Inhibitory Effect of Glycosaminoglycans on Human Mast Cell Stimulation by IL-33

A thesis submitted by

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in partial fulfillment of the requirements for the degree of

PhD

in

Pharmacology & Experimental Therapeutics

Tufts University

Sackler School of Graduate Biomedical Sciences

February 2019

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ABSTRACT

Glycosaminoglycans (GAGs) are linear, highly negatively charged carbohydrate chains present in connective tissues, primarily known for their functional role in connective tissue, joint lubrication and anticoagulant activity. Chondroitin sulfate (CS) and heparin (Hep) are also found in the numerous secretory granules of mast cells (MC), hematopoietic tissue immune cells involved in allergic and inflammatory reactions, and are thought to store biogenic amines, such as histamine, due to their negative charge. It was previously shown that CS and Hep may inhibit secretion of histamine from rat connective tissue MC, but their effect on human MC remains unknown. Immortalized human LAD2 MC were pre-incubated with CS before stimulation by either the peptide substance P (SP, 2 μM) or the cytokine IL-33 (10 ng/mL). The data indicated that pre-incubation with CS had no detectable effect on MC degranulation stimulated by SP, but significantly inhibited TNF and CXCL8 secretion from LAD2 MC stimulated by SP or IL-33. In order to determine if the molecular weight (MW) and structure affected the inhibitory activity of GAGs, the effects of MC pre-incubation with Hep or dermatan sulfate (DS) on mediator secretion from MC stimulated with IL-33 were studied. DS and Hep, both unfractionated (UF) and low molecular weight (LMW), inhibited IL-33-stimulated secretion of TNF and CXCL8 to a similar extent as CS. Studies were conducted at 4°C and 37°C to determine if fluorescein-conjugated CS (CS-F) was taken up at a physiologically relevant temperature. LAD2 MC associated with CS-F only at 37°C, but not 4°C, indicating its uptake. In further pursuit of determining the mechanism of inhibitory action of GAGs on MC stimulated with IL-33, gene expression of TNF and

CXCL8, intracellular calcium ion influx, as well as effect on expression of the IL-33 surface receptor, ST2, were carried out. None of the GAGs tested had any effect of these processes. Neutralization of the hyaluronan receptor CD44 did not detectably affect the uptake of CS-F. To determine if CS interferes with proteins associated with vesicle fusion, the effect of CS on fluorescence associated with soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE) was investigated. CS appeared to interfere with the fluorescence associated with the SNARE protein SNAP-23, but not VAMP-8, in LAD2 MC. In contrast to the LAD2 cells stimulated by IL-33, GAGs did not detectably affect TNF or CXCL8 secretion from primary hCBMC stimulated with IgE/anti-IgE. The data here suggest that GAGs can inhibit secretion of TNF and CXCL8 from human mast cells stimulated by IL-33, a crucial alarmin involved in inflammation. GAGs from the tissue microenvironment or secreted by MC, themselves, upon activation, could inhibit the secretion of mediators from MC in an autocrine fashion. GAGs could be formulated for systemic or topical treatment of allergies or inflammation.

DEDICATION

I would like to dedicate my thesis to my dear grandfathers who are greatly missed: Edward Gross, Elmer Tannenbaum and Jack Zeitlin. Also, to my strong and loving grandmothers, Irene Gross and Lynn Tannenbaum, who are so proud to see the day their granddaughter is granted her PhD.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere appreciation for my thesis advisor and mentor Dr. Theoharis Theoharides. His passion and dedication to the field of mast cell inflammation research has been contagious; his support and encouragement to creatively explore my thesis project have been instrumental in shaping my graduate school career and curiosity as a scientist. I would like to thank my thesis committee members, Dr. Angelo Azzi, Dr. John Castellot, Dr. Athar Chishti and, particularly, my committee chair, Dr. Laura Liscum, for their knowledgeable experimental and theoretical insights which greatly helped me to complete this thesis project. Above all, their faith in me as a scientist in a time of turmoil truly motivated me to stay focused on my goals. I would also like to thank my outside examiner, Dr. Susan Leeman, who is both an innovative pioneer in neuroendocrinology and a great inspiration to me and all other female scientists.

I would like to thank all faculty members of the Program in Pharmacology & Experimental Therapeutics (PPET) who taught me how to thoroughly dissect a scientific journal article and how to think critically about my research project. I am grateful for my past and present PPET classmates, especially Christina Deliyiannis Acetta, Tamar Ledoux and Alexandra Taracanova. Their support has made my graduate school experience a great one and I know we have lifelong friendships ahead of us. Moreover, I would like to thank members of the Tufts community for sharing their technical expertise, specifically Christopher Talbot, Alenka Lovy, Stephen Kwok and Albert Tai.

In addition, I would like to thank all past and present Theoharides Lab members, particularly Arti Patel, who taught me so much about experimental design and believed in my ideas when I doubted them myself. A special thank you to my current lab mates Mona Bawazeer and Eirini Tsilioni for the encouragement given and laughter brought in and out of the lab.

Most of all, I would like to thank all my loving and supportive family, especially my parents, Laurie and Kenneth, and my stepfather Charlie, for being there for me every step of the way along my educational journey—from pre-school to graduate school. As a nurse practitioner and a neurologist, my parents fostered my interest in science and translational medicine. Finally, I want to thank my wonderful girlfriend, Tori, who makes me smile daily and has given me an endless supply of encouragement and love over the past two years.

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LIST OF ABBREVIATIONS

Ab	Antibody
Aβ	Amyloid β proteins
AD	Alzheimer's disease
BMMC	Bone marrow-derived MC
CRH	Corticotropin releasing hormone
CS	Chondroitin sulfate
CS-F	Fluorescein-conjugated CS
Cromolyn	Disodium cromoglycate
DMSO	Dimethyl sulfoxide
DS	Dermatan sulfate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FcεRI	High affinity IgE receptor
FITC	Fluorescein isothiocyanate
GAGs	Glycosaminoglycans
GPCR	G protein-coupled receptors
HA	Hyaluronan
HaCaT	Human keratinocyte cell line
hCBMC	Human cord blood derived MC
Hep	Heparin
HMC-1	Human mast cell-1
hr	Hours
HSC	Haematopoietic stem cells
IgE	Immunoglobulin E
IL	Interleukin
IL-1RAcP	IL-1 receptor accessory protein
IRAK4	IL-1 receptor-associated kinase 4
LAD2	Laboratory of allergic diseases 2
LMW	Low molecular weight
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MC	Mast cell(s)
MCP-1	Monocyte chemoattractant protein 1
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NT	Neurotensin
PE	Phycoerythrin
PG	Proteoglycan(s)
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinases

Ps	Psoriasis
qRT-PCR	Quantitative real time-polymerase chain reaction
RBL-1	Rat basophilic leukemia cells
rhSCF	Recombinant human stem cell factor
SNAP23	Synaptosomal-associated protein 23
SNARE	Soluble NSF attachment protein receptor
SP	Substance P
STX3	Syntaxin 3
STX4	Syntaxin 4
t-SNARE	Target-membrane SNARE
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor-α
TRAF6	TNF receptor-associated factor 6
UF	Unfractionated
v-SNARE	Vesicular SNARE
VAMP8	Vesicle-associated membrane protein 8
VEGF	Vascular endothelial growth factor
WT	Wild-type

CHAPTER 1 : INTRODUCTION

1.1 Mast Cells and Inflammation

1.1.1 Mast Cell Development and Maturation

Mast cells (MC) are tissue immune cells first discovered by Paul Ehrlich in 1878, which earned him the Nobel Prize in Physiology or Medicine in 1908[1]. As shown in **Figure 1.1**, MC are derived from haematopoietic stem cells (HSCs) in the bone marrow, travel as MC progenitors (MCP) in the circulation and differentiate in vascularized tissues[2-5]. There, MC undergo maturation greatly due to the presence of stem cell factor (SCF), the ligand for c-kit, a tyrosine kinase-activated receptor[6]. In addition to maturation, SCF also promotes mast cell adhesion, migration, proliferation, and survival[7]. MC can differentiate into mucosal MC, containing only tryptase in humans and found in the mucosa, or connective tissue MC, containing both tryptase and chymase and found in the submucosa and muscularis propria. Tissue-specific exposure of MC to certain cytokines and growth factors, such as interleukin (IL)-3, IL-4, IL-9, transforming growth factor (TGF)-β and nerve growth factor (NGF), can affect their differentiation fate. In particular, IL-4, in combination with SCF, augments proliferation rate and mediator release of human intestinal MC[8]. The life span of MC is quite long, as they can survive for months or even years[9].

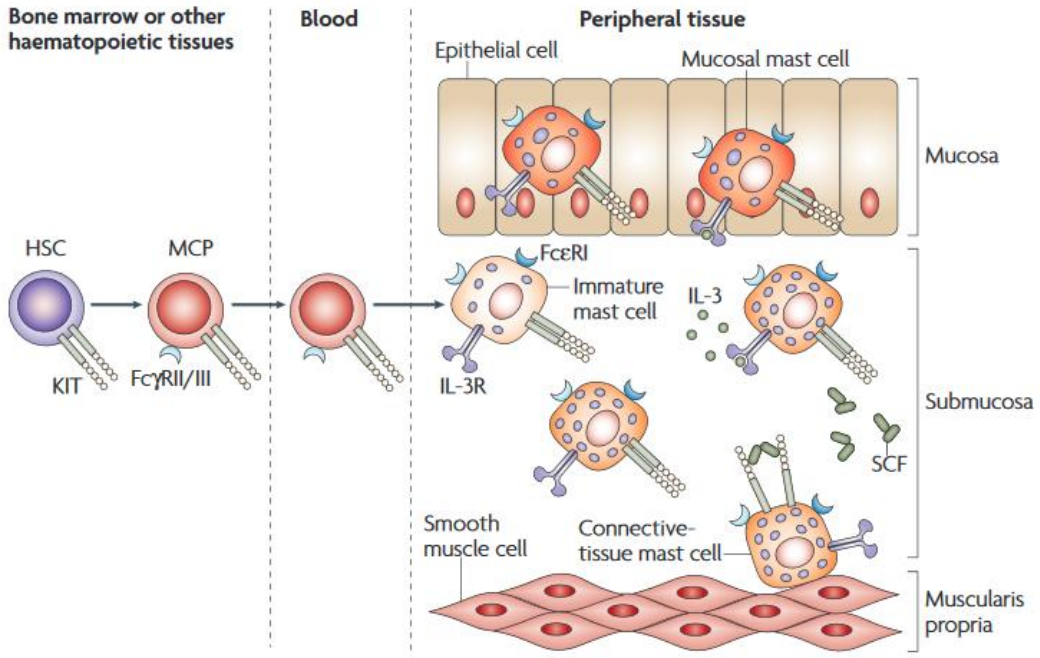


Figure 1.1. Mast cell maturation and development. *Reprinted with permission from: Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol 2008; 8(6):478-486.*

1.1.2 Allergic and Non-Allergic Stimulation of Mast Cells

MC are activated via the classic allergic pathway, mediated by immunoglobulin E (IgE)[10-13]. In response to an exogenous antigen, B cells produce antigen-specific IgE[14], which then binds to its high-affinity heterotetrameric receptors, FcεRI, expressed on the MC surface[15, 16]. Re-exposure to the antigen leads to aggregation and cross-linking of FcεRI receptors by the IgE-bound antigen, inducing rapid secretion of preformed mediators[17], a process called degranulation[18]. IgE levels are highly correlated with allergic symptoms, particularly in asthma[19-22]. The degranulation process begins at the *trans*-Golgi network (TGN), where small, immature pro-granules bud off into the cytoplasm, where maturation occurs. MC maturation is largely determined by the

maturation of its granules, which is strongly dependent on the presence of proteoglycans (PG), mainly serglycin, with heparin or chondroitin sulfate side chains[23]. FcεRI crosslinking leads to the phosphorylation and activation of signaling molecules, such as phosphoinositide 3-kinase (PI3K), and then Ca^{2+} mobilization[24, 25]. Granules translocate along microtubules from the cytoplasm towards the plasma membrane[26]. In this translocation process, granules fuse with other granules and eventually the plasma membrane, which leads to granule exocytosis mediated by different soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs)[27].

In contrast to allergic stimulation via IgE, MC can also be involved in inflammatory processes without degranulation, through "differential" or "selective" secretion of mediators[28, 29]. Non-allergic triggers of MC include neuropeptides, particularly substance P (SP)[30, 31] and neurotensin (NT)[32-34], cytokines, complement components and numerous other endogenous and exogenous peptides, such as endothelin 1 or peptides derived from venom or bacteria[35, 36]. MC express the G protein-coupled receptors (GPCR) receptors[37-39] for SP, corticotropin releasing hormone (CRH) and NT on their cell surface[40]. These peptides are released from peripheral sensory neurons and stimulate MC when bound to their respective receptor. For instance, the neurokinin 1 receptor (NK-1R) for SP[41, 42]. Cytokine stimulation of MC is often selective as well[43]. For example, IL-1 stimulates human MC to selectively release IL-6 without degranulation through small vesicles (40–80 nm), different and much smaller than the secretory granules (800–1000 nm)[44]. Additionally, human cultured MC selectively secrete vascular endothelial growth factor (VEGF) in response to CRH[45].

1.1.3 Mast Cell Mediators

MC reside in vascularized tissues in contact with the external environment, including the skin, respiratory tract and gut[43], where they participate in allergy, innate and acquired immunity[36, 46, 47], autoimmunity[48], and inflammation[49-51] through secretion of various mediators. MC granules contain numerous preformed mediators, such as histamine, enzymes (β-hexosaminidase, tryptase and chymase), proteoglycans (heparin and chondroitin sulfate) and TNF[52], which are released upon rapid degranulation (within 5-30 min)[26] (**Fig. 1.2**). MC can also undergo *de novo* synthesis and secretion (6-24 hours later) of various cytokines, including TNF, IL-1β, IL-4, IL-6, IL-17, interferon-γ, as well as chemokines ,such as CXCL8 and chemokine (C-C motif) ligand 2 (CCL2), and growth factors, such as SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF), NGF and VEGF[41] **(Fig. 1.2)**. The secretion of these lipid mediators, such as leukotrienes, cytokines and preformed histamine, can greatly alter the vascular permeability and adhesiveness of the vascular endothelium, allowing other inflammatory cells to adhere and to migrate into the inflamed tissue[9, 53]. It should also be noted that the contents of MC granules vary by tissue type; connective tissue mast cells contain chymase and tryptase, while connective tissue mast cells and mucosal mast cells contain only tryptase[54].

1.1.4 IL-33 and Mast Cells in the Pathogenesis of Psoriasis

IL-33 is one of the newest members of the IL-1 family of inflammatory cytokines[55] that plays a crucial role in regulation of the innate and immune systems,[56, 57]

as well as many inflammatory diseases[55, 58-60]. IL-33 is secreted by fibroblasts and endothelial cells,[55] as well as from basophils in asthma patients[61]. IL-33 augments the effect of IgE on MC and basophils[59, 62] and also has synergistic effects with inflammatory neuropeptides such as SP [63].

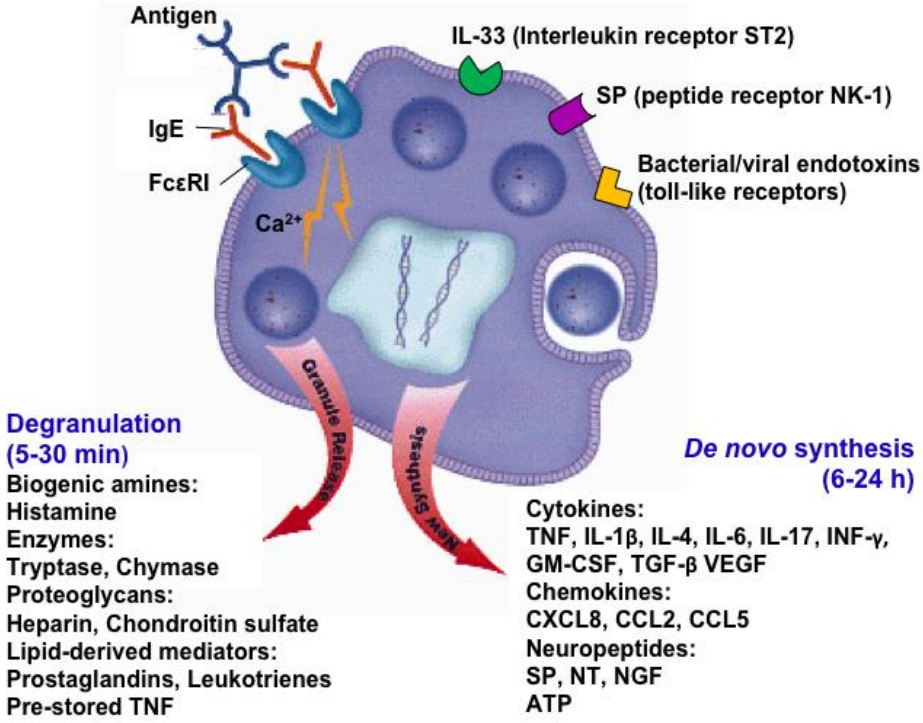


Figure 1.2. Schematic representation of mast cell degranulation and de novo synthesis of various mediators. *Created by Dr. Theoharis Theoharides, Theoharides Lab.*

However, IL-33 can also have an effect on its own, acting as an "alarmin" against injury-induced stress, pathogens or cell death by activating local immune cells[64, 65,58, 62]. IL-33 is the main ligand for the ST2 receptor, which is expressed on the surface of MC, epithelial cells and fibroblasts[55]. The ST2 receptor forms a complex with the IL-1 receptor accessory protein (IL-1RAcP)[66]. ST2 is found in either the more abundant,

transmembrane ST2L form or in the cytoplasmic, soluble sST2 form[55]. IL-33 binding leads to the recruitment and activation of the adaptor protein MyD88, along with IL-1 receptor-associated kinase 4 (IRAK4) and TNF receptor-associated factor 6 (TRAF6)[67, 68]. This signaling cascade then leads to the stimulation of mitogen-activated protein kinase (MAPK) and the transcription factor NF-κB, resulting in its nuclear translocation and pro-inflammatory mediator gene transcription[69]. This leads to the eventual secretion of mediators, such as TNF, CXCL8, IL-6, prostaglandin D_2 and monocyte chemoattractant protein 1 (MCP-1)[60], which exacerbates allergic diseases (**Fig. 1.3**).

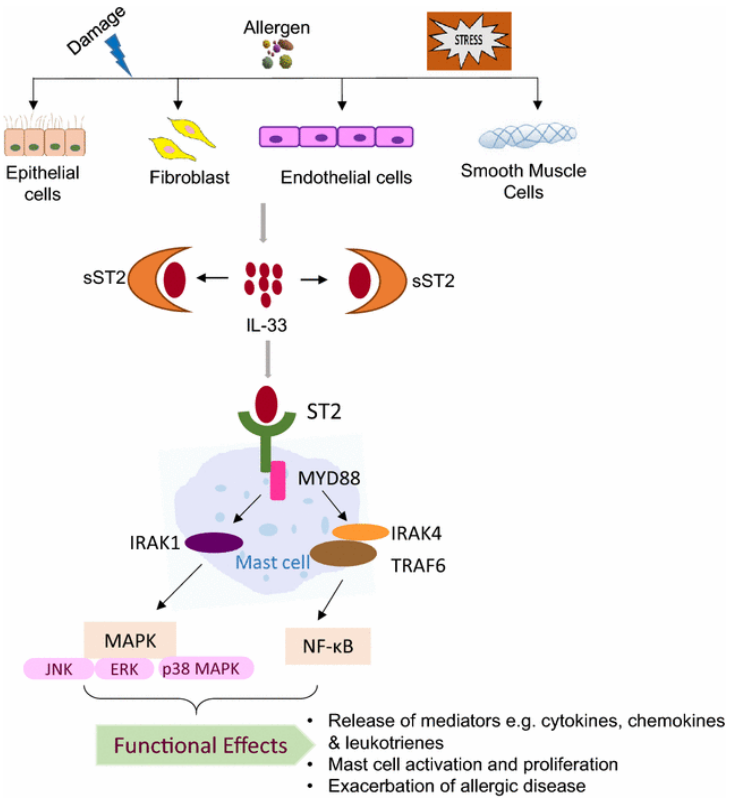


Figure 1.3. The IL-33/ST2 signaling pathway in mast cells. *Reprinted with permission from: Saluja R, Khan M, Church MK, Maurer M. The role of IL-33 and mast cells in allergy and inflammation. Clin Transl Allergy 2015; 5:33.*

Psoriasis (Ps) is a chronic autoimmune skin disease affecting approximately 2-3% of the world's population, with an annual cost of approximately 11 billion USD in 2013[70, 71]. Ps is characterized by keratinocyte hyperproliferation, increased skin capillaries,

oxidative stress, and chronic inflammation[72-74]. Besides infiltration of T cells, neutrophils, dendritic cells and macrophages[74, 75], an increased number of infiltrating mast cells in Ps lesional skin has been reported[76-78]. The nervous system plays a pivotal role in Ps; for example, Ps can be exacerbated by acute stress[79-82]. Furthermore, neuropeptides[83], particularly SP[84], which can activate MC[85], are highly involved in the pathogenesis of Ps. In fact, SP-positive nerve fibers are denser in Ps lesions and are localized close to MC in the dermis[82, 86-88].

In terms of IL-33 in Ps, gene expression of IL-33 and its receptor ST2 are increased in the skin of patients with localized Ps[89]. IL-33 serum levels have also recently been found to be elevated in patients with Ps, compared to healthy controls[90]. We, as well as others, have reported that IL-33 stimulates selective secretion of MC mediators such as TNF, IL-1β, IL-6, IL-31, VEGF and CXCL8[31, 62, 91-94], which have potent vasodilatory, inflammatory and pruritic properties[95]. Our laboratory previously reported that IL-33 augments the effect of the peptide SP on secretion of VEGF[31] and TNF[92] from human MC. TNF, a key cytokine involved in the initiation and progression of Ps[96, 97], can stimulate keratinocytes to produce IL-1[98], IL-6, CXCL8 and VEGF[99, 100] and can activate NF-κB, which is upregulated in Ps lesional skin[101]. Overexpression of both mRNA and protein levels of CXCL8 have been reported in lesional Ps skin[102-104]. In addition, there is a correlation between the expression of CXCL8 and dermal mast cell density in lesional Ps skin as compared to nonlesional Ps skin[105].

1.2 Glycosaminoglycans

Glycosaminoglycans (GAGs) are long, linear, negatively charged polysaccharides, which consist of repeating disaccharide units, involved in a variety of biological processes, such as wound healing and modulation of inflammation[106-108]. GAGs are divided into two groups, nonsulfated [chondroitin and hyaluronan (HA)] and sulfated [heparin (Hep), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate and keratan sulfate][109]. Besides HA, GAGs are typically covalently bound to a protein core, forming a proteoglycan (PG), such as decorin, versican, aggrecan and serglycin[110].

1.2.1 Structural Differences of Glycosaminoglycans

One unique aspect of CS, Hep and DS is not only their negative charge, but also their sulfated moieties, as seen in **Figure 1.4**[111]. The sulfation pattern of CS has a mixture of 4 and 6 sulfation positions (CS from shark cartilage is a mixture of CS-A and CS-C), while DS is sulfated strictly at position 4 and Hep can be sulfated at the 2, 3, 6, or nitrogen positions. Hep is more sulfated than both CS and DS and thus more negatively charged.

CS, DS and Hep also differ in their amino sugar and uronic acid components (**Fig. 1.4)**. DS and Hep differ in their amino sugar components (*N*-acetylgalactosamine is present in DS, while *N*-acetylglucosamine is present in Hep). While DS and CS share the same amino sugar *N*-acetylgalactosamine, they differ in their uronic acid components;

DS, like Hep, contains iduronic acid (IdA), the C-5 epimer of D-glucuronic acid (GlcA), the only uronic acid component of CS[112].

The chain length of GAGs can vary greatly, ranging from 1 to 25,000 disaccharide units, and their molecular weights (MW) vary over three orders of magnitude[113]. CS from shark cartilage has an average MW of ~40 kDa[114], DS from porcine intestinal mucosa has an average MW of ~21-30 kDa[115] and Hep has an average MW of ~17-19 kDa (Millipore Sigma).

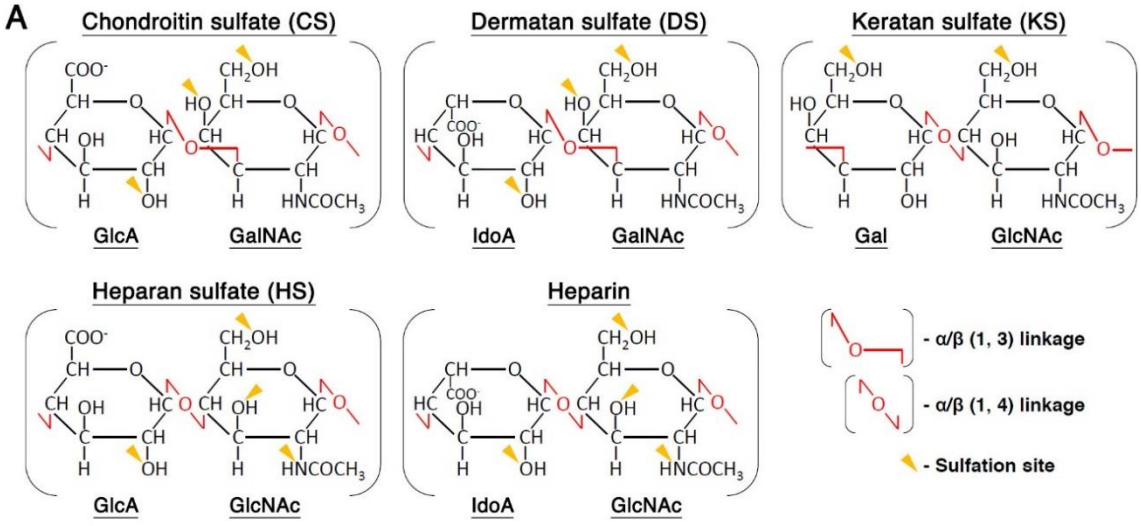


Figure 1.4. Schematic of the structural differences between GAGs. GAGs are long unbranched polysaccharide chains made of the following monosaccharides: galactose (Gal), glucuronic acid (GlcA), iduronic acid (IdoA), N-acetyl-glucosamine (GlcNAc), N-acetyl-galactosamine (GalNAc). *Reprinted with permission from: Mihov D, Spiess M. Glycosaminoglycans: Sorting determinants in intracellular protein traffic. Int J Biochem Cell Biol 2015; 68:87-91.*

1.2.2 Identification and Function of Mast Cell Glycosaminoglycans

MC have long been known to contain high concentrations of GAGs in secretory granules[116]. In fact, the GAG heparin is synthesized and stored exclusively in MC granules[117, 118] and is thought to electrostatically bind and store biogenic amines, such as histamine[119], MC proteases, such as chymase[120, 121] and chemokines, such as CXCL8[122, 123]. Different MC subclasses differ in the expression of GAGs in their granules, but serglycin is consistently the dominating PG species expressed by MC[124, 125]. For example, human cord blood derived MC (hCBMC) have a GAG composition of 25-35% Hep and 65-75% CS[126], while human lung MC have a GAG composition of ~60-70% Hep and ~30-40% CS-E, or "oversulfated" CS[116, 127].

1.2.3 Anti-Inflammatory Effects of Glycosaminoglycans *In Vitro*

Previous studies had reported that CS can inhibit the secretion of histamine from rat connective tissue MC when stimulated with the degranulation trigger Compound 48/80[128]. There have also been reports that Hep can inhibit isolated canine[129], cultured rat peritoneal[130] and basophilic leukemia[131] MC. In addition, in isolated human uterine MC, Hep inhibited histamine secretion induced by anti-IgE, but not by the calcium ionophore A23187[132]. Apparently, LMW Hep preferentially inhibited TNF production in a mouse bone marrow-derived MC[131]. DS is the predominant GAG present in the skin, and has been identified in rat basophilic leukemia (RBL-1), a rat MC-like cell line[133], and in rat bone marrow-derived MC (BMMC)[124, 134]. DS has been mainly implicated in wound

repair and fibrosis[135], but like CS and Hep, also exhibits anti-inflammatory properties. For example, in a mouse model of unilateral ureteral obstruction (UUO), DS was been found to reduce the expression of the chemokine MCP-1, the cytokine TGF-β, as well as macrophage accumulation in the obstructed kidney[136, 137]. Though non-sulfated, another GAG, HA, found in cartilage and also primarily known for its wound-healing properties[138], inhibited RBL-1 MC degranulation and histamine secretion.

There have also been some reports that CS may have anti-inflammatory actions in cells other than MC. For example, a recent study showed that CS can inhibit the release of pro-inflammatory cytokines from coronary endothelial cells and monocytes stimulated with TNF[139]. CS has also been shown to reduce IL-1β-induced NF-κB nuclear translocation, a key transcription factor involved in pro-inflammatory gene expression, in human chondrocytes[140]. Another group showed that CS inhibits NF-κB activation, STAT3 translocation to the nucleus, and the release of some key pro-inflammatory mediators such as TNF, CXCL8, IL-6, and CCL27 from normal human keratinocytes[141].

