Regulation of Interleukin-1β and Tumor Necrosis Factor Secretion from Human Mast Cell

A thesis submitted by

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ABSTRACT

Mast cells are haematopoietically-derived tissue immune cells that participate in allergy, immunity and inflammation through secretion of numerous pro-inflammatory mediators. The peptide substance P (SP) and the cytokines interleukin (IL)-1β and tumor necrosis factor (TNF) have been implicated in inflammatory processes. Here we report that IL-33, a member of the IL-1 family of cytokines, together with SP markedly increase IL-1β and TNF gene expression in cultured human LAD2 and primary mast cells derived from umbilical cord blood. SP and IL-33 in combination also greatly stimulate IL-1β and TNF secretion. Two different Neurokinin-1 (NK-1) receptor antagonists and a ST2 receptor-neutralizing antibody inhibit IL-1β and TNF secretion stimulated by SP and IL-33. Additionally, NK-1 siRNA and ST2 siRNA decrease TNF secretion when stimulated by SP and IL-33 in cultured human mast cells. Surprisingly, NK-1 antagonists also inhibit IL-1β and TNF secretion when stimulated only by IL-33; ST2 receptor reduction also decreases SP-stimulated TNF secretion, suggesting an interaction between NK-1 and ST2 receptors. Additionally, IL-33 increases the expression of NK-1 gene and surface protein expression, as well as phosphorylation of IKβ-α. Methoxyluteolin inhibits IL-1β and TNF gene expression and secretion, as well as phosphorylation of p-IKB-α stimulated by SP and IL-33. These findings identify a unique amplification process of IL-1β and TNF synthesis and secretion via interaction of NK-1 and ST2 receptors inhibitable by methoxyluteolin.

We also investigated the secretion of IL-1β from cultured human mast cells and its regulation by the NLRP3 inflammasome, which is crucial in inflammatory diseases.

We found that in addition to increasing IL-1β synthesis, SP and IL-33 also increase caspase-1 gene expression and pro-IL-1β protein expression, as well as caspase-1 activity in the supernatant fluids. Interestingly, active caspase-1 is present in unstimulated cultured human mast cells and is secreted after stimulation, suggesting an alternative regulation of NLRP3 inflammasome.

Inflammatory responses are often characterized by elevated levels of cytokines, but the complex interplay among peptides and cytokines is not often considered. Here we report that the cytokine IL-33 administered in combination with the pro-inflammatory peptide SP causes a marked increase of IL-1β and TNF synthesis and secretion from cultured human mast cells. These responses are mediated via the activation of the SP receptor, NK-1, and the IL-33 receptor, ST2, and can be inhibited by the natural flavonoid methoxyluteolin. Our findings reveal novel interactions that increase the understanding of inflammation and offer new directions for the development of anti-inflammatory drugs.

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LIST OF ABBREVIATIONS

AP-1	Activator protein-1
ASC	Apoptosis-associated speck-like protein containing CARD
ATP	Adenosine triphosphate
CAPS	Cryoprin-Associated periodic Syndromes
CARD	Caspase activation and recruitment domain
CCL2	C-C motif chemokine ligand 2
CGRP	Calcitonin gene-related peptide
CLR	C-type lectin receptor
COX-2	Cyclooxygenase 2
CRH	Corticotropin-releasing hormone
Cromolyn	Disodium cromoglycate
CTMC	Connective tissue mast cell
DAMP	Danger-associated molecular pattern
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FcεRI	High affinity IgE receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
HaCaT	Human keratinocyte cell line
hCBMCs	Human umbilical cord blood-derived cultured mast cells
HMC-1	Human mast cell-1

HSC	Haematopoietic stem cells
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	IL-1R kinase
JNK	c-Jun N-terminal kinase
LAD2	Laboratory of allergic diseases 2
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LTs	Leukotrienes
MAPK	Mitogen-activated protein kinase
mBMMC	Mouse bone marrow-derived cultured mast cells
MMC	Mucosal mast cell
MSU	Monosodium urate crystals
NACHT	Central nucleotide domain
NF-κB	Nuclear factor-kappa B
NFKB1	Gene encoding NF-κB p50 subunit
NGF	Nerve growth factor
NK	Neurokinin
NLRP3	Nod-like receptor pyrin domain containing protein 3
NO	Nitric oxide

NOD	Nucleotide-binding oligomerization domain
NT	Neurotensin
PAMP	Pathogen-associated molecular pattern
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PI3K	Phosphoinositide 3-kinases
PRR	Pattern recognition receptor
PYD	Pyrin domain
qRT-PCR	Quantitative real time-polymerase chain reaction
RELA	Gene encoding NF-κB p65 subunit
rhSCF	Recombinant human stem cell factor
RLR	RIG-I-like receptor
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SNARE	Soluble NSF attachment protein receptor
SP	Substance P
TGFβ	Transforming growth factor β
TLR	Toll-like receptor
TNF	Tumor necrosis factor-α
TRAF	TNF receptor-associated factor
Treg	T regulatory cells
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

1.1 Mast Cells and Inflammation

1.1.1 Mast Cell Development

Mast cells are haematopoietically-derived immune cells that take their origin from the bone marrow and mature in the vascularized tissues [1-3]. These cells were first identified by Paul Ehrlich in 1878, who was awarded the Nobel Prize in Physiology or Medicine in 1908.

As in representative Figure 1.1, haematopoietic stem cells (HSC) from the bone marrow enter the bloodstream and migrate to the vascularized tissues, where they differentiate into connective tissue mast cells (CTMC) and mucosal mast cells (MMC) following the instructive signals of tissue-specific growth factors and cytokines [2, 3]. Presence and exposure to stem cell factor (SCF), which is the ligand of the tyrosine kinase c-kit receptor, and interleukin (IL)-3 largely affect the maturation of mast cells [4-6]. Moreover, IL-4, which is another crucial regulator of mast cell development, together with SCF increase proliferation and mediator secretion of human intestinal mast cells [7].

1.1.2 Mast Cell Mediators

Mast cells participate in allergy, innate and acquired immunity [8-10], autoimmunity [11], and inflammation [12-14] via secretion of various mediators. Mast cells typically reside in tissues with close proximity to the mucosal lining including respiratory tract, intestines and blood vessels [13]. Depending on the localization and role, mast cells greatly differ in the size and content of their granules, as well as in the cytokine and receptor expression, since they can produce and release many different mediators [7, 15-18]. Upon activation, mast

cells rapidly release preformed mediators through degranulation, as well as the delayed secretion of newly synthesized cytokines, chemokines and growth factors [19] (Figure 1.2).

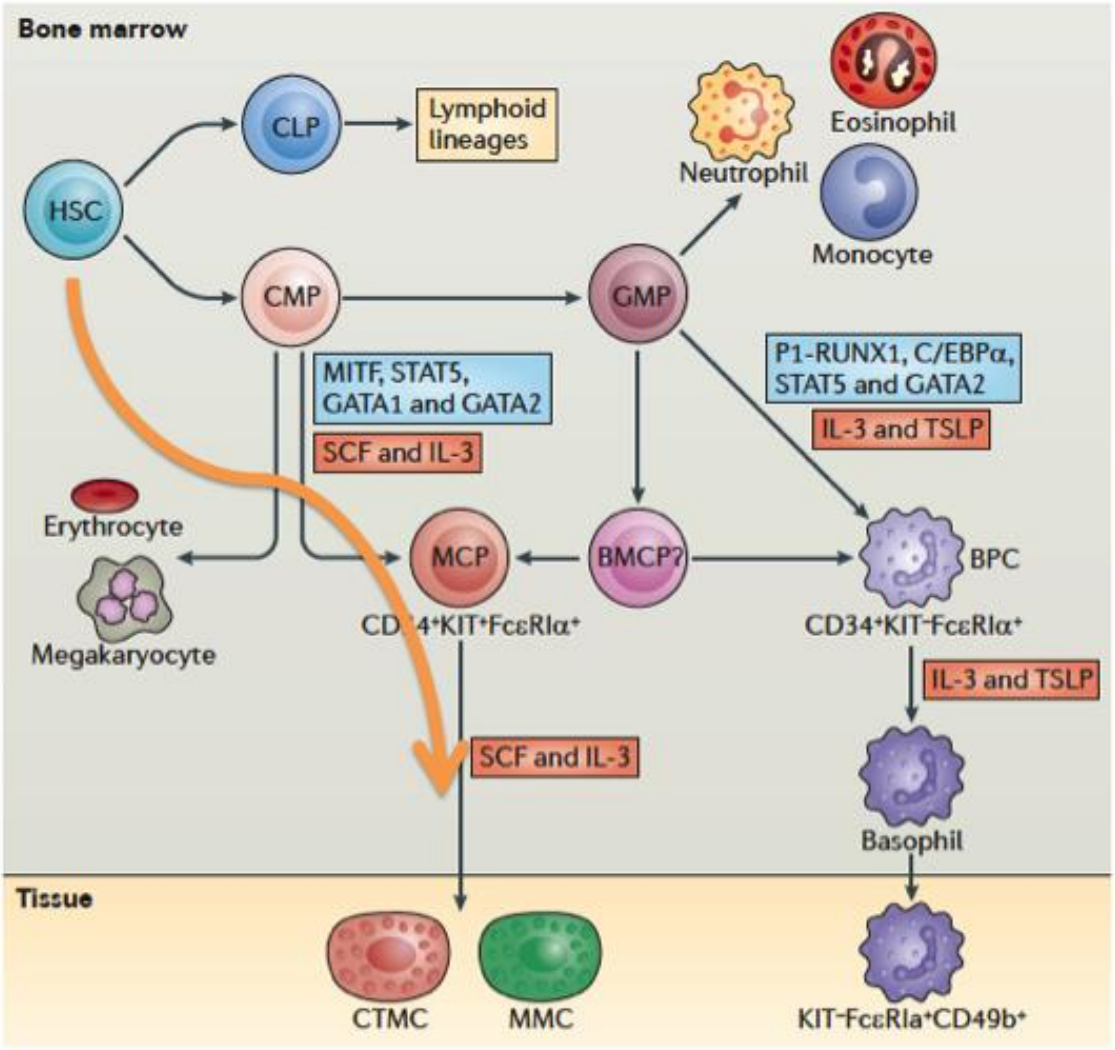

Figure 1.1. Schematic representation of mast cell maturation. *Adopted and modified from Voehringer, D et al. Nature Reviews Immunology, May 2013*

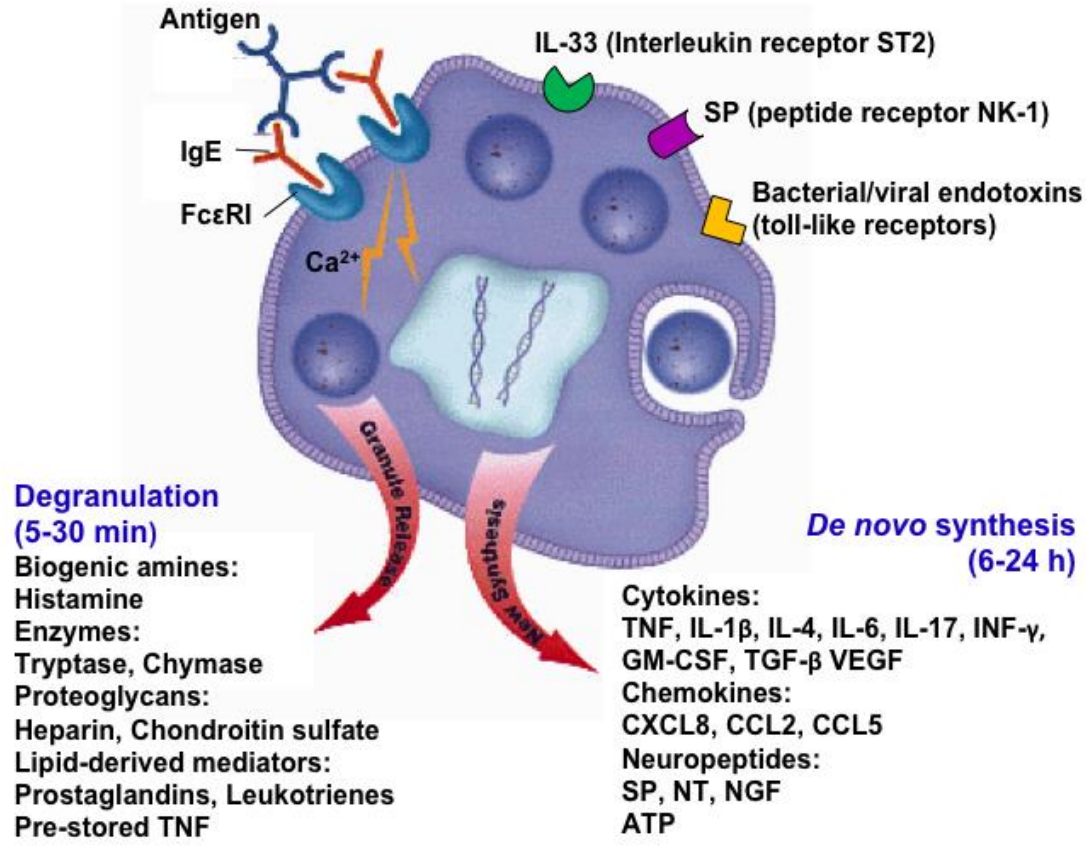

Figure 1.2. Schematic representation of mast cell degranulation and *de novo* synthesis of various mediators. *Adopted from Theoharides Lab.*

Mast cell granules contain various preformed mediators, including biogenic amines (histamine and serotonin), enzymes (β-hexosaminidase, tryptase and chymase), proteoglycans (heparin, chondroitin sulfate and hyaluronic acid), as well as the preformed cytokine tumor necrosis factor (TNF) [20], which can be released within 5-30 min upon stimulation [21]. Mast cell activation also induces *de novo* synthesis and secretion (6-24 hours later) of various cytokines and chemokines [22], including TNF, IL-1β, IL-4, IL-6, IL-17, interferon-γ, IL-8 (or CXCL8) and chemokine (C-C motif) ligand 2 (CCL2), as well as growth factors such as SCF, granulocyte-macrophage colony-stimulating factor (GM-CSF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF)

[18]. Additionally, mast cell granule content varies based on the type of mast cell. For instance, both connective tissue mast cells and mucosal mast cells contain tryptase, but only connective tissue mast cells also contain chymase [23].

1.1.3 Allergic and Non-Allergic Stimulation of Mast Cells

Mast cells are essential for the generation of the allergic reactions, which are mediated by immunoglobulin E (IgE) [24-27]. They are activated through the classic pathway, which signals via crosslinking of the immunoglobulin (IgE) and high affinity IgE receptor (FcεRI) that is expressed on the mast cell surface [28, 29]. B cells produce IgE in response to the exogenous exposure to antigen that binds to the FcεRI receptors on mast cell surfaces [30]. Additionally, IgE can be produced on specific tissues, like lungs, where it initiates local allergic reactions [31, 32]. Re-exposure to the specific antigen leads to the crosslinking of FcεRI receptors by the IgE-bound antigen, resulting in rapid secretion of preformed mediators [33]; this process is called mast cell degranulation. There is a strong correlation between the IgE levels and the allergic symptoms [31, 34, 35]. The degranulation process involves translocation of cytoplasmic granules along microtubules towards the plasma membrane followed by the calcium-dependent assembly of "docking proteins" that belong to the same family as the Soluble NSF Attachment Proteins (SNAREs) [36], resulting in membrane fusion and granule exocytosis [21].

In addition to stimulation via FcεRI (allergic response), mast cells are also activated by neuropeptides such as substance P (SP) [37, 38] and neurotensin (NT) [39-41] that result in selective release of mediators [18] (inflammatory response).

Neuropeptides like SP, corticotropin releasing hormone (CRH), calcitonin gene-related peptide (CGRP) and NT released from peripheral sensory neurons stimulate mast cells [18, 42] via binding to their G protein-coupled receptors (GPCR) [43-45] that are expressed on the mast cell surface [46].

Particularly, SP can activate mast cells through the neurokinin 1 receptor (NK-1R), which leads to nuclear factor-kappa B (NF-κB) activation as well as JNK kinase signaling [47] and subsequent cytokine production [48]. Increased levels of SP have been associated with psoriasis [49], mastocytosis [50], rheumatoid arthritis [51] and other inflammatory diseases [52, 53].

Cytokines, such as IL-33, can stimulate mast cells as well. IL-33 was discovered as a main ligand to ST2 (IL-1R4) receptor, which is mostly expressed on the surface mast cells, epithelial cells and fibroblasts [54]. The ST2 receptor is found in either the transmembrane ST2L form, which is the more abundant form, or in the cytoplasm as the soluble sST2 form, which may be acting as decoy by binding and neutralizing IL-33 [54]. The receptor complex is comprised of the ST2 and IL-1 receptor accessory protein [55]. IL-33 binding recruits the IL-1RAcP co-receptor, the adaptor protein MyD88, along with the associated protein IL-1R kinase (IRAK). ST2 activation leads to stimulation of the mitogen-activated protein kinase (MAPK) via TNF receptor-associated factor 6 (TRAF6), which can signal the activator protein-1 (AP-1) via c-Jun N-terminal kinases (JNKs). TRAF6 can also activate NF-κB, resulting in its nuclear translocation and pro-inflammatory gene transcription [56].

1.1.4 Mast cells in inflammation

In addition to IgE-mediated allergic reactions, mast cells s also participate in innate and acquired immunity [9, 57], autoimmunity [11] and inflammation [58]. Mast cells are now recognized as a crucial participant in the pathogenesis of a number of systemic and brain inflammatory diseases, including asthma, autism, multiple sclerosis, interstitial cystitis, obesity and psoriasis [59-61] possibly through the "selective" release of mediators [57]. It is interesting that the diseases discussed above worsen with stress [22, 62-64] and mast cells are activated by CRH secreted under stress [65, 66]. Mast cells uniquely store preformed TNF [20], which is rapidly released upon stimulation and in turn recruits and affects activation of T cells [67]. Mast cell-derived VEGF increases local vascular permeability and promotes angiogenesis [68]. Due to the great diversity of the cell content and their distribution in various tissues, mast cells actively interact with their surroundings. Therefore, mast cells play an important role in the generation and propagation of various immune and inflammatory responses.

1.2 Mast Cells and Pathogenesis of Psoriasis

Psoriasis is a chronic autoimmune skin disease affecting approximately 2-3% of the world's population, of which 35% have moderate to severe psoriasis. The annual cost of psoriasis was estimated at 11 billion USD in 2013 [69, 70]. The conventional treatments for the management of mild psoriasis include the topical application of glucocorticosteroids and Vitamin D analogs [71, 72]. Moderate psoriasis is usually treated with a combination of these and phototherapy, such as ultraviolet A/B to inhibit epidermal keratinocyte proliferation. Severe psoriasis, resistant to topical drugs or

phototherapy, is usually treated with a combination of retinoids with either methotrexate or cyclosporine, which are immunosuppressive. New biologic treatments have been developed recently, among which are inhibitors of TNF (etanercept, adalimumab, infliximab) [73, 74], IL-12 and IL-23 [75] (ustekinumab), as well as of IL-17A (secukinumab) [76].

