage phenotypes *in vivo* that promoted resolution of chronic inflammation [635, 664,671]. This is the first study in which IL-33 has been directly linked to efferocytosis as a mechanism by which it aids in resolving inflammation. In contrast to the data presented in this study, recent work with macrophages isolated from women with recurrent spontaneous abortion and cultured *in vitro* had predominantly “M1” macrophage populations that enhanced efferocytosis when IL-33 was inhibited by macrophage-derived sST2 [672]. Such *in vitro* analysis of macrophages in isolation may not fully represent the complex signaling that occurs in tissue microenvironments.

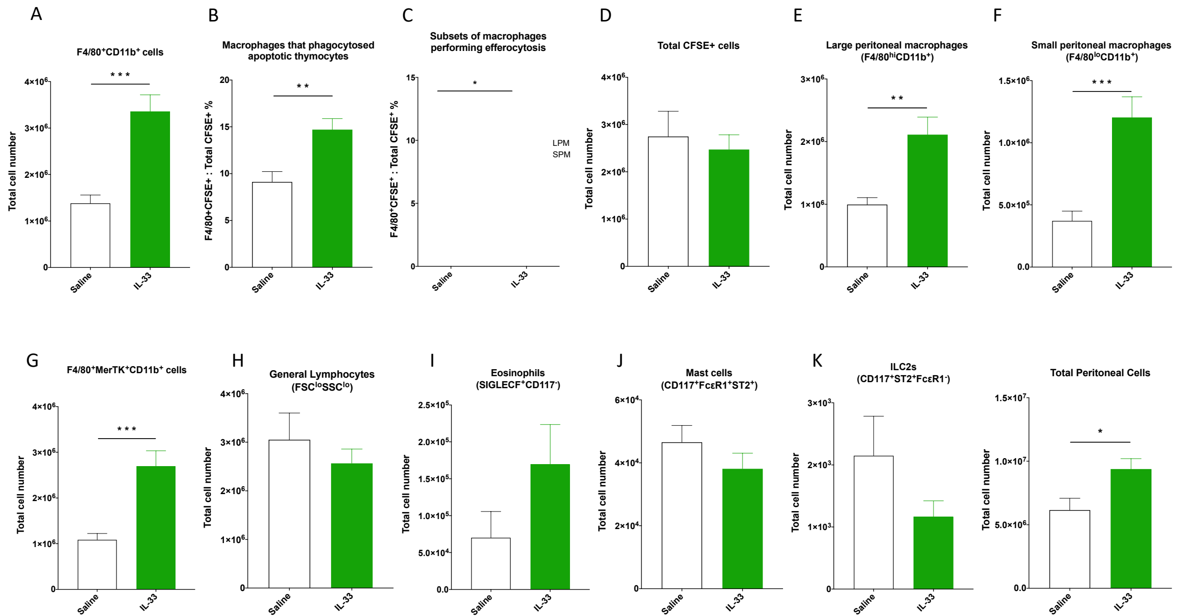


Figure 5.4. – IL-33 increases efferocytosis and associated macrophage phenotypes in the presence of apoptotic cells. Mice were injected i.p. with 0.5µg IL-33 (green, n=10) or saline (white, n=9) for 48h, followed by i.p. injection of 2.5 x 10^7 CFSE-labeled apoptotic thymocytes (30—35% Annexin V$^+$) i.p. for 30 minutes in three independent experiments. Data represented as mean ± SEM. Leukocyte populations were assessed by flow cytometry. Overall macrophage numbers were assessed (A). Efferocytosis activity was measured as % of macrophages that phagocytosed apoptotic thymocytes (B). Within the F4/80$^+$CFSE$^+$ populations, LPMs and SPMs were quantified (C). Total CFSE$^+$ cells were consistent between both groups (D). LPMs (E), SPMs (F) and overall MerTK$^+$ macrophages were assessed (G). General lymphocytes (H), eosinophils (I), mast cells (J) and ILC2s (K) were also examined by flow cytometry, while overall peritoneal cell content was counted in lavage fluid (L). * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

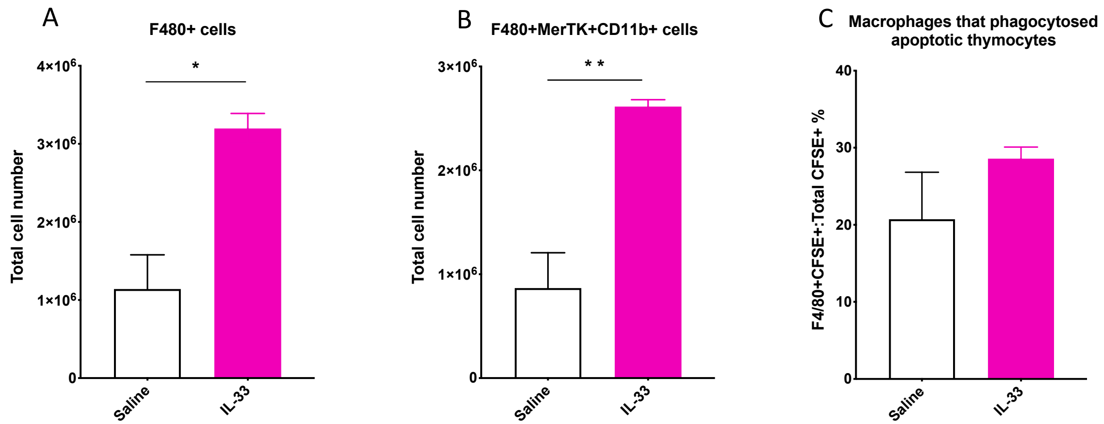


Figure 5.5. – Mast cells are dispensable in promoting MerTK+ macrophages after IL-33 treatment and apoptotic cell administration. Female $Kit^{Wsh/Wsh}$ mice on a C57BL/6 background were treated i.p. with IL-33 (pink, n=3) or saline (white, n=2) for 48h prior to i.p. administration of apoptotic cells. $F4/80^+$ cells (A) and $MerTK^+$ (B) macrophages were significantly increased in the peritoneal cavity of these mice in a similar manner as their C57BL/6 counterparts, while efferocytosis was not significantly increased (C). * $p<0.05$, ** $p<0.01$.

5.2.3 – IL-33 induces broad IL-13 production in the peritoneal microenvironment

We sought to examine potential mechanisms by which IL-33 might enhance efferocytosis in macrophage populations. Peritoneal lavage fluid from IL-33 and saline treated animals at 3h, 6h, 24h, and 48h was assayed for the presence of several cytokines, chemokines, and growth factors via multiplex immunoassay. IL-33-treated mice at 3h and 6h post injection had increased IL-13 (Fig. 5.6A, 3h p=0.0004, 6h p<0.0001), and GM-CSF (Fig. 5.6B, 3h p=0.0011, 6h p<0.0001) levels in lavage fluid compared to saline treated animals. CCL2 was significantly increased in lavage samples early at 3h but not at 6h (Fig. 5.6C, p=0.011). The mediator response was rapid and transient, as none of the cytokines or chemokines assayed were detectable in lavage fluid samples taken 24h or 48h after IL-33 administration. IL-4, IL-10, and CCL5 were undetectable, while no changes were observed in M-CSF (IL-33 vs. saline, 3h p=0.19, 6h p=0.23) and VEGF-A (IL-33 vs. saline, 3h p=0.27, 6h p=0.58) content between conditions.

Given the key role demonstrated for IL-13 in promoting efferocytosis in other systems [350, 521,641], potential sources of this cytokine were examined. Macrophages, lymphocytes, and eosinophils were sorted by FACS from IL-33-treated mice 3h post IL-33 injection (Fig. 5.6D) to assess *Il13* expression by qPCR and ddPCR, as these populations have been identified to produce IL-13 after IL-33 administration [50, 211, 214, 380, 567, 612,659–663]. Three hours after injection of IL-33, increased *Il13* mRNA expression was observed in the eosinophil (Fig. 5.6E, p=0.0286) and lymphocyte (Fig. 5.6F, p=0.0025) populations compared to saline treated mice. *Il13* mRNA was not consistently upregulated in macrophages from IL-33 treated mice, as only two sets had detectable *Il13* mRNA by ddPCR (Fig. 5.6F).

IL-33 has been shown to induce IL-13 production by several leukocytes [50, 211, 380, 567, 612,659–663]. Eosinophils (Fig. 5.6F) and lymphocytes (Fig. 5.6E) had strong increases in *Il13* mRNA after IL-33 treatment. Lymphocytes were gated based on size parameters, and include T and B cells, as well as ILCs. While Treg cell responses to IL-33 have been identified as important sources of IL-13 [521,661], these cells are likely not the sole sources of IL-13 in the peritoneal cavity. Mast cell-derived IL-13 produced in response to IL-33 has been shown to polarize macrophages to an alternatively activated phenotype to suppress inflammation in experimental autoimmune encephalomyelitis [635]. IL-33 has also been shown to promote IL-13 production by eosinophils that contribute to airway inflammation [673]. ILC2 cells are crucial, in several settings, for IL-13 production in response to IL-33. This process is relevant to helminth clearance, allergic inflammation, and control of Treg recruitment [659,660,674]. All of these populations are present in the peritoneal cavity at rest (Fig. 5.1, Fig. 5.8), including Treg cells[675]. Treg cells make up a small population of CD4+ T cells in the peritoneal cavity, ~1% at rest[675]. As other cells respond robustly to IL-33 via IL-13 production, there are likely more contributors than CD4+ Tregs. The redundancy of multiple cell types contributing to the IL-13 signal induced by IL-33 supports the importance of this response for induction of efferocytosis. In light of the increasing use of biologics that limit the action of IL-13 in allergic airway inflammation [676–678], there may be a need to further evaluate the impact of clinical IL-13 blockade on efferocytic responses.