1.2.4 Clinical Relevance of Glycosaminoglycans

Clinical studies have shown that CS can modulate inflammation in osteoarthritis,[142-146] and may also be useful in improving the clinical symptoms of patients with Ps[147]. CS has also been explored as a treatment for coronary artery disease (CAD). One study showed that when rabbits were treated prophylactically with CS prior to atherosclerosis induction, pro-inflammatory molecules C-reactive protein (CRP) and IL-6 were lowered in the serum and fewer rabbits developed atherosclerosis[148]. In

humans, early studies showed treatment with CS (n=60) led to a seven-fold lower incidence of coronary events[149], as well as significantly reduced mortality rates, compared to the control group[150]. There have been reports that Hep can inhibit MC-dependent diseases, such as exercise-induced asthma[151, 152]. Hep also inhibited allergic histamine secretion, as well as immediate cutaneous reaction and bronchoconstriction in sheep[132]. LMWH has been shown to have therapeutic effects on dextran sulfate sodium (DSS)-induced colitis by downregulating IL-1β and IL-10 mRNA levels in the colon[153]. DS has been found to inhibit P-selectin[154], thus attenuating metastasis, inflammation and thrombosis in respective experimental mouse models[155]. DS has also been positively explored as a potential therapeutic in mouse models of unilateral ureteral obstruction[136], arterial injury[156, 157], oral mucositis[158] and a rat model of colitis[137].

Endogenous GAGs have actually been implicated in the pathogenesis of several neurodegenerative disorders, such as Alzheimer's disease (AD)[159, 160], mucopolysaccharidoses[161] and Parkinson's disease[162]. For instance, in AD, binding of PGs and Hep to amyloid β proteins (Aβ) is known to enhance both amyloid aggregation and neurotoxic amyloid fibril formation[163-165]. Interestingly, exogenous GAGs also have shown to exhibit neuroprotective properties[166], thought to be due to competitively inhibiting the interaction between endogenous GAGs and Aβ[167]. Low molecular weight heparin (LMWH) has been reported to dramatically inhibit Aβ fibril formation in a mouse amyloidosis model[168, 169]. CS and a specific CS disaccharide can prevent abnormal amyloid fibril formation[170] and protect against neuronal toxicity[171], respectively.

1.3 SNARE Proteins in Mast Cells

Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are highly conserved eukaryotic proteins[172, 173] that play a central role in vesicle trafficking between intracellular compartments, as well as to and from the plasma membrane by enabling membrane fusion[174]. SNARE machinery involves sets of proteins on either side of the plasma membrane that form a trans-SNARE complex. The role of SNAREs in MC degranulation has been well-studied[175-178]. For example, upon FcεRI activation, secretory granules translocate and dock to the plasma membrane, where typically one vesicular SNARE (v-SNARE), such as VAMP8, pairs with two plasma membrane target-membrane SNAREs (t-SNAREs), such as Syntaxin 4 (STX4) and synaptosome-associated protein of 23 (SNAP-23), to form a typical trans-SNARE complex[27] (**Fig. 1.5**). However, the role of SNAREs in the release of *de novo* synthesized MC mediators, such as cytokines or chemokines, has not been as adequately investigated.

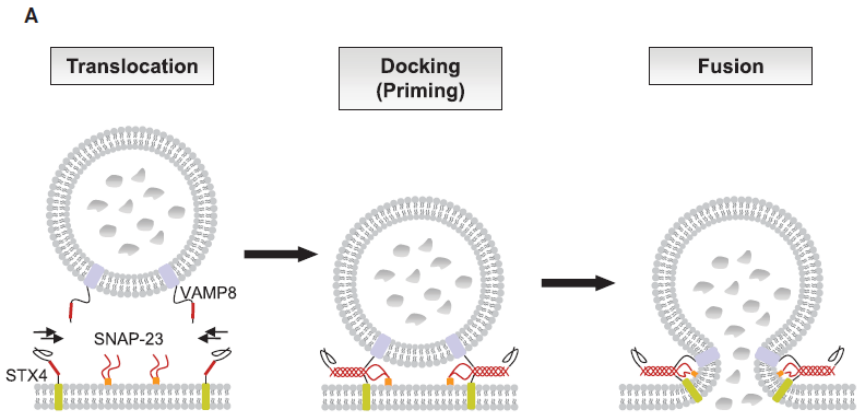


Figure 1.5. SNARE catalyzed granule fusion in mast cells. Upon activation through FcεRI, secretory granules translocate to and dock at the plasma membrane where the t-SNAREs SNAP-23 and STX4 together with the v-SNARE VAMP8 form stable tetrameric complexes. *Reprinted with permission from: Lorentz A, Baumann A, Vitte J, Blank U. The SNARE Machinery in Mast Cell Secretion. Front Immunol 2012; 3:143.*

1.3.1 Mast Cell Mediator Secretory Pathways and Function of SNAREs

Although much is still unknown, recent studies have begun to elucidate the secretory pathways of cytokine and chemokine secretion and the crucial involvement of v-SNAREs and t-SNAREs[27]. One issue leading to this gap in knowledge is the fact that since *de novo* synthesized cytokines and chemokines take several hours to be synthesized and be maximally secreted, they are not easily traceable for imaging studies[179]. The precise secretory pathways necessary for degranulation and *de novo* synthesized cytokine and chemokine secretion in MC are thought to be distinct. The exocytosis of secretory granules and associated secretion of pre-formed mediators, including TNF, occurs through rapid "regulated" or "granule-mediated" exocytotic pathways[180]. However, *de novo* synthesized cytokines, including TNF, are thought to be secreted via either piecemeal degranulation, wherein vesicles bud off from larger secretory granules to be released more gradually[179], or constitutive exocytosis, wherein cytokines synthesized in the rough endoplasmic reticulum (ER) are packaged into secretory vesicles at the TGN and transported into recycling endosomes or directly to the cell surface[181, 182] (**Fig. 1.6**).

While the secretory pathways differ, there are both similarities and differences among the SNAREs necessary for MC degranulation and *de novo* synthesized mediator secretion. For instance, both VAMP-8 and SNAP-23 have been shown to be necessary for degranulation in human[177], murine[175] and rat[178, 183, 184] MC. However, while inhibition of SNAP-23 abolished IgE receptor-mediated secretion of the chemokines CXCL8, CCL2, CCL3 and CCL4 from human MC, inhibition of VAMP-8 only decreased IgE receptor-mediated secretion of CXCL8[185]. Interestingly, there were also no differences in

TNF, IL-6 and MIP-1α secretion between BMMC derived from VAMP-8-deficient mice and those derived from wild-type mice[186]. Another v-SNARE, VAMP-3, has been well-studied in macrophages for its role in trafficking TNF from recycling endosomes (RE) to the plasma membrane, where it binds to the complex of SNAP-23 and STX4, for secretion[187, 188]. While the exact secretory mechanism of TNF is still to be determined in MC, it has been reported that TNF, secreted from WT murine MC stimulated with IL-1β, accumulated at the plasma membrane where it colocalized with vesicular compartments positive for VAMP-3[186]. However, VAMP-3 was not involved in either IgE receptor-mediated histamine[175, 177] or chemokine secretion[185].

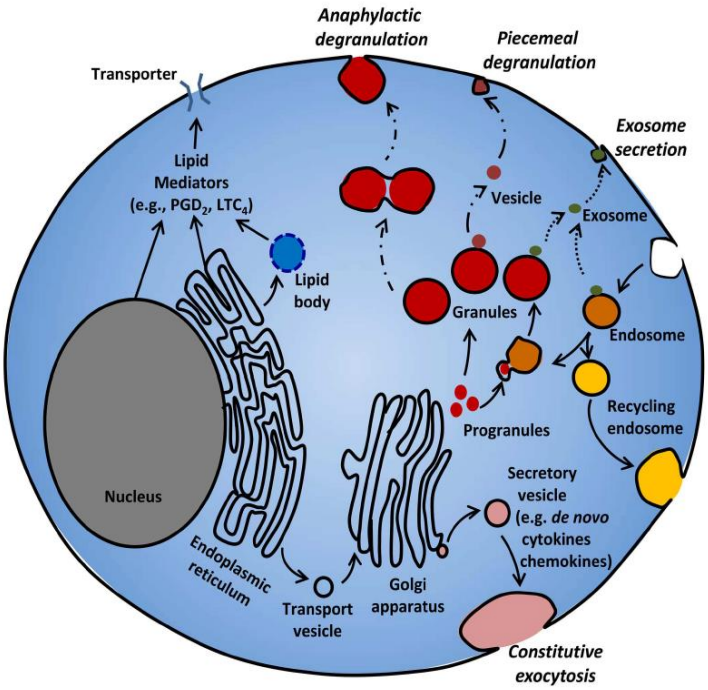


Figure 1.6. Mechanisms of mediator release from MC. MC rapidly release pre-stored granule contents by piecemeal or anaphylactic degranulation. *De novo* synthesized mediators are packaged in secretory vesicles are released through constitutive exocytosis. *Reprinted with permission from: Moon TC, Befus AD, Kulka M. Mast cell mediators: their differential release and the secretory pathways involved. Front Immunol 2014; 5:569.*

1.4 Hypothesis

It was hypothesized that GAGs inhibit the secretion of pro-inflammatory mediators TNF and CXCL8 from human MC stimulated by the cytokine IL-33. In addition, it was hypothesized that GAGs exert their inhibitory action by one or more of the following mechanisms: inhibiting gene expression of either TNF or CXCL8, inhibiting calcium influx, blocking surface expression of the IL-33 receptor, ST2, or interfering with MC SNARE functioning. It was also hypothesized that CS binds to the MC cell surface and/or is taken up into MC, possibly via the hyaluronan receptor CD44.

1.5 Thesis Objectives and Summary

Almost twenty years ago, our laboratory had found that CS can inhibit the secretion of histamine from rat connective tissue MC when stimulated with the degranulation trigger Compound 48/80[128]. Recently, our laboratory reported that human LAD2 MC secrete TNF in response to the potent trigger IL-33[92]. However, it was unknown whether CS or other GAGs were able to inhibit secretion of cytokines and chemokines from human MC, specifically those stimulated by IL-33. Thus, the objective of this study was to determine if there was an inhibitory effect of CS and other GAGs on the secretion of TNF and CXCL8 from human LAD2 MC stimulated by IL-33, and if so, to determine its mechanism of action.

In Chapter 3, the first aim was to determine the effect, both in terms of degranulation and secretion of TNF and CXCL8, of CS on MC stimulated by both SP and

IL-33. The optimal time and concentration of CS necessary to elicit a maximal inhibitory response on MC was then investigated. The manner in which fluorescein-bound CS (CS-F) associates with MC and how it localizes was studied. Using flow cytometry and confocal microscopy, the effect of concentration and temperature on the association of CS-F with MC was investigated. The role of the HA receptor CD44 on the uptake of CS-F by MC was studied. The expression of the SNAREs SNAP-23 and VAMP-8 in unstimulated and IL-33-stimulated MC were compared and it was investigated whether CS affects their expression.

In Chapter 4, the first aim was to compare the inhibitory effect of dermatan sulfate (DS) and heparin (Hep) with that of CS on TNF and CXCL8 secretion from MC stimulated with IL-33. The optimal concentration of DS and Hep necessary to elicit a maximal inhibitory response on MC was investigated. The effect of unfractionated (UF) DS and Hep were compared to their low molecular weight (LMW) counterparts on the secretion of TNF and CXCL8 from MC stimulated with IL-33. The effect of CS, DS and Hep on ionomycin-induced β-hexosaminidase secretion, gene expression of TNF and CXCL8 in IL-33-stimulated MC and expression of the IL-33 receptor, ST2, were studied. Finally, the potential effect of GAGs on secretion of TNF and CXCL8 from primary human cord blood-derived MC (hCBMC) stimulated with anti-IgE was examined. The present findings indicate that CS, DS and Hep can inhibit the release of TNF and CXCL8 from human MC stimulated by IL-33, with CS possibly acting intracellularly. CS exerts few or no side effects[189] and there are no known drug-drug interactions[190]. It is, therefore, an attractive therapeutic option for development of treatments for allergic and inflammatory diseases.

CHAPTER 2 : MATERIALS AND METHODS

2.1 Materials

Chondroitin sulfate (CS) sodium salt from shark cartilage, heparin (Hep) sodium salt and dermatan sulfate (DS) both from porcine intestinal mucosa, SP and ionomycin from *Streptomyces conglobatus* were purchased from Millipore Sigma (St. Louis, MO). Low molecular weight (LMW) Hep and DS were purchased from AMSBIO, Cambridge, MA. Recombinant human stem cell factor (rhSCF, Stemgen) was kindly supplied by Swedish Orphan Biovitrum AB (Stockholm, Sweden). Recombinant human IL-33 and IL-3, as well as ELISA kits for TNF (DY210) and CXCL8 (DY208) were purchased from R&D Systems (Minneapolis, MN). Recombinant human IL-6 was purchased from STEMCELL Technologies (Cambridge, MA). Cell permanent Fura-2 AM was purchased from Invitrogen (Waltham, MA). Fluorescein-labeled CS (CS-F) and dextran (Dex-F) were purchased from Creative PEGWorks (Chapel Hill, NC). RNeasy Mini (Qiagen, Valencia, CA) and iScript cDNA synthesis kits were purchased from BioRad (Hercules, CA). Taqman gene expression primers/assays for TNF (Hs00174128_m1), CXCL8 (Hs00174103_m1) and GAPDH endogenous control (4310884E) were purchased from Thermo Fisher Scientific (Waltham, MA).

Monoclonal (clone # IM7.8.1R) rat IgG2B anti-human/mouse CD44 Alexa-Fluor® 488-conjugated antibody (Ab) and polyclonal goat IgG anti-human ST2/IL-33R PE-conjugated Ab, along with their respective isotype controls, were purchased from R&D Systems. Monoclonal rat IgG2a anti-human H-CAM (CD44) Ab used for CD44 neutralization was also purchased from Thermo Fisher Scientific. Abs for SNARE immunofluorescence, anti-human SNAP-23 mouse IgG1 monoclonal antibody, mouse

IgGκ binding protein-FITC and anti-human FITC-bound VAMP-8 mouse monoclonal Ab, were purchased from Santa Cruz Biotechnology (Dallas, Texas). Human recombinant IgE and anti-IgE were purchased from EMD Millipore (Billerica, MA). All reagents were dissolved in deionized water except for Fura-2 AM and ionomycin, which were dissolved in DMSO, the final concentration of which was <0.1%.

2.2 Methods

2.2.1 Culture of human MC

LAD2 cells, derived from a patient with MC leukemia[191], were kindly supplied by Dr. A Kirshenbaum (NIH, Bethesda, MD) and were cultured in Gibco StemPro-34 medium (Invitrogen) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml rhSCF. Cells were maintained at 37ºC in a humidified incubator at an atmosphere of 95% O_2/5% CO_2. LAD2 cells doubled within 2 weeks in the presence of rhSCF (100 ng/mL). Even though LAD2 cells are an immortalized cell line, these cells closely resemble $CD34^+$-derived primary human MC due to their ability to respond to SCF and express functional high affinity receptors for IgE (FcεRI)[191].

Human umbilical cord blood was obtained after normal deliveries in accordance with established institutional guidelines (Tufts HIRB approval #12152) to culture primary human cord blood mast cells (hCBMC)[192]. Mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (INC Biomedical). CD34+ progenitor cells were isolated by means of positive selection of AC133

(CD133+/CD34+) cells by using magnetic cell sorting (CD133 Microbead Kit; Miltenyi Biotech). For the first 6 weeks, CD34+ progenitor cells were cultured in Iscove modified Dulbecco medium (Life Technologies) supplemented with 0.1% BSA, 1% insulin-transferrin-selenium, 50 ng/mL rhIL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. In addition, for the first 3 weeks, cells were supplemented with 10 ng/mL rhIL-3. After 6 weeks, the cells were cultured in Iscove modified Dulbecco medium supplemented with 10% FBS, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/ streptomycin, and 100 ng/mL rhSCF. hCBMC cultured for at least 12 weeks were used for experiments.

Cell viability was measured by Trypan blue (0.4%) exclusion[63] at all concentrations tested.

2.2.2 Mast cell treatments with GAGs

LAD2 MC (3.0×10^5) were incubated with varying concentrations of GAGs for 1 hr in 12-well flat bottom Falcon cell culture plates from Becton Dickinson (Franklin Lakes, NJ) and then washed twice with ice-cold PBS + EDTA (1 μM). LAD2 MC were next seeded into 48-well flat bottom Falcon cell culture plates at a density of 3.0×10^5 cells/well. MC were triggered as described below; both supernatants and cell pellets were collected.

2.2.3 Inflammatory mediator quantification by ELISA

LAD2 MC were triggered with SP (2 μM) or IL-33 (10 ng/mL) for 1 hr or 24 hrs, respectively. Control cells were treated with the same volume of culture media. Supernatant fluids were collected and both TNF and CXCL8 were measured by ELISA using commercial kits from R&D Systems (Minneapolis, MN) as per the instructions. The minimum detectable level is 5 pg/mL.

2.2.4 BCA assay

After collecting the supernatant fluids, cell pellets were washed once with PBS and then lysed using protein lysis radio-immuno precipitation (RIPA) buffer (Millipore Sigma) in the presence of Halt protease and phosphatase inhibitor cocktail (1X, Thermo Fisher Scientific). Total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific) method using bovine serum albumin (BSA) as standard.

2.2.5 β-Hexosaminidase assay

β-hexosaminidase release, as an index of rapid mast cell degranulation, was assayed using a fluorometric assay as previously reported. Briefly, the supernatant fluid and cell lysates (0.5×10^5 cells/tube, was lysed with 1% Triton X-100 to measure residual cell-associated β-hexosaminidase) were incubated with substrate solution (p-nitrophenyl-N-acetyl-β-D-glucosaminide, Sigma-Aldrich) in 0.1 M NaOH/0.2 M glycine in order to

measure β-hexosaminidase activity. Absorbance was read at 405 nm using a SpectraMax® spectrophotometer (Molecular Devices, San Jose, California), and the results are expressed as percentage of β-hexosaminidase activity released over the total.

2.2.6 RNA isolation and quantitative real time-PCR (qRT-PCR)

LAD2 MC (5.0×10^5) were treated with the indicated GAGs and washed twice with ice-cold PBS (+EDTA), then stimulated with IL-33 (10 ng/mL, 3 hr). Total mRNA was extracted with an RNeasy Mini kit (Qiagen) in accordance with the manufacturer's intructions. An iScript cDNA synthesis kit (BioRad) was used for reverse-transcription of each mRNA sample. qRT-PCR was performed using Taqman gene expression assays for TNF and CXCL8 (Thermo Fisher Scientific). Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems, Foster City, CA). Relative mRNA abundance was determined with the $2^{\Delta\Delta Ct}$ method, using GAPDH as a reference gene. Mediator and GAPDH Ct values from GAG + IL-33 conditions were compared to respective Ct values from IL-33 alone conditions (assigned 1.0), to give the result as expression fold change ($2^{\Delta\Delta Ct}$) over control. Efficiency was confirmed from standard curves run with each experiment.

2.2.7 CS cellular localization by confocal microscopy

LAD2 MC (2.0×10^5) were incubated with either fluorescein-labeled CS (CS-F, Creative PEGWorks) or fluorescein-labeled dextran (Dex-F, Creative PEGWorks) at the

indicated concentrations in Gibco StemPro-34 media for 1 hr in either a 37ºC 5% CO_2 incubator or in a 4ºC cold room, and then washed twice with ice-cold PBS (+EDTA). Cells were then fixed in 4% paraformaldehyde in PBS, washed twice, re-suspended in 20 µL PBS, and then dried on a Fisherbrand™ Superfrost™ Plus microscope slide (Fisher Scientific, Pittsburgh, PA) before imaging with Leica SPE confocal microscope (Leica Microsystems, Buffalo Grove, IL) at 40x. Confocal images represent a maximum stacking of all imaged slices taken in a 3D Z-stack of approximately 10 µm.

2.2.8 Measurement of intracellular calcium

LAD2 MC (1.0×10^5) were first pre-incubated with or without the specified GAGs (50 µg/mL) for 1 hr at 37°C, before being washed twice with PBS (+EDTA) and loaded with cell permanent Fura-2 AM (3 µg/mL, Invitrogen) for 45 min at 37°C. Cells were washed and allowed to stay at 22°C for 30 min to allow for de-esterification of Fura-2 AM. LAD2 MC were then washed again and kept on ice until baseline intracellular calcium levels were measured for 5 min with the FlexStation™ II (Molecular Devices, Sunnyvale, CA) using an 335/363 nm excitation ratio, with emission wavelengths at 505 nm and 512 nm, respectively. After 5 min, MC were stimulated with or without either the calcium ionophore ionomycin (5 µg/mL, Millipore Sigma) or IL-33 (50 ng/mL) for 20 min, while intracellular calcium influx was measured continuously at 37°C.

2.2.9 FACS analysis

LAD2 MC (8.0×10^5) were incubated with varying concentrations of CS-F in Gibco StemPro-34 media for 1 hr at 37ºC, and then washed twice with ice-cold PBS (+EDTA). Cells were then resuspended in 500 µL of PBS on ice, and either analyzed with a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA) or sorted with a MoFlo cytometer (DakoCytomation, Santa Clara, CA) using the appropriate instrument settings and protocol. To evaluate the effect of CD44 neutralization/blocking on CS-F uptake, LAD2 MC were first pre-incubated with monoclonal (clone # Hermes-1) rat IgG_{2a} anti-human H-CAM (CD44, Thermo Fisher Scientific) Ab at the concentrations indicated. To determine the expression of CD44 and ST2 on LAD2 MC, monoclonal (clone # IM7.8.1R) rat IgG_{2B} anti-human/mouse CD44 Alexa-Fluor® 488-conjugated Ab and polyclonal goat IgG anti-human ST2/IL-33R PE-conjugated Ab were used after blocking MC with 5% goat serum (R&D Systems). Expression of these receptors on LAD2 MC was compared to the expression of the antibody's respective isotype control, monoclonal rat IgG_{2B} Alexa-Fluor® 488-conjugated Ab or polyclonal goat IgG PE-conjugated Ab (R&D Systems).

2.2.10 Dimethylmethylene blue GAGs assay

The color reagent was prepared by dissolving 16 mg dimethylmethylene blue in 1 L water containing 3.04 g glycine, 2.37 g NaCl and 95 ml 0.1 M HCI, to give a solution at pH 3.0, with $A_{25} = 0.31$. Chondroitin sulfate GAG standards from 0-40 µg/mL of were

prepared in clear RPMI 1640, consisting of 137 mL of 0.1M NaH_2PO_4 and 63 mL of 0.1M Na_2HPO_4, pH 6.5. Cells were suspended for the indicated time period in clear RPMI 1640, which was used to measure GAGs in supernatants. 80 μL of either standards or samples and 250 μL of dye were added to each well of a 96-well plate and measured at a wavelength of 525 nm.

2.2.11 SNARE protein expression by immunofluorescence

2.2.11.1 SNAP-23

LAD2 MC were pre-incubated with or without CS (50 μg/mL) for 30 min, then washed and stimulated with or without IL-33 (10 ng/mL) for either 30 min or 3 hr. After the indicated stimulation time, MC were washed, and then fixed and permeabilized with 10ºC methanol for 5 min. MC were then washed twice and then allowed to air dry on the slide. Slides were blocked overnight with 10% serum in PBS. The next day, slides were treated with anti-human SNAP-23 mouse IgG1 monoclonal antibody (Santa Cruz) for 1 hr in 22ºC, washed 3 times in PBS. Next, slides were incubated with mouse IgGκ binding protein-FITC (Santa Cruz) for 1 hr in 22ºC, washed 3 times in PBS, and then treated with mounting medium with Hoescht to prepare for fluorescence microscopy.

2.2.11.2 VAMP-8

Methods were the same as SNAP-23, except anti-human VAMP-8 mouse monoclonal Ab was already conjugated to FITC, so no secondary Ab was necessary.

2.2.12 Statistical analysis

All conditions were performed in duplicate, at least, and all experiments were repeated at least three times (n=3), unless noted otherwise. Results are presented as mean±SEM. Data were compared using one-way ANOVA followed by Dunnett's or Tukey's multiple comparison test as indicated, unless sample set(s) did not follow a normal distribution; in this case, the Kruskal-Wallis one-way ANOVA was used instead of an ordinary one-way ANOVA (GraphPad Prism 7, San Diego, CA). Significance is denoted by: *=$p<0.05$, **=$p<0.01$, *=$p<0.001$, *=$p<0.0001$.

CHAPTER 3 : INHIBITORY EFFECT OF CHONDROITIN SULFATE ON SECRETION OF TNF AND CXCL8 FROM LAD2 MAST CELLS

3.1 CS does not interact directly with triggers or mediators of MC

An initial concern before beginning experiments was that, even after one washing, there may be remnants of CS in the culture medium that may potentially inhibit the action of MC triggers or ELISA for the mediators TNF and CXCL8. While there is some CS remaining after one wash in the CS-treated group, it is comparable to that "remaining" in the condition with no CS added (**Fig. 3.1**). After two washes, the CS concentrations measured in both conditions were below the level of detection; hence, this data is not shown.

More specifically, when the average CS "remaining" in the control group is subtracted from the average CS remaining in the CS-treated group, the result is 0.24 µg of CS. The typical dose of CS used in my experiments is 100 µg/mL. In this case, 500 µg/mL was added to MC to increase assay detection. This leads to an approximate value of 0.049 µg CS remaining in the medium after MC are washed once following pre-incubation with CS (100 µg/mL) in my experiments. The MC are then typically resuspended in around 3 mL prior to seeding, giving an estimated final leftover CS concentration of 0.016 µg/mL after 1 wash.

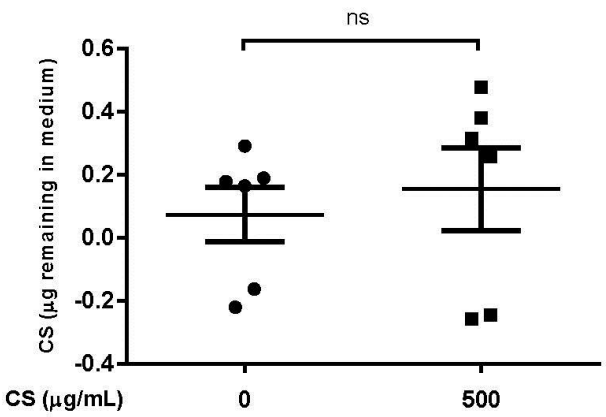


Figure 3.1. Measurement of CS detected in the culture medium after treatment of MC with CS and 1 wash. LAD2 MC were treated with or without CS (500 µg/mL) and then washed once with PBS + EDTA. CS remaining in the medium was determined via the dimethylmethylene blue (DMMB) colorimetric assay. Negative values indicate concentrations below limit of detection. Each condition was performed in duplicate, n=3; ns=not significant.

The concern that CS left in the test wells, if any, may interfere with the potency of the MC triggers, particularly the positively charged SP, was investigated directly. "Spiking" the culture wells with CS (0.01-1 µg/mL) simultaneously while adding SP (1 µM) did not significantly decrease the secretion of (**Fig. 3.2A**) TNF or (**Fig. 3.2B**) CXCL8 from MC. Similarly, when MC were "spiked" with CS (0.01-1 µg/mL) and stimulated with IL-33 (10 ng/mL), the secretion of (**Fig. 3.2C**) TNF and (**Fig. 3.2D**) CXCL8 was not significantly decreased.