Conventional therapies like methotrexate and cyclosporine are cost effective, but require monitoring for toxicities. New biologics, especially anti-TNF therapy, have dramatically changed the management of psoriasis, albeit at additional cost [77]. However, increasing reports indicate that anti-TNF therapy can increase the risk of opportunistic infections [78, 79], cancer [80], and paradoxical inflammation [81]. Recently anti-IL-17 therapy has been developed, but clinical studies using anti-IL-17 therapy were neither large, nor long enough to assess risks of infection or cardiovascular events [82].

Recent studies on the pathogenesis of psoriasis have revealed a genetic predisposition [83, 84] and the role of immune dysfunction [85], as a consequence of an imbalance in polarized T-helper subsets (T_h1-T_h2-T_h17) [72, 86], activation of keratinocytes [86-88] skin-resident infiltrating mast cells [38, 88-91] and other immune cells, including dendritic cells and macrophages [92]. Psoriasis is characterized by keratinocyte hyperproliferation, oxidative stress, and chronic inflammation [88, 93, 94]. IFN-γ can stimulate keratinocytes to release IL-33 [95] and IL-33 concentration is elevated in the skin of the psoriasis patents [96]. IL-33 alone and in combination with SP can induce the release of pro-inflammatory cytokines and chemoattractants such as IL-1β, IL-6, VEGF and TNF [97]. The nervous system also plays a role in psoriasis, including increased nerve fibers and their secreted neuropeptides, such as SP in psoriatic patients [98, 99]. In addition, SP has

been shown to induce IL-1β release from keratinocytes [100].

Recent studies show increased number of infiltrating mast cells in psoriatic lesional skin [90, 91, 101]. Mast cells can be activated by different triggers, such as cytokines, chemokines and neuropeptides, to release multiple mediators with potent vasodilatory, inflammatory and pruritic properties [22], including histamine, IL-1β, IL-6, IL-8, TNF and VEGF, leading to local vascular activation and subsequent immune cell recruitment [14]. Mast cells are the only immune cells that store preformed TNF [102], which could stimulate keratinocytes. Recent evidence shows that mast cells also release IL-17 [103, 104] and IL-33 [105]. We showed that SP and IL-33 have synergistic actions on increasing vascular permeability and VEGF release [47]. In addition, activated mast cells could stimulate T cells through TNF [30, 106]. In fact, mast cells can function as immunomodulatory cells [107]. Moreover, mast cells counteract Treg cell suppression and promote the development of Th17 cells involved in autoimmune diseases [108]. According to these studies, there are significant interactions between keratinocytes and mast cells (Figure 1.3). For example, activated keratinocytes could produce IL-33, which triggers mast cells to release IL-1β. IL-1β could act back on keratinocytes to release a number of other inflammatory mediators. The interactions between these two cell types would result in keratinocyte hyperproliferation and mast cell-mediated inflammation that contribute to psoriasis. Inhibiting hyperactivation of mast cells could be effective prophylactic therapy for psoriasis.

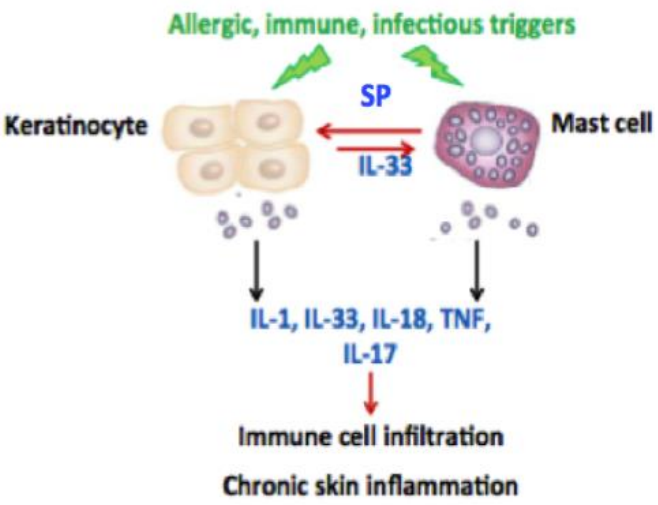

Figure 1.3 Diagrammatic representation of the proposed steps showing mast cell-keratinocyte interactions in skin inflammation and psoriasis. Triggers (such as IFN-γ and SP) stimulate synergistic cell activation and release of inflammatory mediators, as well as keratinocyte hyperproliferation and mast cell-mediated inflammation that contribute to psoriasis. *Adopted from Theoharides Lab*

1.3 Regulation of Interleukin-1β Secretion

Interleukins are crucial cytokines in the signaling and regulation of immune and inflammatory reactions [109]. The contribution of IL-1 family (IL1F) of cytokines production is well recognized in psoriasis [110]. The interleukin-1 cytokine family follows the transcriptional control as other cytokines. IL-1β is present in the cytoplasm in a biologically inactive form. It is activated by the proteolytic cleavage of caspase-1, which is also present in the cytoplasm in pro-form, and it is activated by the multiprotein complex known as NLRP3 inflammasome [111]. IL-1β activation is controlled by at least two independent signals for induction and maturation [112], which will be described later. IL-1β and IL-33 play a crucial role in the regulation of innate and adaptive immune systems [111, 113].

IL-33 is a newly identified IL1F cytokine, which is synthesized in its pro-form and can also be processed by caspase-1. However, pro-IL-33 contains the nuclear localization sequence (NLS) and can induce production of Th2-associated cytokines [114]. In addition, IL-33 can stimulate activation of mast cells with further release of several

mediators such as TNF, IL-6, prostaglandin D_2, and monocyte chemoattractant protein 1 (MCP-1) [114]. IL-33 also has synergistic effects with inflammatory neuropeptides such as SP [47]. IL-33 acts as alarmin against injury-induced stress, pathogens, or cell death by activating local immune cells [115, 116].

IL-33 is synthesized in its pro-form (30 kDa), but its processing does not appear to involve the NLRP3 inflammasome [117]. In contrast, caspase-1 cleaves pro-IL-33 into an *inactive* form [118]. Moreover, unlike pro-IL1-α, the pro-form of IL-33 is biologically active and also contains nuclear localization sequences (like pro-IL1α) allowing it to act both as an intracellular nuclear factor and as an extracellular cytokine [119]. Proteases such as calpain, cathepsin G and elastase can cleave pro-IL-33 into more potent mature forms [120, 121].

1.4 NLRP3 Inflammasome Activation

NLRP3 inflammasome is crucial in the inflammatory response of a cell since it is an upstream activator and regulator of caspase-1 and IL1F cytokines. NLRP3 has a wide range of activators: whole pathogens (Influenza virus, Sendai virus, Adenovirus), pathogen-associated molecules (bacterial pore-forming toxins, hemazoin), environmental insults (silica, asbestos, skin irritants, UV light), and endogenous danger signals (ATP, glucose, MSU, amyloid β) [117]. It is hypothesized that there is an intermediate signal that triggers NLRP3 activation due to a wide array of the stimulating molecules [122]. Therefore, activation of NLRP3 inflammasome requires two signals. The first signal is provided by microbial or endogenous molecules that activate NLRP3 and pro-IL-1β expression (signal 1), which is induced by NF-κB activation.

Microbial or endogenous molecules are recognized by immune cells via Toll- like receptors (TLRs), which belong to the family of pattern recognition receptors (PRRs). In addition to TLRs, other PRRs include nucleotide-binding oligomerization domain protein (NOD)-like receptors (NLRs) [123, 124], RIG-I-like receptors (RLRs) [125], and the cell-surface C-type lectin receptors (CLRs) [126]. PRRs signal to activate intracellular pathways mediated by NF-κB, MAPKs and interferon regulatory factors (IRFs) [127, 128]. PPRs are expressed by the immune cells and are associated with pathogen-associated molecular patterns (PAMPs) [129] of bacteria, parasites, fungus and viruses [130, 131].

NOD1 and NOD2 are cytosolic proteins that recognize bacterial peptidoglycan fragments and activate NF-κB pathway [123], while some NLRs assemble inflammasomes, leading to activation of caspase-1 which cleaves and releases IL-1β and IL-18 [132, 133].

Signal 1 of NLRP3 inflammasome activation and subsequent IL-1β involve activation of IKK complex, degradation of IKβ-α, and translocation of NF-kB to the nucleus to produce proinflammatory cytokines (Figure 1.4.)

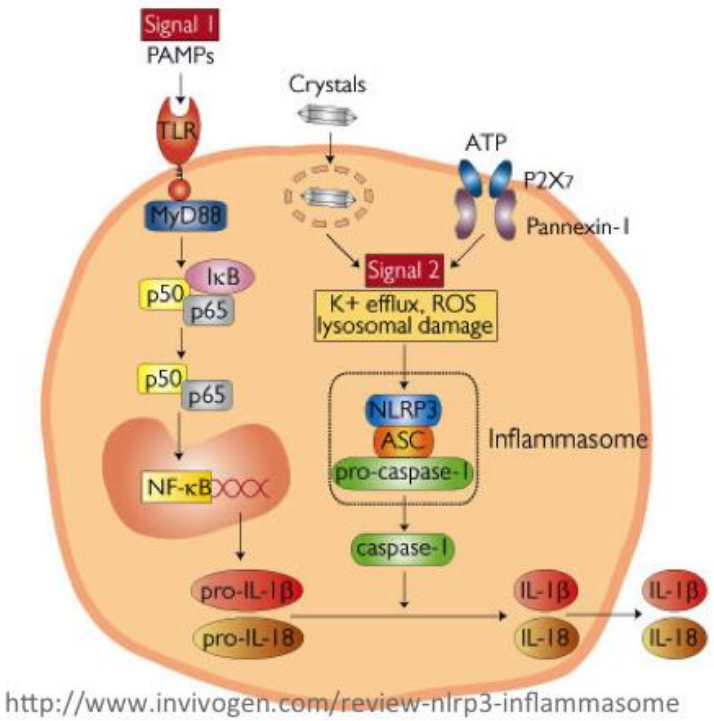

Figure 1.4 NLRP3 Inflammasome requires 2 signals for its activation. Signal 1 is stimulated by PAMPs to activate NF-κB that results in pro-inflammatory cytokine transcription. Signal 2 stimulates NLRP3 Inflammasome oligomerization to release active caspase-1 and process pro-IL-1β. *Adopted from* *http://www.invivogen.com/*

NLRP3 protein belongs to NOD-like receptor (NLR) family of cytosolic proteins and its structure is represented by a tripartite architecture containing a C-terminal region characterized by a series of LRRs (Leucine-rich repeats), a central nucleotide domain (NACHT) [134], and an N-terminal effector domain, which is pyrin domain (PYD)[109] specifically for NLRP3 protein. The LRR domain has been involved in ligand sensing and autoregulation of NLRP3, when oligomerization of the NACHT domain is believed to be crucial step in the NLRP3 activation [134, 135] (Figure 1.5).

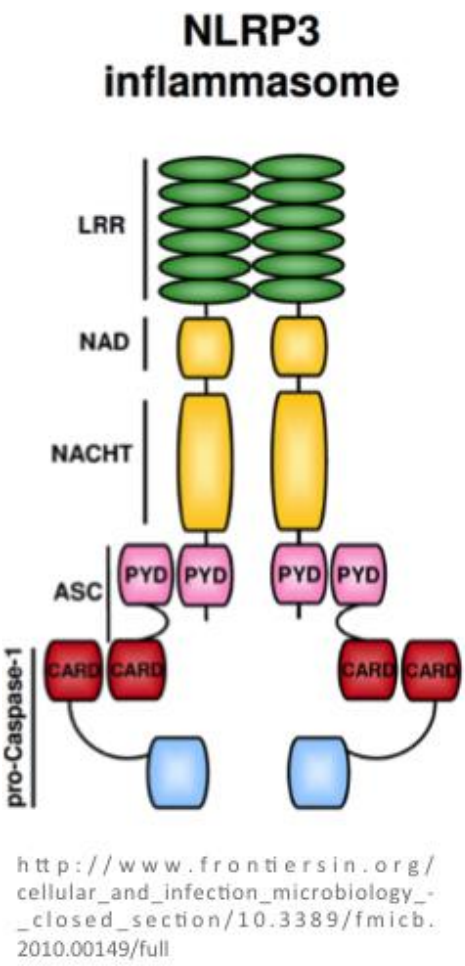

Figure 1.5 Schematic representation of NLRP3 Inflammasome. NLRP3 inflammasome consists of three proteins: (1) NLRP3, (2) ASC and (3) pro-caspase-1. *Adopted from Eitel et al. Front Microbiol. 2010*

NLRP3 inflammasome is crucial in the inflammatory response of a cell since it is an upstream activator and regulator of caspase-1 and IL-1β. The second signal initiates recruitment of the adaptor apoptosis-associated speck-like protein containing a CARD (ASC), which subsequently activates caspase-1 from its inactive pro-form. Caspase-1 activation leads to cleavage of pro-form of IL-1β and results in subsequent IL-1β activation and secretion [136] (Figure 1.4).

There are several theories suggesting potential mechanisms of cellular signaling responsible for NLRP3 activation (signal 2); they include (1) a change in the intracellular concentration of potassium and sodium ions, (2) rupture of lysosomes and (3) the

production of reactive oxygen species (ROS) [112, 136]. In some instances, IL-1β processing can happen independently of NLRP3 inflammasome [137].

NLRP3 activation and IL-β secretion were found to be essential participants in a number of pathologies. The diseases can be genetically defined syndromes like familial Mediterranean fever, Muckle-Well syndrome, and cryopyrin–associated periodic syndromes (CAPS) [138]. Some disorders are defined by NLRP3 activation by danger signals: gout, pseudogout, Alzheimer's disease, asbestosis, silicosis, and type 2 diabetes mellitus [139]. It has been reported that genetic polymorphisms of NLRP3 inflammasome are associated with psoriasis susceptibility [110]. However, no studies have investigated the actual mechanism of NLRP3 activation at the protein level in this disease.

1.5 Flavonoids

Dysregulated mast cell activation contributes not only to the pathogenesis of psoriasis, but is also implicated in mastocytosis [58, 140, 141], asthma [142, 143], atopic dermatitis [144] and autism spectrum disorders (ASD) [145]. The inhibition of mast cells and/or the mediators secreted by these cells would be advantageous for development of novel therapeutics.

There are several endogenous molecules that have mast cell inhibitory actions. For instance, nitric oxide decreases FcεRI-mediated mast cell cytokine secretion [146]. Chondroitin sulfate and heparin, the major constituents of mast cell granules, inhibit human mast cell mediator release [147]. IL-10, which is known for its anti-inflammatory properties, has been shown to inhibit IgE-triggered histamine and TNF secretion from hCBMCs [148].

Disodium cromoglycate (cromolyn) is the only clinically availble mast cell "stabilizer"; however it is ineffective for inhibition of human mast cells [149, 150]. Moreover, the side-effects associated with cromolyn treatment include tachyphylaxis [151] and contact dermatitis [152, 153].

We searched for molecules that may block as many of the pathogenic processes suspected of contributing to psoriasis as possible and at the same time have a strong safety and efficacy profile. It turns out that the natural flavone luteolin, purified from chamomile and artichoke, has several favorable characteristics: (1) anti-oxidant [154], (2) anti-inflammatory [154], (3) mast cell degranulation inhibitor [155], (4) mast cell cytokine release inhibitor [156], and (5) auto-immune T cell activation inhibitor [106, 157]. Luteolin is generally safe [158-161] and can even protect against chemically-induced liver toxicity, a common consequence of many drugs [162]. In our studies, we used methoxyluteolin, which is a derivative of luteolin and has its four hydroxyl groups replaced by methyl groups (Figure 1.6). This could minimize the possibility of any unwanted side effects in subjects with "phenol intolerance" related to luteolin, while permitting greater absorption and metabolic stability [163]. Additionally, methoxyluteolin-based local or systemic formulations could be novel treatment for psoriasis.

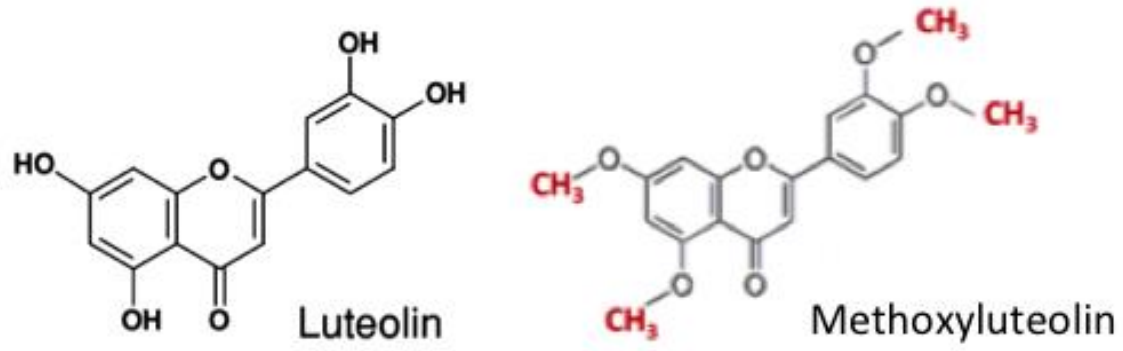

Figure 1.6 Structures of Luteolin and Methoxyluteolin. *Adopted from Theoharides Lab.*

1.6 Hypothesis

Based on the research evidence from the literature, we hypothesized that the neuropeptide substance P and interleukin (IL)-33 separately or in combination can stimulate human mast cells to secrete IL-1β and TNF, which could contribute to a potent pro-inflammatory response and that can be inhibited by the naturally occurring flavonoid methoxyluteolin.

1.7 Thesis Summary and Objectives

Previously, our laboratory reported that in addition to the allergic activation of mast cells[164, 165], SP can stimulate mast cell degranulation and release of newly-synthesized pro-inflammatory cytokines and chemokines [165, 166]. Moreover, increased levels of SP and IL-33 have been associated with psoriasis [47, 49].

In chapter 2, our studies investigated whether SP, IL-33 or their combination could stimulate the activation of human mast cells using cultured human primary umbilical cord blood-derived mast cells (hCBMCs) and the immortalized Laboratory of

Allergic Disease 2 (LAD2) human mast cell line. We determined whether SP and IL-33 could increase the gene expression and release of the pro-inflammatory cytokine TNF from human cultured mast cells. We investigated the mechanism of synergistic interaction between the neuropeptide SP and the cytokine IL-33 by assessing their effects on each other's gene and protein expression, as well as the expression of their corresponding receptors, NK-1 and ST2. Additionally, we investigated the potential interactions of these two receptors as a way to explain the synergistic effect of SP and IL-33 combination. Our findings indicate a unique interaction between NK-1 and ST2 receptors.

In chapter 2, our studies continued by looking at the signaling pathways implicated in the stimulation of human cultured mast cells with SP and IL-33. We screened ten major inflammatory signaling pathways with particular focus on the activation of two, JNK kinase and IKβ-α/NF-κB signaling. Next we investigated whether methoxyluteolin can inhibit TNF secretion from human cultured mast cells by looking at the TNF gene expression, as well as protein secretion after the stimulation with SP and IL-33. Our findings provide evidence to support the strong inhibitory effect of methoxyluteolin on mast cells activation and identify a potential mechanism of action for this inhibition via IKβ-α.