Increases in macrophage number following IL-33 administration (Fig. 5.1A) require substantial cell recruitment activity to expand the population. Following IL-33 administration peritoneal lavage contained significant amounts of GM-CSF (Fig. 5.6B),

and CCL2 (Fig. 5.6C) which are both known to act as chemoattractant signals [679–682] for myeloid cells in peritoneal inflammation. Therefore, in addition to IL-13, there is a contribution by IL-33-induced GM-CSF and CCL2 to recruit myeloid cells to the peritoneal cavity that contribute to local efferocytic responses.

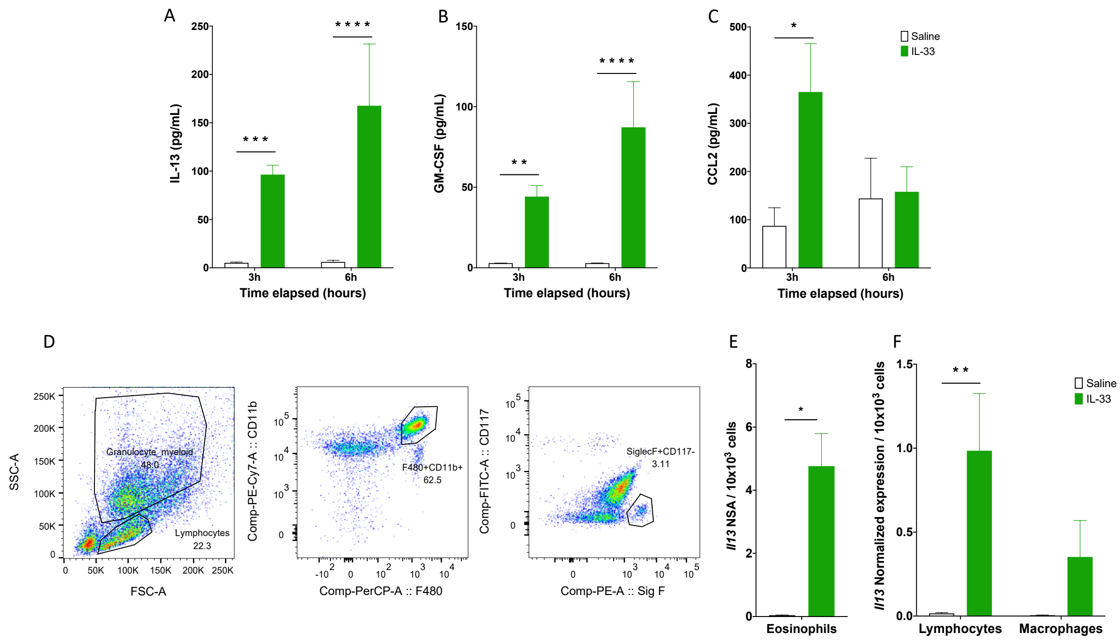


Figure 5.6. – IL-33 induces IL-13 in the peritoneal cavity. Female C57BL/6 mice were injected i.p. with 0.5µg IL-33 or saline for 3h (IL-33 n=17, saline n=16, five separate experiments) or 6h (IL-33 n=7, Saline, n=7, three separate experiments) prior to sacrifice. Results are shown as mean ± SEM. Lavage fluid from IL-33-treated mice contained IL-13 (A), GM-CSF (B), and CCL2 (C). General lymphocytes ($FSC^{lo}SSC^{lo}$), macrophages ($SSC^{int/hi}F4/80^{+}CD11b^{+}$) and eosinophils ($SSC^{int/hi}SIGLECF^{+}CD117^{-}$) were sorted on a FACSAria III flow cytometer for gene expression analysis (D, 2 separate experiments). After IL-33 treatment, eosinophils (E) and lymphocytes (F) had increased *Il13* expression compared to saline. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

5.2.4 – Free mitochondria augment the response to IL-33 in the peritoneal cavity

IL-33 is one of many DAMPs released by dead or dying cells during tissue damage or infection. Therefore, we considered the possibility that the presence of other DAMPs independent of IL-1 family cytokines may impact efferocytosis. A common feature of necrotic cell death is the release of free mitochondria (FM) [421,683] . As such, we sought to determine if a second DAMP may impact the $MerTK^+$ macrophage profile in the peritoneal cavity. Mitochondria were isolated from murine liver and administered i.p. with or without IL-33 48h prior to harvest of peritoneal lavage. FM increased populations of eosinophils (Fig. 5.7F, $p<0.01$), mast cells (G, $p<0.001$) and ILC2s (H, $p<0.05$) in the peritoneal cavity, but did not impact macrophage populations of interest (Fig. 5.7A-D). FM does potentiate IL-33 impacts on these macrophage populations (Fig, 4A-D), as well as lymphocytes (Fig. 5.7E) and overall peritoneal lavage cells (Fig. 5.7I). Although FM total cell counts were not significantly increased at 48h, they were significantly increased at earlier times (e.g., 16h saline $33.76 \times 10^5 \pm 3.28$ cells, FM $46.24 \times 10^5 \pm 1.80$ cells, $p=0.0037$), indicating differential kinetics in the absence of IL-33.

Observed increases in LPMs, SPMs and $MerTK^+$ macrophages were selective to IL-33-treated animals, and not observed in response to alternate DAMP (FM)-induced inflammation (Fig. 5.7A-D). This indicates that the increase in $MerTK^+$ macrophages, and by association efferocytosis, is mediated selectively by IL-33. FM trigger PRRs known to exert largely pro-inflammatory effects [576,683]. In contrast, IL-33 has a wider range of impacts on cellular responses, as leukocytes have been shown to respond in multiple ways depending on the tissue microenvironment and presence of other signals [182]. Importantly, FM have not been shown to induce production of mediators associated

with efferocytosis, such as IL-10 [259,639,640], IL-13 [350, 521,641], resolvins, and maresins [458,465]. The unique position of IL-33 as a dual function cytokine allows it to exert functions outside of the typical inflammatory cascade after damage and contribute to enhancing inflammation resolution.