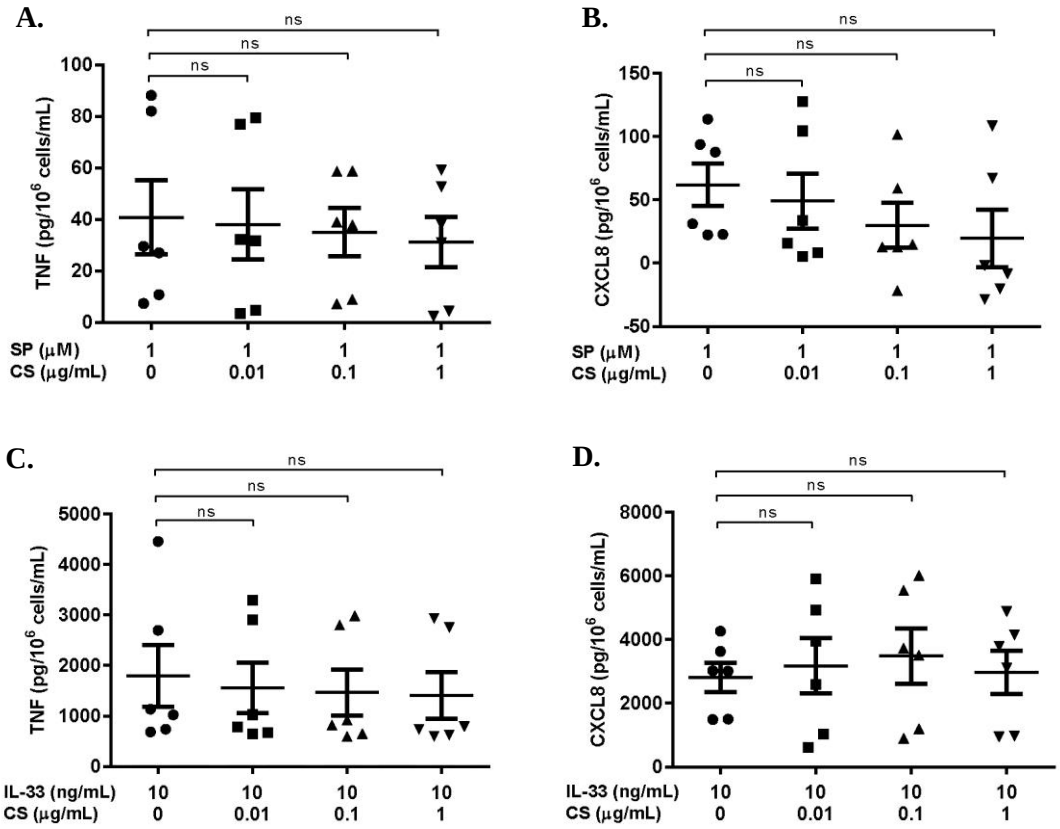


Figure 3.2. Lack of effect of CS in the medium on the ability of SP or IL-33 to stimulate TNF and CXCL8 secretion from MC. LAD2 MC were treated with CS (0.01-1 µg/mL) and then, without being washed, stimulated either with (A, B) SP (1µM) or (C, D) IL-33 (10 ng/mL). The subsequent secretion of (A, C) TNF and (B, D) CXCL8 was measured via ELISA. Each condition was performed in duplicate, n=3.

Another concern was that CS left in the test wells, if any, may interfere with the detection of the MC mediators during ELISAs. "Spiking" the culture wells (no MC present) with CS (0.01-1 µg/mL) in the presence of recombinant TNF (500 pg/mL) for 24 hr did not significantly decrease the detection of TNF (**Fig. 3.3A**). Similarly, when the culture wells were "spiked" with CS (0.01-1 µg/mL) in the presence of recombinant CXCL8 (500 pg/mL) for 24 hr, the detection of CXCL8 was not decreased (**Fig. 3.3B)**. Surprisingly, these low concentrations of CS sometimes slightly increased the detection of both TNF and CXCL8. For an unknown reason, when recombinant TNF or CXCL8 was added to the culture wells, the ELISA reading was always lower than the calculated value.

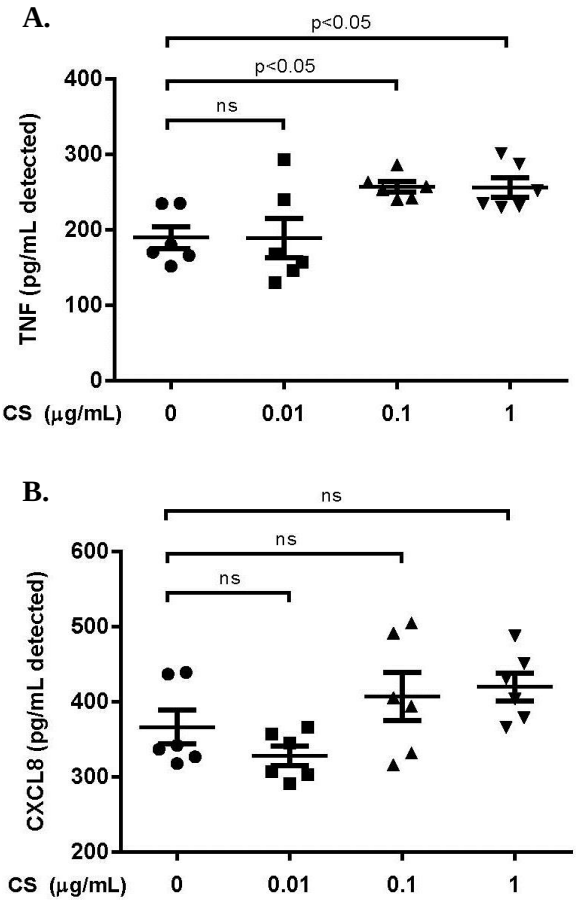


Figure 3.3. Lack of effect of CS on the ELISA for TNF or CXCL8 detection. CS (0.1-1 µg/mL) or water (control) was added to culture medium without LAD2 MC, which was then "spiked" with TNF or CXCL8 (500 pg/mL). (A) TNF and (B) CXCL8 were measured using ELISA. Each condition was performed in duplicate, n=3; ns=not significant.

3.2 CS does not inhibit β-hexosaminidase secretion from MC stimulated by SP

Since IL-33 alone does not lead to LAD2 MC degranulation (results not shown), SP was used to stimulate β-hexosaminidase, a secretory granule enzyme rapidly secreted after stimulation and used as index of degranulation. SP (2 μM) stimulated about 14% β-hexosaminidase secretion. Pre-incubation with CS (50 μg/mL) for 1 hr, followed by two washes and stimulation with SP (2 μM) for 1 hr did not inhibit β-hexosaminidase release from LAD2 MC under the experimental design used (**Fig. 3.4**).

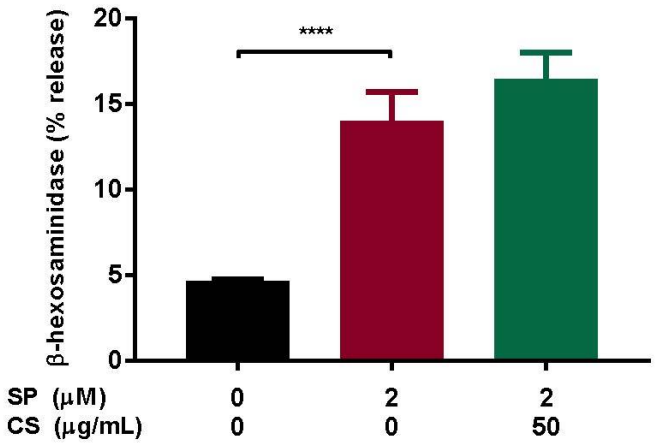


Figure 3.4. No effect of CS on β-hexosaminidase secretion from SP-stimulated human MC. LAD2 MC were pre-incubated with or without CS (50 μg/mL) for 1 hr, washed twice, and then incubated with or without SP (2 μM) for 1 hr. Secretion of β-hexosaminidase was measured as described in Methods. Each condition was performed in triplicate, n=3; ****=p<0.0001.

3.3 CS inhibits TNF and CXCL8 secretion from MC stimulated by SP

Next, the effect of CS on mediator secretion from MC stimulated with SP was investigated. Pre-incubation with CS for 24 hr inhibited SP (1 μM)-induced TNF and CXCL8 release in a dose-dependent manner (**Fig. 3.5**). Specifically, CS (1000 μg/mL) inhibited TNF and CXCL8 release by up to 75% and 60%, respectively. At a lower concentration of 100 μg/mL, CS inhibited TNF and CXCL8 release by about 40% and 32%, respectively.

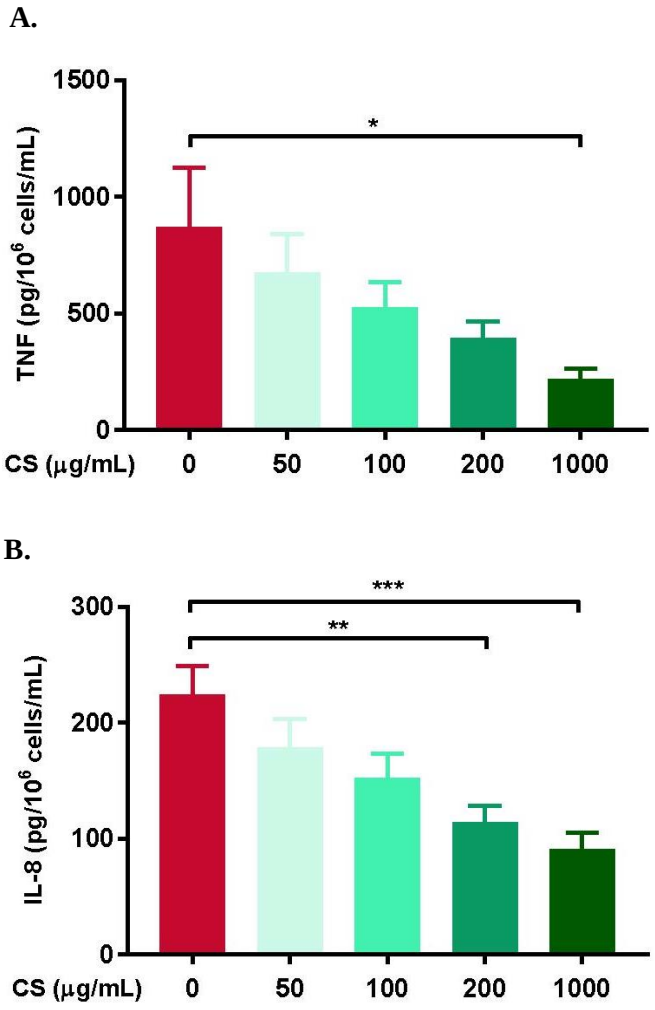


Figure 3.5. Effect of CS on secretion of TNF and CXCL8 from MC stimulated with SP. LAD2 MC were either pre-treated with culture medium (control) or CS for 24 hr prior to one wash, followed by stimulation with SP (1 µM) for 24 hr. Consequential secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in duplicate, n=3; *=p<0.05, **=p<0.01, ***=p<0.001.

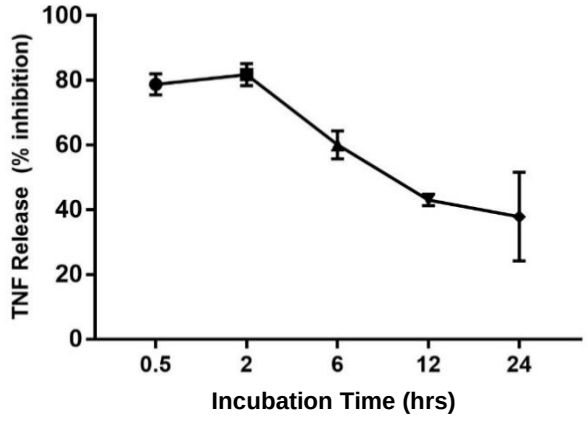
3.4 CS inhibits TNF and CXCL8 secretion from MC stimulated by IL-33

Even though it was earlier stated that CS does not interact electrostatically with the positively charged SP, it was deemed best to avoid SP. Thus, the effect of CS was studied on mediator secretion from MC stimulated with IL-33, since our laboratory had recently reported that IL-33 can stimulate at least TNF release.

3.4.1 Selection of optimal pre-incubation time with CS

Preliminary experiments determined that pre-incubation with CS, followed by one wash with PBS, inhibits the secretion of TNF and CXCL8 from IL-33-stimulated MC in a unique time-dependent manner (**Fig. 3.6A, B**). CS (100 µg/mL) maximally inhibited IL-33-induced TNF secretion between 30 min and 2 hr, and minimally inhibited their secretion at 24 hr (**Fig. 3.6A**). Similarly, CS (100 µg/mL) inhibits IL-33-induced CXCL8 secretion, with maximal inhibition obtained at pre-incubation times between 30 min and 2 hr (**Fig. 3.6B**).

A.



B.

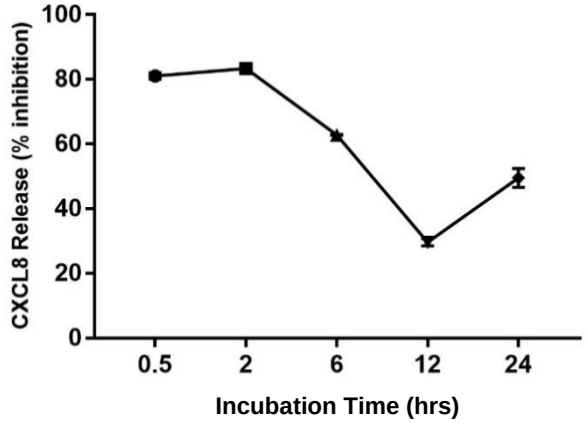


Figure 3.6. Time-course of the inhibitory effect of CS on mediator release from MC stimulated with IL-33. LAD2 MC were pre-incubated with CS (100 µg/mL) and then stimulated with IL-33 (10 ng/mL). The time-course secretion of (A) TNF and (B) CXCL8 was measured. Each condition was performed in duplicate: (A), n=2; (B,) n=1.

3.4.2 Inhibition of TNF and CXCL8 secretion by CS

There was no detectable effect of IL-33 on degranulation (results not shown). Stimulation with IL-33 (10 ng/mL) for 24 hr resulted in significant secretion of both TNF (~900 pg/150 µg protein/mL) and CXCL8 (~2200 pg/150 µg protein/mL) (**Fig. 3.7A, B**). Pre-incubation of MC with various concentrations of CS for 1 hr, washed twice and then stimulated with IL-33 (10 ng/mL) resulted in significant inhibition of TNF and CXCL8 secretion. CS significantly inhibited secretion of TNF at both 75 µg/mL ($p<0.05$) and 150 µg/mL ($p<0.005$; **Fig. 3.7A**), while CXCL8 secretion was only significantly inhibited by CS at 150 µg/mL (**Fig. 3.7B**).

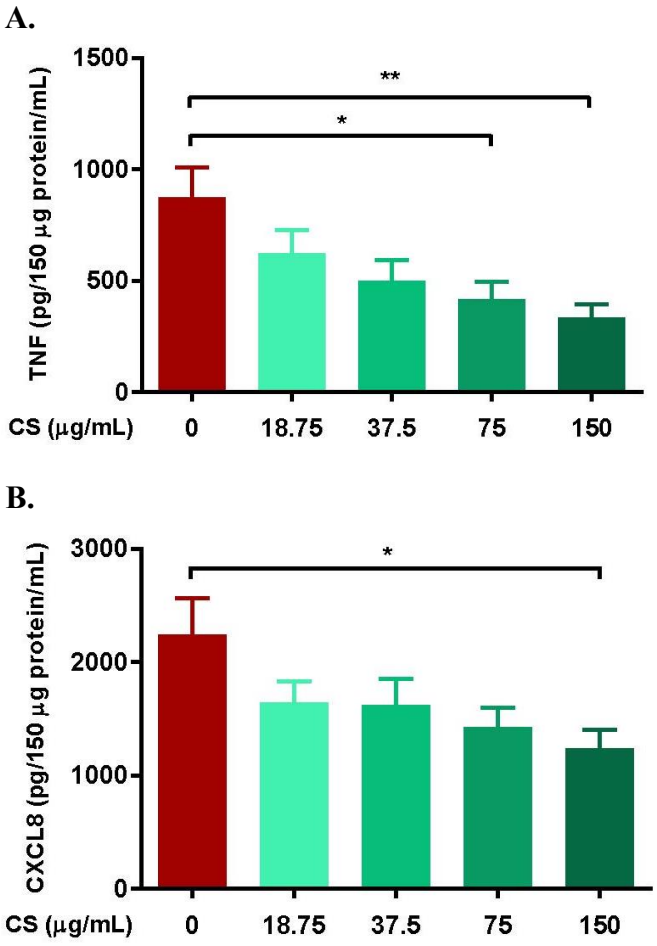


Figure 3.7. Effect of CS on secretion of TNF and CXCL8 from MC stimulated with IL-33. LAD2 MC were either pre-treated with culture medium (control) or CS for 1 hr prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Consequential secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=3; *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

3.5 CS-Fluorescein association with MC

The mechanism of the inhibitory action of CS on human MC was also investigated. First, the cellular localization of fluorescein-bound CS (CS-F) was explored using both FACS and confocal microscopy.

3.5.1 Validation of inhibitory effect of CS-F

In order to confirm that CS-F has the same action as unlabeled CS in terms of inhibitory activity on MC, the effects of CS-F and unlabeled CS on the secretion of TNF and CXCL8 from MC stimulated with IL-33 were compared. Both CS-F and unlabeled CS (both at 50 µg/mL) inhibited secretion of TNF and CXCL8, and there was no statistical significance in the inhibition observed between the two compounds (**Fig. 3.8A, B**).

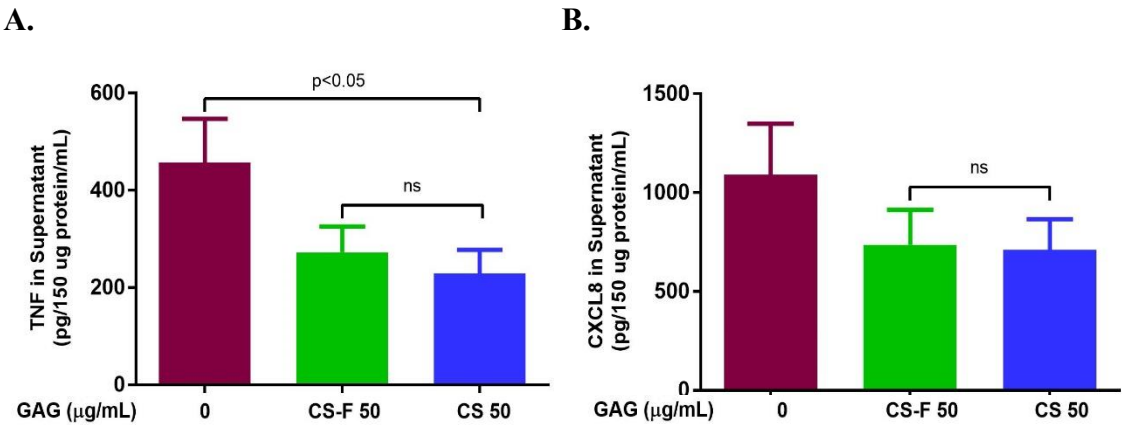


Figure 3.8. Comparison of the inhibitory effect of CS-Fluorescein (CS-F) vs. non-labeled CS on MC. LAD2 MC were treated with CS-F or non-labeled CS (50 µg/mL) for 1 hr, washed twice, and then stimulated with IL-33 (10 ng/mL) for 24 hrs. (A) TNF and (B) CXCL8 secretion was measured via ELISA. Each condition was performed in duplicate, n=3. ns=not significant.

3.5.2 Dose-dependent uptake of CS-F investigated using flow cytometry

In order to determine the cellular uptake of CS-F, MC were pre-incubated with different concentrations of CS-F (2, 10, 50 µg/mL) for 1 hr, washed twice, and then analyzed using flow cytometry. The MC population was plotted as FL1-H, the filter detecting fluorescein fluorescence, vs. FL2-H, an irrelevant control filter. As more CS-F was added, the population of MC associated with CS-F shifted to the right while the unstained population located in the bottom left corner diminished, indicating that the majority of MC were associated with CS-F (**Fig. 3.9**).

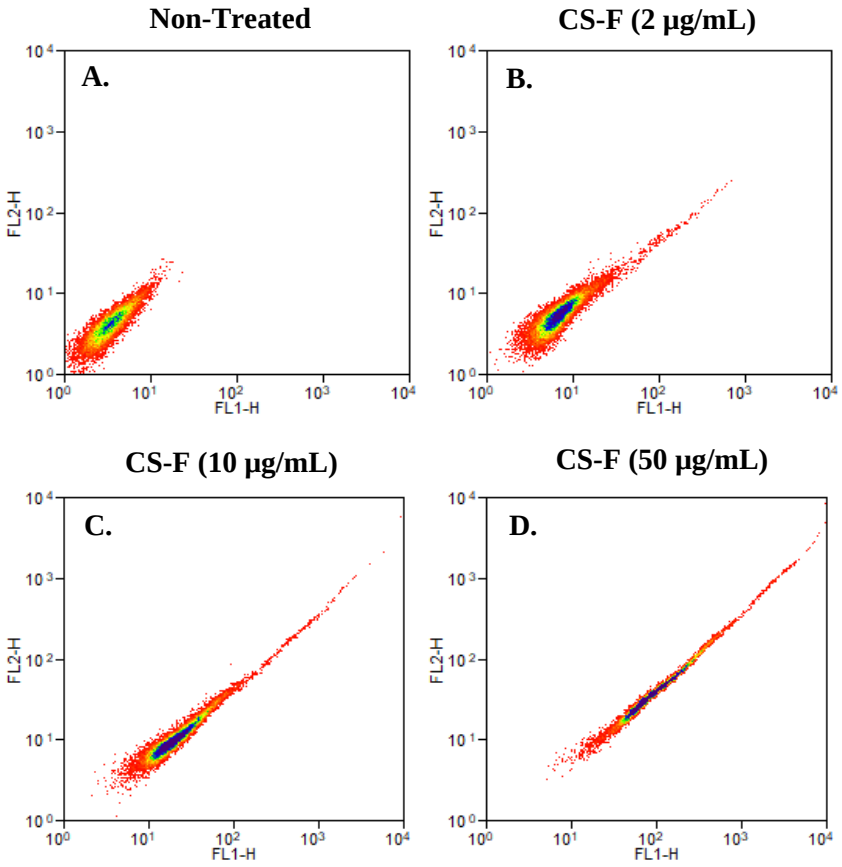


Figure 3.9. Uptake of CS-F in MC measured via flow cytometry. LAD2 MC were treated with culture medium or increasing concentrations of CS-F: (A) non-treated control; (B) CS-F (2 µg/mL); (C) CS-F (10 µg/mL); (D) CS-F (50 µg/mL). Representative experiment, n=2.

3.5.3 Sorting of MC populations treated with CS-F with FACS

No obvious distinct populations were observed with flow cytometry (**Fig. 3.9**). Consequently, it was decided to sort the MC into different populations in order to determine the existence of any sub-populations using confocal microscopy. In **Figure 3.10A**, the plot with an x-axis of FSC-A (forward scatter-area) and a y-axis of SSC-A (side scatter-area) identifies sorted cell populations of interest based on size (FSC) and granularity/complexity (SSC). Typically, cell debris will be located in the bottom left corner of such a plot—in this case, population 4 (P4, purple). Population 3 (P3, royal blue) also consisted of small events, but were more complex. As expected, when populations 3 and 4 were plotted in terms of their fluorescence (**Figure 3.10A**), with the relevant FITC filter (Ex-Max: 494 nm/Em-Max: 520 nm) on the x-axis and an irrelevant fluorescent filter on the y-axis, PerCP-Cy5.5 (Ex-max: 482 nm/Em-max: 676 nm), they consisted of the most fluorescent events. This was corroborated via confocal microscopy observations (**Figure 3.10B-D**). It was concluded that the majority (350,000) of events sorted was those in population 8 (P8, orange), and were healthy cells. It was also determined there was no significant distinction in fluorescence between live cells.

3.5.4 Dose-dependent uptake of CS-F investigated using confocal microscopy

To further investigate the association of CS with MC, they were incubated with increasing concentrations of CS-F (2, 10, 50 μg/mL) for 1 hr at 37°C, then washed twice, before being fixed and mounted onto a slide for confocal imaging. At the lower

concentrations of CS-F (2 and 10 µg/mL), some CS-F was visibly associated only with the cell surface (**Fig. 3.11A, B**). At the higher concentration of CS-F (50 µg/mL), it was localized both at the cell surface and intracellularly (**Fig. 3.11C**). The highest concentration of CS-F was chosen because at concentrations higher than 50 µg/mL, the confocal images appeared "saturated".

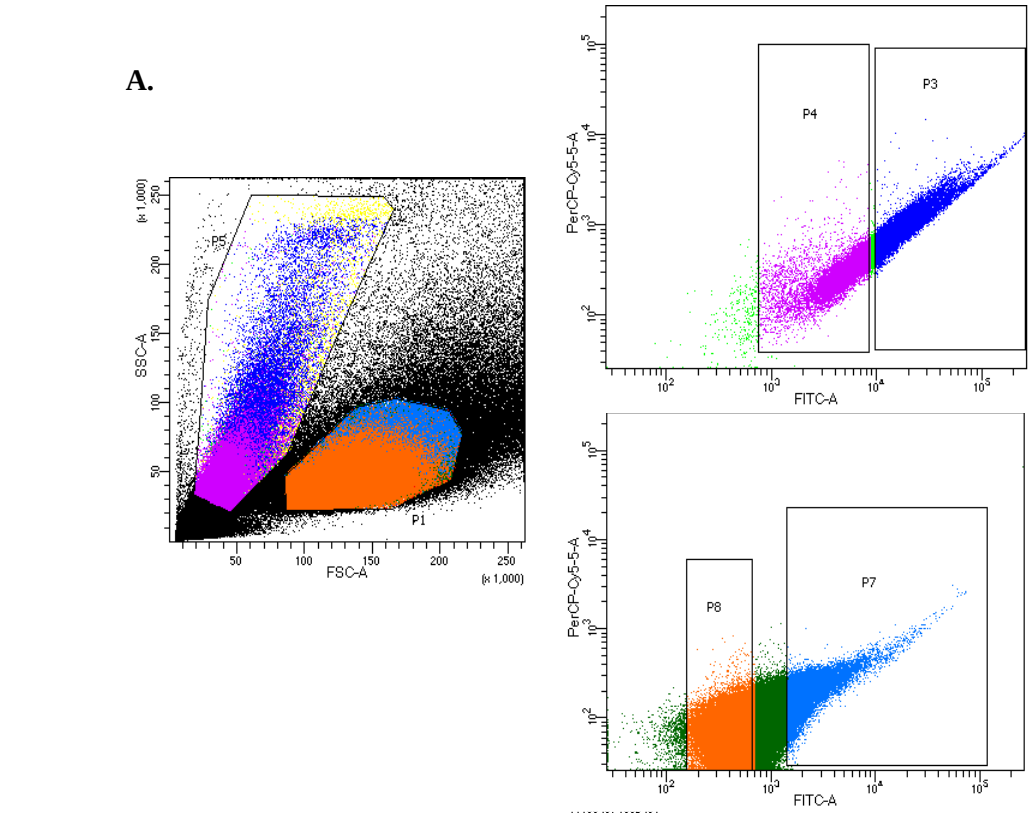


Figure 3.10. Sorting of MC incubated with CS-F via FACS and analysis of sorted populations via confocal microscopy. (A) Visualization of sorted MC populations after incubation with CS-F (5 µg/mL); confocal imaging of sorted MC from the following parent populations seen in (A): (B) P3; (C) P7; (D) P8.

Fluorescein-conjugated dextran (Dex-F), which is not sulfated, was used as control (**Fig. 3.11D**). The concentration of 200 µg/mL was used because it was determined that Dex-F was four times less fluorescent than CS-F (data not shown). In contrast to CS-F, Dex-F, even at this higher concentration, appeared to associate only with the cell surface.

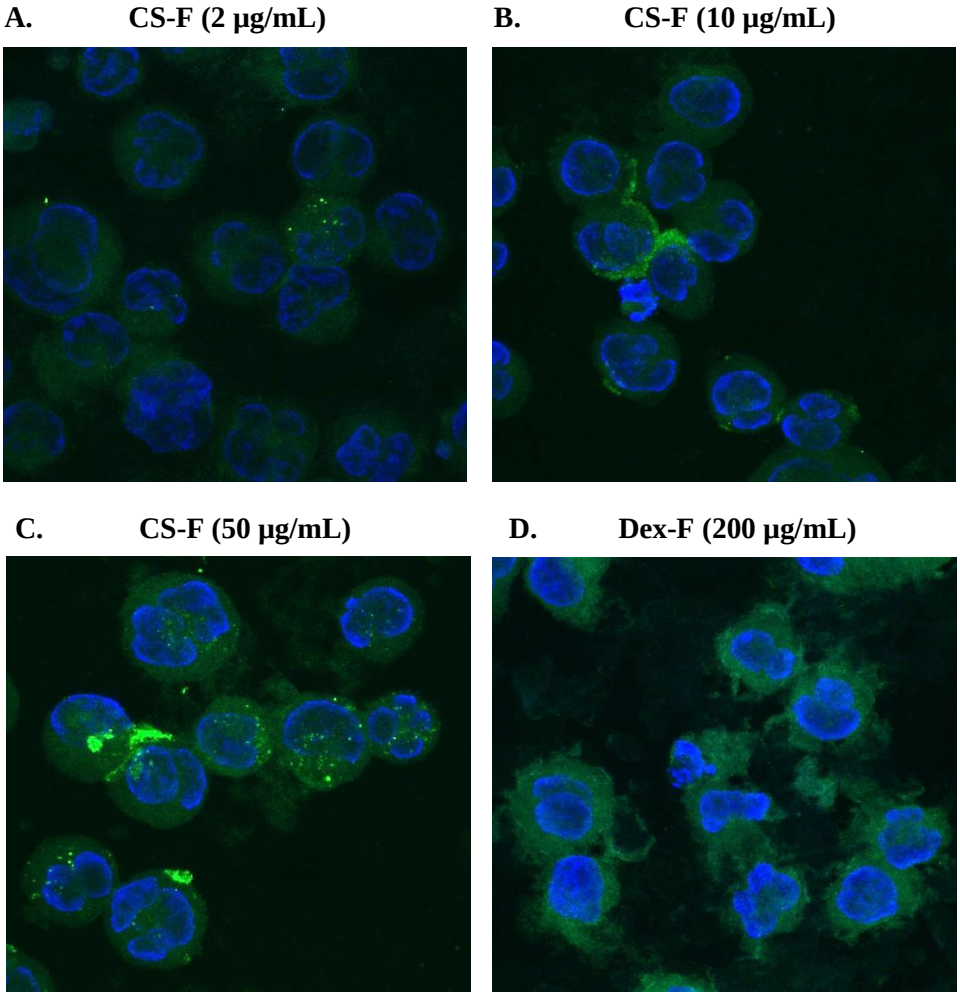


Figure 3.11. Confocal images of MC-associated CS-F compared to dextran-fluorescein (Dex-F) at 37ºC. LAD2 MC were treated with: (A) CS-F (2 µg/mL); (B) CS-F (10 µg/mL); (C) CS-F (50 µg/mL); (D) Dex-F (200 µg/mL). Representative experiment, n=2.