In chapter 3, we investigated the regulation of IL-1β secretion from human cultured mast cells. Our findings identified SP and IL-33 as novel stimuli of IL-1β secretion from human mast cells. We investigated whether this stimulation is regulated by the activation of the NLRP3 inflammasome by using inhibitors that would interfere with the inflammasome activation of different steps. Our findings identified the signaling

pathway of IL-1β secretion in human cultured mast cells, as well as an alternative assembly of the NLRP3 inflammasome. We also investigated whether methoxyluteolin can inhibit IL-1β secretion from human cultured mast cells by looking at the IL-1β gene expression, as well as protein secretion after the combined stimulation with SP and IL-33. Our findings indicate that methoxyluteolin has a strong inhibitory effect on IL-1β secretion, as well as on TNF. Therefore, this naturally occurring molecule may be an advantageous therapeutic target for anti-inflammatory therapy development.

In chapter 4, we investigated alternative regulation of IL-1β secretion from human mast cells by caspase-8. In has been previously reported that caspase-8 can directly process IL-1β, as well as indirectly affect IL-1β secretion by influencing the activation of the NLRP3 inflammasome [167, 168]. Therefore, we investigated IL-1β gene expression and secretion after pharmacologically inhibiting caspase-8 using pan-caspase or capsase-8 specific inhibitors. Our findings indicate how caspase-8 could regulate IL-1β secretion and the NLRP3 inflammasome activation when stimulated by SP and IL-33 in human cultured mast cells.

Chapter 2: Substance P and IL-33 together Markedly Enhance TNF Synthesis and Secretion from Human Mast Cells Mediated by their Receptor Interaction and Inhibited by Methoxyluteolin

2.1 Background

Substance (SP), a peptide originally isolated from the rat brain and characterized by Leeman and Chang [169], has been implicated in inflammatory processes [48, 52, 170-173]. SP has also been shown to stimulate mast cells to secrete histamine [37], and tumor necrosis factor (TNF) [174-176].

Mast cells are hemopoietically-derived tissue immune cells involved in allergic diseases [58], innate and acquired immunity [9], autoimmunity [11] and inflammatory responses through the release of pro-inflammatory mediators. In addition to histamine and TNF, these mediators include: IL-1β, IL-6, IL-8 and vascular endothelial growth factor (VEGF) [22, 47]. We had previously reported that SP and IL-33 in combination increase vascular permeability of the skin and VEGF release from cultured human mast cells [47]. In fact, murine mast cells derived from bone marrow secrete hemokinin-1, which is structurally related to SP, and augments IgE-stimulated mast cells in an autocrine fashion [177].

IL-33 belongs to the interleukin-1 (IL-1) family of cytokines and plays a crucial role in the regulation of the innate and adaptive immune systems [113, 178], as well as in a number of autoimmune, allergic and inflammatory diseases [179, 180]. IL-33 promotes mast cell proliferation and release of pro-inflammatory mediators[97, 181]; it also augments the effect of IgE and nerve growth factor (NGF) on HMC-1 human leukemic mast cells [182]. It is interesting that serine proteases (chymase and tryptase) secreted from mast cells generate a shorter, mature and more active form of IL-33 [183]. IL-33 had been reported to also enhance allergic responses [184] and allergic bronchoconstriction via activation of mast cells in mice [185]. IL-33 is expressed in the epidermis [186] and in the human keratinocytes [187]. Moreover, IL-33 and has been implicated in the pathogenesis of psoriasis via

keratinocyte and mast cell activation [96]. Also IL-33 was reported to be elevated in the serum of generalized psoriasis patients and correlated with high serum TNF [188]. In addition to the newly synthesized TNF secretion reported here, mast cells are the only immune cells that also store and rapidly secrete preformed TNF [8, 20, 106, 189, 190]. Given these findings we decided to investigate whether the interactions between SP and IL-33 may affect human mast cell secretion of TNF.

We previously reported that 5,7,3',4'-tetramethoxyflavone, methoxyluteolin, in which four hydroxyl groups are replaced by methyl groups, is a more potent mast cell inhibitor than 5,7,3',4'-tetrahydroxyflavonol, luteolin [191].

In this study, we report that IL-33, administered in combination with SP, potently enhances TNF synthesis and secretion in cultured human mast cells. These effects are mediated via interaction of NK-1 and ST2 receptors, and are inhibited by methoxyluteolin. These findings provide novel directions for the understanding and treatment of inflammatory diseases.

2.2 Materials and Methods

SP (S6883) was purchased from Sigma-Aldrich (St Louis, MO). Human recombinant IL-33 was obtained from R&D Systems (Minneapolis, MN). Human IgE was purchased from EMD Millipore (Billerica, MA), while anti-IgE was obtained from Sigma-Aldrich (St Loius, MO). NK-1 antagonist L-733,060 was purchased from Sigma-Aldrich (St Louis, MO) and NK-1 antagonist CP-96345 was obtained from Tocris Biosciences (Bristol, UK). ST2 neutralizing antibody and non-specific IgG antibody were purchased from R&D Systems (Minneapolis, MN). The proteasome inhibitor PS 341 was obtained from Tocris Biosciences (Bristol, UK). The flavonoids luteolin and methoxyluteolin were obtained from Pharmascience Nutrients (Clear Water, FL). Silencer® Ambion Select siRNAs targeting human NK-1 receptor and ST2 receptor, as well as scramble siRNA non-targeting control were purchased from Life Technologies (Grand Island, NY). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits were purchased from BioRad (Hercules, CA). Taqman gene expression primers/assays for *TNF* (Hs99999043_m1), *TACR1* (Hs00185530_m1), *IL1RL1* (Hs00249384_m1) and *GAPDH* endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kit for TNF (DY210) was purchased from R&D Systems (Minneapolis, MN). The Allophylocyanin (APC)-conjugated human NK-1 receptor antibody and Phycoerythrin (PE)-conjugated human ST2 receptor antibody, as well as APC-isotype and PE-isotype controls were purchased from R&D Systems (Minneapolis, MN). PathScan Inflammation Sandwich ELISA kit, IKβ-α, phospho-IKβ-α and β-actin primary antibodies were obtained from Cell Signaling Technology (Danvers, MA). Rabbit anti-human NK-1, rabbit anti-human ST2 antibodies were purchased from

Abcam (Cambridge, MA) and goat anti-human IL-1RacP was obtained from R&D Systems (Minneapolis, MN).

Culture of human mast cells

LAD2 mast cells, derived from a human mast cell leukemia [192], were kindly supplied by Dr. A Kirshenbaum (NIH, Bethesda, MD), and were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human stem cell factor (rhSCF, Stemgen), kindly supplied by Swedish Orphan Biovitrum AB (Stockholm, Sweden). Cells were maintained at 37ºC in a humidified incubator at 95% O_2/5% CO_2 atmosphere. LAD2 cells were doubling within 2 weeks in the presence of 100 ng/mL of SCF showing slow proliferation rates. Even though LAD2 cells are an immortalized proliferating cell line, this cell culture closely resembles $CD34^+$-derived primary human mast cells due to its ability to respond to SCF and express functional FcεRI receptors [192]. Cell viability was measured by Trypan blue exclusion [47] as well as by Propidium Iodide at all SP and IL-33 concentrations tested.

Human umbilical cord blood was obtained after normal deliveries in accordance with established institutional guidelines to culture primary hCBMCs [193]. 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 IL-6, 0.1% β-mercaptoethanol,

1% penicillin/streptomycin, and 100 ng/mL rhSCF. 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. hCBMCs cultured for at least 12 weeks were used for experiments. Cell viability was determined by means of trypan blue (0.4%) exclusion. Toluidine blue was used to stain for mast cell purity.

Mast Cell Treatments

LAD2 cells and/or hCBMCs were stimulated with various concentration of SP (0.01-1 μM, Sigma-Aldrich) and IL-33 (1-30 ng/mL; R&D Systems) alone or in combination. In some experiments LAD2 cells were stimulated with human IgE (1 μg/mL; EMD Millipore) overnight and then triggered with anti-IgE (10 ng/mL; Life Technologies). In some experiments, LAD2 cells were pretreated with the NK-1 antagonists L-733,060 (10 μM; Sigma-Aldrich) and CP-96345 (10 μM; Tocris Biosciences), a ST2 neutralizing antibody (0.3 μg/mL-10 ug/mL; R&D Systems) or non-specific IgG antibody (0.3 μg/mL-10 ug/mL; R&D Systems), the proteasome inhibitor PS 341 (1-50 μM; Tocris Biosciences) and methoxyluteolin (1-100 μM) (Skyherbs Lab). Silencer Select siRNA targeting either NK-1 or ST2 receptors, as well as control scramble siRNA (10-100 nM, Life Technologies) were used in Lipofectamine RNAiMAX and OPTI-MEM medium (Life Technologies) to treat LAD2 cells for 72-96 hr to inhibit gene expression of respective receptors.

TNF assays

LAD2 cells (1×10^5 cells/well) were treated with various concentration of SP (0.01-1 μM) and IL-33 (1-30 ng/mL) alone or in combination for 24 hr. Control cells were treated with the same volume of culture media alone. Supernatants were collected and assayed using TNF DuoSet ELISA kits (R&D Systems).

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

LAD2 cells (1×10^6 cells) were stimulated with either SP (1 μM, 6 h), IL-33 (30 ng/mL, 6 h) or their combination. Total mRNA was extracted with an RNeasy Mini kit (Qiagen Inc) 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, NK-1 receptor, and ST2 receptor (Applied Biosystems). Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems). Relative mRNA levels were determined from standard curves run with each experiment. The mRNA gene expressions were normalized to GAPDH endogenous control (Applied Biosystems).

Fluorescence-Activated Cell Sorting (FACS)

LAD2 cells (1×10^6 cells) were treated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 h, centrifuged at 500 x g for 5 min and washed 3 times in phosphate-buffered saline (PBS). Cells were treated with 1 μg of mouse IgG1/human IgG 10^5 cells

for 15 min at room temperature prior to staining to block non-specific binding. The Allophylocyanin (APC)-conjugated human NK-1 receptor antibody and Phycoerythrin (PE)-conjugated human ST2 receptor antibody (R&D Systems) were added to stimulated cells for 45 min at 2-8°C. Following the incubation, unreacted antibodies were removed by washing the cells 3 times with PBS. Finally, cells were resuspended in 750 µl of PBS for flow cytometry analysis. Cell surface expressions of NK-1 and ST2 receptors were determined using the FACSCalibur flow cytometer (BD Biosciences).

Multiple Kinase Phosphorylation assay

LAD2 cells (2×10^6 cells) were stimulated with SP (1 µM) and IL-33 (30 ng/mL) in a time-dependent manner for 5, 10, 30 min and 1, 2, 4, 24 hr. Phosphorylation of SAPK/JNK (Th183/Tyr185), NF-κB p65 (Ser536), and IKβ-α (Ser32) were detected by the PathScan Inflammation Sandwich ELISA kit (#7276, Cell Signaling Technology) according to the instructions provided. Whole cell lysates were assayed at a protein concentration of 1 mg/mL. Absorbance was read at 450 nm using a LabSystems Multiskan RC microplate reader (Fisher Scientific). Relative phosphor-SAPK/JNK, phospho-NF-κB p65, and phospho- IKβ-α levels were normalized to control cells.

Immunoprecipitation and Western blot assays

LAD2 cells (1×10^6 cells) were pre-incubated with the proteasome inhibitor PS 341 (1-50 µM) or methoxyluteolin (1-50 µM) for 2 hrs and then stimulated with SP (1 µM), IL-33 (30 ng/mL) and/or their combination for 1 hr. The reaction was stopped by addition of ice-cold PBS. Cells were washed once with PBS and then lysed using protein

lysis radio-immuno precipitation (RIPA) buffer (Sigma-Aldrich) in the presence of protease inhibitor cocktails. For immunoprecipitation (IP), LAD2 cells ($5x10^6$) were treated with SP (1 μM), IL-33 (30 ng/mL) and/or their combination for 30 min. Cells were washed once with PBS and then lysed using Cell Lysis buffer (Cell Signaling Technology) supplemented with PMSF and EDTA (Cell Signaling Technology). Total protein concentration was determined by bicinchoninic acid assay (BCA) (Thermo Fisher Scientific Inc.) method using bovine serum albumin (BSA) as standard. For IP, 200 ug of protein was incubated with rabbit anti-human NK-1 primary antibody (Abcam) at 1 : 50 ratio overnight at 4° C and then incubated with 30 μl of 50% Protein A bead slurry (Cell Signaling Technology) for 2 hr, washed with Cell Lysis buffer and boiled for 5 min in 2X sodium dodecyl sulfate (SDS). The total cellular proteins (20 μg aliquots) and IP samples were separated using 4-20 % Mini Protean TGX gels (Biorad) under SDS denaturing conditions and electrotransfered onto polyvinylidene fluoride (PVDF) membranes (Biorad). Blocking was carried out with 5% BSA in Tris-buffered saline containing 0.05% Tween-20. The membranes were probed with the following primary antibodies at 1:1,000 dilutions: IKβ-α, phospho-IKβ-α (Cell Signaling Technology) and IL-1RacP (R&D Systems), 1:250 dilution ST2 (Abcam), 1:10,000 dilution NK-1 (Abcam). For loading control, β-actin was probed. For detection, the membranes were incubated with the appropriate secondary HRP-conjugated antibody (Cell Signaling Technology)) at 1:1,000 dilution and the blots were visualized with enhanced chemiluminescence SuperSignal West Pico Substrate (Thermo Scientific).

Statistics

All experimetns were performed in triplicate, and were repeated for at least three times (n=3). Data are presented as mean ± SD. Results were analyzed using the unpaired, 2-tailed, Student's *t*-test. Significance of comparisons between conditions is denoted by * $p<0.05$, ** $p<0.01$,*** $p<0.001$, and **** $p<0.0001$, respectively.

2. 3 Results

Selection of the optimal doses to study TNF secretion stimulated by SP and IL-33 when administered in combination

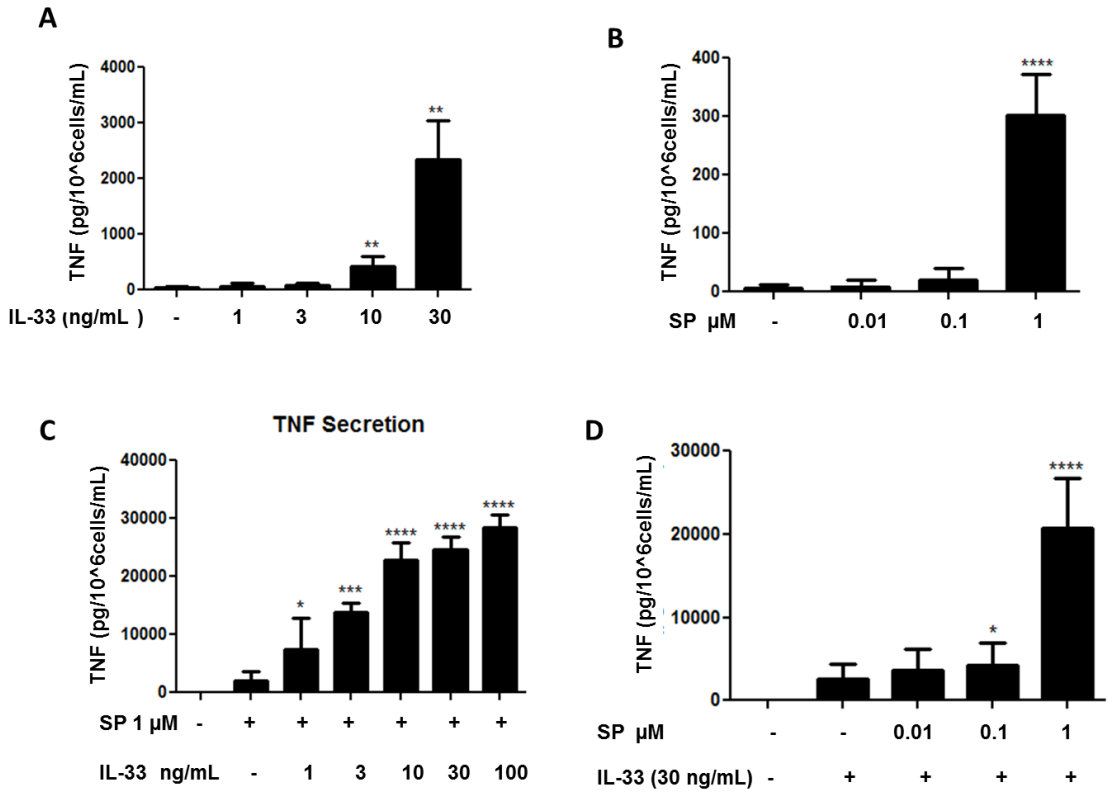

Figure 2.1. Selection of the optimal doses to study TNF secretion stimulated by SP and IL-33 when administered in combination. LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and stimulated with (A) IL-33 (1-30 ng/mL) alone, (B) with SP (0.1-1 µM) alone, or (C-D) with SP (0.01-1 µM), IL-33 (1-100 ng/mL) or their combination as shown for 24 hr. Supernatant fluids were collected at the end of the incubation period and assayed by TNF ELISA. (n=3,* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$)

Stimulation of LAD2 cells by IL-33 alone (1-100 ng/mL) results in a maximum secretion of approximately 2,500 pg/mL of TNF at 30 ng/mL ($p<0.01$) (Figure 2.1A) and SP alone (0.01-1 µM) stimulates about 400 pg/mL of TNF at 1 µM ($p<0.001$) (Figure 2.1B). The combination of IL-33 (30 ng/mL) and SP (1 µM) produces a robust

augmentation of TNF secretion around 25,000 pg/mL ($p<0.001$) (Figure 2.1C-D); therefore, this combination was selected for further experiments.

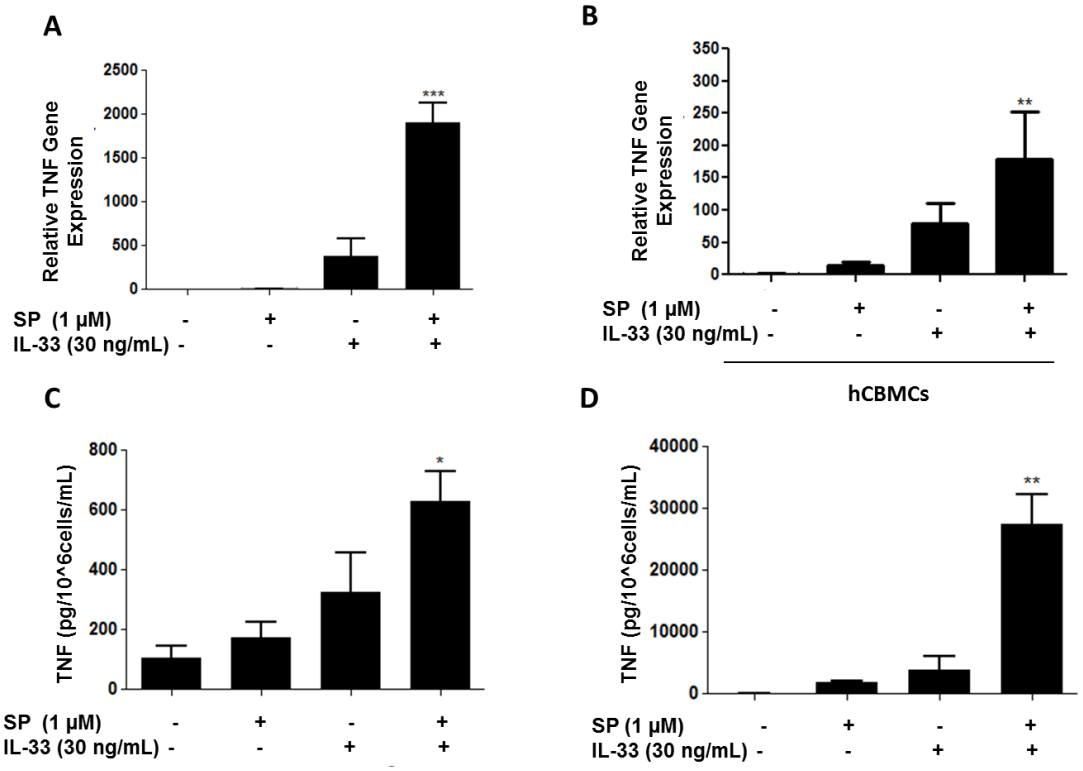

Figure 2.2. SP and IL-33 markedly enhance TNF gene expression and secretion in human mast cells. (A) LAD2 cells (1×10^6 cells per well) and (B) hCBMCs (0.3×10^6 cell per well) were seeded in 12-well culture plate and stimulated with SP (1 µM), IL-33 (30 ng/mL) or their combination for 6 hr. TNF mRNA expression levels were measured by qRT-PCR and normalized to human GAPDH endogenous control. (C-D) LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and stimulated with SP (1 µM), IL-33 (30 ng/mL) or their combination as shown for 24 hr. Control cells were treated with culture media only. Supernatant fluids (D) and cell lysates (C) were collected at the end of the incubation period and assayed by ELISA (n=3, * $p<0.05$ and ** $p<0.01$).