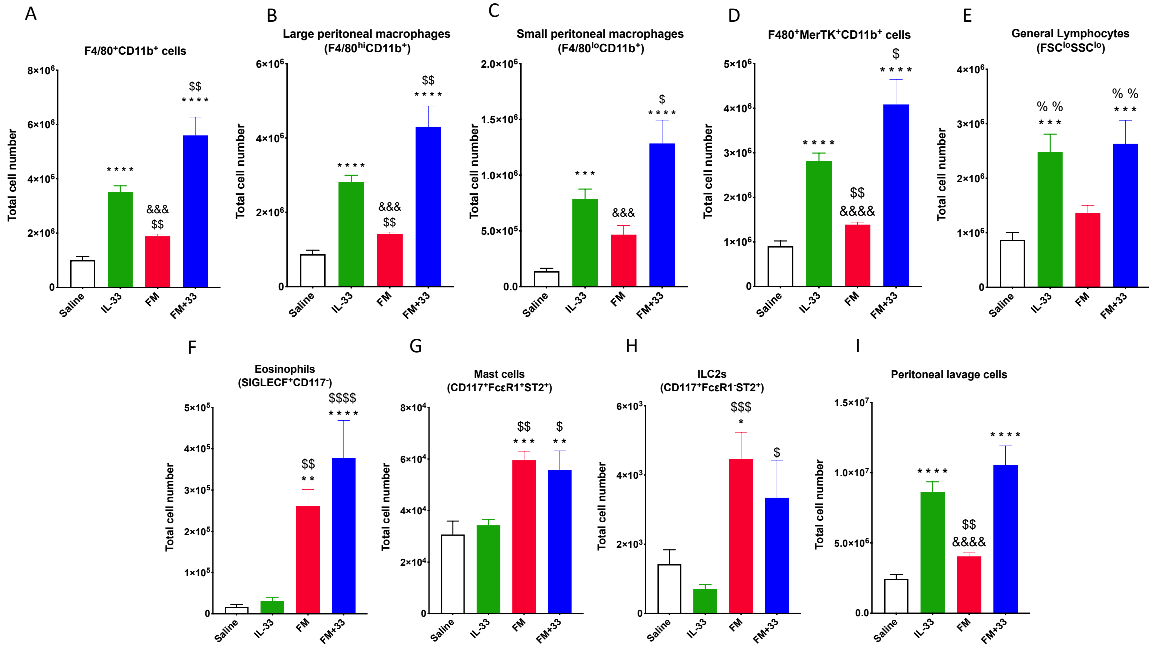
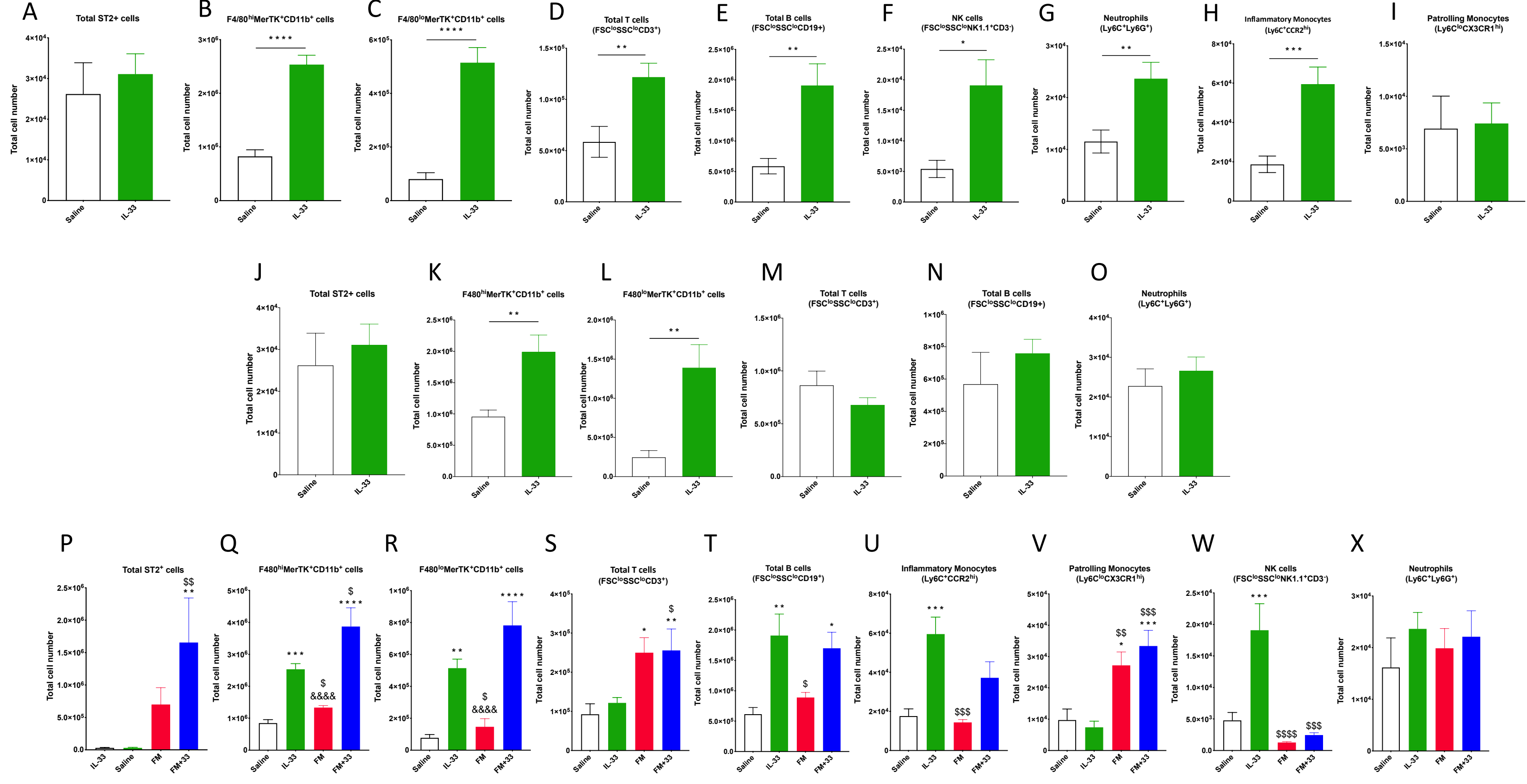


Figure 5.7. – Free mitochondria do not induce MerTK+ macrophages in the peritoneal cavity but have some synergistic impacts with IL-33. Female C57BL/6 mice were injected with 50 μg of free mitochondria (red, FM, n=11 three independent experiments) with or without 0.5 μg IL-33 (blue, FM+33, n=12, three independent experiments) i.p. for 48h prior to sacrifice. Leukocyte populations were assessed by flow cytometry. Total macrophages (A), LPMs (B), SPMs (C) and MerTK^{+} macrophages (D) were assessed in the myeloid compartment. General lymphocytes (E), eosinophils (F), mast cells (G) and ILC2s (H) were also examined. Total peritoneal lavage cells were determined by cell counts from the lavage fluid (I). * indicates significant difference compared to saline, $ compared to IL-33, % compared to FM, and & compared to FM+33. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, and applies to other symbols.



A
Total ST2+ cells
B
F4/80hiMerTK+CD11b+ cells
C
F4/80loMerTK+CD11b+ cells
D
Total T cells (FSCloSSCloCD3+)
E
Total B cells (FSCloSSCloCD19+)
F
NK cells (FSCloSSCloNK1.1+CD3-)
G
Neutrophils (Ly6C+Ly6G+)
H
Inflammatory Monocytes (Ly6C+CCR2hi)
I
Patrolling Monocytes (Ly6CloCX3CR1hi)
J
Total ST2+ cells
K
F480hiMerTK+CD11b+ cells
L
F480loMerTK+CD11b+ cells
M
Total T cells (FSCloSSCloCD3+)
N
Total B cells (FSCloSSCloCD19+)
O
Neutrophils (Ly6C+Ly6G+)
P
Total ST2+ cells
Q
F480hiMerTK+CD11b+ cells
R
F480loMerTK+CD11b+ cells
S
Total T cells (FSCloSSCloCD3+)
T
Total B cells (FSCloSSCloCD19+)
U
Inflammatory Monocytes (Ly6C+CCR2hi)
V
Patrolling Monocytes (Ly6CloCX3CR1hi)
W
NK cells (FSCloSSCloNK1.1+CD3-)
X
Neutrophils (Ly6C+Ly6G+)
Total cell number
Saline
IL-33
FM
FM+33

Figure 5.8. – The lymphocyte and granulocyte compartment in female mice after experimental treatment. Total numbers are represented as mean ± SEM. Leukocyte populations were assessed by flow cytometry. Leukocytes from female mice treated i.p. with IL-33 for 48h were assessed for $ST2^{+}$ cells (A). In the myeloid compartment, $MerTK^{+}$ LPM (B), and SPM (C) were examined. In the lymphocyte compartment total T cells (D), total B cells (E), and NK cells (F) were identified. In the granulocyte/myeloid compartment, neutrophils (G), inflammatory monocytes (H), and patrolling monocytes (I) were assessed. Leukocytes from female mice treated i.p. with IL-33 for 48h, and then with CFSE-labeled apoptotic thymocytes for 30 minutes were assessed for $ST2^{+}$ cells compared to controls (J). $MerTK^{+}$ LPM (K), and SPM (L) populations were also examined. In the lymphocyte compartment, total T cells (M), and total B cells (N) were identified, and in the granulocyte/monocyte compartment neutrophil populations were assessed (O). Leukocytes from female mice treated with FM or FM+IL-33 (FM+33) for 48h were assessed for $ST2^{+}$ cells compared to saline (P). $MerTK^{+}$ LPMs (Q), and $MerTK^{+}$ SPMs (R) were identified. Total T cells (S), total B cells (T) and NK cells (W) in the lymphocyte compartment were examined (T). The granulocyte/monocyte compartment was probed for inflammatory monocytes (U), patrolling monocytes (V) and neutrophils (X). * indicates significant difference compared to saline, $ compared to IL-33, % compared to FM, and & compared to saline. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and applies to other symbols.

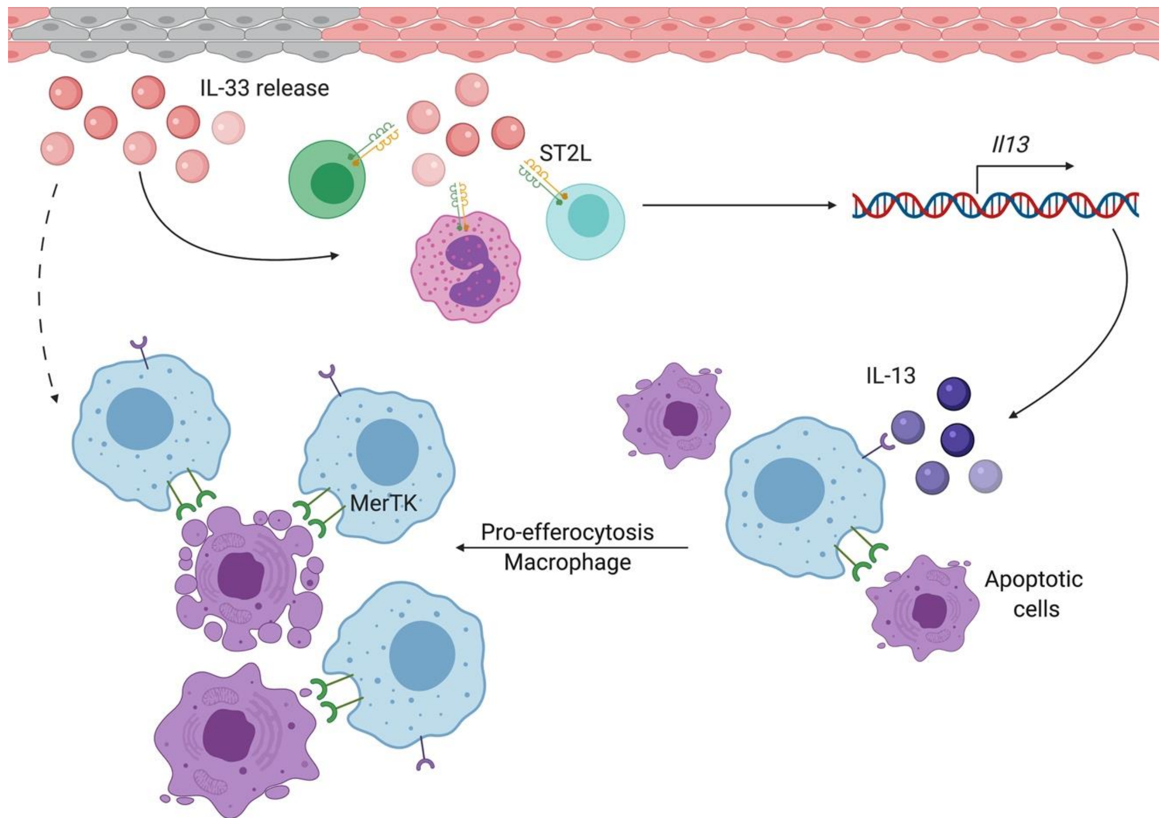


Figure 5.9. – Overview of the mechanism for IL-33 promotion of efferocytosis. IL-33 released as a DAMP activates local leukocyte populations after damage and promotes the selective recruitment of macrophages. Lymphocytes and eosinophils increase production of IL-13 in response to IL-33. This acts to increase the number of MerTK^{+} macrophages to allow efferocytosis to occur, thereby removing apoptotic cells.