3.5.5 Temperature-dependent uptake of CS-F investigated using confocal microscopy

To determine the effect of temperature on the association of CS-F with MC, they were incubated with or without CS-F (50 µg/mL) at either 4ºC or 37ºC for 1 hr, washed twice and then fixed with PFA, before being mounted onto a slide for confocal imaging (**Fig. 3.12**). At 4ºC, CS-F appeared to be only weakly associated with MC (**Fig. 3.12B**), while at 37°C, CS-F was associated with both the cell surface and the cytoplasm (**Fig. 3.12C**). Cells handled and treated with CS added on ice, allowed to reach 37°C for 30 min and then washed, were not inhibited when stimulated with IL-33 (results not shown).

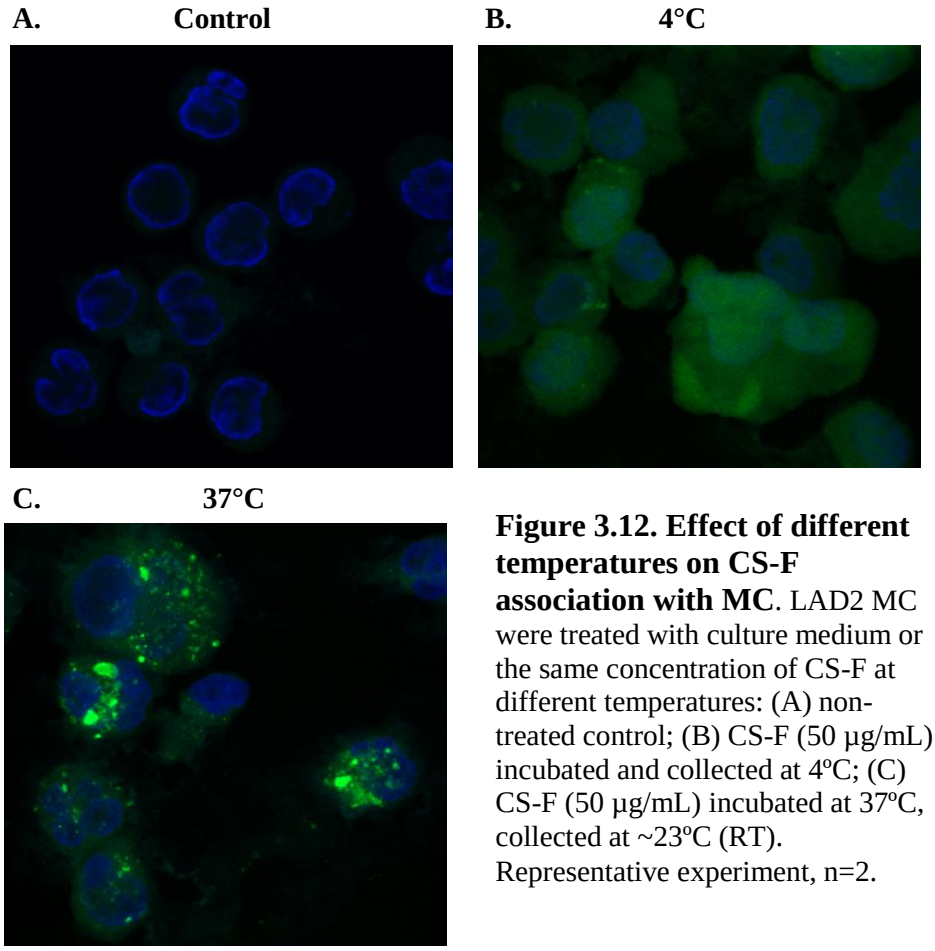


Figure 3.12. Effect of different temperatures on CS-F association with MC. LAD2 MC were treated with culture medium or the same concentration of CS-F at different temperatures: (A) non-treated control; (B) CS-F (50 µg/mL) incubated and collected at 4ºC; (C) CS-F (50 µg/mL) incubated at 37ºC, collected at ~23ºC (RT). Representative experiment, n=2.

3.5.6 CD44 is not involved in CS-F association with MC

CD44 is the receptor for hyaluronan, a non-sulfated GAG. It was next decided to study its potential involvement in the binding and uptake of CS-F. Expression of CD44 on MC was first confirmed via flow cytometry analysis. The MC population was plotted as FL1-H, the filter detecting fluorescein fluorescence, vs. FL2-H, an irrelevant control filter. The rectangle shown, R2, is a subjective area that lies tangent and to the right of the isotype control population. Thus, compared to the isotype control, LAD2 MC strongly expressed CD44 (**Fig. 3.13**).

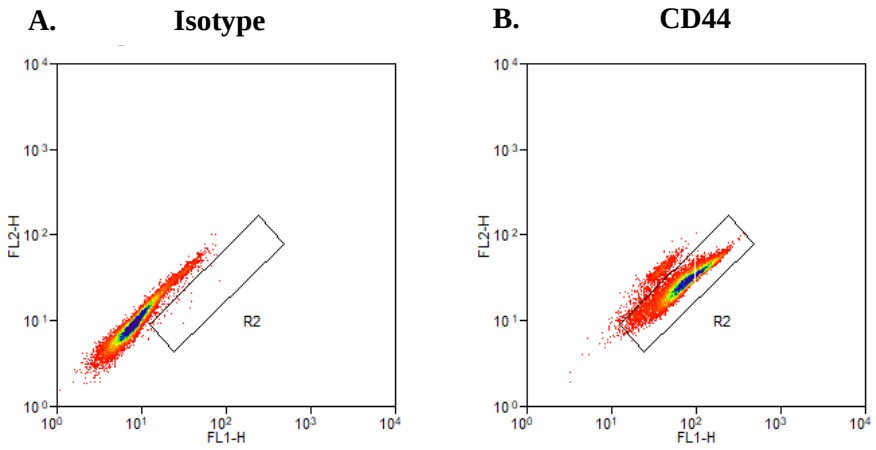


Figure 3.13. CD44 expression in MC. LAD2 MC were analyzed for ST2 expression using flow cytometry: (A) LAD2 MC treated with isotype control rat IgG_{2B} Alexa-Fluor® 488-conjugated Ab; (B) LAD2 MC treated with anti-human/mouse CD44 Alexa-Fluor® 488-conjugated Ab. Representative experiment, n=2.

3.5.6.1 Blocking CD44 does not affect CS-F uptake

To analyze the potential effect of blocking CD44 on uptake of CS-F, MC were incubated with or without anti-human HCAM (homing cell adhesion molecule, CD44)

monoclonal antibody at increasing concentrations (10-40 µg/mL) for 1 hr at 37°C. After washing twice with PBS, MC were then incubated with or without CS-F (10 µg/mL) for 1 hr at 37°C and washed twice again with PBS. Although the blocking of CD44 was not validated in this experiment, the anti-human HCAM Ab used did not detectably interfere with the uptake of CS, as judged by the lack of any shift of the cell population towards the control population, or to the left of the rectangle (R2) shown (**Fig. 3.14**). It was, therefore, concluded that ST2 is not involved in the uptake of CS-F by MC.

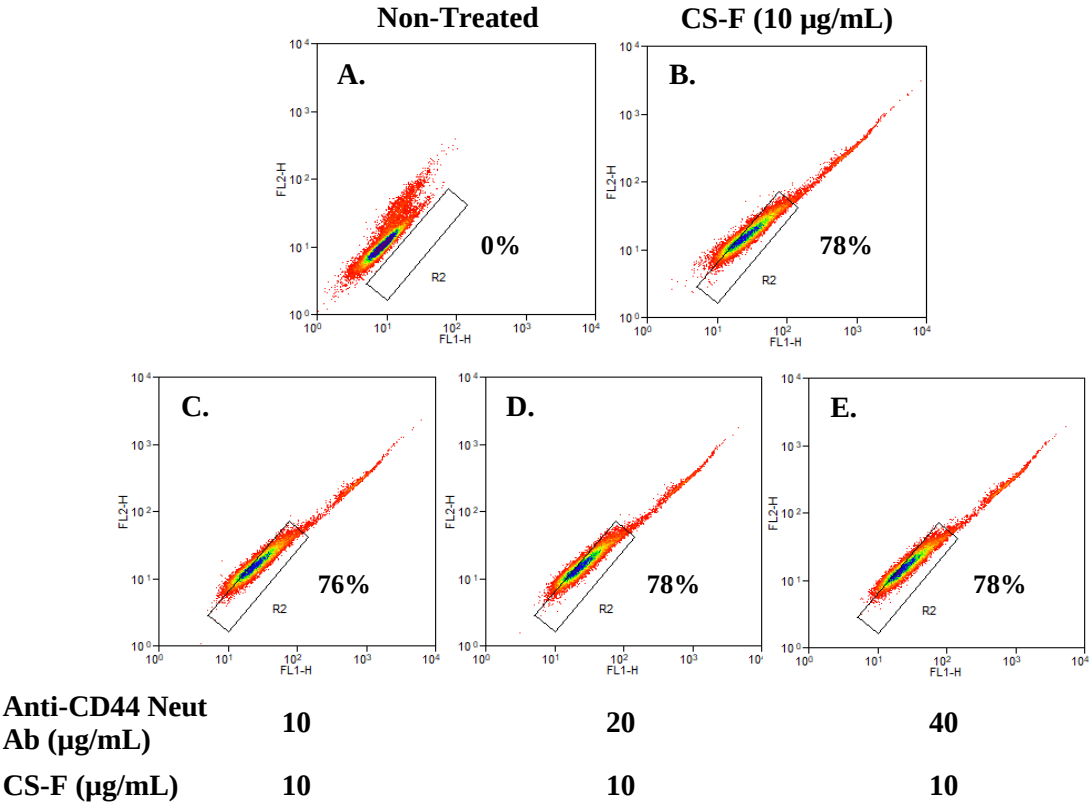


Figure 3.14. No effect of anti-CD44 antibody on uptake of CS-F by MC. LAD2 MC were pre-incubated with or without increasing concentrations of anti-CD44 antibody for 1 hr at 37ºC and washed twice before being incubated with CS-F (10 µg/mL) and analyzed via FACS: (A) non-treated control; (B) CS-F; (C) anti-CD44 (10 µg/mL) + CS-F; (D) anti-CD44 (20 µg/mL) + CS-F; (E) anti-CD44 (40 µg/mL) + CS-F. Representative experiment, n=2.

3.5.6.2 Hyaluronan does not affect CS-F uptake

The possible role of hyaluronan, the known ligand for CD44, on uptake of CS-F was then investigated. MC were pre-incubated with hyaluronan (500 µg/mL) for 1 hr at 37°C, washed twice with PBS and then incubated with or without CS-F (10 µg/mL) for 1 hr at 37°C and washed twice again with PBS. Although the blocking of CD44 was not validated in this experiment, hyaluronan did not detectably interfere with the uptake of CS, as judged by the FACS readout of the FL-1 vs. FL-2 fluorescence filters (**Fig. 3.15**). In other words, there was no shift of the cell population towards the control population, or to the left of the rectangle (R2) shown.

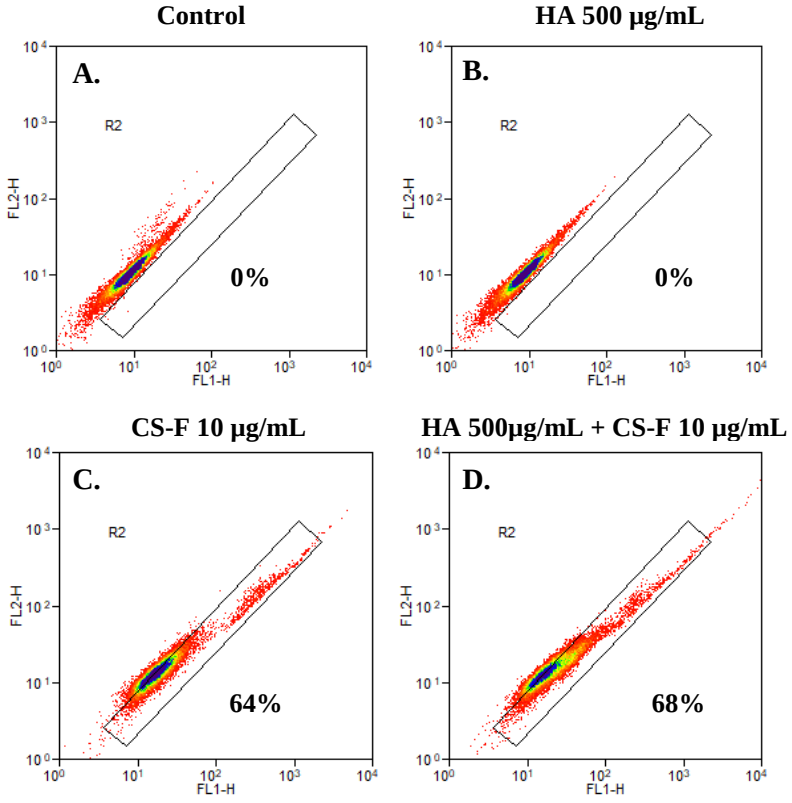


Figure 3.15. No effect of hyaluronan (HA) saturation on uptake of CS-F by MC. LAD2 MC were pre-incubated with or without HA for 1 hr at 37ºC and washed twice before being incubated with CS-F (10 µg/mL) and analyzed via flow cytometry: (A) non-treated control; (B) HA (500 µg/mL); (C) CS-F (10 µg/mL); (D) HA (500 µg/mL) + CS-F (10 µg/mL). Representative experiment, n=1.

3.6 Effect of CS on SNARE proteins

3.6.1 Effect of CS on SNAP-23 expression

SNAP-23 is a target membrane SNARE (t-SNARE) expressed in human MC and is involved in MC degranulation, as well as secretion of histamine and chemokines. It was decided to examine the expression and localization of SNAP-23 in an unstimulated state, after 30 min or 3 hr stimulation with IL-33. The effect of CS on the expression and localization of SNAP-23 was then explored using fluorescence microscopy.

3.6.1.1 Effect of SNAP-23 expression after 30 min stimulation

To determine the potential effect of CS on rapid secretory vesicle protein expression, possible changes in SNAP-23 expression or localization were examined in MC after 30 min stimulation with IL-33. MC were first incubated with or without CS for 1 hr at 37ºC, washed once and then stimulated with or without IL-33 (10 ng/mL) for 30 min. In the unstimulated control, no SNAP-23 fluorescent signal was detected (**Fig. 3.16A**). When MC were stimulated with IL-33 only, SNAP-23-associated fluorescence was apparent and appeared to be mainly localized around the MC surface (**Fig. 3.16B**). When MC were pre-incubated with CS (50 µg/mL) prior to IL-33 stimulation, the SNAP-23 fluorescent signal was still visible, but much less apparent than with IL-33 alone (**Fig. 3.16C**).

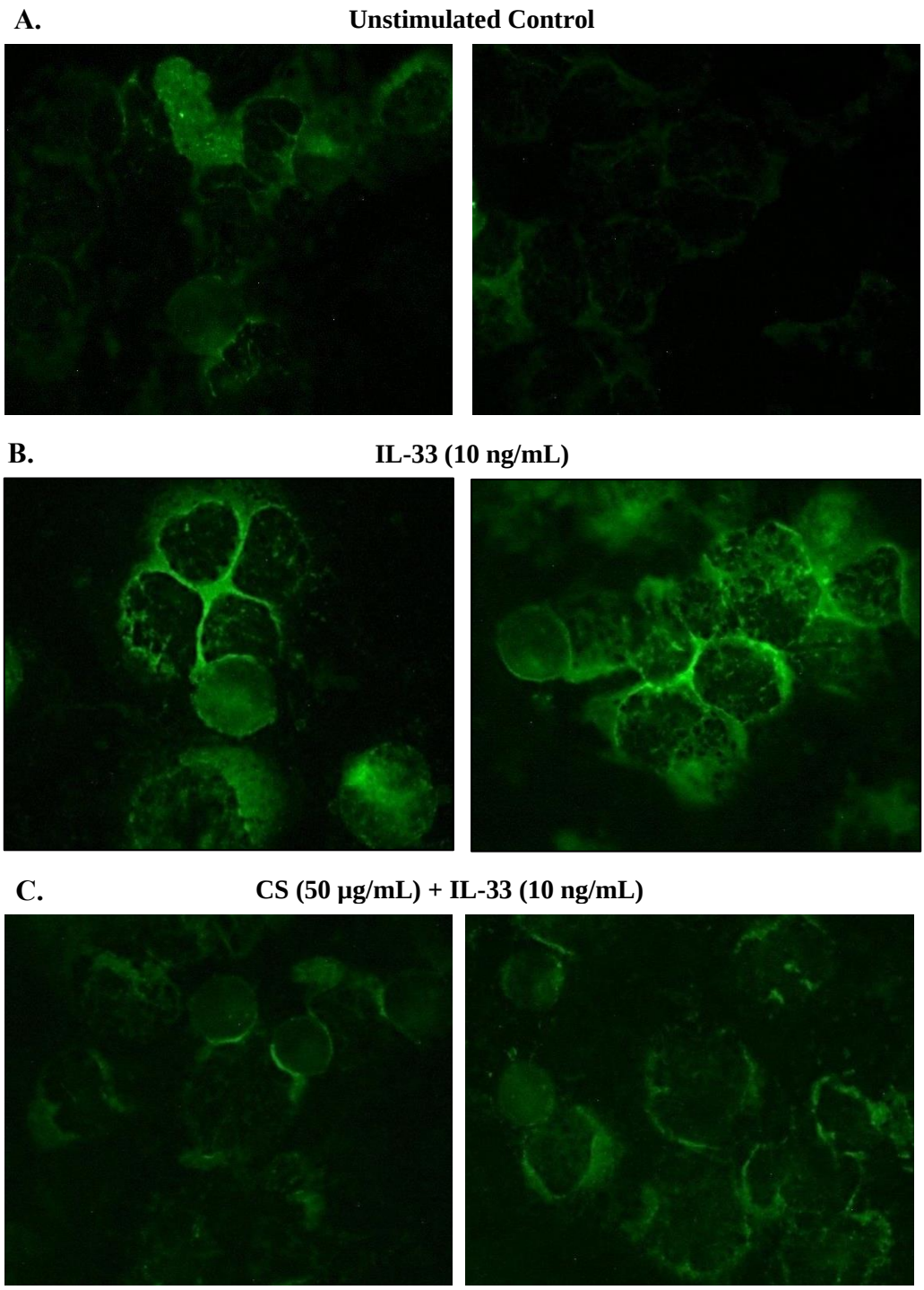


Figure 3.16. Effect of CS on expression of SNAP-23 in MC stimulated with IL-33 for 30 min. LAD2 MC were pre-incubated with or without CS (50 µg/mL) for 1 hr at 37ºC and washed twice before being stimulated with culture medium or IL-33 (10 ng/mL, 30 min). MC were then fixed and permeabilized with ice-cold methanol, washed and treated with anti-human SNAP-23 Ab: (A) unstimulated control; (B) IL-33 (10 ng/mL); (C) CS (50 µg/mL) + IL-33 (10 ng/mL). Photomicrographs are representative of two random populations per condition, n=1.

3.6.1.2 Effect of CS on SNAP-23 expression after 3 hr stimulation

To determine the potential effect of CS on more prolonged secretory granule or vesicle fusion, possible changes in SNAP-23 expression or localization were examined in MC after 3 hr stimulation with IL-33. MC were first incubated with or without CS (50 µg/mL) for 1 hr at 37ºC, washed once and then stimulated with or without IL-33 (10 ng/mL) for 3 hr. In the unstimulated control, SNAP-23 fluorescent signal was somewhat stronger than in the control after 30 min, with some visible punctate spots (**Fig. 3.17A**). It is unclear why the unstimulated control expressed SNAP-23 after 3 hr; it is possible that SNAP-23 is constitutively expressed in MC. When MC were stimulated with IL-33 only, SNAP-23-associated fluorescence was still localized to the cell surface, but was also evident as many punctate spots (**Fig. 3.17B**). When MC were pre-incubated with CS prior to IL-33 stimulation, SNAP-23 was still visible, but less apparent than IL-33 alone, with fewer punctate spots (**Fig. 3.17C**).

3.6.2 Effect of CS on VAMP-8 expression

VAMP-8 is vesicular SNARE (v-SNARE) expressed in human MC that forms a trans-SNARE complex with SNAP-23. Like SNAP-23, VAMP-8 is also involved in MC degranulation, as well as histamine and CXCL8 secretion. It was decided to examine the expression of VAMP-8 in an unstimulated state, as well as after 30 min or 3 hr of stimulation with IL-33. The effect of CS on the expression of VAMP-8 was then explored using fluorescence microscopy.

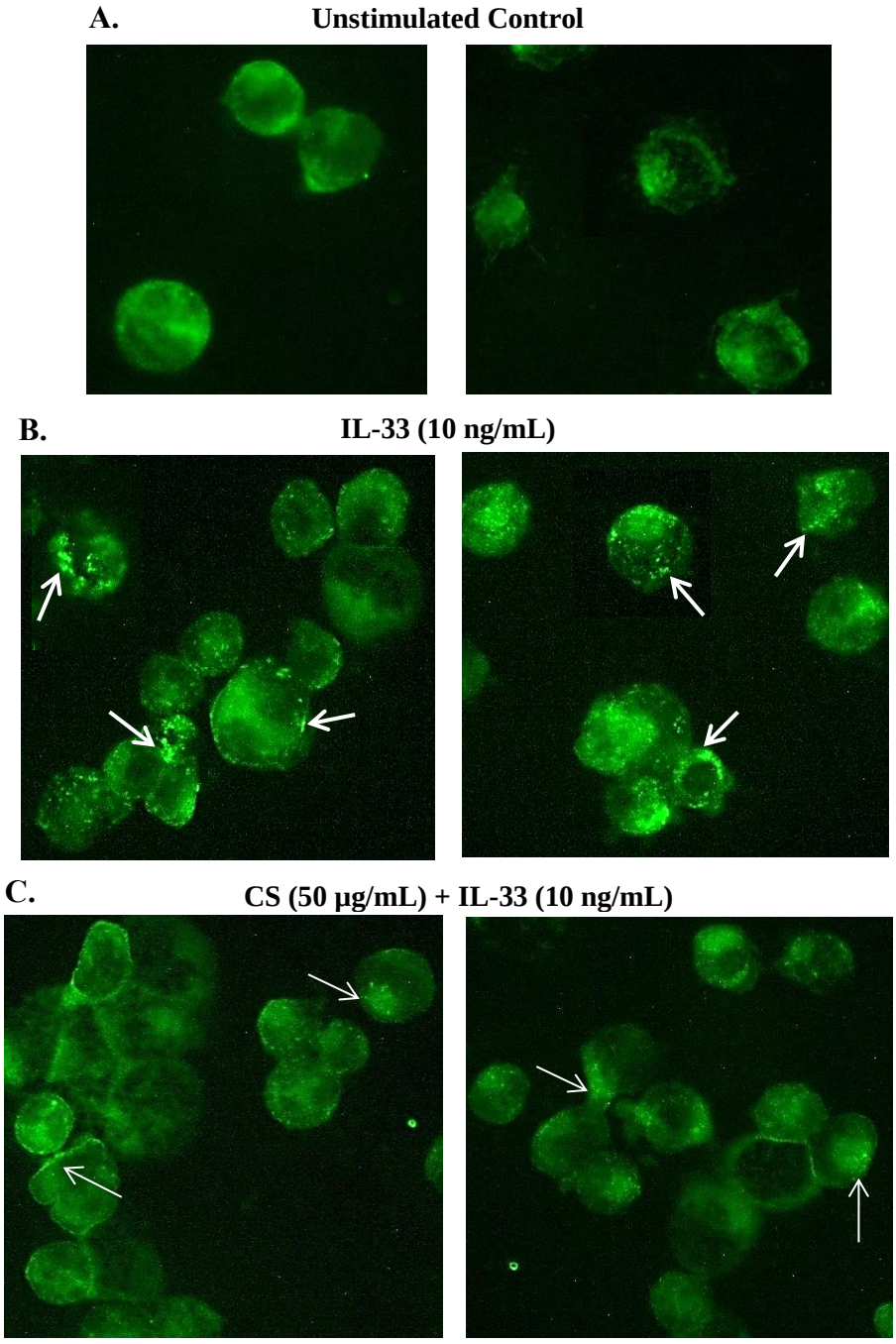


Figure 3.17. Effect of CS on expression of SNAP-23 in MC stimulated with IL-33 for 3 hr. LAD2 MC were pre-incubated with or without CS (50 µg/mL) for 1 hr at 37ºC and washed twice before being stimulated with culture medium or IL-33 (10 ng/mL, 3 hr). MC were then fixed and permeabilized with ice-cold methanol, washed and treated with anti-human SNAP-23 Ab: (A) unstimulated control; (B) IL-33 (10 ng/mL); (C) CS (50 µg/mL) + IL-33 (10 ng/mL). Photomicrographs are representative of two random populations per condition, n=1.

3.6.2.1 Effect of CS on VAMP-8 expression after 30 min stimulation

To determine the potential effect of CS on rapid secretory vesicle fusion, possible changes in VAMP-8 expression or localization were examined in MC after 30 min stimulation with IL-33. MC were ncubated with or without CS (50 µg/mL) for 1 hr at 37ºC, washed and then stimulated with or without IL-33 (10 ng/mL) for 30 min. In the unstimulated control, weak VAMP-8 fluorescent signal was detected (**Fig. 3.18A**). When MC were stimulated with IL-33, VAMP-8-associated fluorescence was apparent and localized around the cell surface (**Fig. 3.18B**). When MC were pre-incubated with CS prior to IL-33 stimulation, VAMP-8-associated fluorescence pattern and intensity appeared to be equivalent to that seen in IL-33-stimulated MC alone (**Fig. 3.18C**).

3.6.2.2 Effect of CS on VAMP-8 after 3 hr stimulation

To determine the potential effect of CS on more prolonged secretory granule or vesicle fusion, possible changes in VAMP-8 expression or localization were examined in MC after 3 hr stimulation with IL-33. MC were incubated with or without CS (50 µg/mL) for 1 hr at 37ºC, washed and then stimulated with or without IL-33 (10 ng/mL) for 3 hr. In the unstimulated control, VAMP-8 fluorescent signal was slightly stronger after 3 hr than after 30 min, with some "half moon" appearance (**Fig. 3.19A**). It is unclear why the unstimulated control expressed VAMP-8; it is possible that VAMP-8 is constitutively expressed in MC. When MC were stimulated with IL-33 only, VAMP-8-associated fluorescence pattern and intensity appeared to be similar to that seen in the unstimulated control (**Fig. 3.19B**). When MC were pre-incubated with CS prior to IL-33 stimulation, VAMP-8-associated fluorescence pattern and intensity appeared to be equivalent to that seen in control and IL-33-stimulated MC alone (**Fig. 3.19C**).