The combination of IL-33 (30 ng/mL) and SP (1 µM) administered in combination significantly ($p<0.001$) increases TNF mRNA gene expression by 1,000-fold (Figure 2.2A) in LAD2 cells and by 100-fold ($p<0.001$) in human umbilical cord blood-derived mast cells (hCBMCs) (Figure 2.2B). IL-33 and SP also significantly

increase TNF cellular protein by 600-fold ($p<0.05$) and secretion by 4,500-fold ($p<0.01$) in LAD2 cells (Figure 2.2C-D).

NK-1 receptor antagonists, L-733,060 and CP-96345, inhibit TNF secretion stimulated by SP and IL-33 administered in combination

Next we investigated whether the enhancing effect of IL-33 and SP is mediated via the neurokinin 1 receptor (NK-1). LAD2 cells were pre-incubated with the NK-1 antagonist L-733,060 (10 μM) or CP-96345 (10 μM) for 30 min and then stimulated with SP (1 μM) alone, IL-33 (30 ng/mL) alone or the combination of both for 24 hr. Pre-incubation with either antagonist significantly ($p<0.0001$) inhibits TNF release by approximately 50% when IL-33 and SP are administered in combination (Figure 2.3A). These results were confirmed when we transiently decreased NK-1 receptor expression by 92% using NK-1 siRNA (50 μM) (Figure 2.5A). NK-1 receptor knockdown significantly ($p<0.001$) decreases TNF release by 50% when stimulated by IL-33 and SP in combination (Figure 2.3B).

ST2 receptor neutralizing antibody inhibits TNF secretion stimulated by the combination of SP and IL-33

Then we investigated whether IL-33 receptor, ST2, blockade will diminish the enhancing effect of IL-33 and SP. Due to the absence of ST2 receptor antagonists, LAD2 cells were pretreated with a ST2 receptor neutralizing antibody and a non-specific IgG control over a number of concentrations (Figure 2.6). LAD2 cells were pre-incubated with the ST2 neutralizing antibody (3 μg/mL) or IgG control (3 μg/mL) for 2 hr and then

stimulated with SP (1 μM) alone, IL-33 (30 ng/mL) alone or the combination of both for 24 hr. Pre-incubation with the ST2 neutralizing antibody significantly ($p<0.0001$) inhibits TNF release by 50% when IL-33 and SP were administered in combination and by 34% when stimulated by IL-33 alone (Figure 2.3C). Additionally, transient 70% reduction of ST2 receptor gene expression using siRNA (Figure 2.5B) significantly ($p<0.05$) decreases TNF release by approximately 30% when IL-33 and SP were administered in combination (Figure 2.3D).

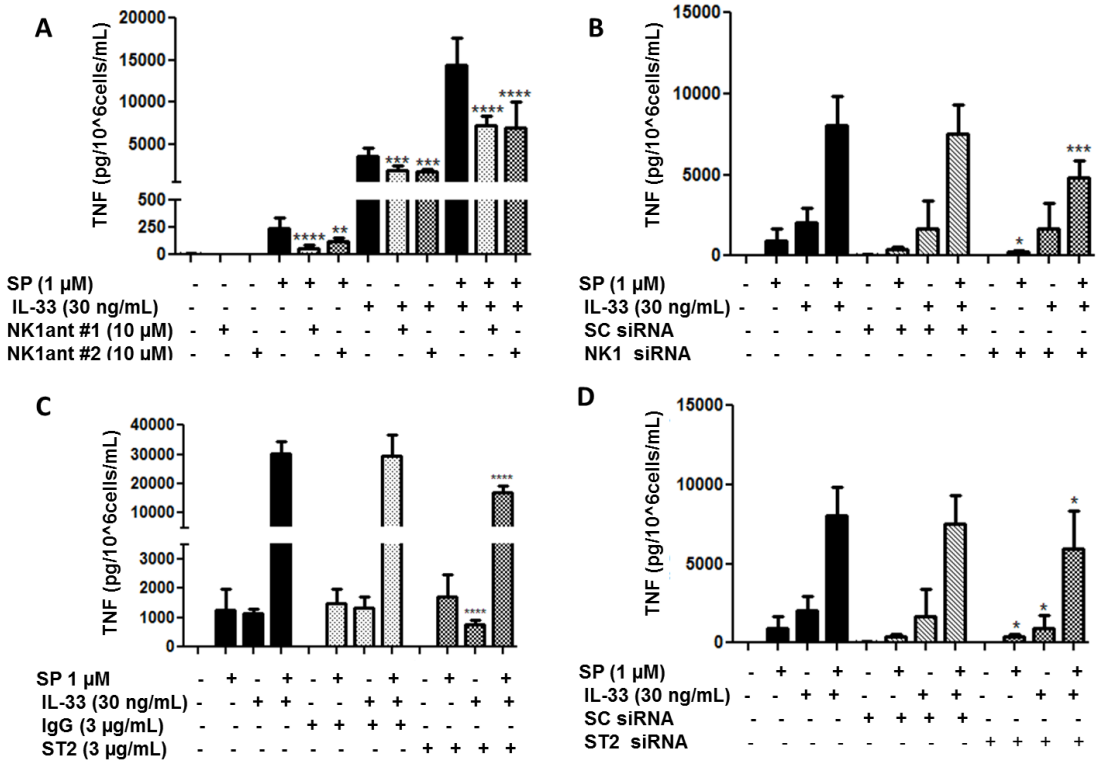

Figure 2.3. NK-1 receptor antagonists and ST2 neutralizing antibody inhibit TNF secretion. (A) LAD2 cells were pretreated with NK-1R antagonists L-733,060 (10 μM) and CP-96345 (10 μM) for 30 min and then were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. (C) LAD2 cells were pre-incubated with ST2 neutralizing antibody (3 ng/mL) or IgG control (3 ng/mL) for 2 hr and then were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. LAD2 cells were pretreated with (B) NK-1 receptor siRNA (50 μM), (D) ST2 receptor siRNA, or (B,D) scrambled siRNA (SC) (50 μM) for 72-96 hr and then stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. Collected supernatant fluids were assayed by TNF ELISA (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$).

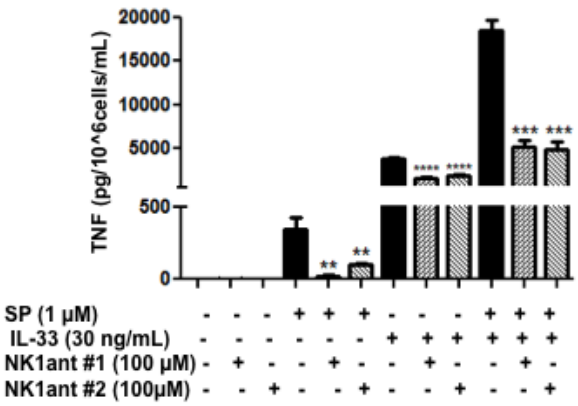

Figure 2.4. NK-1 receptor antagonists at 100 µM do not completely inhibit TNF secretion. LAD2 cells were pretreated with NK-1R antagonists L-733,060 (100 µM) and CP-96345 (100 µM) for 30 min and then were stimulated with SP (1 µM), IL-33 (30 ng/mL) or their combination for 24 hr. Collected supernatant fluids were assayed by TNF ELISA (n=3, * p<0.05, ** p<0.01, *** p<0.001 and ****p<0.0001).

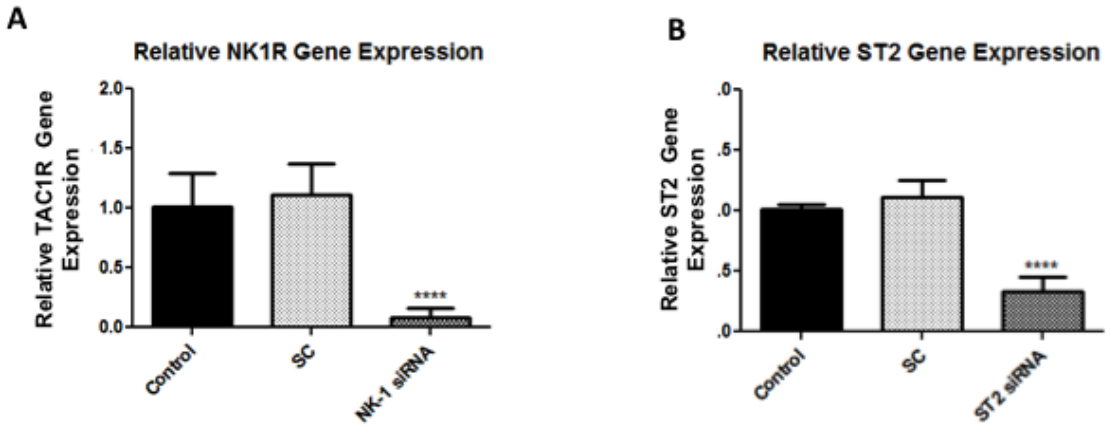

Figure 2.5. NK-1 and ST2 transient gene knockdown. LAD2 cells were treated with NK-1, ST2 or scramble (SC) siRNA (50 µM) for 72-96 hr. **(A)** NK-1 and (B) ST2 mRNA expression levels were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3,* p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001).

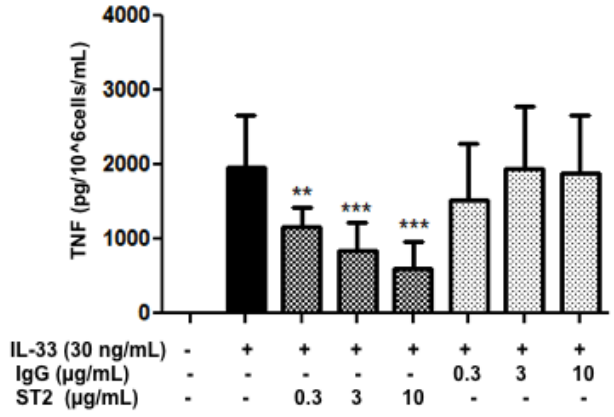

Figure 2.6. Dose-dependent blockade of ST2 receptor. LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with ST2 neutralizing antibody (0.3µg/mL- 10 µg/mL) or IgG control (0.3µg/mL- 10 µg/mL) for 2 hr and then stimulated with IL-33 (30 ng/mL) for 24 hr. Supernatant fluids were collected at the end of the incubation period and assayed by TNF ELISA (n=3,* p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001).

NK-1 and ST2 receptor-receptor interactions

LAD2 cells were pre-incubated with NK-1 antagonists, L-733,060 (10 μM) or CP-96345 (10 μM), for 30 min and then stimulated with IL-33 (30 ng/mL) alone. An interesting new finding we observed that both NK-1 antagonists also significantly ($p<0.001$) inhibit the effect of IL-33 alone by approximately 50% (Figure 2.3A) suggesting an interaction between NK-1 and ST2 receptors. Moreover, ST2 receptor knockdown significantly ($p<0.05$) decreases SP-stimulated TNF release by 30% (Figure 2.3D), again suggesting that the decrease in ST2 expression results in the decrease of NK-1 activation. Interestingly NK-1 antagonists L-733,060 (100 μM) or CP-96345 (100 μM) do not completely inhibit TNF secretion even at 100 μM (Figure 2.4).

To explore further interactions between NK-1 and ST2 receptors we co-immunoprecipitated (co-IP) LAD2 cells using an antibody against NK-1 receptor and assayed for ST2 and IL-33 co-receptor, the IL-1RacP. We found that even in unstimulated LAD2 cells, NK-1 co-immunoprecipitates with ST2 and IL-1RacP (Figure 2.7) suggesting a complex formation among them. Interestingly, IL-1RacP protein expression is markedly increased when stimulated by the combination of SP and IL-33.

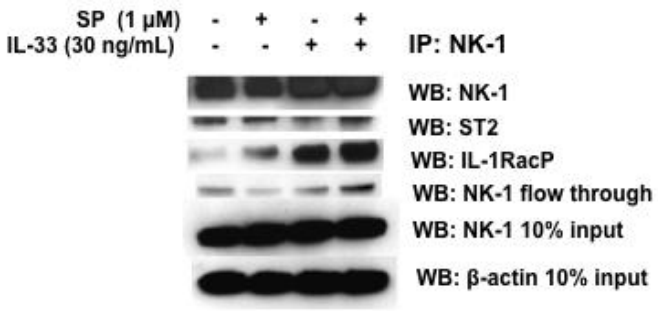

Figure 2.7. NK-1 receptor co-immunoprecipitates with ST2 and IL-1RacP. LAD2 cells (5 x 10^6 cells) were stimulated with the combination of SP and IL-33. Cell lysates were collected after 30 min and immunoprecipitated for an antibody against NK-1 receptor. IP-samples as well as 10% loading control samples were assayed for protein levels of NK-1, ST2, IL-1RacP and β-actin using Western blot (n=3).

IL-33 and SP administered separately or in combination increase their receptor expression

We investigated whether SP alone, IL-33 alone or the combined effect of both on NK-1 and ST2 receptor gene and protein expression. Stimulation with SP (1 μM), IL-33 (30 ng/ml) or their combination for 24 hrs significantly increases NK-1 ($p<0.05$) and ST2 ($p<0.05$) receptor gene expression by 2-fold (Figure 2.8A-B) in LAD2 cells and by 150-fold NK-1 ($p<0.0001$) and by 3-fold ST2 ($p<0.01$) in hCBMCs (Figure 2.8B-C).

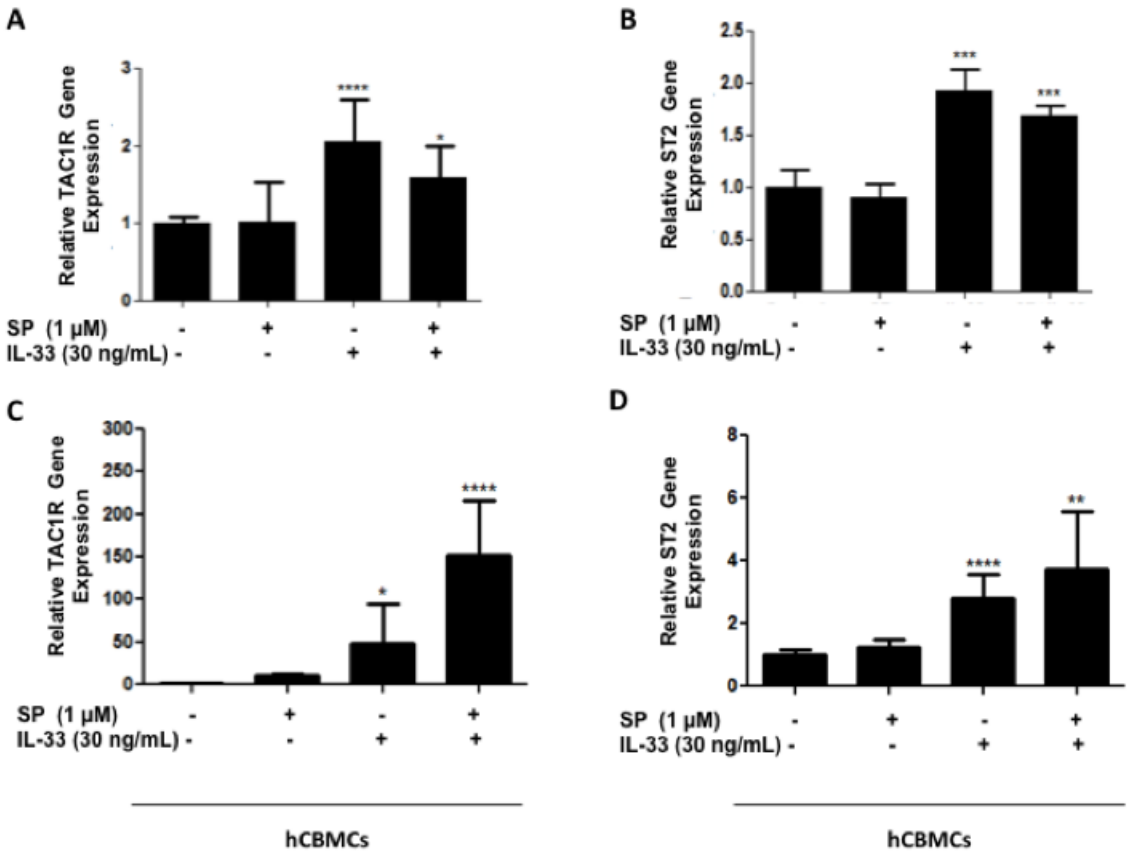

Figure 2.8. The effect of SP and IL-33 on NK-1 and ST2 gene expression. (A-C) LAD2 cells (1×10^6 cells per well) and (C-D) hCBMCs (0.3×10^6 cell per well) were seeded in 12-well culture plate and stimulated with SP (1μM), IL-33 (30 ng/mL) or their combination for 24 hr. NK-1and ST2mRNA expression levels were measured by qRT-PCR and normalized to human GAPDH endogenous control. (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$)

Total protein expression by Western Blot analysis shows that IL-33 (30 ng/mL) in combination with SP (1 µM) increases protein expression of both ST2 and NK-1 receptors (Figure 2.9).

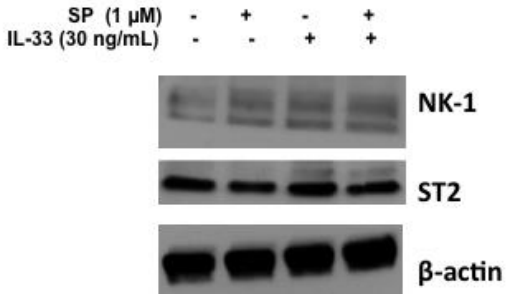

Figure 2.9. IL-33 increases NK-1 total protein. LAD2 cells (1 x 10^6 cells) were stimulated with the combination of SP (1 µM and IL-33 (30 ng/mL). Cell lysates were collected after 24 hr and protein levels of NK-1 and ST2 were measured by Western blot; β-actin was used as a loading control (n=3).