Resolution of inflammation is crucial to return tissues to homeostasis, and the phagocytosis of apoptotic neutrophils by macrophages is an important step in this process [459,461]. As such, initial DAMP release after injury could play a useful role in providing signals to promote resolution. IL-33 is a DAMP that has been shown to act consistently on several leukocytes [178] to induce production of IL-13 [182]. IL-13 increased MerTK^{+} macrophage populations and efferocytosis ability in macrophages *in vitro* [350,641] and *in vivo* in peritonitis and acute lung injury [521], resulting in macrophage populations with tissue repair phenotypes. IL-33 exerts its effects early after cell death, as it is highly susceptible to inactivation by oxidation [198] and proteolytic processing [201,203] once in the extracellular space. Acutely, its proteolytic processing can also amplify its local bioactivity [203]. This would suggest that actions induced by IL-33 would be localised to and potent within the damaged tissue microenvironment. There was an additive effect of administering IL-33 with FM on total macrophage number and MerTK^{+} macrophage number in the peritoneal cavity that was not observed in animals treated with FM alone (Fig. 5.6A-D). Additionally, increases in MerTK^{+} macrophages persisted in the peritoneal cavity after IL-33 and apoptotic thymocyte administration (Fig. 5.4G), where populations of lymphocytes (Fig. 5.4H), and eosinophils (Fig. 5.4I) did not. Together these data would indicate that IL-33 has a robust impact on increasing MerTK^{+} macrophage populations independent of other signals to promote efferocytosis.

By increasing macrophage populations and upregulating local IL-13 production, IL-33 promotes an environment that encourages macrophage efferocytosis of apoptotic neutrophils. This provides clarity to several recent reports identifying positive roles for IL-33 in promoting resolution of inflammation via actions on macrophages. Li and

colleagues showed that in the context of cardiac allograft rejection, graft IL-33 is an important signal within the first three days after transplant to limit pro-inflammatory macrophage actions and promote phenotypes of repair and regulation via IL-33-ST2 signaling in the graft [384]. Tu et al. demonstrated that macrophages from the peritoneal cavity of IL-33 i.p. treated mice reduced pro-inflammatory signaling and disease severity when transferred to mice with colitis [664]. Finally, Lu et al. showed that *Il33*$^{-/-}$ mice have impaired red blood cell removal by splenic red pulp macrophages restored by exogenous IL-33 administration [684]. It has been well established that following efferocytosis, macrophages transition to a pro-reparative phenotype [483–490], while it has been shown *in vitro* that IL-33 does not directly activate macrophages to 'alternative' activation phenotypes [635,664]. Therefore, IL-33 induction of local IL-13 for efferocytosis may provide a mechanism by which macrophages can switch to this pro-repair phenotype.

Macrophages in the peritoneal cavity are crucial facilitators of wound repair within local organs [685,686]. Fat-associated lymphoid clusters, or milky spots, have been shown to be important sources of IL-33 in the peritoneal cavity [180], as have mesothelial cells in the peritoneum [181]. Upon tissue damage in the peritoneal cavity, these cells have been shown to release IL-33 into the tissue microenvironment [181]. IL-33 is important in the peritoneal cavity for reducing inflammation in colitis [664], enhancing macrophage-mediated *Candida albicans* responses [687] and promoting type 2 immune environments in ovarian cancer models that limit metastasis [665]. The findings of the current study further support this work and identify a new mechanism by which IL-33 may be exerting its effects.

In conclusion, the current study shows that IL-33 promotes efferocytosis in the peritoneal cavity by macrophages (Fig. 5.9). This is mediated by the induction of IL-13

production, with contributing signals from eosinophils and lymphocytes. Clinical evidence has shown that increased presence of the soluble IL-33 receptor sST2 in the plasma of patients with allergic airway disease [688,689], systemic lupus erythematosus [688,689], and cardiovascular disease [228,395–399] is predictive of adverse outcomes. Efferocytosis has important implications in these disease settings [460, 529, 655,690–692], indicating clinical relevance of these findings. Future work should focus on the utility of exogenous IL-33 in inducing macrophage efferocytosis in chronic inflammatory models. Our work identifies a novel role for IL-33 to promote macrophage efferocytosis and subsequently resolution of inflammation, which could be targeted in chronic inflammatory settings.

CHAPTER 6: DISCUSSION

6.1 – Summary of major findings

The work outlined in this thesis sought to expand upon previous knowledge in the field of cardiac fibrosis. Namely, to better understand the impact mast cells have on cardiac fibrosis with relevance to human patients, to elucidate the relationship between mast cells and IL-33, and to determine how said relationship could contribute to proper inflammation resolution and wound healing in the context of CVD.

Cardiac fibrosis is a complex process of overlapping phases that progresses from inflammation, to repair, to maturation. Multiple immune and stromal cells respond to a myriad of signals in temporally dependent processes. It would be naïve to assume one cell population or cytokine is the linchpin of such a process, and reductive to present it as such. Rather, through this work we have identified evidence for a potential beneficial mast cell role, and mechanisms by which mast cells and IL-33 can contribute to resolution of inflammation and cardiac fibrosis (Fig. 6.1). We have shown that IL-33 can act on murine leukocyte populations in the peritoneal cavity to promote increases in $MerTK^+$ macrophage populations (Fig. 5.1D) and efferocytosis (Fig. 5.4B) following local induction of IL-13 (Fig. 5.6A). As IL-33 is a cardiac DAMP [371,372, 376,381], it is possible that a similar process could occur in the cardiac microenvironment. Furthermore, we have demonstrated that human mast cells can respond to IL-33 activation via induction of a unique cocktail of mediators that may promote angiogenesis in endothelial cells, and efferocytosis in macrophages (Fig. 4.3D). Whether this is the mechanism at work in human hearts is unknown, but we have shown that patients with increased atrial mast cell content at the time of surgery had less collagen content compared to their low mast cell containing peers (Fig. 3.5C) and the cohort as a whole (Fig. 3.5D). High atrial mast cell density was also associated with improved functional outcomes indicated by reduced incidence of NHYA III/IV reclassification and mortality post-operative.