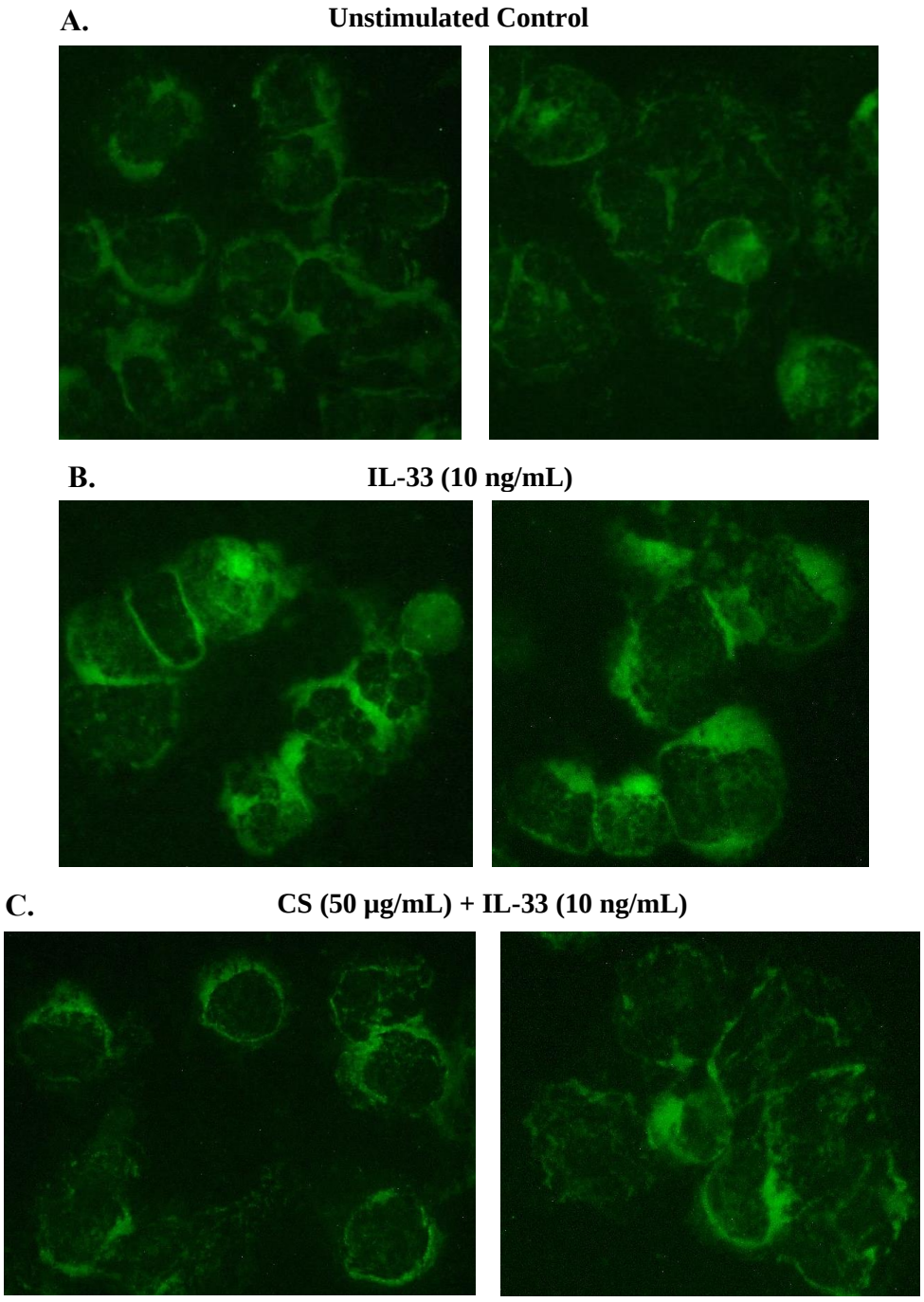


Figure 3.18. Effect of CS on expression of VAMP-8 in MC stimulated with IL-33 for 30 min. LAD2 MC were pre-incubated with or without CS (50 µg/mL) for 1 hr at 37ºC and washed twice before being stimulated with culture medium or IL-33 (10 ng/mL, 30 min). MC were then fixed and permeabilized with ice-cold methanol, washed and treated with anti-human VAMP-8 Ab: (A) unstimulated control; (B) IL-33 (10 ng/mL); (C) CS (50 µg/mL) + IL-33 (10 ng/mL). Photomicrographs are representative of two random populations per condition, n=1.

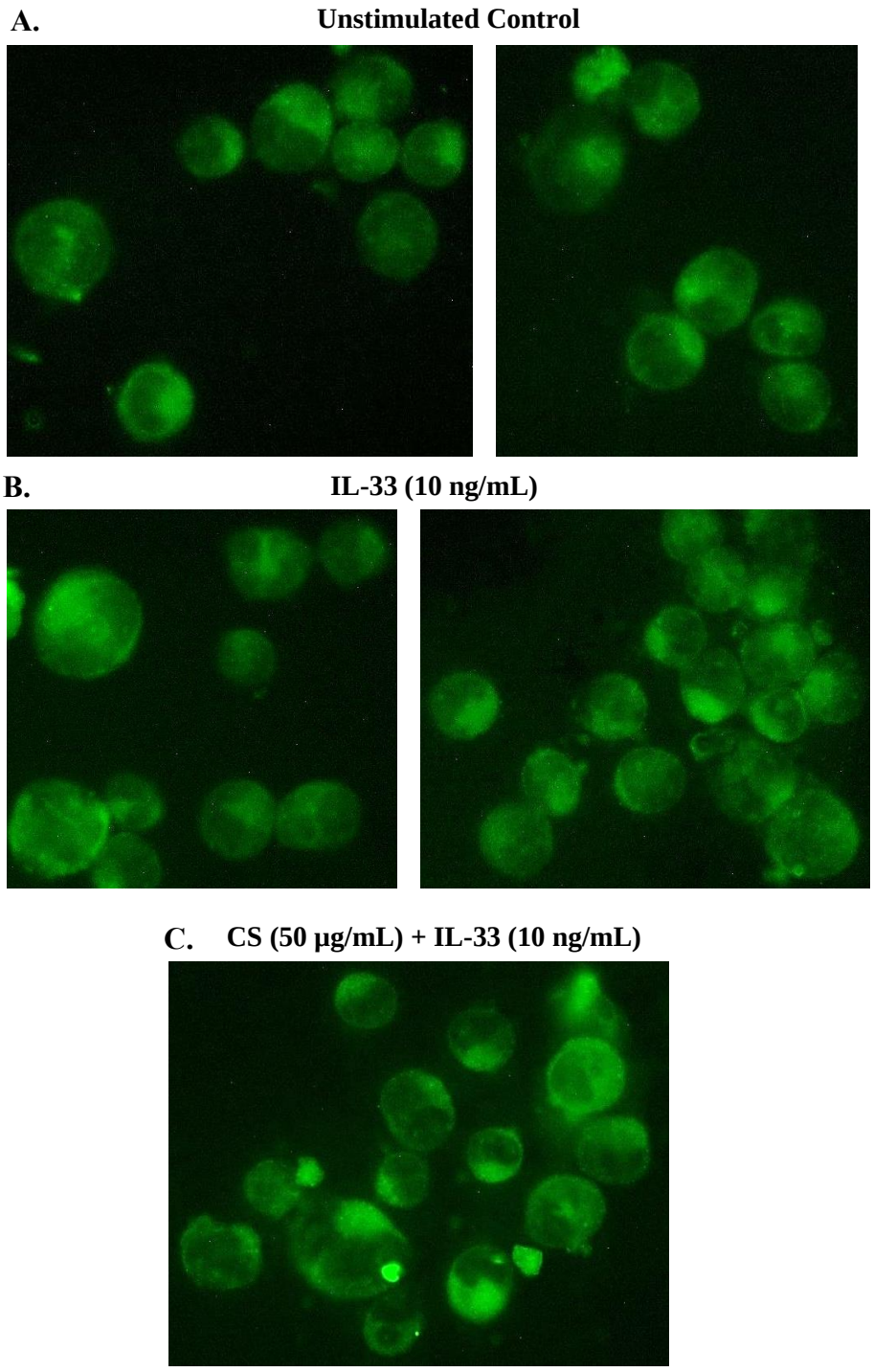


Figure 3.19. Effect of CS on expression of VAMP-8 in MC stimulated with IL-33 for 3 hr. LAD2 MC were pre-incubated with or without CS (50 µg/mL) for 1 hr at 37ºC and washed twice before being stimulated with culture medium or IL-33 (10 ng/mL, 3 hr). MC were then fixed and permeabilized with ice-cold methanol, washed and treated with anti-human VAMP-8 Ab: (A) unstimulated control; (B) IL-33 (10 ng/mL); (C) CS (50 µg/mL) + IL-33 (10 ng/mL). Photomicrographs are representative of two random populations per condition, n=1.

3.7 Discussion

While the anti-inflammatory activity of CS on other cell types, particularly chondrocytes, has already been established[144], its effect on MC cytokine and chemokine release was previously unknown. The findings in this report show, for the first time, that CS can inhibit TNF and CXCL8 secretion from human cultured LAD2 MC stimulated by both SP and IL-33. The CS concentrations used for these studies were selected because CS had previously been used in this range in studies of mediator secretion from rabbit chondrocytes[140] and human keratinocytes[141]. These concentrations may be physiologically relevant, as sulfated GAG concentrations in human synovial fluid range from 80 to 330 μg/ml[193].

Interestingly, it required higher CS concentrations to significantly reduce the secretion of TNF and CXCL8 from MC stimulated with SP, compared to with IL-33. This was particularly true concerning the secretion of TNF in SP-stimulated MC; however, this disparity in significance appears to be partially attributed to the higher variance of TNF secretion. It is important to note that CS did not simply better inhibit secretion of the lowest-secreted mediator at baseline. SP stimulated a mean baseline TNF secretion of almost 4 times that of CXCL8, while IL-33 stimulated a mean baseline CXCL8 secretion of about 2.5 times that of TNF. While CS inhibits the secretion of TNF and CXCL8 relatively equally, percent-wise, in SP-stimulated MC, CS is much more effective at inhibiting TNF than CXCL8 in IL-33-stimulated MC. However, it is possible that CS is unable to affect any mediator secreted at such high concentrations, over 2000 pg/10^6 MC/mL of CXCL8 in IL-33-stimulated MC.

The precise mechanism of the inhibitory action of CS on TNF and CXCL8 secretion from human MC stimulated by SP or IL-33 is presently unknown. Our data show that CS is taken up by MC in a dose-dependent and temperature-dependent manner, with the hyaluronic acid receptor, CD44, appearing to be uninvolved in this process. While altering the temperature conditions is a sufficient way to begin studying CS uptake, to truly determine if the cause of the inhibitory effect observed with CS on mediator secretion is due to its internalization, and not simply MC membrane association, it would be necessary to test endocytic inhibitors, such as dynasore or filipin.

Although CS did not detectably inhibit β-hexosaminidase secretion from secretory granules (1,000 nm), chemokines and cytokines are apparently secreted from small secretory vesicles (50-100 nm)[41], as our laboratory had shown for secretion of IL-6[44]. CS appears to interfere with the fluorescence associated with the MC soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor (SNARE), SNAP-23, but not VAMP-8. A possibility for the inhibition of this decreased t-SNARE (SNAP-23)-associated fluorescence, but not this v-SNARE (VAMP-8) is that perhaps IL-33 does not utilize VAMP-8 while activating MC. VAMP-8 may be constitutively expressed in "stressed" MC, especially because after 3 hr incubation in a tube, the unstimulated control MC exhibited seemingly equal VAMP-8-associated fluorescence as those stimulated with IL-33. Recent studies have begun to elucidate the involvement of SNAREs in MC mediator secretion[27]. However, to the best of our knowledge, the involvement of SNAREs in non-IgE receptor-mediated MC mediator secretion has not been studied. The only relevant report studied the co-localization, but not impact on secretion, of TNF with VAMP-3 and VAMP-8 in MC stimulated with IL-1β[186].

CHAPTER 4 : COMPARISON OF THE EFFECT OF CS WITH THAT OF DERMATAN SULFATE AND HEPARIN IN MC

4.1 DS and Hep inhibit TNF and CXCL8 secretion from MC stimulated by IL-33

Just as the appropriate concentrations and pre-incubation times were determined for CS, it was decided to do the same for the structural analogs of CS, the GAGs dermatan sulfate (DS) and heparin (Hep).

4.1.1 Selection of optimal dose of DS and Hep

When MC were pre-incubated with various concentrations of DS for 1 hr, washed twice and then stimulated with IL-33 (10 ng/mL), the secretion of TNF (**Fig. 4.1A**) and CXCL8 (**Fig. 4.1B**) were inhibited to different degrees. Unlike CS, which required higher concentrations (75-150 µg/mL; **Fig. 3.7**) to significantly inhibit the secretion of both TNF and CXCL8 from MC, DS significantly ($p<0.01$) inhibited the secretion, up to about 70%, of TNF from IL-33-stimulated MC at much lower concentrations (e.g. 37.5 µg/mL) (**Fig 4.1A**). There was no significant difference in TNF inhibition between the highest concentration (150 µg/mL), and the lowest concentration (18.75 µg/mL) (**Fig. 4.1A**). DS was more effective than CS in inhibiting CXCL8 secretion, with only 37.5 µg/mL necessary to exert a significant inhibitory effect (54%, $p<0.05$) (**Fig. 4.1B**).

Since DS inhibited the secretion of both TNF and CXCL8 from MC even at 18.75 µg/mL, it was decided to investigate the effects of even lower doses of DS, and to compare these effects to Hep at the same concentrations. MC were pre-incubated with various concentrations of either DS or Hep for 1 hr, washed twice and then stimulated with IL-33 (10 ng/mL, 24 hrs).

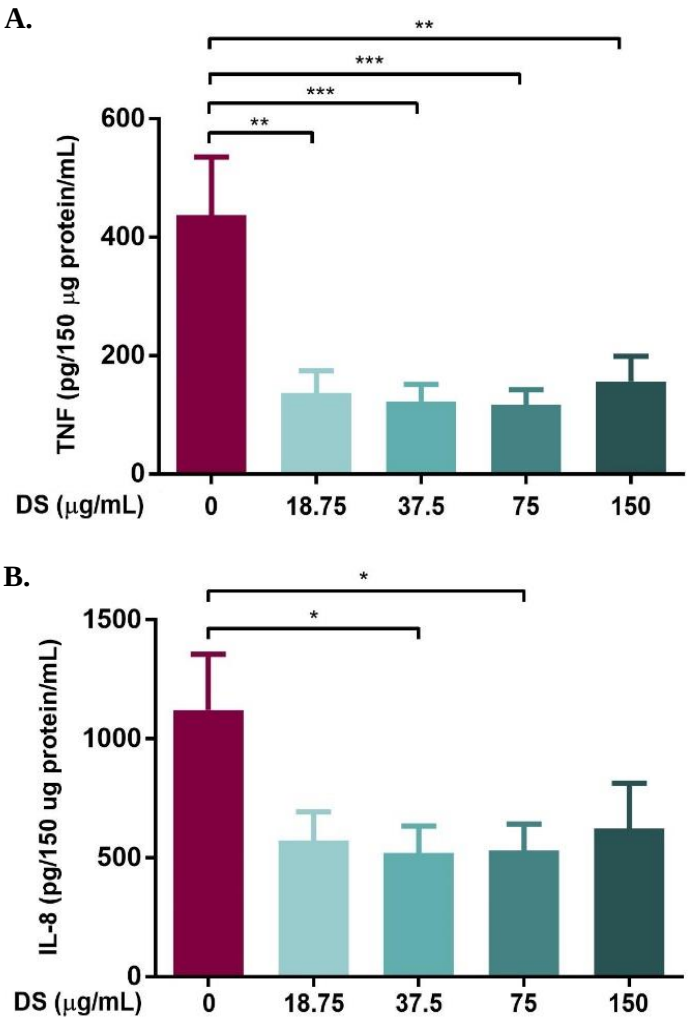


Figure 4.1. Effect of DS on secretion of TNF and CXCL8 from MC stimulated with IL-33. LAD2 MC were pre-treated with increasing concentrations of UF DS for 1 hr at 37°C prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=4; *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Optimal doses for each GAG were decided after initial testing of pre-incubation with 10, 50 and 100 µg/mL of either Hep or DS (data not shown). For DS, maximal inhibition of both TNF and CXCL8 secretion was observed at 10 µg/mL (data not shown). For Hep, there was no apparent difference in inhibition of either TNF or CXCL8 secretion between 50 and 100 µg/mL (data not shown).

Pre-incubation with DS at lower concentrations (2.5, 5 and 10 µg/mL) inhibited secretion of both TNF (46%, $p<0.05$) and CXCL8 (32%, $p<0.05$), with maximal at 2.5 µg/mL, but no apparent difference between 5 and 10 µg/mL (**Fig. 4.2**). Hep only significantly inhibited TNF and CXCL8 secretion from MC at 40 µg/mL, by approximately 40% ($p<0.05$) and 31% ($p<0.01$), respectively (**Fig. 4.3**).

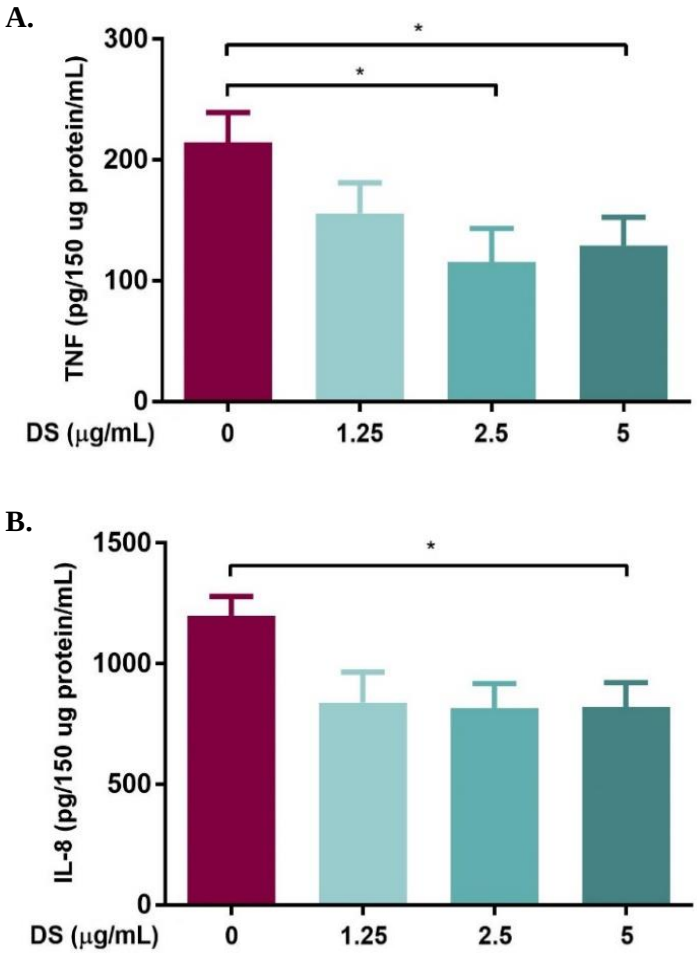


Figure 4.2. Effect of low doses of DS on secretion of TNF and CXCL8 from MC stimulated by IL-33. LAD2 MC were pre-treated with increasing concentrations of DS for 1 hr at 37°C prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=4; *=$p<0.05$.

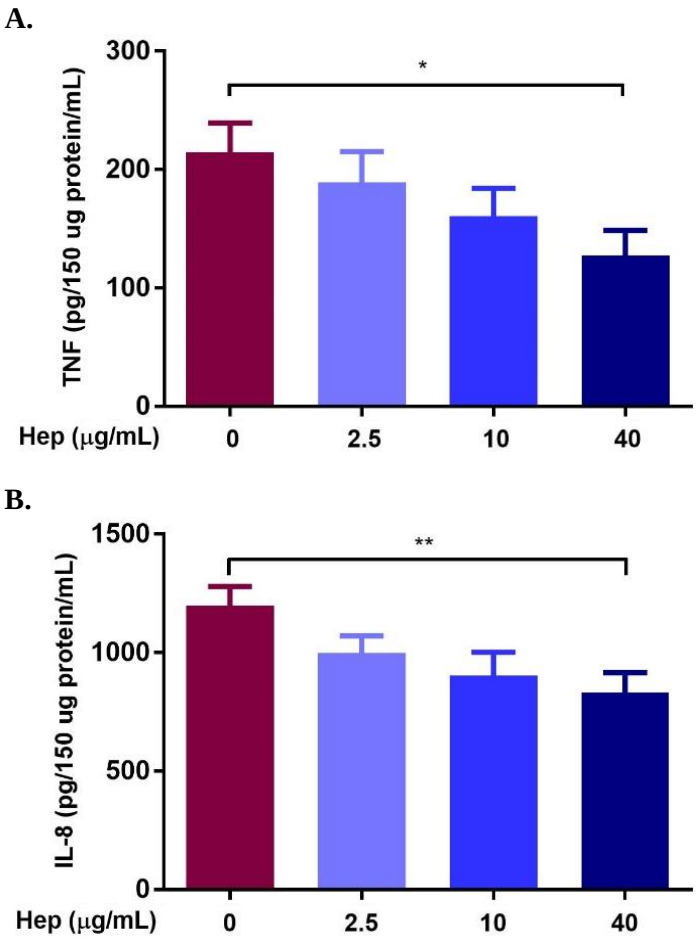


Figure 4.3. Effect of Hep on secretion of TNF and CXCL8 from MC stimulated with IL-33. LAD2 MC were pre-treated with increasing concentrations of Hep for 1 hr at 37°C prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=4; *=$p<0.05$, **=$p<0.01$.

4.1.2 Comparison of effect of UF and LMW DS and Hep on TNF and CXCL8 secretion

To compare CS [unfractionated (UF), ~40 kDa] with other GAGs, MC were pre-incubated with either 2.5 or 50 μg/mL of either UF or low molecular weight (LMW, ~5 kDa) DS or Hep for 1 hr, washed twice, and then stimulated with IL-33 (10 ng/mL) for

24 hr. At 50 µg/mL, LMW DS significantly inhibited the secretion of both TNF ($p<0.001$) and CXCL8 ($p<0.0001$) by about 50%, while UF DS significantly inhibited TNF and CXCL8 ($p<0.0001$) by about 70% (**Fig. 4.4**). At both low and high concentrations, LMW Hep significantly inhibited the secretion of both TNF ($p<0.001$) and CXCL8 ($p<0.0001$) by about 60% (**Fig. 4.5**), while UF DS significantly inhibited TNF ($p<0.05$) and CXCL8 ($p<0.0001$) by about 40% (**Fig. 4.4**).

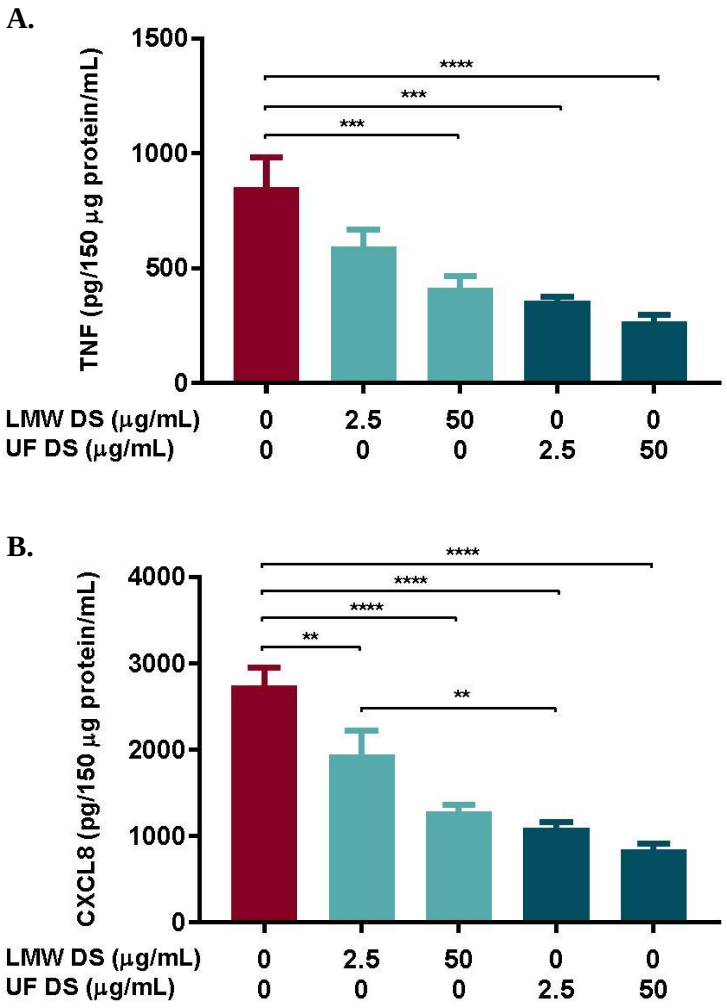


Figure 4.4. Comparison of the effect of UF vs. LMW DS on secretion of TNF and CXCL8 from MC stimulated by IL-33. LAD2 MC were pre-treated with either culture medium or a low (2.5 µg/mL) or high (50 µg/mL) concentration of either UF or LMW DS for 1 hr at 37°C prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=4; **=p<0.01, ***=p<0.001, ****=p<0.0001.

There were no significant differences in inhibition of TNF secretion between both LMW and UF DS (**Fig. 4.4A**) or LMW and UF Hep (**Fig. 4.5A**). However, at low concentrations, there were significant differences in inhibition of CXCL8 secretion between both sizes of DS and Hep, with UF DS being more potent than LMW DS ($p<0.01$; **Fig. 4.4B**), and the reverse being true for Hep ($p<0.01$; **Fig. 4.5B**).

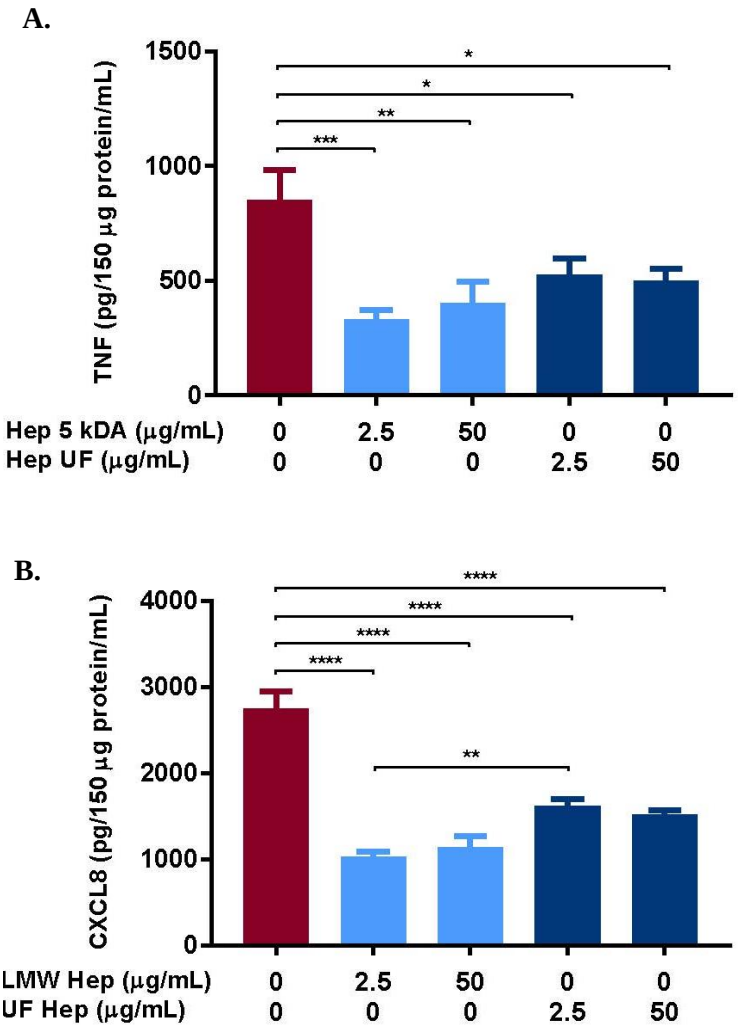


Figure 4.5. Comparison of the effect of UF vs. LMW Hep on secretion of TNF and CXCL8 from MC stimulated by IL-33. LAD2 MC were pre-treated with either culture medium or a low (2.5 µg/mL) or high (50 µg/mL) concentration of either UF or LMW Hep for 1 hr at 37°C prior to two washes and IL-33 (10 ng/mL) stimulation for 24 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in triplicate, n=4; **=p<0.01, ***=p<0.001, ****=p<0.0001.

4.2 GAGs do not inhibit β-hexosaminidase secretion from MC stimulated by ionomycin

Since IL-33 alone does not lead to LAD2 MC degranulation (results not shown), the ionophore ionomycin was used to stimulate secretion of β-hexosaminidase, a secretory granule used an index of degranulation. Ionomycin (5 μM) alone stimulated about 32% secretion of β-hexosaminidase. Pre-incubation with either CS, DS or Hep (50 μg/mL) for 1 hr, two washes and stimulation with ionomycin (5 μM) for 1 hr did not detectably inhibit β-hexosaminidase secretion from LAD2 MC (**Fig. 4.6**).

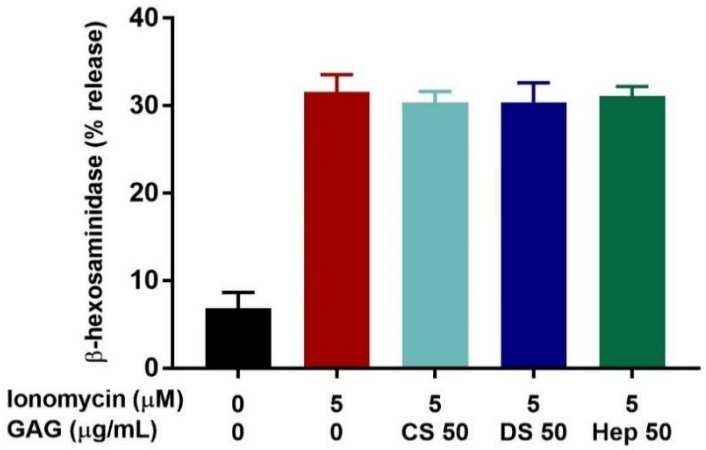


Figure 4.6. Lack of effect of GAGs on secretion of β-hexosaminidase from MC stimulated by ionomycin. LAD2 MC were pre-incubated with or without the GAGs indicated (50 μg/mL) for 1 hr, washed, and then incubated with or without the ionophore ionomycin (5 μM) for 1 hr. Secretion of β-hexosaminidase was measured as described in Methods. Each condition was performed in duplicate, n=2.