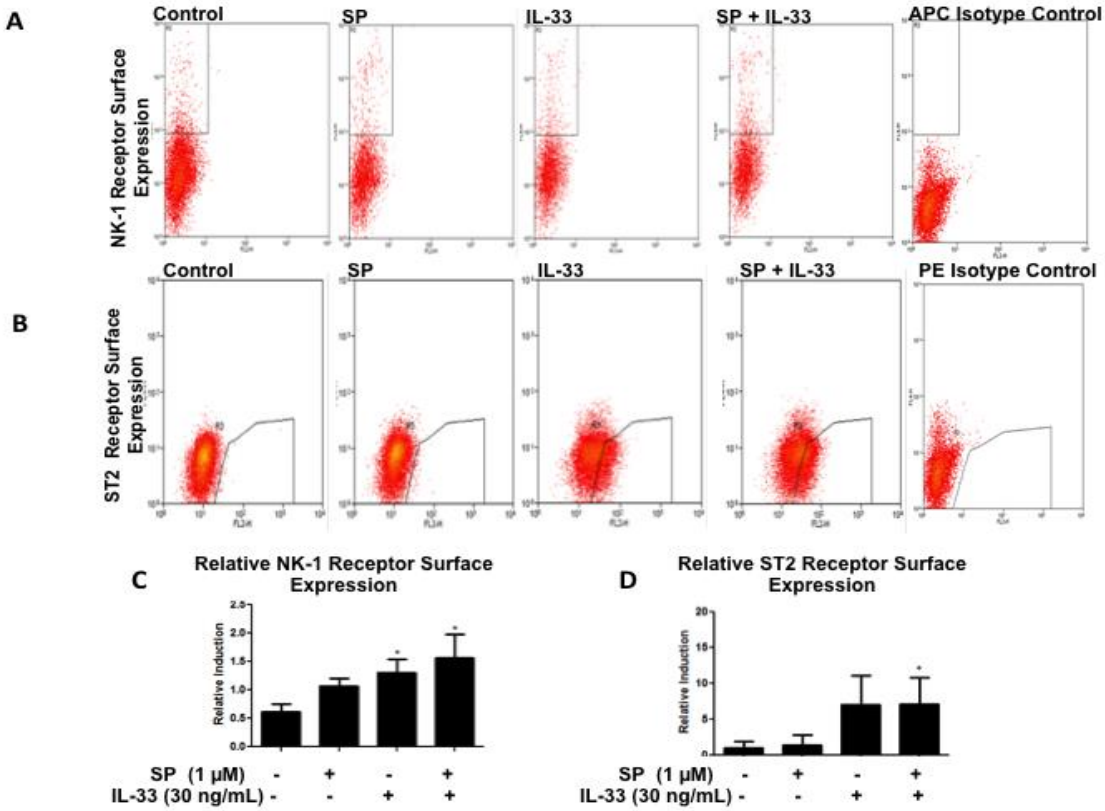

Figure 2.10. IL-33 increases NK-1 and ST2 expression. (A) LAD2 cells (1 × 10^6 cells per well) were seeded in 12-well culture plate were stimulated with SP (1 µM), IL-33 (30 ng/mL) or their combination for 24 hr. Collected cells were probed for APC-conjugated NK-1 receptor and (B) PE-conjugated ST2 receptor (representative of one experiment). (C-D) Relative NK-1 and ST2 expression on cell the surface compared to control (n=3, * $p<0.05$).

Also FACS analysis data shows that IL-33 (30 ng/mL) increases its own ST2 receptor surface expression (7-fold) and NK-1 surface expression (1.4-fold) (Figure 2.10C-D). In contrast, SP (1 μM) increases its own NK-1 receptor surface expression (1.1-fold), but does not affect ST2 receptor surface expression. The combination of IL-33 (30 ng/mL) and SP (1 μM) increases NK-1 receptor protein surface expression by 1.5-fold ($p<0.05$) (Figure 2.10C).

NF-κB and SAPK/JNK signaling pathways are involved in TNF gene expression and secretion

Next we investigated the signaling pathways involved in the effect of SP and IL-33 administered in combination on TNF gene expression and secretion. PathScan ELISA performed on 7 different kinases (ERK1/2, p38 MAPK, MEK1/2, IKβ-α, NF- κB, SAPK/JNK, and STAT3) shows activation of the NF-κB and the SAPK/JNK pathways. We observed a differential time-course of the signaling pathways activation throughout 24 hr with SP and IL-33 combined stimulation. The SAPK/JNK pathway is activated as early as 5 min after stimulation and is maintained for the next 4 hrs (Figure 2.11B). In contrast, activation of NF-κB, as shown by phosphorylation of IKβ-α, is observed starting at 1 hr (Figure 2.11A) and is also maintained for the next 4 hrs. Next, LAD2 cells were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 1 hr to determine which component of the combination triggers phosphorylation of IKβ-α. Western blot assay shows that IL-33 is responsible for the phosphorylation of IKβ-α and the combination of SP and IL-33 increases it even further (Figure 2.11C).

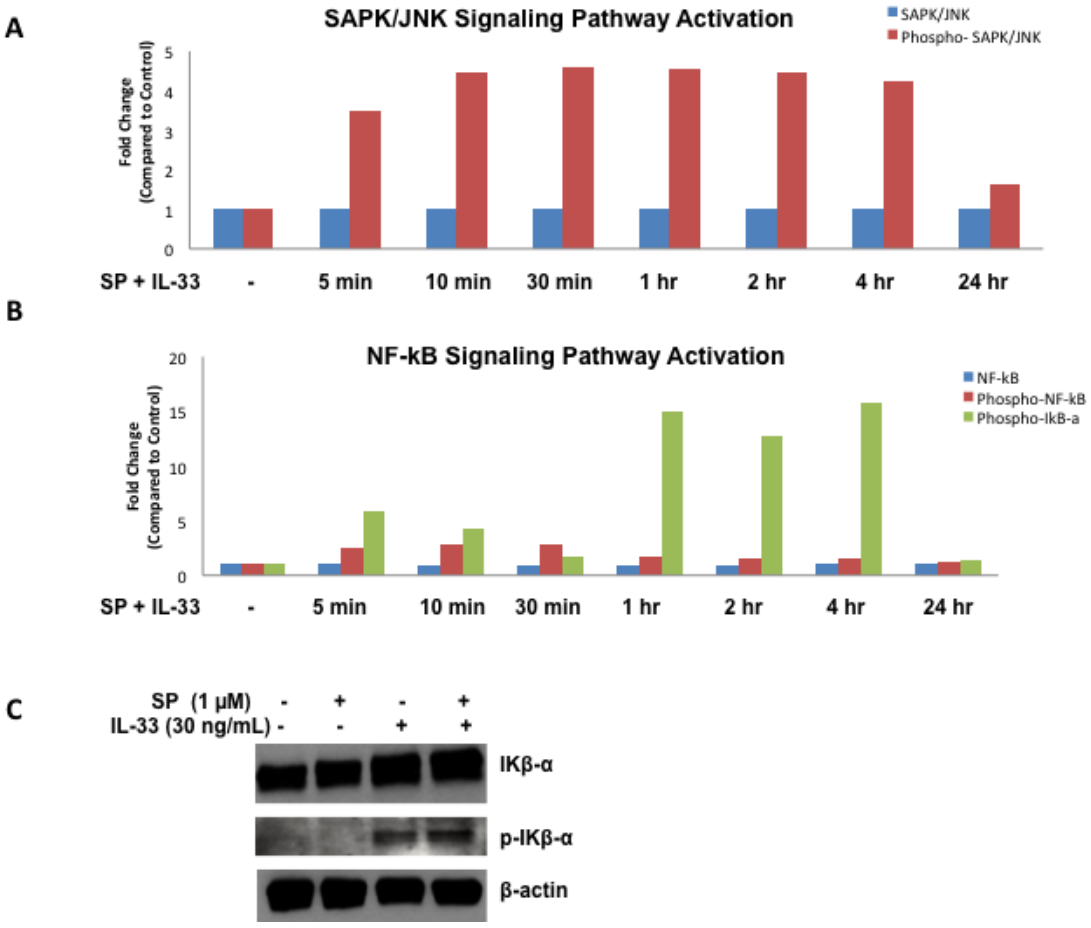

Figure 2.11. NF-κB and SAPK/JNK signaling pathways are involved in TNF gene expression and secretion. (A-B) LAD2 cells (2×10^6 cells) were stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 5, 10, 30 min and 1, 2, 4, 24 hr. Phosphorylation of NF-κB p65 (Ser536), IKβ-α (Ser32) and SAPK/JNK (Th183/Tyr185) were detected by the PathScan Inflammation Sandwich ELISA kit. Whole cell lysates were assayed at a protein concentration of 1 mg/mL. (C) LAD2 MCs were stimulated with SP (1 μM), IL-33 (30 ng/mL), and their combination. Cell lysates were collected after 1 hr and protein levels of IKβ-α and phospho-IKβ-α were measured by Western blot; β-actin was used as loading control (n=3).

Methoxyluteolin inhibits TNF gene expression and secretion

LAD2 cells were pre-incubated (2hr) with luteolin (Figure 2.12) or methoxyluteolin (1-100 μM, for 2 hr) and then stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL) for 24 hrs. TNF cellular protein is significantly ($p<0.05$) inhibited by 45% at the lowest flavonoid concentrations (1 μM) and TNF secretion is

significantly ($p<0.0001$) inhibited by 50% at 25 μM and above (Figure 2.13A, B). SP and IL-33-induced TNF mRNA gene expression is significantly ($p<0.001$) inhibited by 98% at 50 μM (Figure 2.13C). Methoxyluteolin was more potent than luteolin inhibiting TNF secretion throughout.

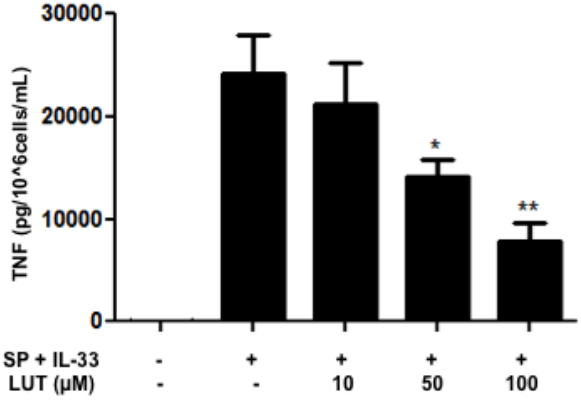

Figure 2.12. Luteolin inhibits TNF secretion. LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with luteolin (Lut) (10-100 μM) then stimulated with the combination of SP (1μM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with 0.1% DMSO, the highest concentration corresponding to that of 100 μM Lut. Supernatant fluids were assayed for TNF by ELISA (n=3, Student's t-test,* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

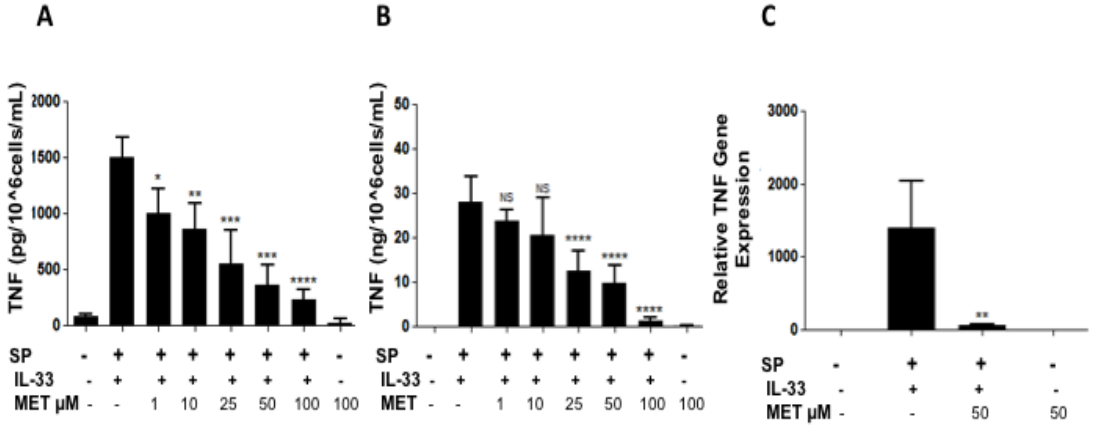

Figure 2.13. Methoxyluteolin inhibits TNF gene expression and secretion. (A-B) LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with methoxyluteolin (Methlut) (1-100 μM) then stimulated with the combination of SP (1μM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with 0.1% DMSO, the highest concentration corresponding to that of 100 μM Methlut. Collected supernatant fluids and cell lysates were assayed by TNF ELISA. (C) LAD2 cells (1×10^6 cells per well) were seeded in 12-well culture plate and pre-incubated with Methlut (50 μM) then stimulated with the combination of SP (1μM) and IL-33 (30 ng/mL) for 6 hr. TNF mRNA expression levels were measured by qRT-PCR and

normalized to human GAPDH endogenous control (n=3,* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

We also investigated the effect of methoxyluteolin on IKβ-α phosphorylation. LAD2 mast cells were pre-incubated with the proteasome inhibitor PS 341 (1, 10, and 50 μM) as a positive control or methoxyluteolin (1, 10, 50 μM) and then stimulated with the combination of IL-33 and SP. Cell lysates were collected after 1 hr and protein levels of IKβ-α and phospho-IKβ-α were assayed by Western blot. Pre-incubation with methoxyluteolin (1-50 μM) markedly inhibits IKβ-α phosphorylation induced by the combination of IL-33 (30 ng/mL) and SP (1 μM) in LAD2 cells (Figure 2.14).

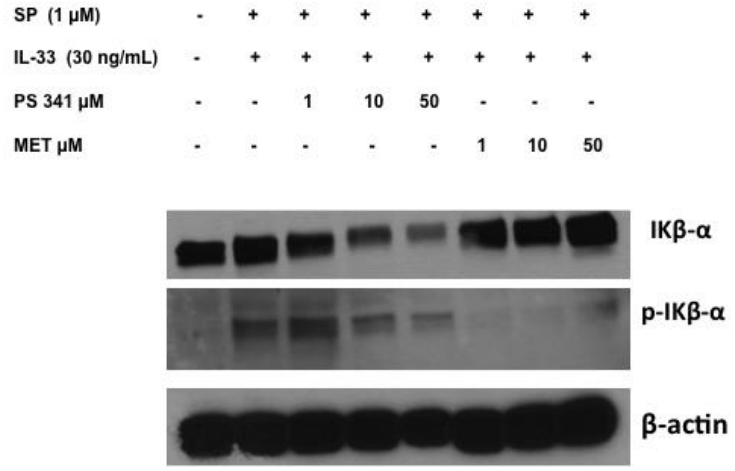

Figure 2.14. Methoxyluteolin inhibits IKβ-α phosphorylation. LAD2 cells (1 x 10^6 cells) were pre-incubated with proteasome inhibitor PS 341 (1, 10, and 50 μM) or Methlut (1, 10, 50 μM) and then stimulated with the combination of SP and IL-33. Cell lysates were collected after 1 hr and protein levels of IKβ-α and phospho-IKβ-α were measured by Western blot; β-actin was used as loading control (n=3).

Additionally, we investigated whether SP, IL-33 or their combination can have an enhancing effect on allergic stimulation with IgE/anti-IgE. LAD2 cells triggered with SP and IL-33 in combination and also challenged with IgE/anti-IgE do not show significantly different release of TNF (Figure 2.15).

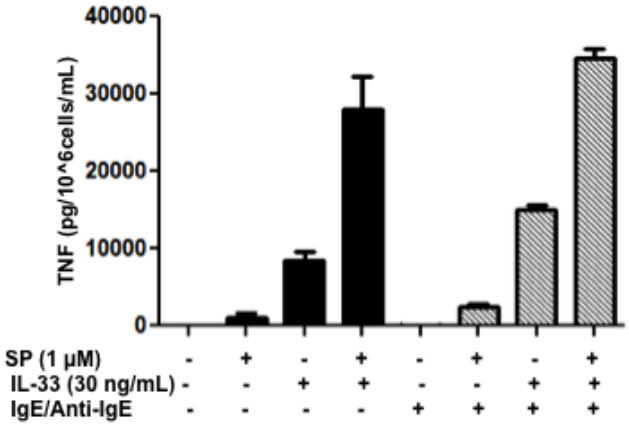

Figure 2.15. IgE/anti-IgE does not increase SP and IL-33 stimulated secretion of TNF. LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with human IgE (1 µg/mL) or medium overnight and next day were stimulated with anti-IgE (10 ng/mL) for 2 hr and/or SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. Supernatant fluids were collected at the end of the incubation period and assayed by TNF ELISA (n=3,* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

The purity of hCBMCs was confirmed by immunohistochemical staining for tryptase (Figure 2.16A) and c-kit (Figure 2.16B). To understand better morphology of hCBMC, we compared the pictures taken using EMT at different time points of culture of hCBMCs and normal mast cells isolated from detrusor muscle (Figure 2.17A-C). It is evident from the pictures that hCMBCs differ in the quality, content and texture of the secretory granules.

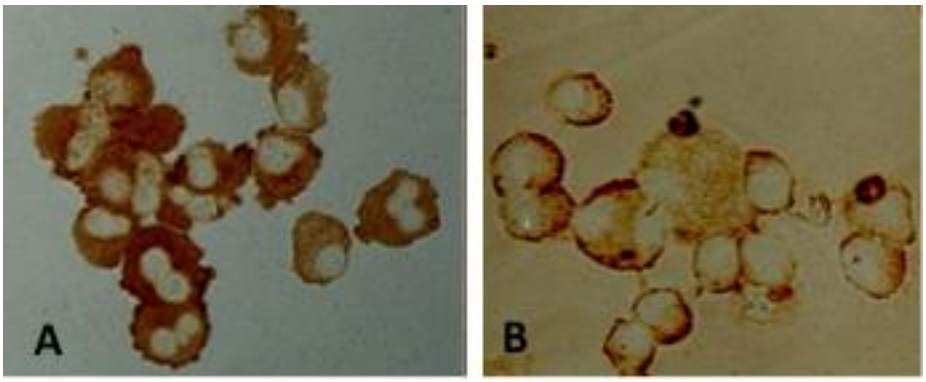

Figure 2.16. hCBMCs contain tryptase and express surface c-kit receptor. Photomicrographs of hCBMCs stained by immunohistochemistry at 12 weeks for (A) tryptase content and (B) c-kit receptor surface expression. Magnification: x 1000.