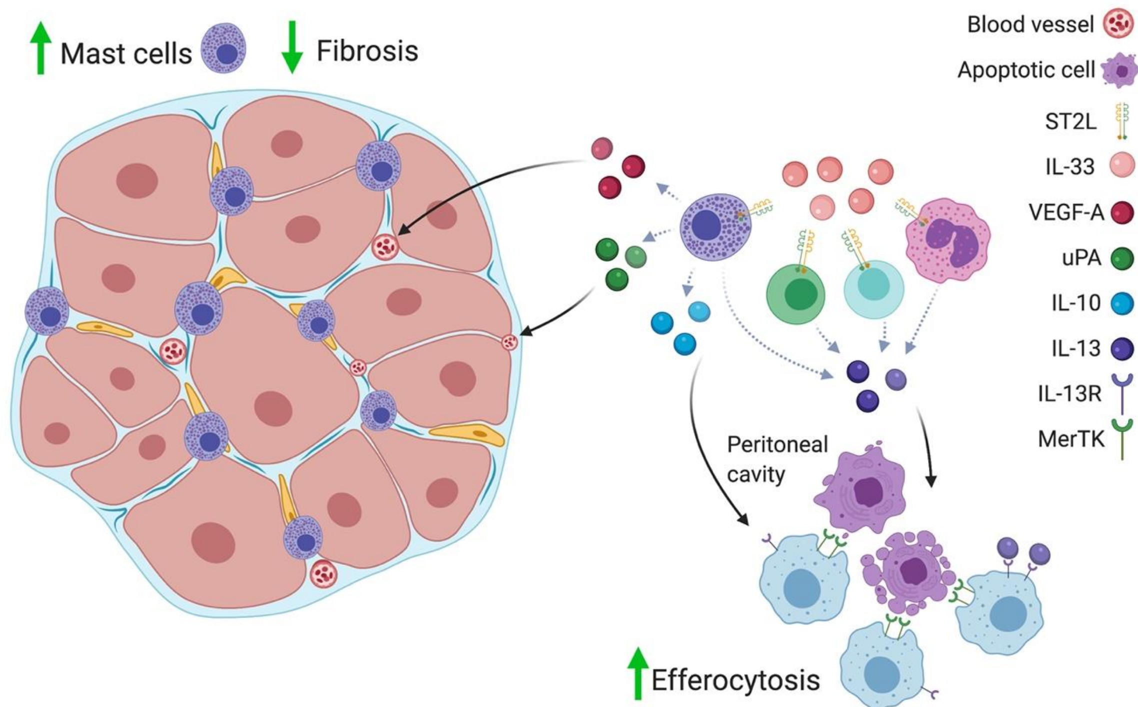


Figure 6.1. – Summary of findings. We sought to assess how mast cells and IL-33 may contribute to cardiac fibrosis and resolution of inflammation. In human atrial tissue samples, increased mast cell presence was associated with decreased fibrotic tissue. We examined mast cell responses to the cardiac DAMP IL-33 *in vitro* and found a significant increase in angiogenic growth factors VEGF-A and uPA, and in pro-efferocytosis cytokines IL-10 and IL-13. To model efferocytosis, mice received IL-33 via intraperitoneal injection, where it was observed that MerTK$^+$ macrophage number and efferocytosis were increased. IL-13 was shown to be increased early after IL-33 administration in the peritoneal cavity, and lymphocytes and eosinophils were identified as potential sources. This data provides several lines of inquiry to pursue in order to better understand and therapeutically target fibrotic remodelling in the heart.

6.2 – Implications and relevance of major findings

Mast cells were associated with beneficial effects in the human heart as it relates to collagen deposition (Fig. 3.5C,D, Fig. 3.8A,B) and functional outcomes (Fig. 3.5H,I, Fig. 3.8E). These observations were in contrast to previously published data in murine models [75]. While we were unable to identify a direct mechanism responsible for our *ex vivo* human observations, the answer likely lies in cardiac mast cell activity. For example, we observed a significant increase in the percentage of degranulating mast cells in patients with high collagen content (Fig. 3.5E) compared to low collagen content. Mast cell degranulation was not significantly different between high and low mast cell density

groups (Fig. 3.7). Mast cell granule contents are known to be largely pro-fibrotic, while *de novo* synthesis activities are generally associated with anti-fibrotic actions [75]. Therefore, gaining a better understanding of what actions mast cells exert in the cardiac microenvironment would be important for better therapeutic targeting of their actions.

Our finding that increased mast cell density is associated with decreased collagen content in human cardiac tissue is in direct contrast with several studies, where mast cell density increases were associated with increased remodelling, as was discussed in chapter 3 [361, 363,598]. Our work benefited from a large cohort (n=112) of patients who could be followed post-operative, and tissue obtained from live subjects. Typically, in human heart studies, work focuses on smaller patient groups (n<30), and often tissues are acquired post-mortem which prevents assessment of functional outcomes in relation to tissue findings. Atrial appendage tissues that were obtained in our study are medical waste during cardiac procedures and are an underutilized resource to study human cardiac tissue. Future work should capitalize on these samples to probe questions such as the nature of the resident leukocyte population in the heart, interactions between leukocytes and stromal cells, and the expression of PRRs on resident cardiac leukocytes. By assessing mast cell content in atrial tissue, potentially in combination with other fibrotic biomarkers like plasma sST2 [401], clinicians could identify patients that would benefit from anti-fibrotic therapies.

The mechanism by which mast cells are associated with beneficial effects is unknown but could be mediated by IL-33-ST2L signaling. IL-33 is known to be protective in the heart after damage [343–345, 372, 374, 376,378–381], an *in vivo* observation that is emphasized by the accuracy of sST2 as a prognostic biomarker of remodeling and heart failure progression (Fig. 3.6J) [227,395–399]. We have shown *in vitro* that primary human mast cells do not degranulate in response to IL-33 or several other relevant DAMPs (Fig. 4.3A), nor do they produce pro-inflammatory or pro-fibrotic mediators (Fig. 4.3B). Instead, they produce several mediators with relevance to beneficial outcomes in the heart, such as VEGF-A [338–340], IL-10 [331–333], and IL-13 [349,350]. Of interest, mRNA expression of *IL33* and *VEGFA* was positively correlated in human atrial tissue samples taken at the time of surgery (Fig. 4.1E). This could indicate that the induction of *VEGFA* is related to *IL33* in the cardiac microenvironment.

Mast cell production of uPA has a less clear role in this context. Although uPA is an angiogenic growth factor induced by VEGF-A and important for promoting vessel formation and endothelial cell migration [279], it's actions as a plasminogen activating protease have been linked to detrimental fibrotic processes in the heart [625,626] via induction of TGF-β signaling [627]. Conversely, uPA has also been shown to be a beneficial actor in this context by promoting revascularization and VEGF-A upregulation that leads to reduced infarct size, and fibrosis [618–620]. It is possible that targeted induction of uPA by mast cells may have potential therapeutic utility in reversing extensive remodelling, as uPA has been shown to degrade ECM to reduce scar tissue in liver [693] and skin [694]. Given that angiogenesis is a crucial component of the remodeling phase in proper wound repair [231,238], it is also possible that timing of uPA induction could be important in pushing towards pro-angiogenic and pro-repair responses in the myocardium.

In addition to an increase in angiogenic growth factors, mast cells *in vitro* also produced IL-10 and IL-13. Both of these cytokines have been associated with promoting efferocytosis activity in macrophages [259, 350, 521,639–641]. Upregulation of both angiogenic and efferocytic programs is crucial for effective wound healing [642]. Administration of lethally irradiated murine fibroblasts at damaged sites promoted effective wound healing in skin and liver, and angiogenesis in damaged tissues after two weeks compared to *Casp3*$^{-/-}$ fibroblasts [695]. Mice deficient in efferocytosis mediators *Mertk*$^{-/-}$ and *Mfge-8*$^{-/-}$ had impaired angiogenesis and wound healing in both the heart [259] and the skin [696]. It is well established that following efferocytosis macrophages differentiate into wound repair phenotypes with the capacity to promote angiogenesis at damaged sites [458, 463, 487,488, 490, 464–467,483–486]. Thus, mast cell activation of both angiogenic and efferocytic programs in response to the DAMP IL-33 could contribute to proper repair in the cardiac setting. Female and male mice had different responses to IL-33 in the peritoneal cavity (Fig. 5.3). Sex differences in the response to IL-33 have previously been reported in the context of allergic airway inflammation and ILC2 responses [697,698]. While IL-33 itself has not been shown to be influenced by sex in its protective role in cardiac remodelling, sST2 does have differing content in healthy male and female subjects[391–393], though upon development of CVD these differences do not persist [227,394,395]. However, it is well understood that sex has a significant impact on the progression of cardiac remodelling

itself [699,700]. Females appear to be protected from extensive cardiac compared to males, though how different cardiovascular disease risk factors influences this observation has yet to be explored [699]. Additionally, mast cell activity is impacted by male and female sex hormones [701]. It would be of interest to determine how sex hormones influence mast cell activity as it relates to cardiac remodelling, and furthermore if this impacts the beneficial role of IL-33 in this system.

Through this work we have identified a previously undescribed mechanism by which IL-33 contributes to inflammation resolution after damage via induction of efferocytosis. IL-33 induces local IL-13 production (Fig. 5.6A), MerTK$^+$ macrophage populations (Fig. 5.1D) and efferocytosis in the context of apoptotic thymocytes (Fig. 5.4B). Modeling these responses in the peritoneal cavity allowed us to observe IL-33 impacts on efferocytosis *in vivo*, which is difficult at sites where macrophages require isolation from the surrounding solid tissue [461]. This mechanism could be particularly important at sites with high IL-33 release after damage, such as the heart [381]. IL-33 has been shown to have protective effects in the cardiac microenvironment after damage, such as reducing collagen deposition, hypertrophy [379], pro-inflammatory cytokine signaling [372, 374,380] and infarct size [343–345,378], while improving cardiac function and survival [376,381]. These beneficial effects have been attributed to the pro-survival impact of IL-33 on cardiomyocytes under hypoxic conditions [344,375–378]. However, given the myriad resident immune cell populations in the heart [231,238] it is possible that IL-33 could be acting on these populations as well to promote inflammation resolution.

The identity and prevalence of ST2$^+$ leukocytes in the cardiac microenvironment at rest is unknown. Efforts have been made to characterize resident leukocytes in murine hearts using single cell RNA-sequencing techniques (scRNA-seq) [252,699–702]. Macrophages and monocytes make up the majority of resident leukocytes [252,699], while B cells, T cells, NK cells, ILC2s, non-T non-B granulocytes, and DC-like cells [700] have also been identified at rest. Mast cells were not readily identified in resting murine hearts, but were assessed in the context of pressure-overload hypertension [702], where they were determined to be the smallest cluster pulled out of murine myocardium based on the CD45$^+$ cells identified. However, CD45 may not be a qualifying marker for mast cell identity in the heart, as in human hearts mast cells have been shown to be

Tryptase$^+$CD45^{dim} [702]. While there is no data available on the ST2L expression of resident cardiac leukocytes, at distal sites ST2L is known to be expressed on murine mast cells [50], ILC2s [207], T_{regs} [208], and T_{H2} [209] cells at rest. MerTK has been shown to reduce cardiac fibrosis in mouse models of experimental MI and hypertension [258–260]. Thus, IL-33 induction of MerTK via local leukocyte IL-13 production may be a mechanism by which beneficial outcomes could be mediated. The type and abundance of resident leukocytes in the human heart remains to be determined, but mast cells [606], and macrophages [252] are among those known to be present.