4.3 GAGs do not affect gene expression of TNF or CXCL8

Stimulation with IL-33 was determined to elicit maximal gene expression of either TNF or CXCL8 at 3 hr (results not shown). MC were pre-incubated with DS, Hep or CS (50 μg/mL) for 1 hr, washed twice, and then stimulated with IL-33 (10 ng/mL) for either

1.5 hr (**Fig. 4.7A, B**) or 3 hr (**Fig. 4.7C, D)**. Gene expression of neither TNF nor CXCL8 was significantly inhibited compared to IL-33 alone (**Fig. 4.7**). The effect of DS, Hep or CS pre-incubation prior to 6 hr of IL-33 stimulation was also studied. Again, there was no inhibition of either TNF or CXCL8 gene expression at this longer time point (results not shown).

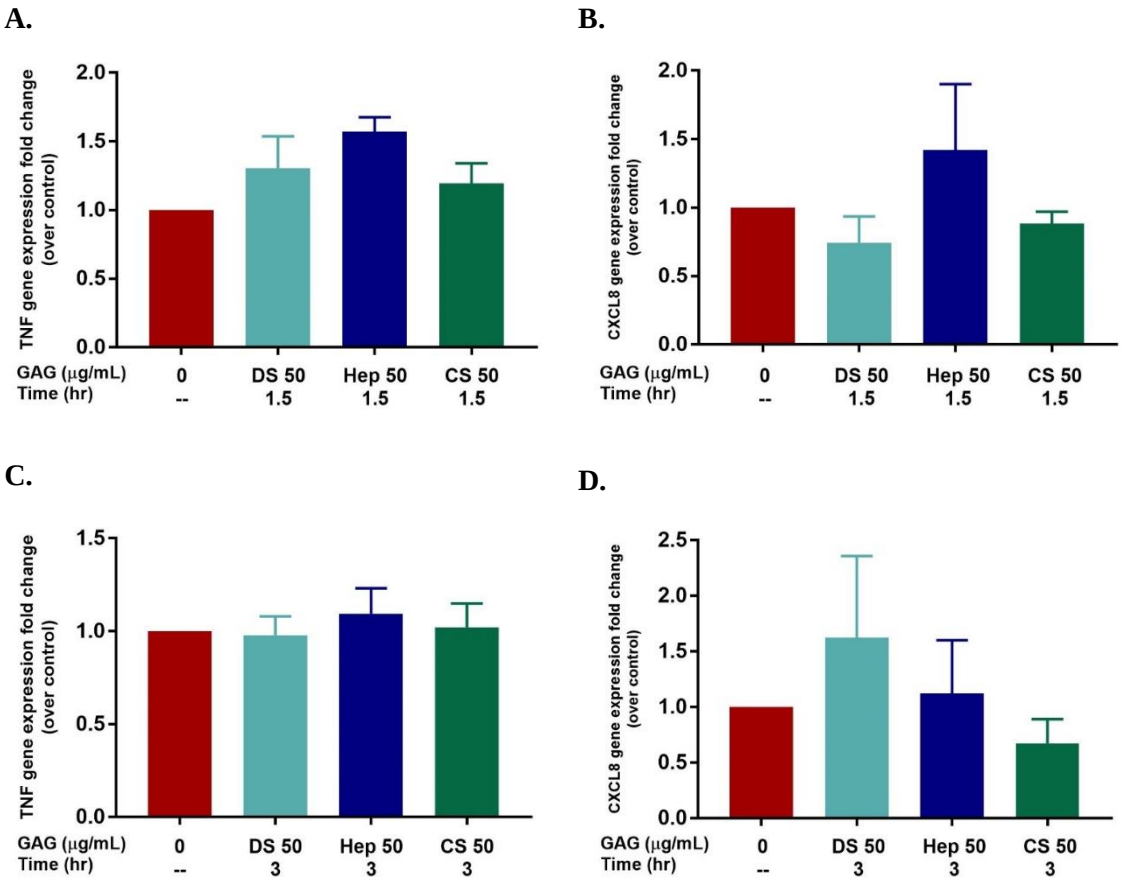


Figure 4.7. No effect of GAGs on gene expression of TNF and CXCL8 from MC stimulated by IL-33. LAD2 MC were pre-treated with or without the specified GAG (50 µg/mL) for 1 hr prior to two washes and IL-33 (10 ng/mL) stimulation for (A, B) 1.5 or (C, D) 3 hr. Relative mRNA expression of (A, C) TNF and (B, D) CXCL8 was determined with the $2^{\Delta\Delta Ct}$ method, using GAPDH as a reference gene. Results are expressed as expression fold change ($2^{\Delta\Delta Ct}$) over IL-33 stimulation without GAGs. Each condition was performed in duplicate, n=3.

4.4 GAGs do not affect ionomycin-induced intracellular calcium ion influx

To investigate whether GAGs interfere with intracellular calcium ion levels, MC were first pre-incubated with or without either DS, CS or Hep (50 µg/mL) for 1 hr and were then loaded with Fura-2 AM (3 µg/mL) for 45 min, with 20 more min to allow for dye de-esterification. Intracellular calcium ion levels were measured pre- and post-stimulation with the calcium ionophore ionomycin (5 µg/mL) or IL-33 (50 ng/mL) for 20 min. IL-33 did not detectably affect intracellular calcium levels, while ionomycin increased the influx of intracellular calcium (**Fig. 4.8**). Neither DS, CS or Hep affected the increase in intracellular calcium levels induced by ionomycin over 20 min (**Fig. 4.8**). The apparent small increase in intracellular calcium levels in the control condition is thought to be due to non-specific photomechanical changes that occur during addition of the trigger.

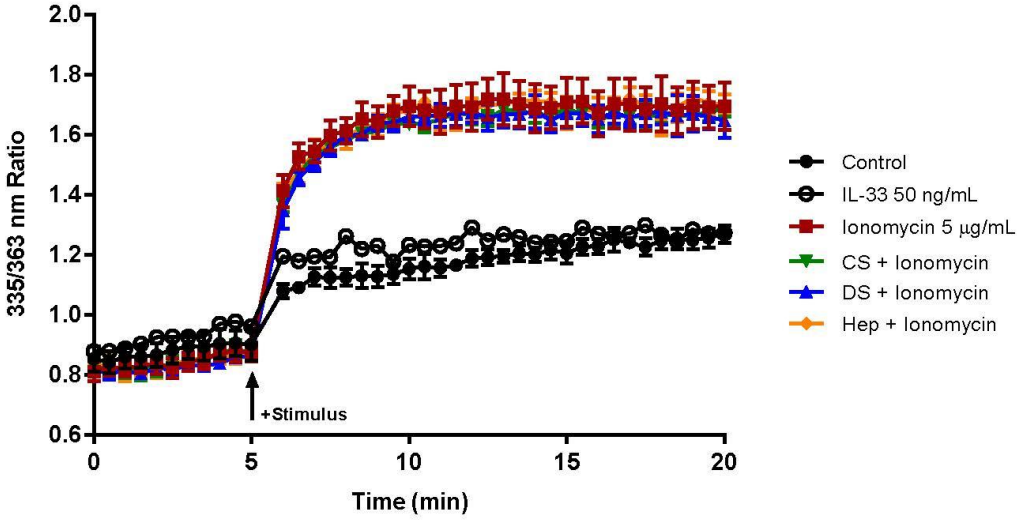


Figure 4.8. No effect of CS, Hep and DS on intracellular calcium ion levels in stimulated MC. LAD2 MC were pre-incubated with or without the aforementioned GAGs for 1 hr at 37°C before being loaded with Fura-2 AM. They were then stimulated by either ionomycin (5 µg/mL) or IL-33 (50 ng/mL). Each condition was performed in duplicate, n=3.

4.5 GAGs do not affect ST2 expression

To further examine the mechanism of action of the inhibitory effect of GAGs on MC stimulated with IL-33, the effect of DS, CS and Hep was studied on the expression of the IL-33 receptor, ST2. MC were pre-incubated with the indicated GAGs (50 µg/mL) for 1 hr and washed twice with PBS. MC were then incubated with anti-human ST2/IL-33R phycoerythrin (PE)-conjugated Ab or its isotype control, goat IgG PE-conjugated Ab. Nonspecific binding was blocked with 0.5% BSA plus 5% goat serum. None of the GAGs had a significant effect on ST2 expression (**Fig. 4.9**).

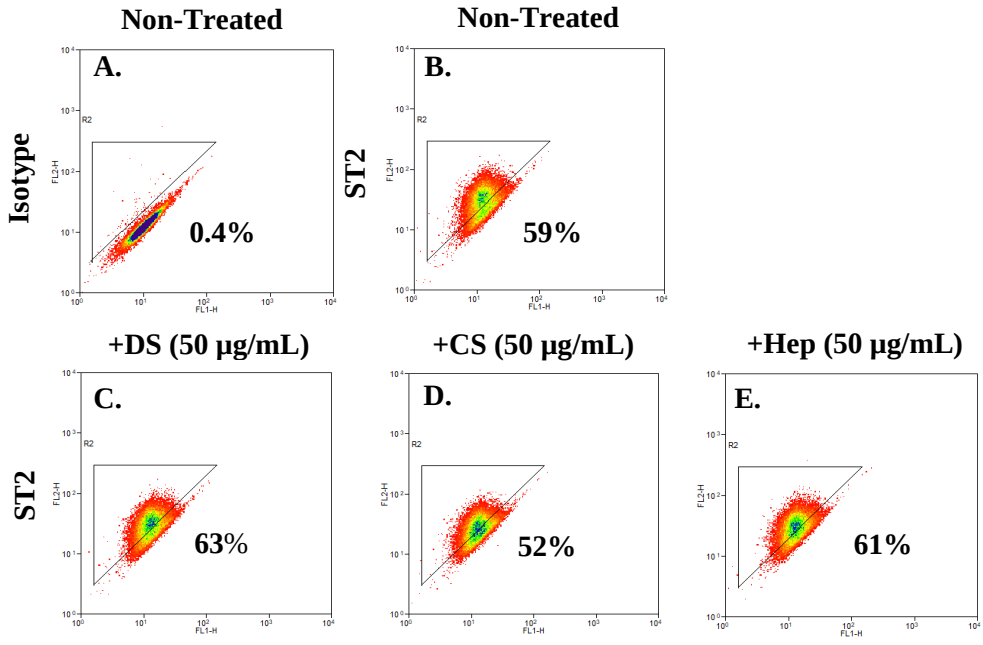


Figure 4.9. No effect of GAGs on expression of ST2 in MC. LAD2 MC were pre-incubated with or without the indicated GAG (50 µg/mL) for 1 hr at 37ºC and washed twice before being analyzed for ST2 expression via FACS: (A) LAD2 MC treated with goat IgG PE-conjugated Ab; (B) LAD2 MC treated with anti-human ST2 PE-conjugated Ab; LAD2 MC pre-treated with (C) DS (50 µg/mL), (D) CS (50 µg/mL) or (E) Hep (50 µg/mL) and then treated with anti-human ST2 PE-conjugated Ab. Representative experiment, n=2.

4.6 GAGs do not inhibit IgE/anti-IgE-mediated TNF & CXCL8 secretion from hCBMC

The effects of GAGs on stimulated primary human cord blood-derived MC (hCBMC) were investigated in order to compare the inhibitory effect of GAGs observed in the immortalized LAD2 MC line. However, IgE/anti-IgE (allergic stimulation) were used to trigger hCBMC because the secretion of TNF in response to IL-33 alone was insufficient to detect (data not shown). hCBMC were first treated with IgE 2 µg/mL overnight, then washed once, pre-incubated with CS, DS or Hep for 30 min, washed again, then stimulated with anti-IgE 15 µg/mL for 6 hr. GAGs do not inhibit IgE/anti-IgE-mediated secretion of either TNF or CXCL8 from hCBMC (**Fig. 4.10**).

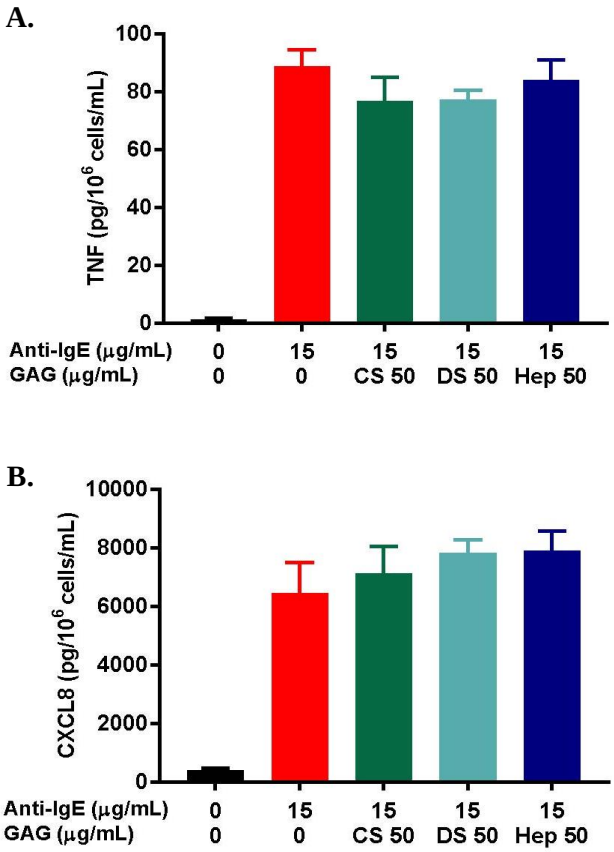


Figure 4.10. No effect of GAGs on TNF and CXCL8 secretion from hCBMC stimulated with IgE/anti-IgE. hCBMC were treated with IgE (2 µg/mL) overnight before being washed and pre-incubated with or without the indicated GAG (50 µg/mL) for 30 min at 37ºC, washed twice, then stimulated with anti-IgE (15 µg/mL) for 6 hr. Secretion of (A) TNF and (B) CXCL8 was measured via ELISA. Each condition was performed in duplicate, n=2.

4.7 Discussion

The findings in this report show, for the first time, that DS and Hep, both UF and LMW, can inhibit TNF and CXCL8 secretion from human cultured LAD2 MC stimulated by IL-33. The inhibitory differences of UF and LMW Hep on cytokine secretion, due to variations in sulfation, disaccharide length and MW, have been studied in MC and other cell types. In murine MC, it appears that a LMW Hep, enoxaparin, is more effective than UF Hep at inhibiting TNF secretion[131]. UF Hep required 100 times higher molarity concentration than enoxaparin; although, molarity is not an ideal method to characterize UF GAG concentrations, due to them being a heterogeneous mixture of varying MW GAG chains[194]. In terms of sulfation, in LPS-stimulated normal human bronchial epithelial cells, fully sulfated Hep significantly suppressed ERK signaling, as well as COX-2 and CXCL8 gene expression, while desulfated Hep had no effects[195].

This report shows that GAGs do not affect ionomycin-induced β-hexosaminidase secretion or intracellular calcium ion influx. It has been reported that binding of 1,4,5-inositoltriphosphate (IP_3) to the ER, leading to the subsequent internal release of calcium, is necessary for degranulation[196, 197], but not for TNF production by MC[198]. There is also a report that UF Hep can inhibit human uterine MC or rat peritoneal MC degranulation induced by anti-IgE, but not by calcium ionophore[132]. However, LAD2 MC do not respond to triggering with IgE/anti-IgE (results not shown).

Since CS, DS nor Hep also did not detectably affect gene expression of IL-33-stimulated MC mediators or ST2 receptor expression, the mechanism of this inhibitory effect is still unknown. CS has been shown to inhibit NF-κB, a key transcription factor

involved in pro-inflammatory gene expression, nuclear activation or translocation in human chondrocytes[140], human THP-1 macrophages[199], normal human keratinocytes[141], mouse melanoma cells[200], human endometrial stromal cells[201] and in mouse skin[202]. Heparin has also found to inhibit NF-κB activation in both T-cells[203] and cerebral endothelial cells[204], as well as NF-κB translocation in human pulmonary microvascular endothelial cells[205]. In MC, IL-33 is known to activate multiple signaling kinases, including NF-κB and MAP kinases, such as Erk1/2, p38 and JNK[67, 206], leading to subsequent mediator gene expression and secretion[66]. However, since CS inhibition of TNF or CXCL8 gene expression in LAD2 MC was not detected, it was chosen not to further pursue the role of these kinases. These findings are in contrast to other studies which found that Hep can affect the gene expression of mediators in different cell types. For example, Hep can inhibit the gene expression of the cytokines, IL-6 and IL-1β, and the cellular adhesion molecules, ICAM-1 and E-selectin, in human endothelial cells stimulated with LPS[205]. Hep also decreased the gene expression of IL-1β, IL-6 and TNF in human mononuclear cells stimulated with LPS, but not IFN-γ[207].

In addition, it was found that, unlike in LAD2 MC, GAGs did not detectably affect TNF or CXCL8 secretion in primary hCBMC stimulated with IgE/anti-IgE. While it would have been preferred to use the same non-allergic trigger as was used on LAD2 MC, IL-33 did not detectably stimulate sufficient TNF release from hCBMC. Some drawbacks of these hCBMC is that they are difficult to obtain and proliferate, and their secretory granules also do not appear to be as mature as adult human MC[92].

CHAPTER 5 : DISCUSSION

5.1 Discussion

The present findings indicate that CS, as well as DS and Hep, both in UF and LMW forms, can inhibit the release of TNF and CXCL8 from human MC stimulated by IL-33. This effect appears to occur intracellularly. In addition, it was found that GAGs do not exert their inhibitory action by either affecting gene expression of TNF or CXCL8, or inhibiting calcium ion influx, or blocking the surface expression of the IL-33 receptor, ST2. Moreover, the hyaluronan receptor CD44 is not involved in the association or uptake of CS-F with MC. Preliminary results show that CS interferes with MC SNARE-associated fluorescence, particularly the t-SNARE SNAP-23, but a Western blot is needed to confirm if CS actually affects their protein expression

Our laboratory had previously reported that CS partially inhibits histamine secretion from rat peritoneal MC[128]. CS inhibited the secretion of pro-inflammatory cytokines from murine coronary endothelial cells and monocytes stimulated with TNF[139]. Hep prevented MC degranulation induced by nasal provocation in atopic subjects[208], while also inhibiting cultured canine MC[129] and rat peritoneal MC[130]. LMW Hep preferentially inhibited TNF production in a mouse bone marrow-derive MC[209].

DS is the predominant GAG present in the skin and has been implicated in wound repair and fibrosis[135]. DS has been identified in RBL-1, a rat MC-like cell line[133], and in rat bone marrow-derived MC (BMMC)[124, 134]. DS has been found to inhibit P-selectin, thus attenuating metastasis, inflammation and thrombosis in respective experimental mouse models,[155] as well as the chemokine monocyte chemoattractant protein 1 (MCP-1), the cytokine TGF-β and macrophage recruitment[136, 137].

The effect of GAGs on MC stimulated with IL-33 had not been reported before. Our present studies comparing UF and LMW GAGs yielded no significant differences in inhibition of TNF secretion. However, interestingly, at low concentrations, there were significant differences in inhibition of CXCL8 secretion between both sizes of DS and Hep, with UF DS being more inhibitory than LMW DS, and the reverse being true for Hep. The clinical differences between UF and LMW Hep, concerning their anti-thrombotic and pharmacokinetic properties, have long been established[210]. Interestingly, anti-coagulant and anti-inflammatory activity does not always go hand-in-hand for differing Hep structures. For example, N-acetylation and C5 epimerization of O-sulfated non-anticoagulant Hep-like molecules inhibited the secretion of pro-inflammatory cytokines from LPS-stimulated human mononuclear cells, while similar, non-N-acetylated Hep-like molecules did not[211]. Additionally, it has been shown that saccharide chain length of LMWH can affect cytokine release from stimulated peripheral blood mononuclear cells (PBMCs) of asthmatic subjects[212]. Smaller fractions, particularly dp4 (four saccharide units), of the LMWH enoxaparin, were responsible for the observed 48% inhibition of TNF secretion, whereas the larger dp22 fractions of another LMWH, dalteparin, were responsible for the 25% increase in TNF release[212]. Thus, while only differing MW of DS and Hep were investigated, factors such as GAG sulfation and chain length may impact their ability to inhibit chemokines, such as CXCL8, in particular.

In contrast to those studies that reported CS and Hep can inhibit the expression and subsequent activation of signaling pathways, particularly NF-κB, and gene expression of mediators[140, 141, 199, 201-205, 207], our experiments showed that neither CS, Hep or DS had an effect on IL-33-stimulated gene expression of TNF or CXCL8 in LAD2 MC. While IL-

33 does indeed signal through NF-κB in MC[67], it was chosen not to further pursue the role of these kinases due to the lack of inhibition observed by GAGs on MC mediator gene expression.

It is shown here that the fluorescence associated with the t-SNARE SNAP-23, but not the v-SNARE VAMP-8, is increased in MC stimulated by IL-33 and that CS may interfere with this involvement. This finding actually corroborates studies in macrophages, where SNARE, specifically syntaxin 6/Vti1b[213] and syntaxin 4[214], synthesis is upregulated upon activation to accommodate the increased trafficking needed for TNF release.

Although it was determined that CS does not interfere with either the detection of MC mediators via ELISA or the potency of SP or IL-33, one possible explanation for the reduced secretion of mediators from IL-33-stimulated LAD2 MC pre-treated with GAGs may be the intracellular binding of TNF and CXCL8 with GAGs, potentially interfering with their secretion. Interactions between cytokines, chemokines and growth factors with GAGs have been well characterized[122, 215] and are not surprising due to the necessary role of GAGs in immune cell migration and production of chemokine gradients[216]. Another possibility is that, in addition to being taken up intracellularly, through binding to the MC plasma membrane, GAGs may biophysically block the portion of the ST2 receptor responsible for IL-33 binding or interfere with the ability of membrane-anchored ST2 to form a heterodimer along with its co-receptor, IL-1RAP[217].

There is still the possibility that GAGs may be inhibiting oxidative stress. It has been shown in different cell types that GAGs, particularly CS, are not only anti-

inflammatory, but exhibit antioxidant and neuroprotective properties as well[218, 219]. More specifically, CS has long been reported to chelate transition metals such as Cu^{++} or Fe^{++} that are subsequently responsible for the production of reactive oxygen species (ROS) production[220]. CS can serve as a free radical scavenger *in vivo* in a rat model of hepatotoxicity[221, 222]. In terms of its neuroprotective characteristics, CS was reported to drastically reduce ROS generation induced by both H_2O_2 (extracellular ROS) and Rot/oligo (intracellular ROS) in human neuroblastoma SH-SY5Y cells[223]. It was recently reported that inflammation induced by IL-33 exacerbated the development of chronic obstructive pulmonary disease (COPD) through the upregulation of oxidative stress indicators[224]. It is, therefore, reasonable to hypothesize that GAGs may interfere with oxidative stress produced by IL-33.

Another possible mechanism of the inhibitory action exerted by GAGs on MC that should be considered is GAG inhibition of MC proteases which enhance the pro-inflammatory activity of IL-33. While caspase-1 has been the protease most studied in the proteolytic processing of the IL-1 family of cytokines, it has recently been reported that extracellular neutrophil- and mast cell-derived proteases, specifically neutrophil elastase, cathepsin-G, chymase and tryptase are responsible for the proteolysis of IL-33[225-227]. The major forms of IL-33 produced by MC proteases secreted by activated MC, chymase and tryptase, were 30-fold more potent than full-length human IL-33 for activation of group-2 innate lymphoid cells (ILC2s) ex vivo[227]. Clinically, human lung MC have been reported to release elastase when stimulated with anti-IgE and ionophore[228]. Moreover, tryptase and cathepsin-G were co-localized in MC derived from the skin of patients with cutaneous mastocytosis[229]. In addition, increased levels of elastase and α_1-antitrypsin

have been reported in the sputum of asthmatic patients[230]. Interestingly, GAGs have been shown to bind to and inhibit the highly cationic proteases, chymotrypsin- and elastase-like enzymes, in human granulocytes[231]. It has also been shown that human lysosomal elastase from polymorphonuclear leucocytes is inhibited by the sulfated GAG Arteparon[232]. Thus, it is reasonable to hypothesize that GAGs could interfere with the proteases responsible for potentiating the activity of IL-33 in MC, particularly *in vivo*, since GAGs were washed off in the present experiments.

5.2 Limitations of Study

CD44 mainly plays a role in differentiation, maturation and proliferation of cutaneous MC[233, 234]. It was also reported that CD34 (+)-derived cultured human peripheral MC can adhere to hyaluronan-coated plates[235]. The CD44 receptor is a cell surface CS proteoglycan, critical for the binding and uptake of hyaluronan[236, 237]. However, CD44 has also been implicated in its ability to bind CSPGs, aggrecan[238], versican[154] and serglycin[239]. One limitation of the present study is whether the anti-human CD44 Ab used truly "neutralizes" the CD44 receptor. It is also possible that CS-F binds to a CD44 epitope other than the epitope recognized and blocked by the anti-human neutralizing CD44 Ab used. It would be best to first study if there is a loss of the functional aspect of the CD44 receptor, such as migration, invasion or adhesion[239, 240], before investigating its role in CS-F uptake. Another viable option would be to use CD44 siRNA to completely knock down receptor expression, and then study CS-F uptake in MC.

While our experiments discovered changes in the fluorescence associated with the t-SNARE SNAP-23 in MC stimulated with IL-33 after 30 min or 3 hr, clear changes in SNARE translocation from the cytoplasm to surrounding the plasma membrane were not observed. VAMP-8 has been reported to translocate to the plasma membrane and form a complex in MC activated by phorbol ester PMA and ionomycin[177]. However, while this degranulation-inducing process occurs rapidly (within 15 min), it is difficult to "catch" the proper time when secretory vesicles are transported to the plasma membrane in MC stimulated with a cytokine that occur over hours.

Unlike in LAD2 MC (data not shown), GAGs did not detectably affect TNF or CXCL8 secretion in primary hCBMC stimulated with IgE/anti-IgE. IL-33 did not detectably stimulate sufficient TNF secretion from hCBMC. It is important to note that primary MC vary phenotypically between each other and between immortalized lines, making it challenging to study "true" MC pathophysiology. Typically, human MC are derived from either lung resections or from foreskin following circumcisions[192]. However, MC derived from foreskin are often morphologically immature. For example, it was reported that SP stimulates TNF release from LAD2 MC, but not from purified human foreskin MC[241]. Our experiments used primary human MC that are isolated from umbilical cord blood, collected from healthy donors after uncomplicated deliveries. Some drawbacks of these hCBMC is that they are difficult to obtain and proliferate, and their secretory granules also do not appear to be as mature as adult human MC[92].

5.3 Clinical Significance of Findings

GAGs play a crucial role in cell-to-cell communication and protein homeostasis. Hence, dysfunction of GAG synthesis or degradation can lead to the development or exacerbation of several diseases, such as Ehlers-Danlos syndrome (EDS)[242], mucopolysaccharidoses[243], cystic fibrosis[244] and rheumatoid arthritis[245]. EDS, a genetic connective tissue disorder characterized by fragility of the soft connective tissues and hypermobility[246], is of particular interest. The prevalence of all forms of EDS is approximately 1 in 5,000 individuals worldwide[247]. EDS is typically caused by a mutation in the gene encoding collagen synthesis; however, there is one subtype of EDS, musculocontractural EDS, which is characterized by loss-of-function mutations in the gene *CHST14*, resulting in dermatan 4-O-sulfotransferase 1 deficiency, leading to impaired DS biosynthesis[242]. Remarkably, a subpopulation of patients with the most common EDS form, hypermobile EDS, have been shown to have MC activation disorders, specifically MC activation syndrome rather than systemic mastocytosis[248].

Several reports have described the comorbidity of inflammatory conditions, such as gastrointestinal disorders[249], eosinophilic gastrointestinal disorders[250] and an increased frequency of asthma[251], naso-ocular symptoms and history of anaphylaxis[252], in patients with CTDs, such as EDS. Interestingly, immunohistochemical analysis identified an increased content of chymase-positive MC in undamaged eyelid skin of patients with CTDs[253].

MC are important for allergies and many inflammatory diseases[254, 255], including psoriasis[73], CAD[256] and even EDS[248], but unfortunately, there are no effective, clinically

available, endogenous MC inhibitors. Disodium cromoglycate (cromolyn) significantly inhibits histamine secretion from rodent MC, but it is a very weak inhibitor of human MC[128, 257, 258] and its mechanism is instead thought to be through inhibition of cutaneous sensory nerves[259]. Furthermore, recent evidence has challenged the inhibitory ability of cromolyn in mice[260]. Some natural flavonoids, such as quercetin and luteolin, have anti-inflammatory actions[261-263] and inhibit MC activation,[264-268] but their oral absorption is limited. Our laboratory had also previously reported that the naturally occurring polyamines, spermine and spermidine, can inhibit rat MC, but they have not been further studied[269].