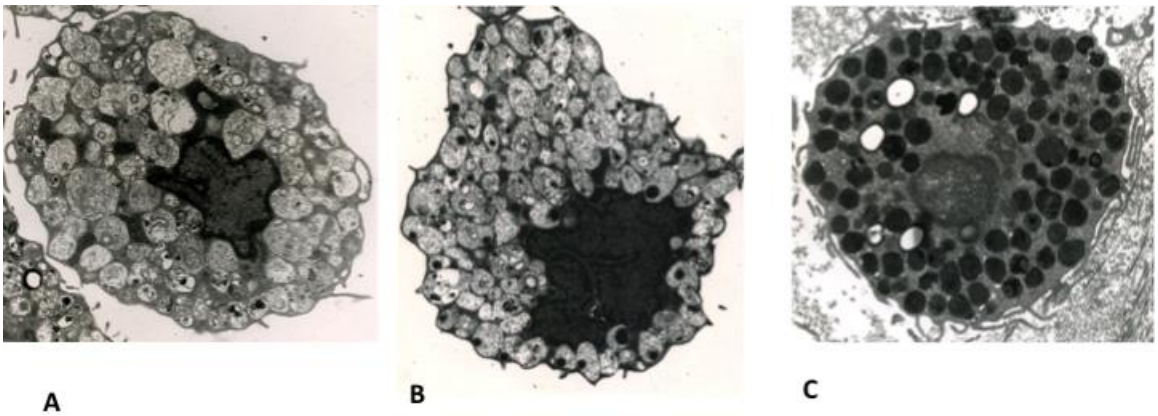

Figure 2.17. Lack of secretory granule content maturity in hCBMCs. Transmission Electron Micrographs of individual human mast cells. (A) hCBMC – 9 wk culture; (B)) hCBMC – 15 wk culture; (C) Normal detrusor muscle mast cell. Magnification: x 10,000.

2. 4 Conclusion

The results presented here indicate novel complex interactions between the peptide SP and the cytokine IL-33 administered in combination that lead to an impressive increase in TNF synthesis and secretion in cultured human mast cells. These responses depend on the activation of both NK-1, the receptor for the peptide SP, and ST2, the receptor for the cytokine IL-33.

An important new finding is that interfering with the NK-1 receptor function results in the decrease of the ST2 receptor activation and decrease in the ST2 receptor expression results in the decrease NK-1 receptor signaling. Treatment with 100X more of the NK-1 antagonists does not completely inhibit the robust TNF secretion that suggests that another mechanism is contributing to this effect. NK-1 inhibition was previously reported to only partially inhibit SP stimulation of skin-derived human mast cells [194], supporting the idea of another pathway that might be participating in this response. Furthermore, the NK-1 antagonists inhibit TNF secretion in mast cells stimulated by IL-33 alone, while the ST2 siRNA also inhibits SP-stimulated TNF, indicating that there may be some receptor-receptor interaction between NK-1 and ST2. A precedent for the receptor-receptor interaction is a report of cross-activation of c-kit, the receptor for stem cell factor, through complexing with ST2 and its co-receptor, the IL-1R accessory protein (IL-RAcP), in murine mast cells [195]. An analogous structural association could occur among NK-1, ST2 and IL-RAcP by a direct contact; therefore we co-immunoprecipitated these proteins. The results of these experiments show that co-immunoprecipitation using an antibody specific to NK-1 recovers both ST2 and IL-1RacP. The expression of IL-1RacP is markedly increased after the stimulation with the combination of SP and IL-33,

suggesting that this protein participates in complexing NK-1 and ST2 receptors. Because co-immunoprecipitation is performed after cell lysis, the association of these proteins cannot be settled just by using this method. These results merit further investigation to assess NK-1, ST2 and IL-1RacP association by techniques that don't require cell lysis.

Mast cells are now recognized as being critical in inflammatory processes [196] and are implicated in inflammatory diseases that include rheumatoid arthritis [197], inflammatory bowel disease [198], psoriasis [199], multiple sclerosis [200] and fibromyalgia syndrome [201]. Independent reports have shown that mast cells, SP, IL-33 and TNF contribute to the inflammatory processes in these diseases [71, 188, 201-204]. Our findings provide new insights into how SP, IL-33, TNF and mast cells may interact in these inflammatory diseases. The combined enhancing effect of SP and IL-33 on robust secretion of TNF from human mast cells could be a major contributor to inflammatory processes that occur in the pathological conditions mentioned.

A major challenge in the studies of mast cell pathophysiology is the source of primary mast cells. Ordinarily, mature human mast cells could come from either clean margins of breast mastectomies, extract tissue from fat reduction, or circumcisions. However, these cells vary enormously in their phenotypic characteristics due to the age of the donor, site of the skin used, the degree of inflammation, or the adipocytokines presence. The primary mast cells that we used are isolated from umbilical cord blood, collected from healthy donors after uncomplicated deliveries. The drawback of these hCBMC cells is that their secretory granules do not appear to have the morphology of mature adult human mast cells. SP had previously been shown to stimulate small amounts of TNF from rat peritoneal mast cells [174, 205] and cultured human skin mast cells [175, 176],

but at much higher SP concentrations (20-100 μM). One major difference between these publications may be the fact that human skin mast cells that respond to SP are derived from adult skin [175], while those cells that did not respond to SP were purified form circumcisions [194]. In addition, it was reported that SP stimulates TNF release from LAD2 cells, but not from purified human skin mast cells [194]. In fact, there was a great variability in the allergic response to IgE among mast cells derived from circumcisions [183 206]. Also it is important to note that unlike previous reports showing that IL-33 augmented allergic IgE-stimulated histamine release, it did not augment inflammatory IgE-stimulated TNF release in human cultured mast cells.

The findings of this study provide new understanding of physiological and pathological interactions between peptides, cytokines and mast cells that may lead to discovery of novel pharmacological approaches for development of anti-inflammatory therapies. We used a naturally occurring molecule, methoxyluteolin, to explore its inhibitory effects on mast cells. Methoxyluteolin inhibits the enhancing effect of SP and IL-33 on TNF gene expression and secretion from human mast cells, indicating that this flavonoid must be acting at some step of signaling pathway following the receptor activation. We previously showed that methoxyluteolin is a more potent inhibitor than luteolin of human mast cells [165] and human keratinocytes [191]. The time course of the key signaling steps of the stimulation with the combination of IL-33 and SP leads to JNK kinase activation within the first 5 minutes, while IKβ-α becomes active within the first hour and both remain active for the next 4 hours of stimulation. Previously, SP/NK-1 receptor signaling has been shown to activate JNK kinase [47], while IL-33/ST2 receptor activation has been shown to signal via NK-κB [207]. One of the ways methoxyluteolin may

inhibit TNF gene expression and secretion is through the inhibition of IKβ-α phosphorylation. SP was previously reported to stimulate NF-κB in murine umbilical cord blood mast cells [205] at 10-100 μM and in hCBMCs at 1 μM [165]. Upon phosphorylation, IKβ-α releases NF-κB, which translocates from the cytoplasm into the nucleus and regulates gene expression of various inflammatory mediators including TNF [208]. IL-33 could contribute to TNF gene expression and secretion stimulated by the combination of IL-33 and SP via translocation into the nucleus, where it could possibly activate some transcription factors, particularly AP-1 [56], an affect that may be inhibited by methoxyluteolin. We had previously shown that both luteolin and methoxyluteolin inhibited NF-κB and also decreased NF-κB p65 DNA-binding activity in the nuclear extract of human mast cells [209]. Both flavonoids also decreased mRNA expression of two genes encoding different subunits in the NF-κB protein complex, NFKB1 (encoding NF-κB p50 subunit) and RELA (encoding NF-κB p65 subunit) [209].

Our results have important clinical implications for the understanding of the complex interplay among SP, IL-33 and mast cells in inflammatory processes. The impressive enhancing effect of IL-33 and SP combination on TNF gene expression and secretion in human mast cells may be a key step in the pathogenesis of inflammatory diseases. The potent inhibitory effect of methoxyluteolin suggests that it could be developed as systemic or local anti-inflammatory treatment. The magnitude of TNF synthesis and secretion, due to interactions between SP and IL-33, suggests new therapeutic approaches through the use of SP and IL-33 receptor antagonists as well as methoxyluteolin.

Chapter 3: Regulation of IL-1β Secretion from Human Mast Cells Stimulated by the Neuropeptide Substance P (SP) and the Cytokine Interleukin (IL)-33 and Inhibited by the Flavonoid Methoxyluteolin

3.1 Background

Mast cells are hemopoietically-derived tissue immune cells involved in allergic diseases [58], innate and acquired immunity [9], autoimmunity [11] and inflammation through the release of pro-inflammatory mediators such as histamine, IL-6, IL-8, TNF and VEGF [58]. Mast cells also promote the development of Th17 cells involved in autoimmune diseases [108] and can also function as immunomodulatory cells [107]. The signaling steps in the stimulation of mast cells through their high affinity surface IgE receptor (FcεRI) have been well documented [210]; however, the steps involved in the stimulation by non-allergic triggers, especially cytokines and neuropeptides, are not well understood. Substance P (SP) is a neuropeptide, originally isolated from the rat brain [169], has been implicated in inflammatory processes [48, 52, 170, 171, 173, 211] and can stimulate mast cells [37]. We had previously reported that SP and IL-33 have a synergistic action in stimulating VEGF release from human cultured mast cells [47].

IL-33 belongs to the interleukin-1 (IL-1) family of cytokines and has emerged as a "danger signal" that may be involved in a number of autoimmune and inflammatory diseases [179, 180, 212]. IL-33 is secreted by fibroblasts and endothelial cells [54], but was also shown to be produced by murine mast cells [213]. IL-33 augments secretion of PGD_2, TNF and CCL2 [97, 180] when stimulated by IgE from leukemic HMC-1 mast cells [182].

The structurally related 5,7,3',4'-tetrahydroxyflavone, luteolin, inhibits mast cells [155] and also has anti-oxidant [154], anti-inflammatory [154], as well as auto-immune T cell inhibitory properties [106, 157]. We recently reported that methoxyluteolin is a potent inhibitor of human mast cell activation [165].

IL-1β plays an important role in the regulation of innate and adaptive immune systems, in inflammatory processes, as well as in inflammatory diseases known as cryoprin-associated periodic syndromes (CAPS) that include the familial Mediterranean fever and the Muckle-Well syndrome [109, 111, 113, 120]. IL-1β is present in the cytoplasm in a biologically inactive form and it is activated by the proteolytic cleavage of caspase-1; this cysteine protease is also present in the cytoplasm in a pro-form and is activated by the multiprotein complex known as NLRP3 inflammasome [111, 214]. Even though many immune cells secrete IL-1β [113], the mechanism of its secretion from mast cells has not been studied.

The NLRP3 inflammasome is activated by numerous triggers such as pathogens, pathogen-associated molecular patterns (PAMPs), environmental agents (silica, asbestos), and endogenous danger signals [117, 212]. NLRP3 activation requires two signals [122, 215]. The first signal is provided by microbial or endogenous molecules that induce the NF-κB transcriptional factor to stimulate NLRP3 and pro-IL-1β protein expression (signal 1); the second signal initiates recruitment and assembly of the adaptor Apoptosis-associated Speck-like protein containing a CARD (ASC) with the NLRP3 protein and pro-capsase-1, subsequently cleaving the inactive pro-caspase-1 into active caspase-1 (p20). Caspase-1 activation leads to cleavage of the pro-form of IL-1β into active mature IL-1β (p17) form, which is then secreted extracellularly (signal 2) [112, 216]. However, it is still not clear how NLRP3 activators regulate NLRP3 assembly. Due to the chemical and structural diversity of the NLRP3 activators, it has been hypothesized that NLRP3 does not interact directly with them, but it may require some intermediate cellular signal [217].

Here we show that SP and IL-33 act synergistically to induce IL-1β gene expression, intracellular protein level and secretion from human cultured mast cells. Moreover, we show that constitutively active caspase-1 and the mature form of IL-1β are present in unstimulated human mast cells suggesting an alternative regulation and assembly of NLRP3 inflammasome and IL-1β secretion. Moreover, we show that these effects are inhibited by the natural flavonoid methoxyluteolin that could be developed as a new therapeutic approach for inflammatory conditions.

3.2. Materials and Methods

SP, LPS, ATP, and AC-YVAD-CMK were purchased from Sigma-Aldrich (St Louis, MO). Recombinant human IL-33, recombinant human TNF, recombinant human Interferon-γ were obtained from R&D Systems (Minneapolis, MN). Nigericin was purchased from Enzo Life Sciences (Framingdale, NY) and Glybenclamide was purchased from InvivoGen (San Diego, CA). Human recombinant IgE and anti-IgE were purchased from EMD Millipore (Billerica, MA). NK-1 antagonist L-733,060 was purchased from Sigma-Aldrich (St Louis, MO) ST2 neutralizing antibody and non-specific IgG antibody were purchased from R&D Systems (Minneapolis, MN). The proteasome inhibitor PS 341 was obtained from Tocris Biosciences (Bristol, UK). The flavonoid methoxyluteolin was obtained from Pharmascience Nutrients (Clear Water, FL). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits were purchased from BioRad (Hercules, CA). Taqman gene expression primers/assays for *NLRP3 (*Hs00918082_m1), *PYCARD (*Hs01547324_gH), *CASP1* (Hs00354836_m1), *IL1B* (Hs01555410_m1) and *GAPDH* endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kits for IL-1β (DY201), IL-33 (DY3625) and TNF (DY210) were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-human primary antibodies for NLRP3, pro-IL-1β, caspase-1, β-actin were purchased from Cell Signaling Technology (Danvers, MA) and mouse anti-human ASC and cleaved IL-1β were obtained from Santa Cruz Biotechnology (Dallas, TX). FLICA® Caspase-1 Activity Assay was purchased from ImmunoChemistry Technologies (Bloomington, MN) and Ac-YVAD-AFC was obtained from Santa Cruz Biotechnology (Dallas, TX).

Culture of human mast cells

LAD2 cells, derived from a human mast cell leukemia [192], were kindly supplied by Dr. A Kirshenbaum (NIH, Bethesda, MD) and were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human stem cell factor (rhSCF, Stemgen), kindly supplied by Swedish Orphan Biovitrum AB (Stockholm, Sweden). Cells were maintained at 37ºC in a humidified incubator at an atmosphere of 95% O_2/5% CO_2. LAD2 cells were doubling within 2 weeks in the presence of 100 ng/mL of SCF showing slow proliferation rates. Even though LAD2 cells are an immortalized proliferating cell line, this cell culture closely resembles $CD34^+$-derived primary human mast cells due to its ability to respond to SCF and express functional FcεRI receptors [192]. Cell viability was measured by Trypan blue exclusion [47] as well as by Propidium Iodide at all SP and IL-33 concentrations tested.

Human umbilical cord blood was obtained after normal deliveries in accordance with established institutional guidelines to culture primary hCBMCs [193]. 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 IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. 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. hCBMCs cultured for at least 12 weeks were used for experiments. Cell viability was determined by means of trypan blue (0.4%) exclusion.

Mast Cell Treatments

LAD2 cells and/or hCBMCs were stimulated with various concentrations of SP (0.01-1 μM, Sigma-Aldrich) and IL-33 (1-30 ng/mL; R&D Systems) alone or in combination. In some experiments LAD2 cells were stimulated with human IgE (1 μg/mL; EMD Millipore) overnight and then triggered with anti-IgE (10 ng/mL; Life Technologies). In some experiments, LAD2 cells were pretreated with the NK-1 antagonists L-733,060 (10 μM; Sigma-Aldrich), a ST2 neutralizing antibody (0.3 μg/mL-10 ug/mL; R&D Systems) or non-specific IgG antibody (0.3 μg/mL-10 ug/mL; R&D Systems), the proteasome inhibitor PS 341 (1-50 μM; Tocris Biosciences) and methoxyluteolin (1-100 μM) (Skyherbs Lab).

IL-1β and TNF assays

LAD2 cells (1×10^5 cells/well) were treated with various concentration of SP (0.01-1 μM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with the same volume of culture media. Supernatant fluids were collected and assayed using IL-1β and IL-33 DuoSet ELISA kits (R&D systems). IL-1β DuoSet ELISA Kit had 6.3% cross-reactivity with precursor human recombinant IL-1β according to the manufacturer's instructions.

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

Mast cells were stimulated with either SP (1 μM, 6 h), IL-33 (30 ng/mL, 6 h) or their combination. Total mRNA was extracted with an RNeasy Mini kit (Qiagen Inc.) 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 IL-1β, NLRP3, ASC and caspase-1 (Applied Biosystems). Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems, Foster City, CA). Relative mRNA levels were determined from standard curves run with each experiment. The mRNA gene expressions were normalized to GAPDH endogenous control (Applied Biosystems).

Western blot analysis

LAD2 cells (1×10^6 cells) were pre-incubated with Glybenclamide (50 μM), AC-YVAD-CMK (50 μM) or methoxyluteolin (50 μM) for 2 hrs and then stimulated with SP (1 μM), IL-33 (30 ng/mL) and/or their combination for 24 hr. The reaction was stopped by addition of ice-cold phosphate buffered saline (PBS). Cells were washed once with PBS and then lysed using protein lysis radio-immuno precipitation (RIPA) buffer (Sigma-Aldrich) in the presence of protease and phosphotase inhibitor cocktails (Thermo Fisher Scientific Inc.). Total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific Inc.) method using bovine serum albumin (BSA) as standard. The total cellular proteins (20 μg aliquots) were separated using 4-20 % Mini Protean TGX gels (Biorad) under SDS denaturing conditions and electrotransfered onto PVDF membranes (Biorad). Blocking was carried out with 5% BSA in Tris-buffered

saline containing 0.05 % Tween-20. The membranes were probed with the following primary antibodies at 1:1,000 dilutions: NLRP3, pro-IL-1β, caspase-1, β-actin (Cell Signaling Technology), ASC and cleaved IL-1β (Santa Cruz Biotechnology). For the loading control β-actin was probed. For detection, the membranes were incubated with the appropriate secondary HRP-conjugated antibody (Cell Signaling Technology) at 1:1,000 dilution and the blots were visualized with enhanced chemiluminescence using SuperSignal© West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.).

FLICA® Caspase-1 Activity Assay

LAD2 cells ($2.5x10^5$) were treated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 h, centrifuged at 500 g for 5 min and washed in Apoptosis Wash Buffer (ImmunoChemistry Technologies). Cells were resuspended in 300 μL 1X FLICA-YVAD (ImmunoChemistry Technologies), fluorescent caspase-1 binding probe, and incubated for 1 hr at 37° C. Propidium Iodide (PI) staining was used as negative control. Following the incubation, unreacted substrate was removed by washing the cells with Apoptosis Wash Buffer. Finally, cells were resuspended in 300 μl of Apoptosis Wash Buffer for flow cytometry analysis. Active caspase-1 was determined using a FACSCalibur flow cytometer (BD Biosciences).

Caspase-1 Activity Assay

LAD2 cells ($5 x 10^4$) were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 h in black 96-well plates (Corning Inc.). After stimulation, 50 μM YVAD-AFC was added to cells and incubated for 2 hr at 37°C (Ac-YVAD-AFC, Santa

Cruz Biotechnology). Fluorescence was measured by a SpectroMax Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The medium background was subtracted from raw fluorescent values and then the values were normalized to control untreated cells.

Statistics

All experiments were performed in triplicate and were repeated for at least three times (n=3). Data are presented as mean ± SD. Results were analyzed using the unpaired, 2-tailed, Student's *t*-test. Significance of comparisons between conditions is denoted by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, **** $p<0.0001$, respectively.