Mast cells readily respond to IL-33 via induction of IL-13 as has been shown in our work (Fig. 4.3D) and by others [50,612]. IL-13 is a known activator of efferocytosis in macrophages via IL-4Rα signaling [350], and local induction of IL-13 by IL-33 can induce phagocytosis of apoptotic cells in the peritoneal cavity by macrophages (Fig 5.4B). However, this system did not appear to be relevant in the peritoneal cavity, as Wsh mice that received apoptotic cells had similar increases in F4/80$^+$CD11b$^+$MerTK$^+$ macrophages as their mast cell sufficient counterparts (Fig. 5.5). This could be due to the fact that most if not all resting ST2$^+$ leukocytes are present in the peritoneal cavity at the time of IL-33 administration, making the contribution of IL-13 from mast cells likely redundant. While this may be the case in the peritoneal cavity, there are other sites where mast cell contribution may be more relevant. Mast cells are resident in human and murine cardiac tissue, with greater abundance in the former, and IL-33 is released in response to damage by cardiomyocytes and cardiac fibroblasts. Therefore, it is possible that mast cells could respond to IL-33 by producing IL-13 to promote MerTK$^+$ macrophages and enhanced efferocytosis capacity. Future work should aim to elucidate this process in the cardiac microenvironment in animal models with mast cell densities representative of human hearts.

6.3 – Limitations of the experimental systems

Mast cells are difficult populations to study in the cardiac tissue setting. They are sensitive cells that degranulate in response to hypoxia [597], and mechanical stimulation [471,472], which impacts the ability to identify their activity without extraction and processing acting as confounding factors. Furthermore, mast cells in murine hearts are distributed differently, and present at lesser densities than in humans [271]. For example, the

murine myocardium contains a median population of 0.56 ± 0.05 mast cells/mm^2 [271], while our study of human atria showed a median population of 7.3 ± 2.67 mast cells/mm^2 (Fig. 3.1B). This almost 14-fold difference in mast cell content would indicate that mast cells could play a more important role in human disease than murine models can effectively show. Furthermore, models of mast cell deficiency that are specific in their loss of mast cells without extraneous overlap in other populations or genes have only recently become prevalent [352]. Work conducted in tandem with our human studies in *Cpa3-Cre; Mcl-1$^{fl/fl}$,* or Hello Kitty, mice showed that a lack of mast cells did not impact cardiac fibrosis in an AngII-mediated model of hypertrophy and scarring (Appendix A). These observations are in keeping with several studies in the literature indicating no role for mast cells in murine cardiac fibrosis models [286,355,356]. In light of these data, as well as the noted confusion in the current literature around mast cell roles in CVD and cardiac fibrosis [75,607], the relevance of murine models to study mast cells in cardiac fibrosis could be questioned. As will be discussed in due course, murine models may need to be modified prior to continuation of such work.

We were fortunate to receive human atrial tissue samples in this study. However, working with human cardiac tissues also comes with several limitations. Atrial appendage samples were obtained from cardiac surgery patients. Therefore, there is only a single point in time for biological sampling, and this point is at the end or latter course of disease progression. As such, the etiology of disease is unknown and there is no control over the instigating event that led to disease or damage, nor the time from instigating event to tissue acquisition. Perhaps most importantly, samples were acquired from the atria, rather than the ventricles where any infarct or extensive remodelling is more likely to have occurred. While it is obviously dangerous to the patient to excise tissue from the ventricles, this does limit conclusions that can be made with regard to direct mast cell roles in infarct contraction and reparative fibrosis.

The IL-33/ST2L pathway is known to be active in cardiac tissues after damage [371–375], however we were unable to explore this pathway thoroughly in relation to mast cells in our human patient samples. This is due in large part to the design of our studies. Patients were collected as part of two studies, one in Halifax, Nova Scotia, and one in St. John, New Brunswick. The Restitution Enhancement in Arthritis and Chronic Heart disease

study in Halifax focused on patients who had a MI within 30 days of undergoing CABG surgery to study outcomes after ischemic incidents. The Inflammation Metabolism Physical Ability and Research Translation study in St. John had broader inclusion criterion, admitting patients undergoing CABG or VR surgeries and assessing outcomes related to obesity and metabolic disorders. As such, our patient cohort was quite broad, and importantly very few tissues were isolated from patients early after ischemic incident, if one had occurred. IL-33 is a DAMP [176] released from stromal cells in the cardiac microenvironment after injury [376,381]. Due to the many levels of bioactivity regulation (Fig. 1.2), IL-33 is thought to be active and potent at sites local to damage for short periods of time [178,198–203]. Therefore, to study IL-33 in this setting, samples would likely need to be excised soon after ischemic injury. These limitations make it quite difficult to study how IL-33 impacts wound healing in human cardiac tissues. We were able to assess the impact of sST2 in this setting and showed a positive correlation between plasma sST2 content and atrial collagen content at the time of surgery (Fig. 3.6J), supporting literature data that sST2 is a prognostic biomarker of cardiac remodelling [226–228]. Future work should focus on design of studies that allow for assessment of IL-33 tissue content, as well as $ST2L^+$ leukocyte content in myocardial tissues.

As a proxy measure, we sought to determine the impacts of IL-33 on human mast cells *in vitro* as they relate to angiogenesis and resolution of inflammation. While we were able to observe significant increases in IL-10, IL-13, uPA and VEGF-A production (Fig 4.3D), it remains to be seen how this response may be relevant *in vivo*. IL-33 is rapidly oxidized [198] and proteolytically processed [201–203] in the tissue microenvironment, but it is unclear if these settings are reflected in culture conditions. Furthermore, multiple DAMPs are released after cardiac damage, including HMGB1 [550], S100A8/A9 [704], HSPs [704], and extracellular ATP [705], that could impact the mast cell response to IL-33. Although we have shown that mast cells do not degranulate in response to these DAMPs (Fig. 4.3A), it would be important to consider the impact of multiple DAMPs or necrotic cardiomyocytes on mast cell actions to be more confident in their *in vivo* responses.

Our observations that IL-33 can drive efferocytosis *in vivo* are quite exciting in identifying a new role for IL-33 after damage. However, it is unclear how strong this

signal would be in the context of larger DAMP release, as briefly touched on above. We were able to show that addition of free mitochondria and IL-33 together did not dampen MerTK$^+$ macrophage populations but rather enhanced them, where free mitochondria alone could not (Fig. 5.7D). However, multiple signals are present in the context of damage, including up-regulated pro-inflammatory signals from local resident immune cells [420]. How these signals may interfere with or contribute to the impact of IL-33 on efferocytosis is unclear.

Efferocytosis is a constant process in tissues and organs throughout the body, as it is crucial to homeostasis [529]. However, efferocytosis is extremely difficult to visualize *in vivo* as phagocytosis of apoptotic cells can occur in as little as 10 minutes [461]. The issue is further complicated in solid tissues, like the heart, where mechanical and enzymatic digestion is necessary to isolate cells prior to progressing with further assays. Therefore, we chose to model efferocytosis in the peritoneal cavity, as it has been shown to be a reliable site for manipulating the inflammatory response and visualizing phagocytosis of apoptotic cells [465, 477, 487,706]. While using this model allowed us to assess the impact of IL-33 on efferocytosis and inflammation resolution, it is difficult to make a direct translation to the cardiac microenvironment. This is due in large part to differences in tissue resident leukocyte populations, in particular macrophages. It has become increasingly recognized that tissue resident macrophages have different functions informed by the locations in which they reside [614]. Furthermore, serous cavity macrophage populations appear to have distinct functions for rapid infiltration from the peritoneal cavity into local solid tissues and organs to co-ordinate repair responses therein [707]. Similar phenotypes have been observed in macrophages population the pericardial cavity as well [708]. In contrast, cardiac tissue resident macrophages remain in the heart, contracting in population size immediately following damage, and proliferating to expand locally as wound repair progresses [252]. The degree of similarity in the response to IL-33 and local IL-13 induction between functionally distinct resident cardiac macrophages [252,346] and resident peritoneal macrophages [709,710] is unknown but may impact how their capacity for efferocytosis can be targeted.

6.4 – Proposed future directions

We have established foundational baselines for mast cell influences in human heart, and IL-33 impacts on inflammation resolution. To define how mast cell-IL-33 interactions contribute to cardiac fibrosis will require further analyses. To do this, we propose the design of a new human study and adapted murine study.