The present findings indicate that GAGs can inhibit the release of TNF and CXCL8 from IL-33-stimulated human MC, possibly by acting intracellularly. GAGs from the tissue microenvironment or secreted by MC, themselves, upon activation, could inhibit the secretion of mediators from MC in a paracrine or autocrine fashion. CS exerts few or no side effects[189] and there are no known drug-drug interactions.[190] It is, therefore, an attractive therapeutic option for for allergic and inflammatory diseases, such as Ps, atopic dermatitis and asthma. One group has reported that CS inhibited antigen-induced IgE production in mice immunized with ovalbumin, suggesting at least an indirect anti-allergic effect[270]. Although CS is estimated to only have a bioavailability of approximately 10-20% in humans[271], clinical studies have shown that orally administered CS can modulate inflammation in osteoarthritis[142-146], Ps[147] and coronary artery disease[149]. However, GAGs may be better suited for topical or inhalant delivery, especially since it has already been reported that Hep can inhibit exercise-induced asthma[151, 152]. Thus, it is plausible that other GAGs such as CS and DS could be effective

treatments for asthma, as well. Perhaps GAGs could be combined with cyclodextrin to shield their highly negative charges, or be formulated as liposomes or nanoparticles, to enhance their solubility, oral absorption and delivery.

5.4 Future Directions

Additional studies are needed to determine if CS actually prevents secretory vesicle fusion with the membrane. To truly determine if the observed inhibitory effect of CS on the secretion of TNF and CXCL8 from MC is due to the uptake of CS, it would be necessary to test inhibitors of endocytosis, but this would not be an easy task since there are multiple pathways for endocytosis: pinocytic, such as clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE), as well as phagocytic[272]. An additional obstacle is that chemical and pharmacological endocytic inhibitors are often non-specific and/or interfere with the actin cytoskeleton[273, 274] that is reprimed for MC secretion. However, it would still be of interest to treat MC with different endocytic inhibitors, such as dynasore (CME inhibitor) or filipin (CIE inhibitor), before pre-incubating MC with CS, stimulating with IL-33 and then investigating the subsequent secretion of TNF and CXCL8.

Future studies should investigate the effects of other GAGs, such as DS and Hep, on the expression of SNAP-23 and VAMP-8, as well as other SNAREs, such as STX3, STX4 and VAMP-3. While immunohistochemistry might be sufficient for analyzing SNARE protein localization, it would be best to perform Western blot analysis and/or immunoprecipitation to quantitatively determine if CS inhibits SNARE protein

expression. Ideally, it would be interesting to pre-incubate MC with CS-F, instead of unlabeled CS used in the present study, then stimulate the cells with IL-33 (10 ng/mL) for an extended time period, from 30 min to 12 hr. Human TNF and CXCL8 Abs would then be used to study the co-localization, if any, between TNF and CXCL8 and SNAREs. If possible, one could use a fluorescently tagged IL-33 molecule to investigate its accumulation, vesicular trafficking and co-staining with SNAREs and CS-F. One study used this approach for studying the trafficking of fluorescently tagged IL-6 and TNF, as well as their interaction with recycling endosomes[275].

Some of the inhibitory action of GAGs may be via binding to the cell surface, in addition to being taken up. Thus, it would be interesting to use the enzymes chondroitinase or heparinase, which should, in theory, cleave and detach GAGs bound to the MC surface[237], prior to stimulation with IL-33. If, after chondroitinase or heparinase treatment, GAGs still exert their inhibitory action of reducing TNF and CXCL8 secretion from MC, then perhaps it is reasonable to suggest that their mechanism is solely through internalization.

CHAPTER 6 : APPENDIX

6.1 Secretion of sulfated GAGs from LAD2 MC stimulated with SP or Compound 48/80

The characterization of sulfated GAGs was established using the dimethylmethylene blue GAG detection assay[276]. Sulfated GAGs are found in high concentrations in LAD2 MC (**Fig. 6.1**). Preliminary studies showed that, after 24 hr, a lower concentration of GAGs was detected in the lysates of LAD2 MC treated with the MC degranulation triggers, SP and Compound 48/80, compared to the lysates of unstimulated control (**Fig. 6.1**).

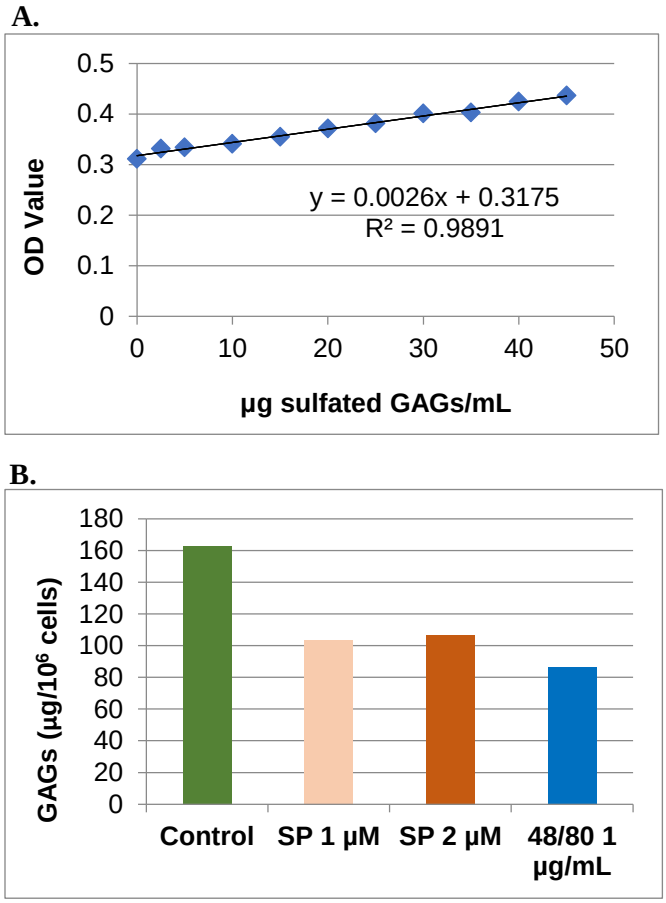


Figure 6.1. Preliminary quantification of sulfated GAGs in LAD2 MC by dimethylmethylene blue assay. (A) Example of a typical sulfated GAGs standard curve. 250 µL of dimethylmethylene blue (DMMB) dye solution is added to 80 µL of varying concentrations (0-40 µg/mL) of CS to produce a linear standard curve. (B) Concentration of sulfated GAGs in LAD2 lysates after 24 hr incubation with MC triggers. Representative experiment, all conditions performed in duplicate.

6.2 Publications

Gross, A.R. & Theoharides, T.C. Manuscript accepted: Chondroitin sulfate inhibits secretion of TNF and CXCL8 from human mast cells stimulated with IL-33. *BioFactors*, Sep. 2018, in press.

CHAPTER 7 : REFERENCES

1. Beaven MA. Our perception of the mast cell from Paul Ehrlich to now. Eur J Immunol 2009; 39(1):11-25.

2. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol 2008; 8(6):478-486.

3. Williams CM, Galli SJ. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J Allergy Clin Immunol 2000; 105(5):847-859.

4. Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. Science 1996; 271:818-822.

5. Chen CC, Grimbaldeston MA, Tsai M, Weissman IL, Galli SJ. Identification of mast cell progenitors in adult mice. Proc Natl Acad Sci U S A 2005; 102(32):11408-11413.

6. Galli SJ, Tsai M, Wershil BK. The c-*kit* receptor, stem cell factor, and mast cells. Am J Pathol 1993; 142:965-974.

7. Okayama Y, Kawakami T. Development, migration, and survival of mast cells. Immunol Res 2006; 34(2):97-115.

8. Bischoff SC, Sellge G, Lorentz A, Sebald W, Raab R, Manns MP. IL-4 enhances proliferation and mediator release in mature human mast cells. Proc Natl Acad Sci USA 1999; 96:8080-8085.

9. Amin K. The role of mast cells in allergic inflammation. Respir Med 2011.

10. Galli SJ. New concepts about the mast cell. N Engl J Med 1993; 328:257-265.

11. Siraganian RP. Mast cell signal transduction from the high-affinity IgE receptor. Curr Opin Immunol 2003; 15:639-646.

12. Blank U, Rivera J. The ins and outs of IgE-dependent mast-cell exocytosis. Trends Immunol 2004; 25:266-273.

13. Kraft S, Rana S, Jouvin MH, Kinet JP. The role of the FcepsilonRI beta-chain in allergic diseases. Int Arch Allergy Immunol 2004; 135(1):62-72.

14. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature 2008; 454(7203):445-454.

15. Ishizaka K. Regulation of IgE synthesis. Annu Rev Immunol 1984; 2:159-182.

16. Metzger H, Alcazar G, Hohman R, Kinet J-P, Pribluda V, Quarto R. The receptor with high affinity for immunoglobulin E. Annu Rev Immunol 1986; 4:419-470.

17. Theoharides TC, Douglas WW. Somatostatin induces histamine secretion from rat peritoneal mast cells. Endocrinology 1978; 102:1637-1640.

18. Galli SJ, Tsai M. IgE and mast cells in allergic disease. Nat Med 2012; 18(5):693-704.

19. Smurthwaite L, Walker SN, Wilson DR et al. Persistent IgE synthesis in the nasal mucosa of hay fever patients. Eur J Immunol 2001; 31(12):3422-3431.

20. Theoharides TC, Kalogeromitros D. The critical role of mast cell in allergy and inflammation. Ann NY Acad Sci 2006; 1088:78-99.

21. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2010; 125(2 Suppl 2):S73-S80.

22. Gould HJ, Sutton BJ, Beavil AJ et al. The biology of IGE and the basis of allergic disease. Annu Rev Immunol 2003; 21:579-628.

23. Abrink M, Grujic M, Pejler G. Serglycin is essential for maturation of mast cell secretory granule. J Biol Chem 2004; 279(39):40897-40905.

24. Rivera J, Olivera A. A current understanding of Fc epsilon RI-dependent mast cell activation. Curr Allergy Asthma Rep 2008; 8(1):14-20.

25. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol 2014; 14(7):478-494.

26. Nishida K, Yamasaki S, Ito Y et al. Fc{epsilon}RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol 2005; 170(1):115-126.

27. Lorentz A, Baumann A, Vitte J, Blank U. The SNARE Machinery in Mast Cell Secretion. Front Immunol 2012; 3:143.

28. Theoharides TC, Bondy PK, Tsakalos ND, Askenase PW. Differential release of serotonin and histamine from mast cells. Nature 1982; 297:229-231.

29. Theoharides TC, Kops SK, Bondy PK, Askenase PW. Differential release of serotonin without comparable histamine under diverse conditions in the rat mast cell. Biochem Pharmacol 1985; 34:1389-1398.

30. Fewtrell CMS, Foreman JC, Jordan CC, Oehme P, Renner H, Stewart JM. The effects of substance P on histamine and 5-hydroxytryptamine release in the rat. J Physiol 1982; 330:393-411.

31. Theoharides TC, Zhang B, Kempuraj D et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. Proc Natl Acad Sci U S A 2010; 107(9):4448-4453.

32. Lazarus LH, Perrin MH, Brown MR. Mast cell binding of neurotensin; iodination of neurotensin and characterization of the interaction of neurotensin with mast cell receptor sites. J Biol Chem 1977; 252:7174-7179.

33. Lazarus LH, Perrin MH, Brown MR, Rivier JE. Mast cell binding of neurotensin; II. molecular conformation of neurotensin involved in the stereospecific binding to mast cell receptor sites. J Biol Chem 1977; 252:7180-7183.

34. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. Immunology 2007; 123:398-410.

35. Theoharides TC, Kalogeromitros D. The critical role of mast cells in allergy and inflammation. Ann N Y Acad Sci 2006; 1088:78-99.

36. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol 2005; 6(2):135-142.

37. Ferry X, Brehin S, Kamel R, Landry Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. Peptides 2002; 23(8):1507-1515.

38. Janiszewski J, Bienenstock J, Blennerhassett MG. Picomolar doses of substance P trigger electrical responses in mast cells without degranulation. Am J Physiol 1994; 267:C138-C145.

39. Tore F, Tuncel N. Mast cells: target and source of neuropeptides. Curr Pharm Des 2009; 15(29):3433-3445.

40. Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. Nat Rev Immunol 2006; 6(3):218-230.

41. Theoharides TC, Kempuraj D, Tagen M, Conti P, Kalogeromitros D. Differential release of mast cell mediators and the pathogenesis of inflammation. Immunol Rev 2007; 217:65-78.

42. Roth S, Kummer W. A quantitative ultrastructural investigation of tyrosine hydroxylase-immunoreactive axons in the hairy skin of the guinea pig. Anat Embryol (Berl) 1994; 190(2):155-162.

43. Theoharides TC, Alysandratos KD, Angelidou A et al. Mast cells and inflammation. Biochim Biophys Acta 2012; 1822(1):21-33.

44. Kandere-Grzybowska K, Letourneau R, Kempuraj D et al. IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells. J Immunol 2003; 171(9):4830-4836.

45. Cao J, Papadopoulou N, Kempuraj D et al. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. J Immunol 2005; 174(12):7665-7675.

46. Gordon JR, Galli SJ. Mast cells as a source of both preformed and immunologically inducible TNF-α/cachectin. Nature 1990; 346(6281):274-276.

47. Mekori YA, Metcalfe DD. Mast cells in innate immunity. Immunol Rev 2000; 173:131-140.

48. Rottem M, Mekori YA. Mast cells and autoimmunity. Autoimmun Rev 2005; 4:21-27.

49. Kinet JP. The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. Annu Rev Immunol 1999; 17:931-972.

50. Theoharides TC, Alysandratos K-D, Angelidou A, Zhang B. Mast cells and tumor microenvironment. In: Bagley JR, editor. The Tumor Microenviroment. Springer; 2010 p. 353-370.

51. Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF. Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 2009; 64(12):1699-1714.

52. Zhang B, Weng Z, Sismanopoulos N et al. Mitochondria distinguish granule-stored from de novo synthesized tumor necrosis factor secretion in human mast cells. Int Arch Allergy Immunol 2012; 159(1):23-32.

53. Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. Blood 2011; 118(20):5383-5393.

54. Bienenstock J, Tomioka M, Matsuda H et al. The role of mast cells in inflammatory processes: evidence for nerve mast cell interactions. Int Arch Allergy Appl Immunol 1987; 82:238-243.

55. Liew FY, Pitman NI, McInnes IB. Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol 2010; 10(2):103-110.

56. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009; 27:519-550.

57. Dinarello CA. Setting the cytokine trap for autoimmunity. Nat Med 2003; 9(1):20-22.

58. Theoharides TC, Petra AI, Taracanova A, Panagiotidou S, Conti P. Targeting IL-33 in autoimmunity and inflammation. J Pharmacol Exp Ther 2015; 354(1):24-31.

59. Saluja R, Ketelaar ME, Hawro T, Church MK, Maurer M, Nawijn MC. The role of the IL-33/IL-1RL1 axis in mast cell and basophil activation in allergic disorders. Mol Immunol 2015; 63(1):80-85.

60. Arend WP, Palmer G, Gabay C. IL-1, IL-8, and IL-33 families of cytokines. Immunol Rev 2008; 223:20-38.

61. Fux M, Pecaric-Petkovic T, Odermatt A et al. IL-33 is a mediator rather than a trigger of the acute allergic response in humans. Allergy 2014; 69(2):216-222.

62. Moulin D, Donze O, Talabot-Ayer D, Mezin F, Palmer G, Gabay C. Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. Cytokine 2007; 40(3):216-225.

63. Theoharides TC, Zhang B, Kempuraj D et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. Proc Natl Acad Sci U S A 2010; 107(9):4448-4453.

64. Lukens JR, Gross JM, Kanneganti TD. IL-1 family cytokines trigger sterile inflammatory disease. Front Immunol 2012; 3:315.

65. Lunderius-Andersson C, Enoksson M, Nilsson G. Mast Cells Respond to Cell Injury through the Recognition of IL-33. Front Immunol 2012; 3:82.

66. Saluja R, Khan M, Church MK, Maurer M. The role of IL-33 and mast cells in allergy and inflammation. Clin Transl Allergy 2015; 5:33.

67. Schmitz J, Owyang A, Oldham E et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 2005; 23(5):479-490.

68. Funakoshi-Tago M, Tago K, Hayakawa M et al. TRAF6 is a critical signal transducer in IL-33 signaling pathway. Cell Signal 2008; 20(9):1679-1686.

69. Kakkar R, Lee RT. The IL-33/ST2 pathway: therapeutic target and novel biomarker. Nat Rev Drug Discov 2008; 7(10):827-840.

70. Vanderpuye-Org, Zhao Y, Lu J et al. Evaluating the economic burden of psoriasis in the United States. J Am Acad Dermatol 2015; 72(6):961-967.

71. Brezinski EA, Dhillon JS, Armstrong AW. Economic Burden of Psoriasis in the United States: A Systematic Review. JAMA Dermatol 2015; 151(6):651-658.

72. Ozdamar SO, Seckin D, Kandemir B, Turanli AY. Mast cells in psoriasis. Dermatology 1996; 192:189-190.

73. Harvima IT, Nilsson G, Suttle MM, Naukkarinen A. Is there a role for mast cells in psoriasis? Arch Dermatol Res 2008; 300(9):461-476.

74. Schon MP, Boehncke WH. Psoriasis. N Engl J Med 2005; 352(18):1899-1912.
75. Deng Y, Chang C, Lu Q. The Inflammatory Response in Psoriasis: a Comprehensive Review. Clin Rev Allergy Immunol 2016; 50(3):377-389.

76. Metz M, Maurer M. Innate immunity and allergy in the skin. Curr Opin Immunol 2009; 21(6):687-693.

77. Paus R, Theoharides TC, Arck PC. Neuroimmunoendocrine circuitry of the 'brain-skin connection'. Trends Immunol 2006; 27(1):32-39.

78. Matsuda H, Ushio H, Paliwal V, Ptak W, Askenase PW. Adoptive cell transfer of contact sensitivity-initiation mediated by nonimmune cells sensitized with monoclonal IgE antibodies. J Immunol 1995; 154:5080-5092.

79. Katsarou-Katsari A, Filippou A, Theoharides TC. Stress and inflammatory dermatoses. Int J Immunopathol Pharmacol 1999; 12:7-11.

80. Fortune DG, Richards HL, Griffiths CE. Psychologic factors in psoriasis: consequences, mechanisms, and interventions. Dermatol Clin 2005; 23(4):681-694.

81. Harvima RJ, Viinamäki H, Harvima IT et al. Association of psychic stress with clinical severity and symptoms of psoriatic patients. Acta Derm Venereol (Stockh) 1996; 76:467-471.

82. Harvima IT, Viinamäki H, Naukkarinen A, Paukkonen K, Neittaanmäki H, Horsmanheimo M. Association of cutaneous mast cells and sensory nerves with psychic stress in psoriasis. Psychother Psychosom 1993; 60:168-176.

83. Saraceno R, Kleyn CE, Terenghi G, Griffiths CE. The role of neuropeptides in psoriasis. Br J Dermatol 2006; 155(5):876-882.

84. Remröd C, Lonne-Rahm S, Nordliond K. Study of substance P and its receptor neurokinin-1 in psoriasis and their relation to chronic stress and pruritus. Arch Dermatol Res 2007; 299:85-91.

85. Scholzen T, Armstrong CA, Bunnett NW, Luger TA, Olerud JE, Ansel JC. Neuropeptides in the skin: interactions between the neuroendocrine and the skin immune systems. Exp Dermatol 1998; 7(2-3):81-96.

86. Naukkarinen A, Jarvikallio A, Lakkakorpi J, Harvima IT, Harvima RJ, Horsmanheimo M. Quantitative histochemical analysis of mast cells and sensory nerves in psoriatic skin. J Pathol 1996; 180:200-205.

87. Chan J, Smoller BR, Raychauduri SP, Jiang WY, Farber EM. Intraepidermal nerve fiber expression of calcitonin gene-related peptide, vasoactive intestinal peptide and substance P in psoriasis. Arch Dermatol Res 1997; 289(11):611-616.

88. Al'Abadie MS, Senior HJ, Bleehen SS, Gawkrodger DJ. Neuropeptides and general neuronal marker in psoriasis--an immunohistochemical study. Clin Exp Dermatol 1995; 20(5):384-389.

89. Meephansan J, Komine M, Tsuda H, Karakawa M, Tominaga S, Ohtsuki M. Expression of IL-33 in the epidermis: The mechanism of induction by IL-17. J Dermatol Sci 2013; 71(2):107-114.

90. Sehat M, Talaei R, Dadgostar E, Nikoueinejad H, Akbari H. Evaluating Serum Levels of IL-33, IL-36, IL-37 and Gene Expression of IL-37 in Patients with Psoriasis Vulgaris. Iran J Allergy Asthma Immunol 2018; 17(2):179-187.

91. Petra AI, Tsilioni I, Taracanova A, Katsarou-Katsari A, Theoharides TC. Interleukin 33 and interleukin 4 regulate interleukin 31 gene expression and secretion from human laboratory of allergic diseases 2 mast cells stimulated by substance P and/or immunoglobulin E. Allergy Asthma Proc 2018; 39(2):153-160.

92. Taracanova A, Alevizos M, Karagkouni A et al. SP and IL-33 together markedly enhance TNF synthesis and secretion from human mast cells mediated by the interaction of their receptors. Proc Natl Acad Sci U S A 2017; 114(20):E4002-E4009.

93. Enoksson M, Lyberg K, Moller-Westerberg C, Fallon PG, Nilsson G, Lunderius-Andersson C. Mast cells as sensors of cell injury through IL-33 recognition. J Immunol 2011; 186(4):2523-2528.

94. Iikura M, Suto H, Kajiwara N et al. IL-33 can promote survival, adhesion and cytokine production in human mast cells. Lab Invest 2007; 87(10):971-978.

95. Theoharides TC, Cochrane DE. Critical role of mast cells in inflammatory diseases and the effect of acute stress. J Neuroimmunol 2004; 146((1-2)):1-12.

96. Arican O, Aral M, Sasmaz S, Ciragil P. Serum levels of TNF-alpha, IFN-gamma, IL-6, IL-8, IL-12, IL-17, and IL-18 in patients with active psoriasis and correlation with disease severity. Mediators Inflamm 2005; 2005(5):273-279.

97. Gottlieb AB, Chamian F, Masud S et al. TNF inhibition rapidly down-regulates multiple proinflammatory pathways in psoriasis plaques. J Immunol 2005; 175(4):2721-2729.

98. Kutsch CL, Norris DA, Arend WP. Tumor necrosis factor-alpha induces interleukin-1 alpha and interleukin-1 receptor antagonist production by cultured human keratinocytes. J Invest Dermatol 1993; 101(1):79-85.

99. Patel A.B., Tsilioni I, Weng Z, Theoharides T.C. TNF stimulates IL-6, CXCL8 and VEGF secretion from human keratinocytes via activation of mTOR, inhibited by tetramethoxyluteolin. Exp Dermatol 2018; 27(2):135-143.

100. Canavese M, Altruda F, Ruzicka T, Schauber J. Vascular endothelial growth factor (VEGF) in the pathogenesis of psoriasis--a possible target for novel therapies? J Dermatol Sci 2010; 58(3):171-176.

101. Lizzul PF, Aphale A, Malaviya R et al. Differential expression of phosphorylated NF-kappaB/RelA in normal and psoriatic epidermis and downregulation of NF-kappaB in response to treatment with etanercept. J Invest Dermatol 2005; 124(6):1275-1283.

102. Lemster BH, Carroll PB, Rilo HR, Johnson N, Nikaein A, Thomson AW. IL-8/IL-8 receptor expression in psoriasis and the response to systemic tacrolimus (FK506) therapy. Clin Exp Immunol 1995; 99(2):148-154.

103. Takematsu H, Tagami H. Quantification of chemotactic peptides (C5a anaphylatoxin and IL-8) in psoriatic lesional skin. Arch Dermatol 1993; 129(1):74-80.

104. Gillitzer R, Berger R, Mielke V, Muller C, Wolff K, Stingl G. Upper keratinocytes of psoriatic skin lesions express high levels of NAP-1/IL-8 mRNA in situ. J Invest Dermatol 1991; 97(1):73-79.

105. Jiang WY, Chattedee AD, Raychaudhuri SP, Raychaudhuri SK, Farber EM. Mast cell density and IL-8 expression in nonlesional and lesional psoriatic skin. Int J Dermatol 2001; 40(11):699-703.

106. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. FASEB J 2006; 20(1):9-22.

107. Raman R, Sasisekharan V, Sasisekharan R. Structural insights into biological roles of protein-glycosaminoglycan interactions. Chem Biol 2005; 12(3):267-277.

108. Souza-Fernandes AB, Pelosi P, Rocco PR. Bench-to-bedside review: the role of glycosaminoglycans in respiratory disease. Crit Care 2006; 10(6):237.

109. Hook M. Cell-surface glycosaminoglycans. Annu Rev Biochem 1984; 53:847-869.

110. Kjellen L, Lindahl U. Proteoglycans: structures and interactions. Annu Rev Biochem 1991; 60:443-475.

111. Mihov D, Spiess M. Glycosaminoglycans: Sorting determinants in intracellular protein traffic. Int J Biochem Cell Biol 2015; 68:87-91.

112. Thelin MA, Bartolini B, Axelsson J et al. Biological functions of iduronic acid in chondroitin/dermatan sulfate. FEBS J 2013; 280(10):2431-2446.

113. Scott JE. Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues. FASEB J 1992; 6(9):2639-2645.

114. Sim J-S, Im A-R, Cho SM, Jang HJ, Jo JH, Kim YS. Evaluation of chondroitin sulfate in shark cartilage powder as a dietary supplement: Raw materials and finished products. Food Chemistry 2007; 101(2):532-539.

115. Sakai S, Kim WS, Lee IS et al. Purification and characterization of dermatan sulfate from the skin of the eel, Anguilla japonica. Carbohydr Res 2003; 338(3):263-269.

116. Metcalfe DD, Lewis RA, Silbert JE, Rosenberg RD, Wasserman SI, Austen KF. Isolation and characterization of heparin from human lung. J Clin Invest 1979; 64(6):1537-1543.

117. Fillion GM, Slorach SA, Uvnas B. The release of histamine, heparin and granule protein from rat mast cells treated with compound 48-80 in vitro. Acta Physiol Scand 1970; 78(4):547-560.

118. Slorach SA. Histamine and heparin release from isolated rat mast cells exposed to compound 48-80. Acta Physiol Scand 1971; 82(1):91-97.

119. Rabenstein DL. Heparin and heparan sulfate: structure and function. Nat Prod Rep 2002; 19:312-331.

120. Sayama S, Iozzo RV, Lazarus GS, Schechter NM. Human skin chymotrypsin-like proteinase chymase. Subcellular localization to mast cell granules and interaction with heparin and other glycosaminoglycans. J Biol Chem 1987; 262(14):6808-6815.

121. Pejler G, Sadler JE. Mechanism by which heparin proteoglycan modulates mast cell chymase activity. Biochemistry 1999; 38(37):12187-12195.

122. Kuschert GS, Coulin F, Power CA et al. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. Biochemistry 1999; 38(39):12959-12968.

123. Handel TM, Johnson Z, Crown SE, Lau EK, Proudfoot AE. Regulation of protein function by glycosaminoglycans--as exemplified by chemokines. Annu Rev Biochem 2005; 74:385-410.

124. Ronnberg E, Melo FR, Pejler G. Mast cell proteoglycans. J Histochem Cytochem 2012; 60(12):950-962.

125. Ronnberg E, Pejler G. Serglycin: the master of the mast cell. Methods Mol Biol 2012; 836:201-217.

126. Nilsson G, Blom T, Harvima I, Kusche-Gullberg M, Nilsson K, Hellman L. Stem cell factor-dependent human cord blood derived mast cells express alpha-and beta-tryptase, heparin and chondroitin sulphate. Immunology 1996; 88:308-314.

127. Thompson H, Schulman E, Metcalfe D. Identification of chondroitin sulfate E in human lung mast cells. J Immunol 1988; 140:2708-2713.

128. Theoharides TC, Patra P, Boucher W et al. Chondroitin sulfate inhibits connective tissue mast cells. Br J Pharmacol 2000; 131:1039-1049.

129. Inase N, Schreck RE, Lazarus SC. Heparin inhibits histamine release from canine mast cells. Am J Physiol 1993; 264(4 Pt 1):L387-L390.

130. Ahmed T, Campo C, Abraham MK et al. Inhibition of antigen-induced acute bronchoconstriction, airway hyperresponsiveness, and mast cell degranulation by a nonanticoagulant heparin: comparison with a low molecular weight heparin. Am J Respir Crit Care Med 1997; 155(6):1848-1855.

131. Baram D, Rashkovsky M, Hershkoviz R et al. Inhibitory effects of low molecular weight heparin on mediator release by mast cells:preferential inhibition of cytokine production and mast cell dependent cutaneous inflammation. Clin Exp Immunol 1997; 110:485-491.