3.3 Results

SP and IL-33 synergistically increase IL-1β secretion

In order to investigate IL-1β secretion, LAD2 cells were first stimulated with known IL-1β triggers such as LPS (100 ng/mL), ATP (5 μM) and their combination, as well as Nigericin (10 μM), TNF (50 ng/mL), IFN-γ (100 U), and IgE (1 μg/mL)/anti-IgE (10 μg/mL). Their responses were compared to stimulations with IL-33 (30 ng/mL) and SP (1 μM), a known mast cell activator. None of these known triggers except Nigericin stimulate IL-1β secretion from LAD2 cells (Figure 3.1). Instead, stimulation of LAD2 cells by SP (1 μM) or IL-33 (30 ng/mL) alone for 24 hrs results in 15 and 35 pg/10^6cell/mL of IL-1β secretion, respectively ($p=0.15$, $p=0.088$) (Figure 3.1). However, the combination of SP (1 μM) and IL-33 (30 ng/mL) produces a potent statistically significant ($p<0.01$) augmentation increasing IL-1β secretion by 20-fold compared to SP-treated cells (Figure 3.1).

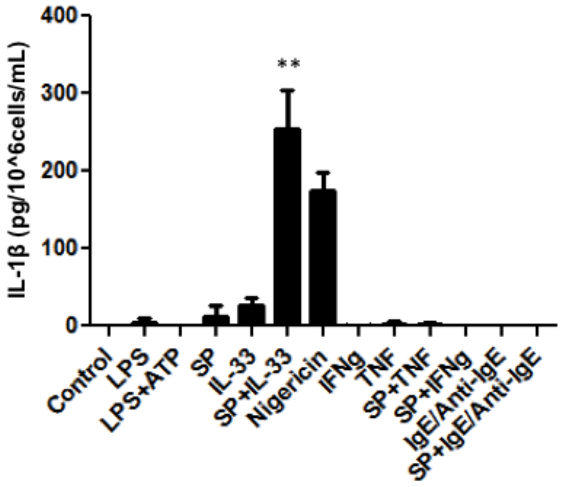

Figure 3.1. SP and IL-33 stimulate IL-1β Secretion. LAD2 cells (1×10^5 cells/well) were seeded in 96-well culture plate and stimulated with LPS (100 ng/mL), ATP (5 μM), SP (1 μM), IL-33 (30 ng/mL), Nigericin (10 μM), TNF (50 ng/mL), IFN-γ (100 U), IgE (1 μg/mL)/anti-IgE (5 μg/mL) or their combinations as shown for 24 hr. Control cells were treated with culture media only. Supernatant fluids were collected at the end of the incubation period. IL-1β secretion was assayed using ELISA (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

To select the optimal doses of SP and IL-33 for the study of IL-1β secretion LAD2 cells were treated with various concentrations of SP and IL-33. Stimulation by SP alone (0.01-1 μM) does not trigger IL-

1β secretion on its own, but in combination with IL-33 (30 ng/mL) it shows a dose-dependent increase (Figure 3.2A) with maximum IL-1β secretion 1 μM SP ($p<0.0001$) (Figure 3.2A). Next LAD2 cells were stimulated with SP (1 μM) and IL-33 (1-100 ng/mL) in combination for 24 hr. The combination of SP (1 μM) and IL-33 (30 ng/mL) produces significant ($p<0.0001$) increase in IL-1β secretion of approximately 400 pg/mL (Figure 3.2B).

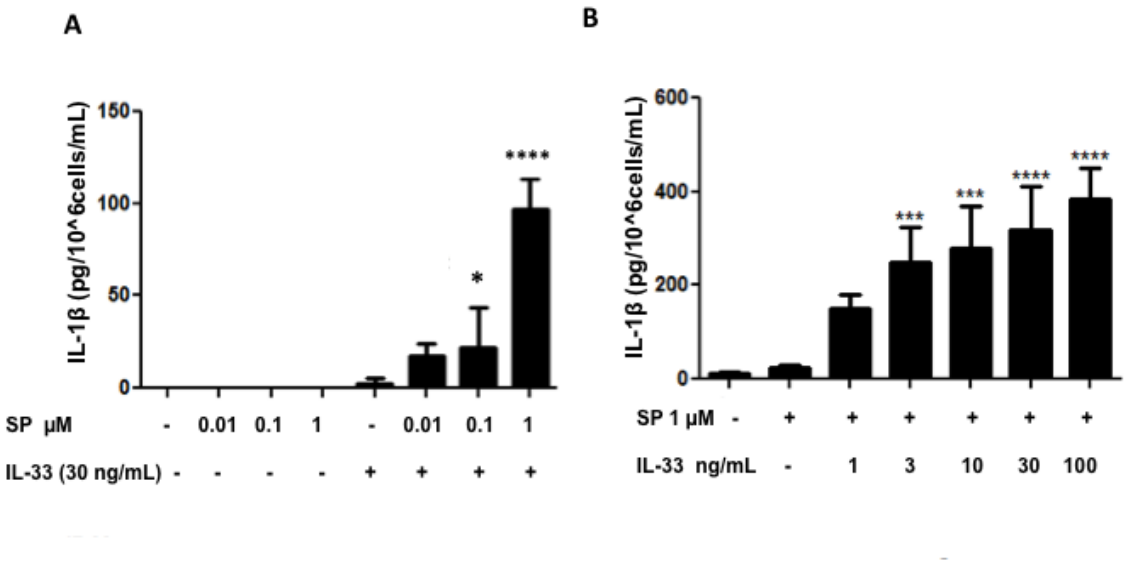

Figure 3.2. Selection of the optimal doses to study IL-1β secretion stimulated by SP and IL-33 when administered in combination. LAD2 cells (1×10^5 cells /well) were stimulated with **(A)** SP (0.01-1 μM), **(B)** IL-33 (1-100 ng/mL) and their combination as shown for 24 hr. Supernatant fluids were collected at the end of the incubation period. IL-1β secretion was assayed using ELISA (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Therefore, this combination was selected for the further studies. A time course study shows that 24 hr of stimulation yields the highest amount of IL-1β secretion (Figure 3.3). Cell viability was tested using propidium iodide (PI) staining. LAD2 cells treated with SP (1μM), IL-33 (30 ng/mL) or their combination for 24 hrs are 96-97% viable (Figure 3.4).

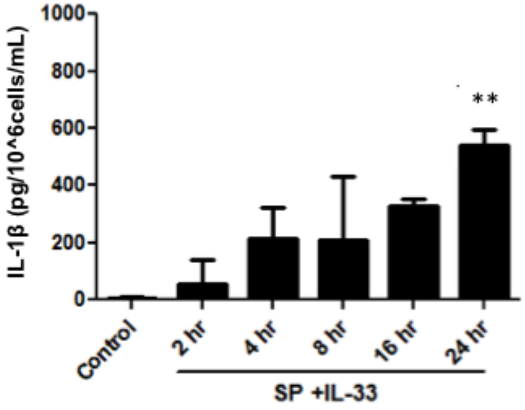

Figure 3.3. Time-dependent IL-1β secretion. LAD2 cells (1×10^5 cells/well) were stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL) for 2-24 hr. Supernatant fluids were collected at the end of the incubation period. IL-1β secretion was assayed using ELISA (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

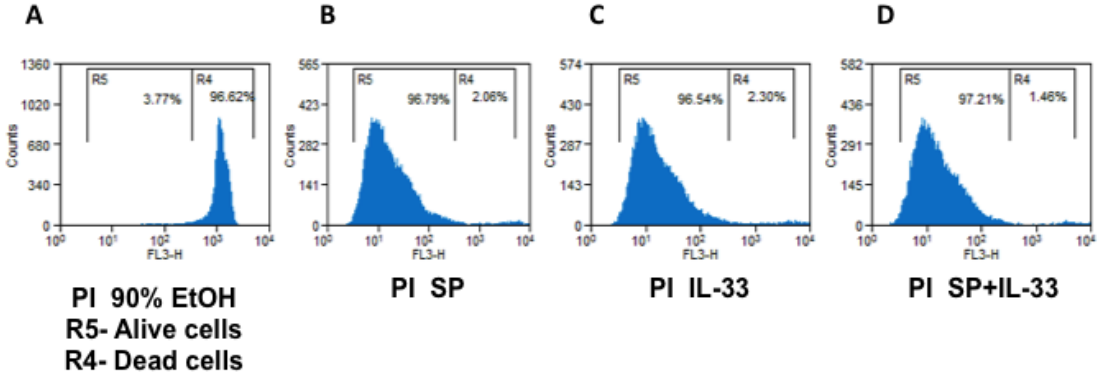

Figure 3.4. SP and IL-33 does not affect cell viability. LAD2 cells (0.25×10^6 cells/well) were stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. Stimulated cells were incubated with Propidium Iodide (PI) for 5 min and PI-positive cells were assessed by flow cytometry (n=3).

We also investigated whether SP, IL-33 or their combination can have a synergistic effect on allergic stimulation with IgE/anti-IgE. Interestingly, LAD2 cells triggered with SP (1 μM) and IL-33 (30 ng/mL) in combination and also challenged with IgE/anti-IgE show significant ($p<0.001$) decrease in IL-1β secretion (Figure 3.5), suggesting that upon this stimulation human mast cells produce more inflammatory response rather than allergic.

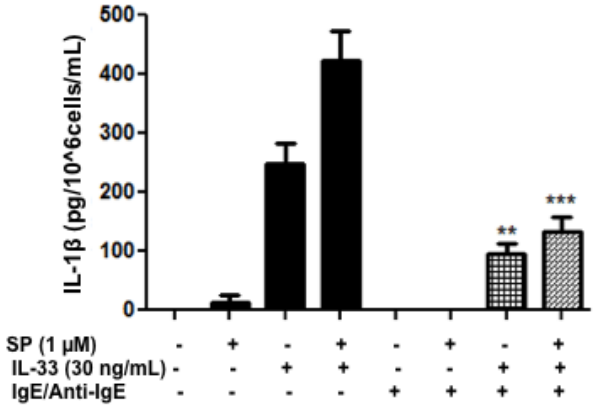

Figure 3.5. IgE/anti-IgE decrease SP and IL-33 stimulated secretion of IL-1β. LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with human IgE (1 μg/mL) or medium overnight and next day were stimulated with anti-IgE (10 ng/mL) for 2 hr and/or SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. Supernatant fluids were collected at the end of the incubation period and assayed by IL-1β ELISA (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

To further investigate this inflammatory response with selected doses of SP and IL-33, we stimulated LAD2 and human umbilical cord blood derived mast cells (hCBMCs) with SP and IL-33 for 6 hrs and measured IL-1β gene expression. SP (1 μM) and IL-33 (30 ng/mL) synergistically increase IL-1β gene expression by 90-fold ($p<0.0001$) in LAD2 cells and by 200-fold ($p<0.0001$) in hCBMCs (Figure 3.6A-B). The combination of SP and IL-33 also significantly increases intracellular protein (Figure 3.6C) and secretion of IL-1β (Figure 3.6D) by 100-fold ($p<0.0001$).

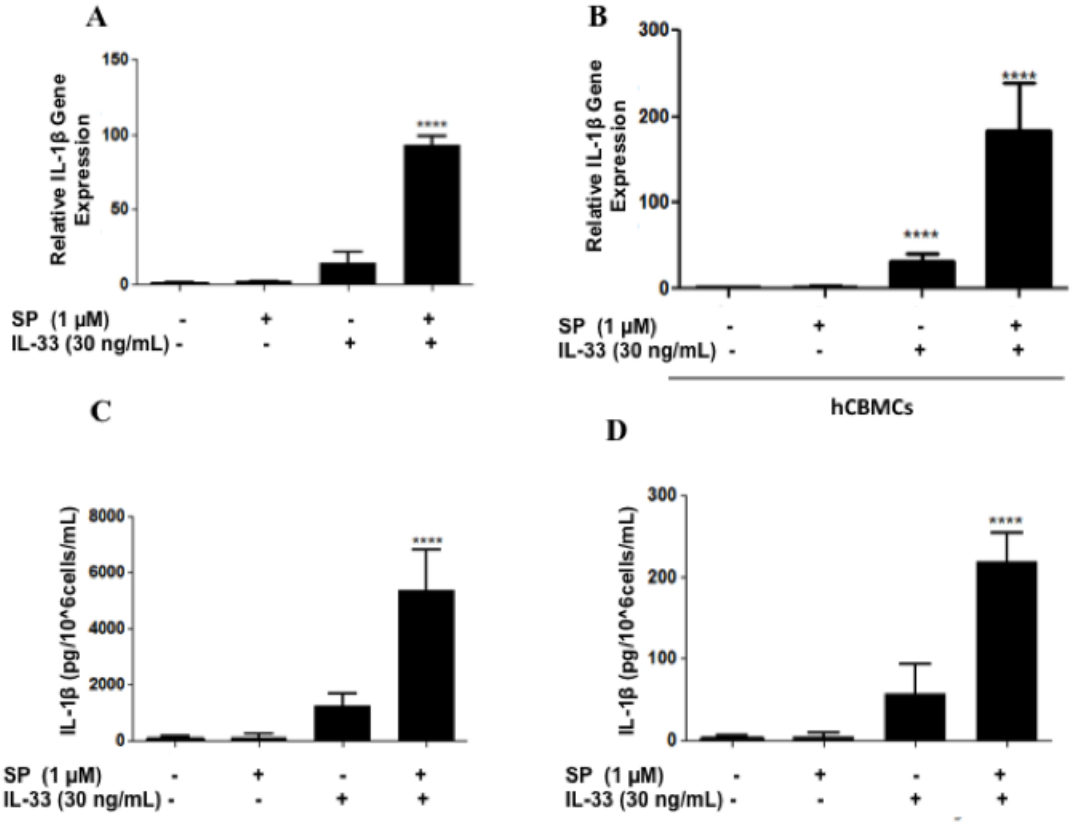

Figure 3.6. SP and IL-33 markedly enhance IL-1β gene expression and secretion in human mast cells. (A) LAD2 cells (1×10^6 cells per well) and **(B)** hCBMCs (0.3×10^6 cell per well) were seeded in 12-well culture plate and stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 6 hr. IL-1β mRNA expression levels were measured by qRT-PCR and normalized to human GAPDH endogenous control. **(C-D)** LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination as shown for 24 hr. Control cells were treated with culture media only. Supernatant fluids **(D)** and cell lysates **(C)** were collected at the end of the incubation period and assayed by IL-1β ELISA (n=3, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

NK-1 receptor antagonists, L-733,060 and CP-96345, inhibit IL-1β secretion stimulated by SP and IL-33

Next we investigated whether the synergistic effect of SP and IL-33 is mediated through the SP receptor, the neurokinin 1 (NK-1R). LAD2 cells were pre-incubated with the NK-1 antagonist L-733,060 (10 μM) or CP-96345 (10 μM) for 30 min and then

stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. Pre-incubation with the NK-1 receptor antagonists significantly ($p<0.001$) inhibits IL-1β secretion by approximately 85% (Figure 3.7). Surprisingly, the antagonists also significantly ($p<0.001$) inhibit the effect of IL-33 alone by the same extent (Figure 3.7) implying that the NK-1 and ST2 receptors may be interacting. (The interaction between NK-1 and ST2 receptor was discussed in Chapter 2).

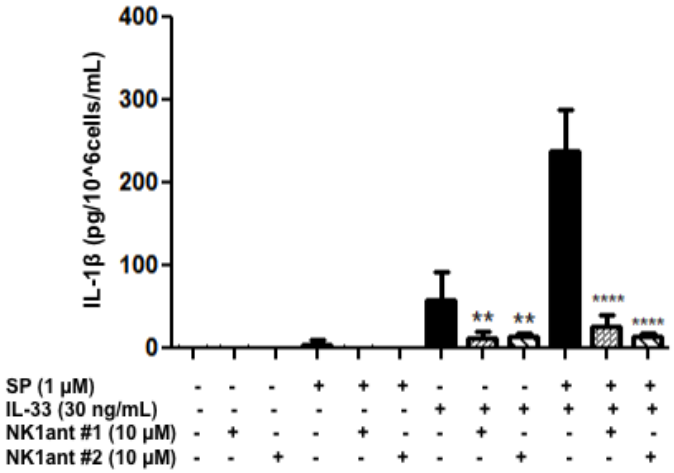

Figure 3.7. NK-1 receptor antagonists inhibit IL-1β secretion. LAD2 cells were pretreated with NK-1R antagonists L-733,060 (10 μM) and CP-96345 (10 μM) for 30 min and then were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. Collected supernatant fluids were assayed by IL-1β ELISA (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

ST2 receptor neutralizing antibody inhibits IL-1β secretion stimulated by the combination of SP and IL-33

Then we investigated whether IL-33 receptor, ST2, inhibition will diminish the synergistic effect of IL-33 and SP. LAD2 cells were pretreated with a ST2 receptor neutralizing antibody and a non-specific IgG control over a number of concentrations (Figure 3.8A) to select the optimal dose of the neutralizing antibody. LAD2 cells were pre-incubated with the ST2 neutralizing antibody (3 μg/mL) or IgG control (3 μg/mL) for 2 hr and then stimulated with SP (1 μM) alone, IL-33 (30 ng/mL) alone or the combination of both for 24 hr. Pre-incubation with the ST2 neutralizing antibody

significantly ($p<0.0001$) inhibits IL-1β secretion by 60% when IL-33 and SP were administered in combination and by 50% when stimulated by IL-33 alone (Figure 3.8B).

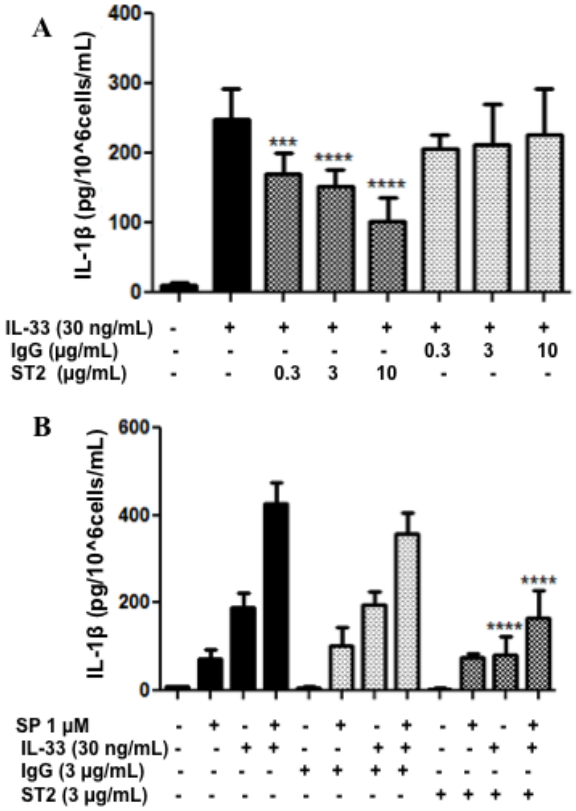

Figure 3.8. ST2 neutralizing antibody inhibits IL-1β secretion. (A) LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with ST2 neutralizing antibody (0.3µg/mL- 10 µg/mL) or IgG control (0.3µg/mL- 10 µg/mL) for 2 hr and then stimulated with IL-33 (30 ng/mL) for 24 hr. **(B)** LAD2 cells were pre-incubated with ST2 neutralizing antibody (3 ng/mL) or IgG control (3 ng/mL) for 2 hr and then were stimulated with SP (1 µM), IL-33 (30 ng/mL) or their combination for 24 hr. Collected supernatant fluids were assayed by IL-1β ELISA (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Active Caspase-1 is expressed in unstimulated mast cells and SP and IL-33 increase its gene expression and activity

Next we investigated whether the combination of SP and IL-33 affects the NLRP3 inflammasome activation resulting in the subsequent increase of IL-1β secretion from human mast cells. IL-33 (30 ng/mL) or the combination of SP (1 µM) and IL-33 (30 ng/mL) stimulate pro-IL-1β protein expression (Figure 3.9) but does not affect the protein levels of NLRP3 and ASC. Interestingly, active forms of capsase-1 (p20) and IL-1β (p17) are present even in unstimulated LAD2 cells (Figure 3.9).