To better understand the impact of mast cells in relation to IL-33 on cardiac fibrosis in human patients, a study should be designed with strict inclusion criteria and long-term follow-up metrics. Recruitment of patients with acute MI undergoing coronary artery bypass grafting within a defined window of 30 days post-MI would allow us to have a more homogeneous population of patients to assess. While this is a more difficult criteria to meet at smaller centres in Atlantic Canada, it could be possible at a larger surgical institution. From these patient samples, atrial appendage tissues and plasma would be obtained for several purposes. Atrial appendage is medical waste in these surgeries, and as such larger sections can be acquired than is possible by biopsy. Tissue samples would be divided into three sections. The first section would be used for histological assessment of collagen content, general pathology, and MANTRA system analyses of MerTK distribution in relation to apoptotic cells. The second would be used for scRNA-seq of mast cells and local leukocytes to gain a better understanding of their activity in atria. The third could be used for protein analyses of mediators directly in tissues. In co-ordination, plasma samples can be assessed for sST2 and other fibrosis-associated biomarkers [226] to better define their relevance in this setting. To assess the IL-33/ST2L system, determination of ST2L expression on the surface of resident leukocytes with MANTRA staining could give an indication of ability to respond to IL-33, while scRNA-seq could give an indication of local cells that may have upregulated IL-33 expression. Finally, efforts should be made to gain access to normal tissues, such as atrial appendage from transplant donor hearts, to assess $ST2L^+$ cell populations at rest.

Functional characterization of mast cell-IL-33 relationships in the heart would likely rely on mouse models. However, the issue of mast cell density discrepancies in the murine system is a still a serious barrier. While canine models have been shown to have similar mast cell distribution and density to humans [271,711], it is obviously more convenient, and cost effective to work with mice. It has been shown that mast cell distribution in murine myocardium is driven by *Mitf* [271]. With the use of Cre-loxP

recombination systems, a mouse strain could be developed to drive cardiac specific [713,714] *Mitf* expression in the hopes of increasing mast cell density in the heart. Models already exist where *Mitf* could be inserted, such as *VIPR2-ERT2CreERT2*, which have specific cardiac targeted insertion from embryonic day 11.5 in mice [713]. These animals could then be crossed to *Il33-/-* or *Il1rl1-/-* mice to assess the impacts of remodelling in mast cell sufficient mice lacking IL-33-ST2L signaling. Furthermore, assessment of MerTK$^+$ macrophage populations and their localization to mast cells by MANTRA staining would allow us to determine the impact of mast cell responses to IL-33 on efferocytosis activity. In this way, mast cell-IL-33 roles in cardiac fibrosis could be more accurately modeled to human disease.

It would be important to assess the potential therapeutic relevance of IL-33 to induce efferocytosis and subsequent resolution pathways. Macrophages isolated from mice treated i.p. with IL-33 and introduced to animals who subsequently underwent TNBS colitis were able to reduce destruction of villi and inflammatory cytokine and chemokine production [664]. However, in this study only CD206$^+$ macrophages were administered, and the authors did not assess efferocytosis marker expression. It would be interesting to repeat this study with the administration of IL-33 itself, or IL-33-induced MerTK$^+$ macrophages to mice with established colitis to determine if inflammation could be resolved by either stimulus.

To better understand the impact of IL-33 in the context of overall tissue damage, future work should focus on models of peritoneal damage, such as surgically induced peritoneal adhesions, in *Il33-/-* and *Il1rl1-/-* animals. While administration of IL-33 can induce efferocytosis in the peritoneal cavity (Fig. 5.4B), it is unclear how robust it would be in the context of multiple DAMPs. By inducing local damage in the context of mice lacking IL-33, a better understanding could be gained about the functional relevance of these observations. Previous work has shown IL-33 to be associated with type 2 skewing of immune cells in the peritoneal cavity with relevance to ovarian cancer [665], endometriosis [714], sepsis [220], and colitis [664], but there has yet to be a clear definition of the actions of IL-33 in the context of damage signals without exogenous administration. This would provide useful information on the persistence of the IL-33 signal strength when additional DAMP 'noise' is present.

6.5 – Conclusions

At the outset of this project, we considered the potential link between mast cells and IL-33 in promoting beneficial outcomes in cardiac fibrosis. Significant new knowledge has been contributed to further probe this question and identify potentially useful therapeutic targets. We have shown *ex vivo* that increased mast cell density is associated with improved remodelling and functional outcomes in human patients, that mast cells produce mediators relevant to angiogenesis and efferocytosis *in vitro*, and that IL-33 promotes efferocytosis *in vivo* by peritoneal macrophages. With future work focused on improving murine models to study cardiac mast cell function, and the relevance of IL-33 in activating resolution pathways in chronic inflammation, we can hope to better therapeutically target cardiac fibrosis to improve the lives of patients with cardiovascular disease.

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APPENDIX A.

Mast cells are present in the murine heart and have been shown to increase prior to and during tissue remodeling [324–326, 330, 356,360]. However the specific role of mast cells in murine models of cardiac fibrosis has not been determined, as various studies have attributed both detrimental and beneficial roles to these cells [75]. This could be an artifact of the commonly used mouse model of mast cell deficiency the $Kit^{Wsh/Wsh}$ (Wsh) model. Wsh mice have an inversion mutation of the gene for c-kit, a crucial mast cell growth factor receptor, that overlaps with several adjacent genes [285], including the gene encoding Corin. Corin is a cardiac-specific blood pressure regulator, and as such naïve Wsh mice have increased susceptibility to aberrancies in blood pressure and cardiac function [286]. Therefore, Wsh mice are a poor model to study mast cells in CVD in general. We sought to determine the role of mast cells in an Angiotensin II (AngII)-mediated model of hypertrophy and cardiac fibrosis that has been well established by our group [265,570]. We used the *Cpa3-Cre;* $Mcl\text{-}1^{fl/fl}$ model, referred to as hello kitty mice, which uses a cre-mediated excision mechanism to remove anti-apoptotic protein Mcl-1 for targeted apoptosis in carboxypeptidase A3 expressing cells [715]. This results in a systemic deficiency in mast cells, as well as a reduction in basophils. Hello kitty mice can then be compared to mice that are *Cpa3-Cre;* $Mcl\text{-}1^{+/fl}$, termed heterozygotes here, that are mast cell sufficient. *Corin* mRNA was detectable in hello kitty, heterozygote, and C57BL/6 mice, but not Wsh (Fig. 1A).

Hello kitty and heterozygous mice received 1.44mg/kg/day AngII systemically via mini-osmotic pumps implanted subcutaneously for 7 or 28 days prior to sacrifice. After 7

days of AngII, body weight change was comparable between both Hello kitty and heterozygous mice (Fig. 1B), as was hypertrophy (Fig. 1C) and collagen content in cardiac sections as determined by SRFG staining (Fig. 1D). Additionally, expression of fibrosis-associated genes *Vegfa, Col3a1, Col1a1* (Fig. 1E), *Tgfb, Mmp9, Ccl2,* and *Acta2* (Fig. 1F) was not impacted by mast cell deficiency after AngII treatment. We increased the length of AngII treatment to 28 days, where we observed similar impacts on body weight change (Fig. 2A), hypertrophy (Fig. 2B), collagen content (Fig. 2C), and fibrosis-associated gene expression (Fig. 2D-E). This would indicate that mast cells do not impact fibrosis in the context of AngII-mediated hypertrophy. However, we were struck during our analysis by the paucity of mast cells in heterozygote and C57BL/6 myocardial tissue in comparison to human samples. During the course of this project, it was shown by Ingason and colleagues that murine mast cell distribution is fundamentally different than that of humans[271], with a higher density of mast cells in the epicardium, compared to their presence being highest in the myocardium in humans. As such, the actions of mast cells on the cardiac microenvironment is likely fundamentally different between the two species. Therefore, we chose to discontinue this project in favour of focusing our efforts on the role of mast cells in human atrial fibrosis as outlined in chapter 3.

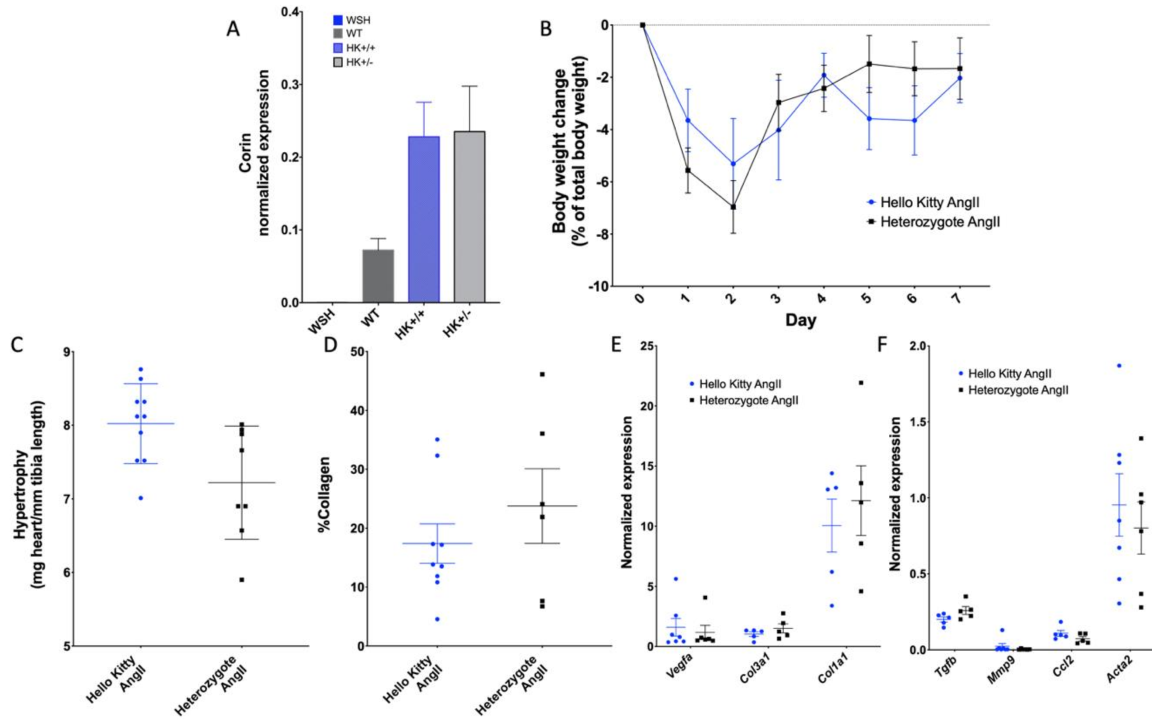

Figure 1. Mast cells do not influence fibrosis or hypertrophy after 7 days of AngII. Mast cell deficient (Hello Kitty, n=9) and sufficient (Heterozygote, n=7) mice experience equivalent AngII-mediated hypertrophy and fibrosis after 7 days. Hello kitty and hello kitty heterozygotes both express *Corin*, which is not expressed by Wsh mice (A). Hello kitty mice and heterozygotes experience similar body weight change over the course of 7 days of AngII (B). Hypertrophy (C), and % collagen (D) were also equivalent between the two groups after 7 days of AngII. Expression of fibrosis-associated genes *Vegfa, Col3a1, Col1a1* (E)*, Tgfb, Mmp9, Ccl2,* and *Acta2* (F) were also similar between both groups.