132. Lucio J, D'Brot J, Guo CB et al. Immunologic mast cell mediated responses and histamine release are attenuated by heparin. J Appl Physiol 1992; 73:1093-1101.

133. Seldin DC, Austen KF, Stevens RL. Purification and characterization of protease-resistant secretory granule proteoglycans containing chondroitin sulfate di-B and heparin-like glycosaminoglycans from rat basophilic leukemia cells. J Biol Chem 1985; 260:11131-11139.

134. Broide DH, Metcalfe DD, Wasserman SI. Functional and biochemical characterization of rat bone marrow derived mast cells. J Immunol 1988; 141:4298-4305.

135. Trowbridge JM, Gallo RL. Dermatan sulfate: new functions from an old glycosaminoglycan. Glycobiology 2002; 12(9):117R-125R.

136. Belmiro CL, Goncalves RG, Kozlowski EO et al. Dermatan sulfate reduces monocyte chemoattractant protein 1 and TGF-beta production, as well as macrophage recruitment and myofibroblast accumulation in mice with unilateral ureteral obstruction. Braz J Med Biol Res 2011; 44(7):624-633.

137. Belmiro CL, Castelo-Branco MT, Melim LM et al. Unfractionated heparin and new heparin analogues from ascidians (chordate-tunicate) ameliorate colitis in rats. J Biol Chem 2009; 284(17):11267-11278.

138. Becker LC, Bergfeld WF, Belsito DV et al. Final report of the safety assessment of hyaluronic acid, potassium hyaluronate, and sodium hyaluronate. Int J Toxicol 2009; 28(4 Suppl):5-67.

139. Melgar-Lesmes P, Garcia-Polite F, Del-Rey-Puech P et al. Treatment with chondroitin sulfate to modulate inflammation and atherogenesis in obesity. Atherosclerosis 2015; 245:82-87.

140. Jomphe C, Gabriac M, Hale TM et al. Chondroitin sulfate inhibits the nuclear translocation of nuclear factor-kappaB in interleukin-1beta-stimulated chondrocytes. Basic Clin Pharmacol Toxicol 2008; 102(1):59-65.

141. Andres RM, Paya M, Montesinos MC et al. Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-kappaB and STAT3 in human keratinocytes. Pharmacol Res 2013; 70(1):20-26.

142. Ronca F, Palmieri L, Panicucci P, Ronca G. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage 1998; 6 Suppl A:14-21.

143. Uebelhart D, Thonar EJ, Delmas PD, Chantraine A, Vignon E. Effects of oral chondroitin sulfate on the progression of knee osteoarthritis: a pilot study. Osteoarthritis Cartilage 1998; 6 Suppl A:39-46.

144. Iovu M, Dumais G, du SP. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage 2008; 16 Suppl 3:S14-S18.

145. Vallieres M, du SP. Modulation of inflammation by chondroitin sulfate. Osteoarthritis Cartilage 2010; 18 Suppl 1:S1-S6.

146. Jordan KM, Arden NK, Doherty M et al. EULAR Recommendations 2003: an evidence based approach to the management of knee osteoarthritis: Report of a Task Force of the Standing Committee for International Clinical Studies Including Therapeutic Trials (ESCISIT). Ann Rheum Dis 2003; 62(12):1145-1155.

147. Verges J, Montell E, Herrero M et al. Clinical and histopathological improvement of psoriasis with oral chondroitin sulfate: a serendipitous finding. Dermatol Online J 2005; 11(1):31.

148. Herrero-Beaumont G, Marcos ME, Sanchez-Pernaute O et al. Effect of chondroitin sulphate in a rabbit model of atherosclerosis aggravated by chronic arthritis. Br J Pharmacol 2008; 154(4):843-851.

149. Morrison LM. Response of ischemic heart disease to chondroitin sulfate-A. J Am Geriatr Soc 1969; 17(10):913-923.

150. Morrison LM, Enrick N. Coronary heart disease: reduction of death rate by chondroitin sulfate A. Angiology 1973; 24(5):269-287.

151. Ahmed T, Garrigo J, Danta I. Preventing bronchoconstriction in exercise-induced asthma with inhaled heparin. N Engl J Med 1993; 329:90-95.

152. Garrigo J, Danta I, Ahmed T. Time course of the protective effect of inhaled heparin on exercise-induced asthma. Am J Respir Crit Care Med 1996; 153(5):1702-1707.

153. Wang XF, Li AM, Li J et al. Low molecular weight heparin relieves experimental colitis in mice by downregulating IL-1beta and inhibiting syndecan-1 shedding in the intestinal mucosa. PLoS ONE 2013; 8(7):e66397.

154. Kawashima H, Hirose M, Hirose J, Nagakubo D, Plaas AH, Miyasaka M. Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. J Biol Chem 2000; 275(45):35448-35456.

155. Kozlowski EO, Pavao MS, Borsig L. Ascidian dermatan sulfates attenuate metastasis, inflammation and thrombosis by inhibition of P-selectin. J Thromb Haemost 2011; 9(9):1807-1815.

156. Godoy JA, Block DB, Tollefsen DM, Werneck CC, Vicente CP. Dermatan sulfate and bone marrow mononuclear cells used as a new therapeutic strategy after arterial injury in mice. Cytotherapy 2011; 13(6):695-704.

157. Godoy JA, Carneiro GD, Sielski MS, Barbosa GO, Werneck CC, Vicente CP. Combined dermatan sulfate and endothelial progenitor cell treatment: action on the initial inflammatory response after arterial injury in C57BL/6 mice. Cytotherapy 2015; 17(10):1447-1464.

158. Gruber S, Arnold M, Cini N et al. Radioprotective Effects of Dermatan Sulfate in a Preclinical Model of Oral Mucositis-Targeting Inflammation, Hypoxia and Junction Proteins without Stimulating Proliferation. Int J Mol Sci 2018; 19(6).

159. Castillo GM, Ngo C, Cummings J, Wight TN, Snow AD. Perlecan binds to the beta-amyloid proteins (A beta) of Alzheimer's disease, accelerates A beta fibril formation, and maintains A beta fibril stability. J Neurochem 1997; 69(6):2452-2465.

160. van HJ, Wesseling P, van den Heuvel LP, de Waal RM, Verbeek MM. Heparan sulphate proteoglycans in Alzheimer's disease and amyloid-related disorders. Lancet Neurol 2003; 2(8):482-492.

161. Ginsberg SD, Galvin JE, Lee VM et al. Accumulation of intracellular amyloid-beta peptide (A beta 1-40) in mucopolysaccharidosis brains. J Neuropathol Exp Neurol 1999; 58(8):815-824.

162. Liu IH, Uversky VN, Munishkina LA, Fink AL, Halfter W, Cole GJ. Agrin binds alpha-synuclein and modulates alpha-synuclein fibrillation. Glycobiology 2005; 15(12):1320-1331.

163. Fraser PE, Nguyen JT, Chin DT, Kirschner DA. Effects of sulfate ions on Alzheimer beta/A4 peptide assemblies: implications for amyloid fibril-proteoglycan interactions. J Neurochem 1992; 59(4):1531-1540.

164. Sipe JD, Cohen AS. Review: history of the amyloid fibril. J Struct Biol 2000; 130(2-3):88-98.

165. Cohlberg JA, Li J, Uversky VN, Fink AL. Heparin and other glycosaminoglycans stimulate the formation of amyloid fibrils from alpha-synuclein in vitro. Biochemistry 2002; 41(5):1502-1511.

166. Dudas B, Rose M, Cornelli U, Pavlovich A, Hanin I. Neuroprotective properties of glycosaminoglycans: potential treatment for neurodegenerative disorders. Neurodegener Dis 2008; 5(3-4):200-205.

167. Ariga T, Miyatake T, Yu RK. Role of proteoglycans and glycosaminoglycans in the pathogenesis of Alzheimer's disease and related disorders: amyloidogenesis and therapeutic strategies--a review. J Neurosci Res 2010; 88(11):2303-2315.

168. Zhu H, Yu J, Kindy MS. Inhibition of amyloidosis using low-molecular-weight heparins. Mol Med 2001; 7(8):517-522.

169. Bergamaschini L, Rossi E, Storini C et al. Peripheral treatment with enoxaparin, a low molecular weight heparin, reduces plaques and beta-amyloid accumulation in a mouse model of Alzheimer's disease. J Neurosci 2004; 24(17):4181-4186.

170. Borysik AJ, Morten IJ, Radford SE, Hewitt EW. Specific glycosaminoglycans promote unseeded amyloid formation from beta2-microglobulin under physiological conditions. Kidney Int 2007; 72(2):174-181.

171. Rolls A, Avidan H, Cahalon L et al. A disaccharide derived from chondroitin sulphate proteoglycan promotes central nervous system repair in rats and mice. Eur J Neurosci 2004; 20(8):1973-1983.

172. Sudhof TC, Rothman JE. Membrane fusion: grappling with SNARE and SM proteins. Science 2009; 323(5913):474-477.

173. Sudhof TC, Rizo J. Synaptic vesicle exocytosis. Cold Spring Harb Perspect Biol 2011; 3(12).

174. Ungar D, Hughson FM. SNARE protein structure and function. Annu Rev Cell Dev Biol 2003; 19:493-517.

175. Puri N, Roche PA. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. Proc Natl Acad Sci U S A 2008; 105(7):2580-2585.

176. Woska JR, Jr., Gillespie ME. SNARE complex-mediated degranulation in mast cells. J Cell Mol Med 2011.

177. Sander LE, Frank SP, Bolat S et al. Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells. Eur J Immunol 2008; 38(3):855-863.

178. Woska JR, Jr., Gillespie ME. Small-interfering RNA-mediated identification and regulation of the ternary SNARE complex mediating RBL-2H3 mast cell degranulation. Scand J Immunol 2011; 73(1):8-17.

179. Blank U, Madera-Salcedo IK, Danelli L et al. Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells. Front Immunol 2014; 5:453.

180. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. Blood 2011; 118(1):9-18.

181. Moon TC, Befus AD, Kulka M. Mast cell mediators: their differential release and the secretory pathways involved. Front Immunol 2014; 5:569.

182. De Matteis MA, Luini A. Exiting the Golgi complex. Nat Rev Mol Cell Biol 2008; 9(4):273-284.

183. Hepp R, Puri N, Hohenstein AC, Crawford GL, Whiteheart SW, Roche PA. Phosphorylation of SNAP-23 regulates exocytosis from mast cells. J Biol Chem 2005; 280(8):6610-6620.

184. Vaidyanathan VV, Puri N, Roche PA. The last exon of SNAP-23 regulates granule exocytosis from mast cells. J Biol Chem 2001; 276(27):25101-25106.

185. Frank SP, Thon KP, Bischoff SC, Lorentz A. SNAP-23 and syntaxin-3 are required for chemokine release by mature human mast cells. Mol Immunol 2011; 49(1-2):353-358.

186. Tiwari N, Wang CC, Brochetta C et al. VAMP-8 segregates mast cell-preformed mediator exocytosis from cytokine trafficking pathways. Blood 2008; 111(7):3665-3674.

187. Murray RZ, Kay JG, Sangermani DG, Stow JL. A role for the phagosome in cytokine secretion. Science 2005; 310(5753):1492-1495.

188. Stow JL, Murray RZ. Intracellular trafficking and secretion of inflammatory cytokines. Cytokine Growth Factor Rev 2013; 24(3):227-239.

189. Hathcock JN, Shao A. Risk assessment for glucosamine and chondroitin sulfate. Regul Toxicol Pharmacol 2007; 47(1):78-83.

190. Andermann G, Dietz M. The influence of the route of administration on the bioavailability of an endogenous macromolecule: chondroitin sulphate (CSA). Eur J Drug Metab Pharmacokinet 1982; 7(1):11-16.

191. Kirshenbaum AS, Akin C, Wu Y et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. Leuk Res 2003; 27:677-682.

192. Kempuraj D, Papadopoulou NG, Lytinas M et al. Corticotropin-releasing hormone and its structurally related urocortin are synthesized and secreted by human mast cells. Endocrinology 2004; 145:43-48.

193. Carroll G. Measurement of sulphated glycosaminoglycans and proteoglycan fragments in arthritic synovial fluid. Ann Rheum Dis 1989; 48(1):17-24.

194. Pomin VH, Mulloy B. Glycosaminoglycans and Proteoglycans. Pharmaceuticals (Basel) 2018; 11(1).

195. Yi NY, Newman DR, Zhang H, Morales JH, Sannes PL. Heparin and LPS-induced COX-2 expression in airway cells: a link between its anti-inflammatory effects and GAG sulfation. Exp Lung Res 2015; 41(9):499-513.

196. Ghosh TK, Eis PS, Mullaney JM, Ebert CL, Gill DL. Competitive, reversible and potent antagonism of inositol 1, 4, 5-trisphosphate-activated calcium release by heparin. J Biol Chem 1988; 263:11075-11079.

197. Ahmed T, Syriste T, Mendelssohn R et al. Heparin prevents antigen-induced airway hyperresponsiveness: Interference with IP_3-mediated mast cell degranulation. J Appl Physiol 1994; 76:893-901.

198. Pendl GG, Prieschl EE, Thumb W, Harrer NE, Auer M, Baumruker T. Effects of phosphatidylinositol-3-kinase inhibitors on degranulation and gene induction in allergically triggered mouse mast cells. Int Arch Allergy Immunol 1997; 112(4):392-399.

199. Stabler TV, Huang Z, Montell E, Verges J, Kraus VB. Chondroitin sulphate inhibits NF-kappaB activity induced by interaction of pathogenic and damage associated molecules. Osteoarthritis Cartilage 2017; 25(1):166-174.

200. Zhao Y, Zhang D, Wang S et al. Holothurian glycosaminoglycan inhibits metastasis and thrombosis via targeting of nuclear factor-kappaB/tissue factor/Factor Xa pathway in melanoma B16F10 cells. PLoS ONE 2013; 8(2):e56557.

201. Spratte J, Meyer zu SH, Endlich N, Zygmunt M, Fluhr H. Heparin inhibits TNF-alpha signaling in human endometrial stromal cells by interaction with NF-kappaB. Mol Hum Reprod 2013; 19(4):227-236.

202. Xu CX, Jin H, Chung YS et al. Chondroitin sulfate extracted from ascidian tunic inhibits phorbol ester-induced expression of Inflammatory factors VCAM-1 and COX-2 by blocking NF-kappaB activation in mouse skin. J Agric Food Chem 2008; 56(20):9667-9675.

203. Hecht I, Hershkoviz R, Shivtiel S et al. Heparin-disaccharide affects T cells: inhibition of NF-kappaB activation, cell migration, and modulation of intracellular signaling. J Leukoc Biol 2004; 75(6):1139-1146.

204. Lee JH, Lee J, Seo GH, Kim CH, Ahn YS. Heparin inhibits NF-kappaB activation and increases cell death in cerebral endothelial cells after oxygen-glucose deprivation. J Mol Neurosci 2007; 32(2):145-154.

205. Li X, Zheng Z, Li X, Ma X. Unfractionated heparin inhibits lipopolysaccharide-induced inflammatory response through blocking p38 MAPK and NF-kappaB activation on endothelial cell. Cytokine 2012; 60(1):114-121.

206. Miller AM. Role of IL-33 in inflammation and disease. J Inflamm (Lond) 2011; 8(1):22.

207. Attanasio M, Gori AM, Giusti B et al. Cytokine gene expression in human LPS- and IFNgamma-stimulated mononuclear cells is inhibited by heparin. Thromb Haemost 1998; 79(5):959-962.

208. Zeng D, Prosperini G, Russo C et al. Heparin attenuates symptoms and mast cell degranulation induced by AMP nasal provocation. J Allergy Clin Immunol 2004; 114(2):316-320.

209. Baram D, Rashkovsky M, Hershkoviz R et al. Inhibitory effects of low molecular weight heparin on mediator release by mast cells: preferential inhibition of cytokine production and mast cell-dependent cutaneous inflammation. Clin Exp Immunol 1997; 110(3):485-491.

210. Hirsh J, Levine MN. Low molecular weight heparin. Blood 1992; 79:1-17.

211. Gori AM, Attanasio M, Gazzini A et al. Cytokine gene expression and production by human LPS-stimulated mononuclear cells are inhibited by sulfated heparin-like semi-synthetic derivatives. J Thromb Haemost 2004; 2(9):1657-1662.

212. Shastri MD, Stewart N, Eapen M et al. Opposing effects of low molecular weight heparins on the release of inflammatory cytokines from peripheral blood mononuclear cells of asthmatics. PLoS ONE 2015; 10(3):e0118798.

213. Murray RZ, Wylie FG, Khromykh T, Hume DA, Stow JL. Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis Factor-alpha. J Biol Chem 2005; 280(11):10478-10483.

214. Pagan JK, Wylie FG, Joseph S et al. The t-SNARE Syntaxin 4 Is Regulated during Macrophage Activation to Function in Membrane Traffic and Cytokine Secretion. Curr Biol 2003; 13:156-160.

215. Coombe DR. Biological implications of glycosaminoglycan interactions with haemopoietic cytokines. Immunol Cell Biol 2008; 86(7):598-607.

216. Tanino Y, Coombe DR, Gill SE et al. Kinetics of chemokine-glycosaminoglycan interactions control neutrophil migration into the airspaces of the lungs. J Immunol 2010; 184(5):2677-2685.

217. Griesenauer B, Paczesny S. The ST2/IL-33 Axis in Immune Cells during Inflammatory Diseases. Front Immunol 2017; 8:475.

218. Egea J, Garcia AG, Verges J, Montell E, Lopez MG. Antioxidant, antiinflammatory and neuroprotective actions of chondroitin sulfate and proteoglycans. Osteoarthritis Cartilage 2010; 18 Suppl 1:S24-S27.

219. Campo GM, Avenoso A, Campo S, Ferlazzo AM, Calatroni A. Chondroitin sulphate: antioxidant properties and beneficial effects. Mini Rev Med Chem 2006; 6(12):1311-1320.

220. Albertini R, De LG, Passi A, Moratti R, Abuja PM. Chondroitin-4-sulfate protects high-density lipoprotein against copper-dependent oxidation. Arch Biochem Biophys 1999; 365(1):143-149.

221. Lee JY, Lee SH, Kim HJ et al. The preventive inhibition of chondroitin sulfate against the CCl4-induced oxidative stress of subcellular level. Arch Pharm Res 2004; 27(3):340-345.

222. Ha BJ, Lee JY. The effect of chondroitin sulfate against CCl4-induced hepatotoxicity. Biol Pharm Bull 2003; 26(5):622-626.

223. Canas N, Valero T, Villarroya M et al. Chondroitin sulfate protects SH-SY5Y cells from oxidative stress by inducing heme oxygenase-1 via phosphatidylinositol 3-kinase/Akt. J Pharmacol Exp Ther 2007; 323(3):946-953.

224. Zou SC, Jiang J, Song J. IL-33 induced inflammation exacerbated the development of chronic obstructive pulmonary disease through oxidative stress. Eur Rev Med Pharmacol Sci 2018; 22(6):1758-1764.

225. Afonina IS, Muller C, Martin SJ, Beyaert R. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. Immunity 2015; 42(6):991-1004.

226. Lefrancais E, Roga S, Gautier V et al. IL-33 is processed into mature bioactive forms by neutrophil elastase and cathepsin G. Proc Natl Acad Sci U S A 2012; 109(5):1673-1678.

227. Lefrancais E, Duval A, Mirey E et al. Central domain of IL-33 is cleaved by mast cell proteases for potent activation of group-2 innate lymphoid cells. Proc Natl Acad Sci U S A 2014; 111(43):15502-15507.

228. Meier HL, Schulman ES, Heck LW, MacGlashan D, Newball HH, Kaplan AP. Release of elastase from purified human lung mast cells and basophils. Identification as a Hageman factor cleaving enzyme. Inflammation 1989; 13(3):295-308.

229. Ribatti D, Nico B, Finato N, Crivellato E, Beltrami CA. Co-localization of tryptase and cathepsin-G in mast cells in cutaneous mastocytosis. Cancer Lett 2009; 279(2):209-212.

230. Vignola AM, Bonanno A, Mirabella A et al. Increased levels of elastase and alpha1-antitrypsin in sputum of asthmatic patients. Am J Respir Crit Care Med 1998; 157(2):505-511.

231. Marossy K. Interaction of the chymotrypsin- and elastase-like enzymes of the human granulocyte with glycosaminoglycans. Biochim Biophys Acta 1981; 659(2):351-361.

232. Baici A, Salgam P, Fehr K, Boni A. Inhibition of human elastase from polymorphonuclear leucocytes by a glycosaminoglycan polysulfate (Arteparon). Biochem Pharmacol 1980; 29(12):1723-1727.

233. Tanaka S. Targeting CD44 in mast cell regulation. Expert Opin Ther Targets 2010; 14(1):31-43.

234. Takano H, Nakazawa S, Shirata N et al. Involvement of CD44 in mast cell proliferation during terminal differentiation. Lab Invest 2009; 89(4):446-455.

235. Fukui M, Whittlesey K, Metcalfe DD, Dastych J. Human mast cells express the hyaluronic-acid-binding isoform CD44 and adhere to hyaluronic acid. Clin Immunol 2000; 94:173-178.

236. Culty M, Nguyen HA, Underhill CB. The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. J Cell Biol 1992; 116(4):1055-1062.

237. Hurt-Camejo E, Rosengren B, Sartipy P, Elfsberg K, Camejo G, Svensson L. CD44, a cell surface chondroitin sulfate proteoglycan, mediates binding of interferon-gamma and some of its biological effects on human vascular smooth muscle cells. J Biol Chem 1999; 274(27):18957-18964.

238. Fujimoto T, Kawashima H, Tanaka T et al. CD44 binds a chondroitin sulfate proteoglycan, aggrecan. Int Immunol 2001; 13(3):359-366.

239. Toyama-Sorimachi N, Sorimachi H, Tobita Y et al. A novel ligand for CD44 is serglycin, a hematopoietic cell lineage-specific proteoglycan. Possible involvement in lymphoid cell adherence and activation. J Biol Chem 1995; 270(13):7437-7444.

240. Dong F, Eibach M, Bartsch JW et al. The metalloprotease-disintegrin ADAM8 contributes to temozolomide chemoresistance and enhanced invasiveness of human glioblastoma cells. Neuro Oncol 2015; 17(11):1474-1485.

241. Guhl S, Lee HH, Babina M, Henz BM, Zuberbier T. Evidence for a restricted rather than generalized stimulatory response of skin-derived human mast cells to substance P. J Neuroimmunol 2005; 163(1-2):92-101.

242. Muller T, Mizumoto S, Suresh I et al. Loss of dermatan sulfate epimerase (DSE) function results in musculocontractural Ehlers-Danlos syndrome. Hum Mol Genet 2013; 22(18):3761-3772.

243. Clarke LA, Russell CS, Pownall S et al. Murine mucopolysaccharidosis type I: targeted disruption of the murine alpha-L-iduronidase gene. Hum Mol Genet 1997; 6(4):503-511.

244. Boat TF, Cheng PW. Epithelial cell dysfunction in cystic fibrosis: implications for airways disease. Acta Paediatr Scand Suppl 1989; 363:25-29.

245. Wang JY, Roehrl MH. Glycosaminoglycans are a potential cause of rheumatoid arthritis. Proc Natl Acad Sci U S A 2002; 99(22):14362-14367.

246. De PA, Malfait F. The Ehlers-Danlos syndrome, a disorder with many faces. Clin Genet 2012; 82(1):1-11.

247. Mao JR, Bristow J. The Ehlers-Danlos syndrome: on beyond collagens. J Clin Invest 2001; 107(9):1063-1069.

248. Seneviratne SL, Maitland A, Afrin L. Mast cell disorders in Ehlers-Danlos syndrome. Am J Med Genet C Semin Med Genet 2017; 175(1):226-236.

249. Fikree A, Aktar R, Grahame R et al. Functional gastrointestinal disorders are associated with the joint hypermobility syndrome in secondary care: a case-control study. Neurogastroenterol Motil 2015; 27(4):569-579.

250. Abonia JP, Wen T, Stucke EM et al. High prevalence of eosinophilic esophagitis in patients with inherited connective tissue disorders. J Allergy Clin Immunol 2013; 132(2):378-386.

251. Morgan AW, Pearson SB, Davies S, Gooi HC, Bird HA. Asthma and airways collapse in two heritable disorders of connective tissue. Ann Rheum Dis 2007; 66(10):1369-1373.

252. Louisias M, Silverman S, Maitland A. Prevalence of allergic disorders and mast cell activation syndrome in patients with Ehlers Danlos syndrome. Annals of allergy, asthma & immunology: official publication of the American College of Allergy, Asthma, & Immunology 2013; 111(5):A12-A13.

253. Luzgina NG, Potapova OV, Shkurupiy VA. Structural and functional peculiarities of mast cells in undifferentiated connective tissue dysplasia. Bull Exp Biol Med 2011; 150(6):676-678.

254. Theoharides TC, Valent P, Akin C. Mast Cells, Mastocytosis, and Related Disorders. N Engl J Med 2015; 373(2):163-172.

255. Sismanopoulos N, Delivanis DA, Alysandratos KD et al. Mast cells in allergic and inflammatory diseases. Curr Pharm Des 2012; 18(16):2261-2277.

256. Theoharides TC, Sismanopoulos N, Delivanis DA, Zhang B, Hatziagelaki EE, Kalogeromitros D. Mast cells squeeze the heart and stretch the gird: Their role in atherosclerosis and obesity. Trends Pharmacol Sci 2011; 32(9):534-542.

257. Okayama Y, Benyon RC, Rees PH, Lowman MA, Hillier K, Church MK. Inhibition profiles of sodium cromoglycate and nedocromil sodium on mediator release from mast cells of human skin, lung, tonsil, adenoid and intestine. Clin Exp Allergy 1992; 22:401-409.

258. Weng Z, Zhang B, Asadi S et al. Quercetin is more effective than cromolyn in blocking human mast cell cytokine release and inhibits contact dermatitis and photosensitivity in humans. PloS One 2012; 7(3):e33805-k.

259. Vieira Dos SR, Magerl M, Martus P et al. Topical sodium cromoglicate relieves allergen- and histamine-induced dermal pruritus. Br J Dermatol 2010; 162(3):674-676.

260. Oka T, Kalesnikoff J, Starkl P, Tsai M, Galli SJ. Evidence questioning cromolyn's effectiveness and selectivity as a 'mast cell stabilizer' in mice. Lab Invest 2012; 92(10):1472-1482.

261. Middleton EJ, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. Pharmacol Rev 2000; 52(4):673-751.

262. Kempuraj D, Tagen M, Iliopoulou BP et al. Luteolin inhibits myelin basic protein-induced human mast cell activation and mast cell dependent stimulation of Jurkat T cells. Br J Pharmacol 2008; 155(7):1076-1084.

263. Corcoran MP, McKay DL, Blumberg JB. Flavonoid basics: chemistry, sources, mechanisms of action, and safety. J Nutr Gerontol Geriatr 2012; 31(3):176-189.

264. Asadi S, Theoharides TC. Corticotropin-releasing hormone and extracellular mitochondria augment IgE-stimulated human mast-cell vascular endothelial growth factor release, which is inhibited by luteolin. J Neuroinflam 2012; 9(1):85.

265. Kandere-Grzybowska K, Kempuraj D, Cao J, Cetrulo CL, Theoharides TC. Regulation of IL-1-induced selective IL-6 release from human mast cells and inhibition by quercetin. Br J Pharmacol 2006; 148:208-215.

266. Weng Z, Patel AB, Panagiotidou S, Theoharides TC. The novel flavone tetramethoxyluteolin is a potent inhibitor of human mast cells. J Allergy Clin Immunol 2015; 135(4):1044-1052.

267. Kimata M, Shichijo M, Miura T, Serizawa I, Inagaki N, Nagai H. Effects of luteolin, quercetin and baicalein on immunoglobulin E-mediated mediator release from human cultured mast cells. Clin Exp Allergy 2000; 30(4):501-508.

268. Kempuraj D, Madhappan B, Christodoulou S et al. Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells. Br J Pharmacol 2005; 145:934-944.

269. Vliagoftis H, Boucher WS, Mak LL, Theoharides TC. Inhibition of mast cell secretion by oxidation products of natural polyamines. Biochem Pharmacol 1992; 43:2237-2245.

270. Sakai S, Akiyama H, Sato Y et al. Chondroitin sulfate intake inhibits the IgE-mediated allergic response by down-regulating TH2 responses in mice. J Biol Chem 2006.

271. Lauder RM. Chondroitin sulphate: a complex molecule with potential impacts on a wide range of biological systems. Complement Ther Med 2009; 17(1):56-62.

272. Besterman JM, Low RB. Endocytosis: a review of mechanisms and plasma membrane dynamics. Biochem J 1983; 210(1):1-13.

273. Dutta D, Donaldson JG. Search for inhibitors of endocytosis: Intended specificity and unintended consequences. Cell Logist 2012; 2(4):203-208.

274. Ivanov AI. Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? Methods Mol Biol 2008; 440:15-33.

275. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL. Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFalpha. J Cell Biol 2007; 178(1):57-69.

276. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986; 883(2):173-177.