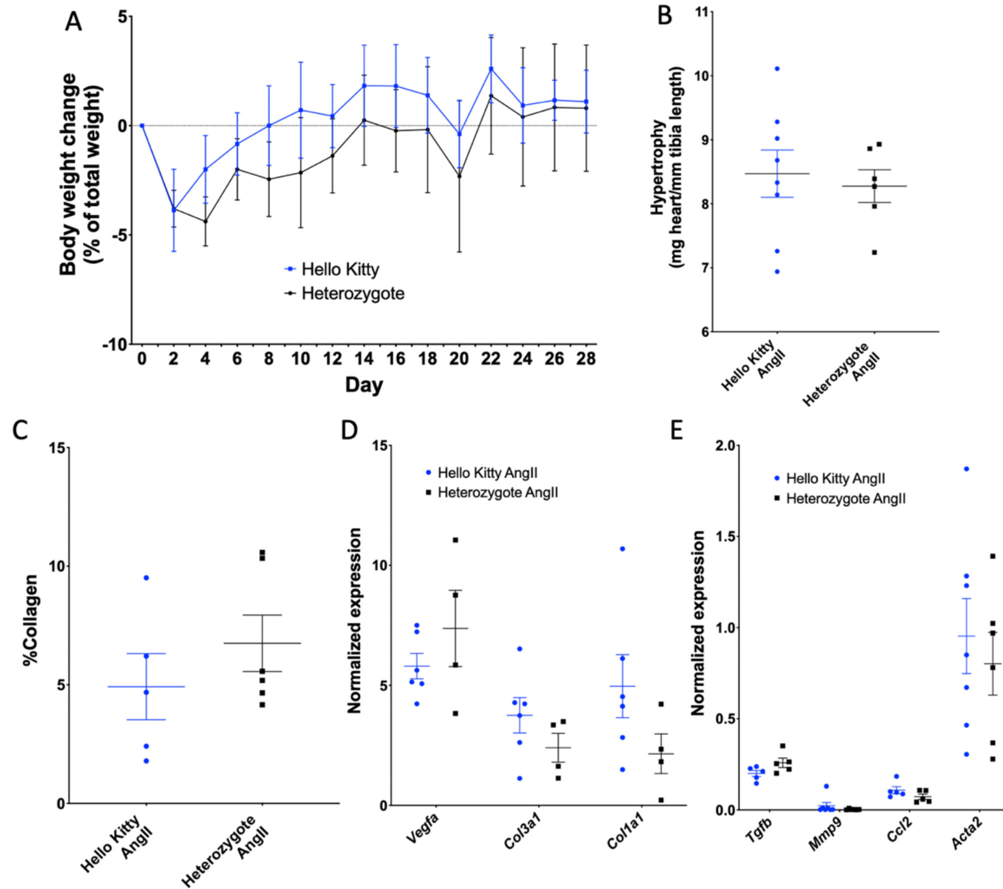

Figure 2. Mast cells do not influence fibrosis or hypertrophy after 28 days of AngII. AngII treatment for 28 days has similar impacts on mast cell deficient and sufficient mice. Hello kitty mice (n=8) and heterozygotes (n=6) experienced comparable body weight change (A), hypertrophy (B), and change in % collagen (C) over the course of 28 days of AngII. Expression of fibrosis-associated genes *Vegfa, Col3a1, Col1a1* (D)*, Tgfb, Mmp9, Ccl2,* and *Acta2* (E) were also similar between both groups.

APPENDIX B.

To determine the optimal dose of IL-33 to activate CBMCs *in vitro*, dose response experiments were conducted with three donors to at 3 ng/mL, 10 ng/mL, and 30 ng/mL IL-33. Production of IL-13, VEGF-A, uPA and IL-10 was assessed in response. IL-33 at 30 ng/mL showed the greatest response across all four mediators assessed (Fig. 3A-D). We then conducted a time course at 3h, 6h, and 24h with our chosen dose of 30 ng/mL, and IL-10, IL-13, VEGF-A and uPA. We found that 24h of IL-33 activation yielded the best response in all four mediators assessed (Fig. 3E-G). Of note, there was no uPA present in media from CBMCs harvested at 3h or 6h and as such we did not include uPA in the resultant figure.

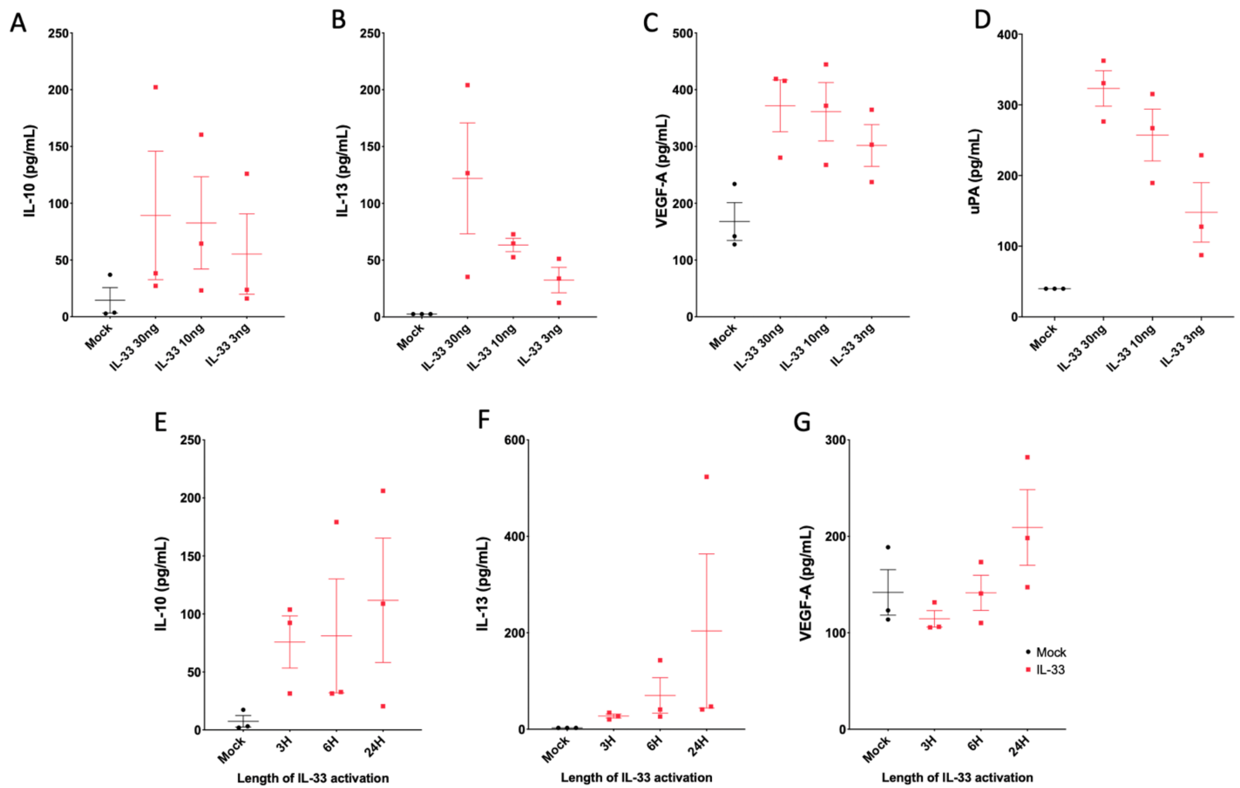

Figure 3. Dose response and time course activations of CBMCs with IL-33. To determine optimal dose of IL-33 to activate CBMCs, cells from 3 donors were cultured for 24h with 3 ng/mL, 10 ng/mL, and 30 ng/mL. Optimal dose was determined by assessing IL-10 (A), IL-13 (B), VEGF-A (C), and uPA (D) in culture media. Time course experiments were then conducted for 3h, 6h, and 24h at 30 ng/mL IL-33. Optimal time for activation was determined by assessing IL-10 (E), IL-13 (F), and VEGF-A (G). Data represented as mean ± SEM.

APPENDIX C

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