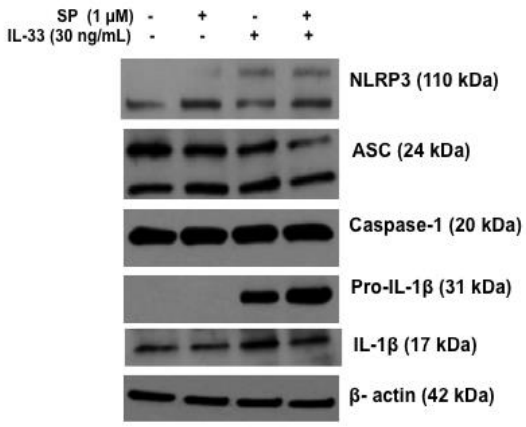

Figure 3.9. NLRP3 Inflammasome components and mature form of IL-1β are expressed in human mast cells. LAD2 cells (1×10^6 cells/well) were seeded in 12-well culture plate and were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. Cell lysates were collected after 24 hrs and protein levels of the NLRP3 inflammasome components (NLRP3, ASC, caspase-1), pro-IL-1β and active IL-1β (p17) were measured by Western blot, using β-actin as loading control (n=3).

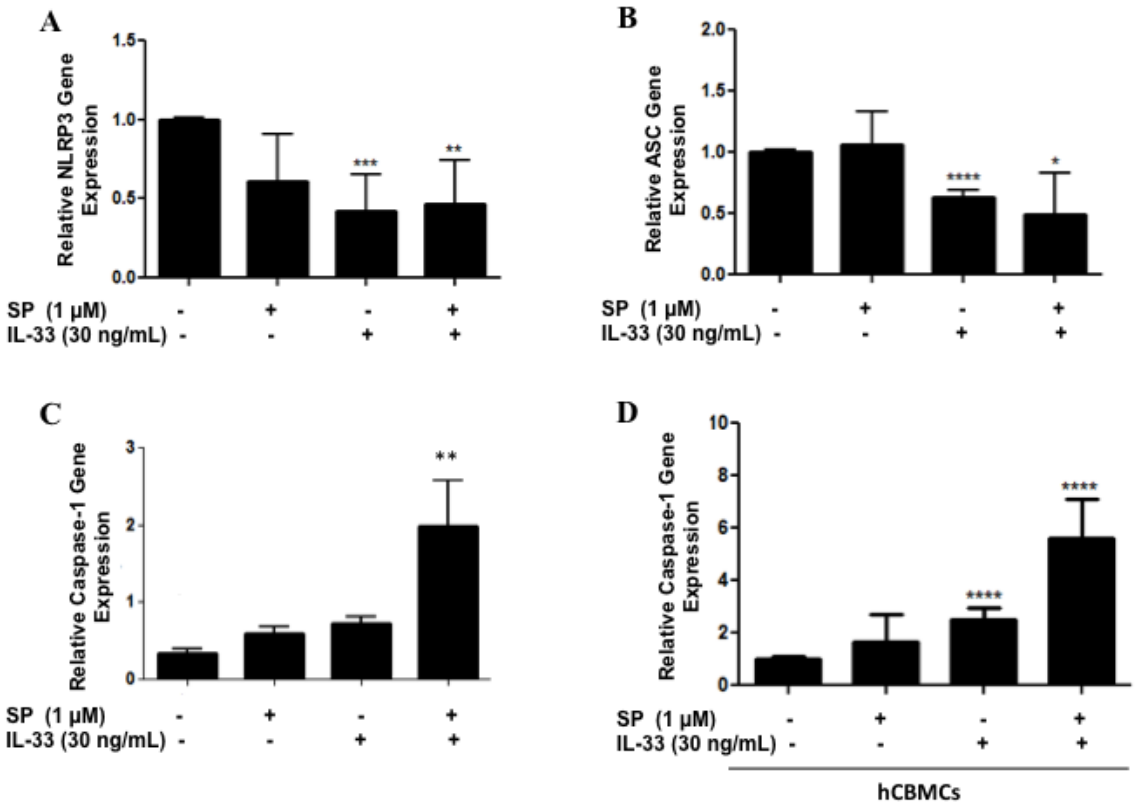

Figure 3.10. SP and IL-33 increase caspase-1 gene expression in human mast cells. LAD2 cells (1×10^6 cells/well) **(A-C)** and hCMBCs (0.3×10^6 cell/well) **(D)** were seeded in 12-well culture plate were stimulated SP (1 μM), IL-33 (30 ng/mL) or their combination for 6 hr. The gene expression of the NLRP3 components, (A) NLRP3, (B) ASC and (C-D) Caspase-1 gene expression were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

This finding prompted us to measure the gene expression of the NLRP3 inflammasome components, NLRP3, ASC and caspase-1, as well as caspase-1 cellular activity. The combination of SP (1 μM) and IL-33 (30 ng/mL) significantly ($p<0.01$) increases caspase-1 gene expression by 5-fold (Figure 3.10) in LAD2 and hCBMCs but does not affect to the same extend NLRP3 and ASC gene expression. Caspase-1 activity is also increased by 2-fold in the supernatant fluids following the stimulation with SP and IL-33 (Figure 3.11). Next, we investigated the presence of the active caspase-1 in unstimulated and stimulated LAD2 cells using FLICA© assay. Untreated control LAD2 cells make up 76.8% of active caspase-1 positive cells (Figure 3.12A). The stimulation by SP (1 μM), IL-33 (30 ng/mL) or their combination does not significantly change the percentage of active caspase-1 positive cells (Figure 3.12B-D), as it does not change the protein expression of active caspase-1 (Figure 3.9).

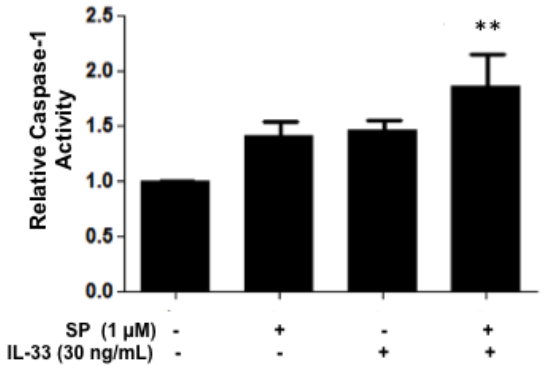

Figure 3.11. SP and IL-33 increase caspase-1 activity. LAD2 cells (0.5×10^5 cells/well) were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr; after stimulation, 50 μM YVAD-AFC substrate was added to cells and incubated for 2 hr. Caspase-1 activity in the supernatant fluids was determined by measuring fluorescence at 405 nm wavelength. Fold-increase in caspase-1 activity was determined by comparing the values to the untreated control samples (n=3).

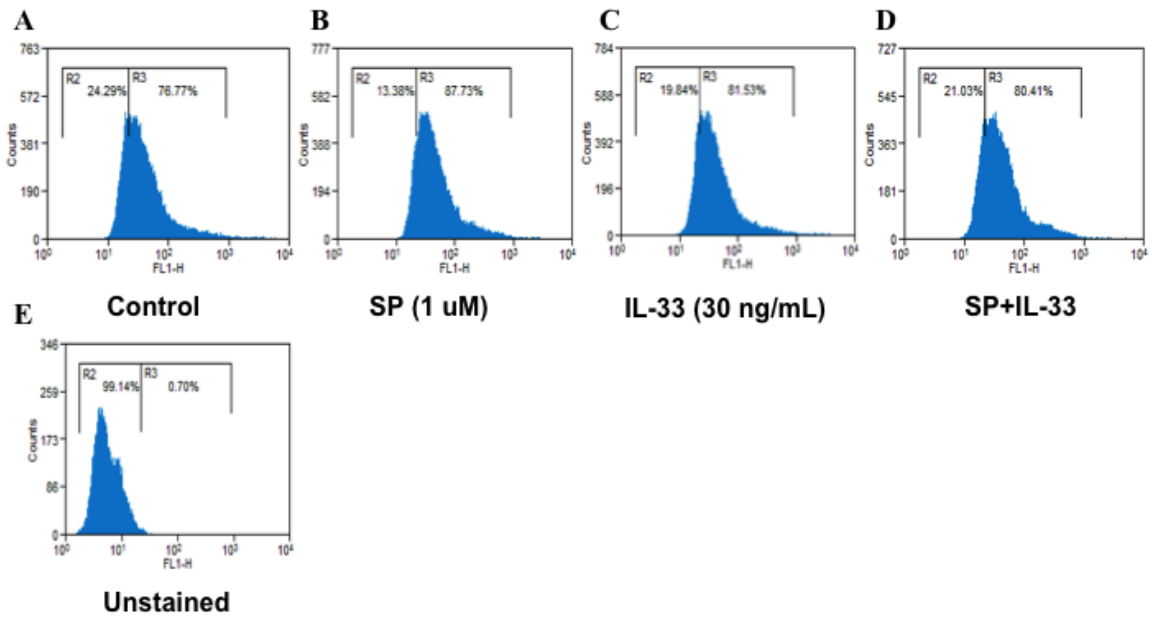

Figure 3.12. Active caspase-1 is constitutively present in human mast cells. LAD2 cells (0.25×10^6 cells/well) were stimulated with SP (1 μM), IL-33 (30 ng/mL) or their combination for 24 hr. Stimulated cells were incubated with FLICA caspase-1 probe for 1 hr and active capsase-1 positive cells were assessed by flow cytometry (n=3).

Methoxyluteolin and Inflammasome inhibitors decrease IL-1β secretion

To investigate the regulation of IL-1β secretion following the combined stimulation of SP and IL-33, we used the selective irreversible caspase-1 inhibitor AC-YVAD-CMK (25-100 μM), the potassium channel blocker Glybenclamide, which is known to block NLRP3 inflammasome activation [218] (25-100 μM), and methoxyluteolin (25-100 μM). For simplicity, AC-YVAD-CMK and Glybenclamide are designated as inflammasome inhibitors. As expected the inflammasome inhibitors do not significantly inhibit gene expression and secretion of TNF (Figure 3.13B-14B), used as a "negative" control as it does not require NLRP3 activation; however, they significantly inhibit IL-1β secretion ($p<0.05$) (Figure 3.13A) and IL-1β gene expression (($p<0.05$) (Figure 3.14A). Methoxyluteolin significantly ($p<0.05$) inhibits IL-1β secretion by 85% and gene

expression by 95% at 50 µM (Figure 3.13A-14A). In fact, methoxyluteolin significantly ($p<0.01$) inhibits IL-1β secretion by 45% at 1 µM (Figure 3.15B).

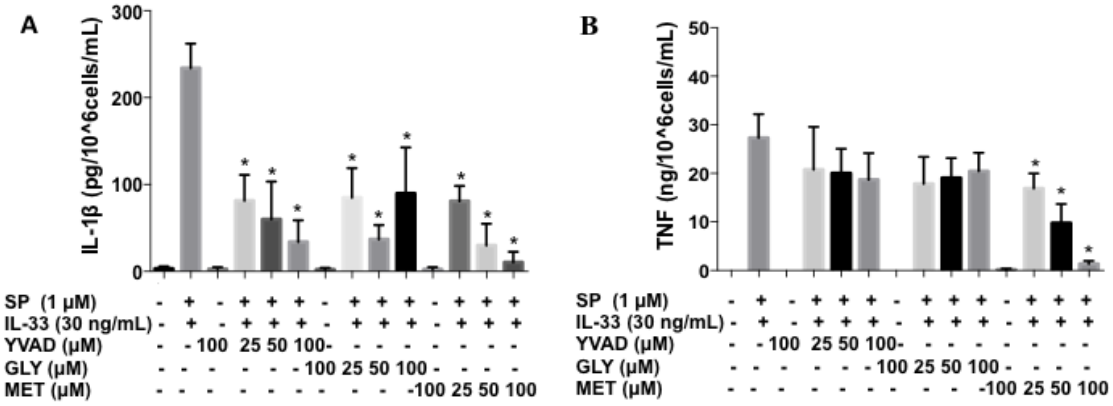

Figure 3.13. Methoxyluteolin and inflammasome inhibitors reduce IL-1β secretion. **(A-B)** LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with AC-YVAD-CMK (YVAD 25-100 µM), Glybenclamide (GLY 25-100 µM), and methoxyluteolin (MET 25-100 µM) for 2 hrs and then stimulated with the combination of SP (1µM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with 0.1% DMSO, the highest concentration corresponding to that of 100 µM. Collected supernatant fluids were assayed using ELISA for IL-1β and TNF, as a negative control. (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

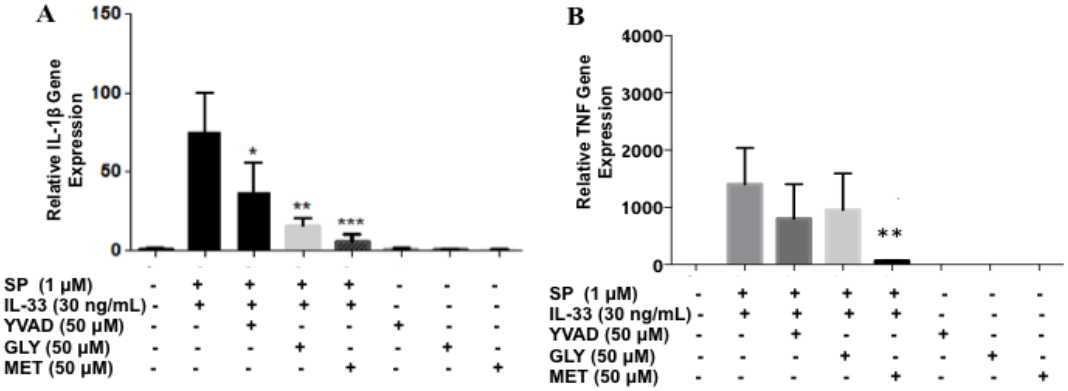

Figure 3.14. Methoxyluteolin and inflammasome inhibitors decrease IL-1β gene expression. LAD2 cells (1×10^6 cells per well) were seeded in 12-well culture plate and pre-incubated with AC-YVAD-CMK (YVAD 50 µM), Glybenclamide (GLY 50 µM), methoxyluteolin (MET 50 µM) and then stimulated with the combination of SP (1µM) and IL-33 (30 ng/mL) for 6 hr. IL-1β and TNF, as negative control, gene expression

levels were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3, * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001).

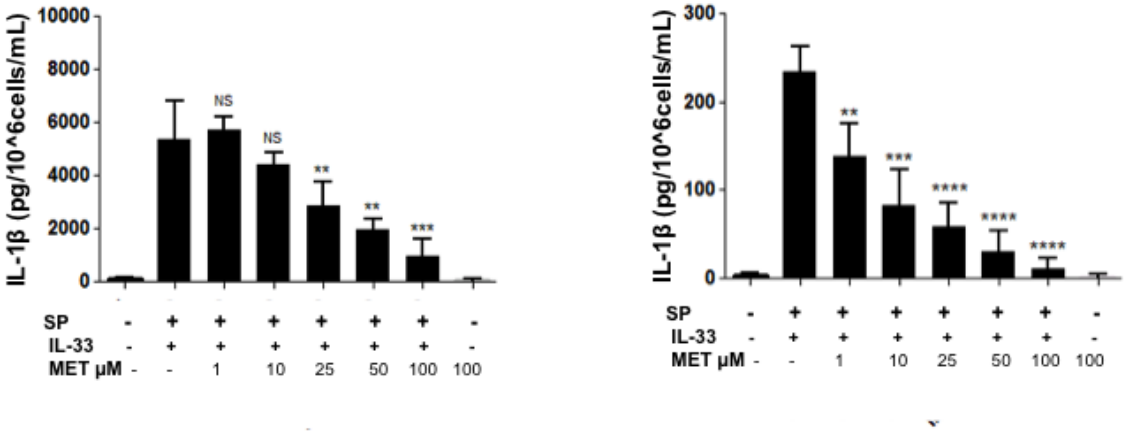

Figure 3.15. Dose-dependent inhibition of IL-1β secretion. LAD2 cells were pretreated with methoxyluteolin (MET 1- 100 µM) for 2 hr and then were stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. Collected lysates (**A)** and supernatant fluids **(B)** were assayed for IL-1β ELISA (n=3, * p<0.05, ** p<0.01, and *** p<0.001).

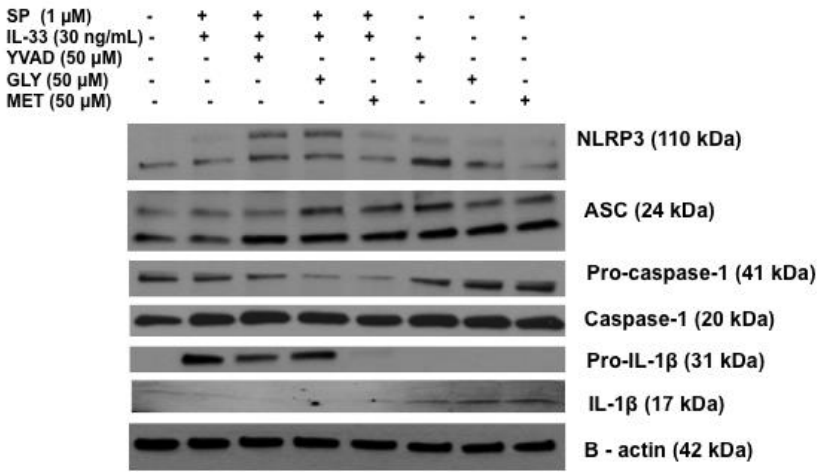

Figure 3.16. Methoxyluteolin decreases caspase-1 gene expression. LAD2 cells (1×10^6 cells/well) were seeded in 12-well culture plate and pre-incubated with AC-YVAD-CMK (YVAD 50 µM), Glybenclamide (GLY 50 µM) or methoxyluteolin (MET 50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 6 hr. The gene expression of the NLRP3 components, **(A)** NLRP3, **(B)** ASC and **(C)** caspase-1 gene expression were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3, * p<0.05, ** p<0.01, and *** p<0.001).

Next we studied the mechanism of NLRP3 inflammasome activation by SP and IL-33. Inflammasome inhibitors and methoxyluteolin, used at the same concentrations as in Figure 3.14, reduce the protein expression of pro-caspase-1, pro-IL-1β and active form

of IL-1β (p17); however the levels of active caspase-1 remain unchanged as well as the protein levels of NLRP3 and ASC (Figure 3.16). Casapse-1 gene expression is significantly ($p<0.05$) inhibited by methoxyluteolin (Figure 3.17C) and when NLRP3 and ASC gene expression is not affected to the same extend (Figure 3.17A-B).

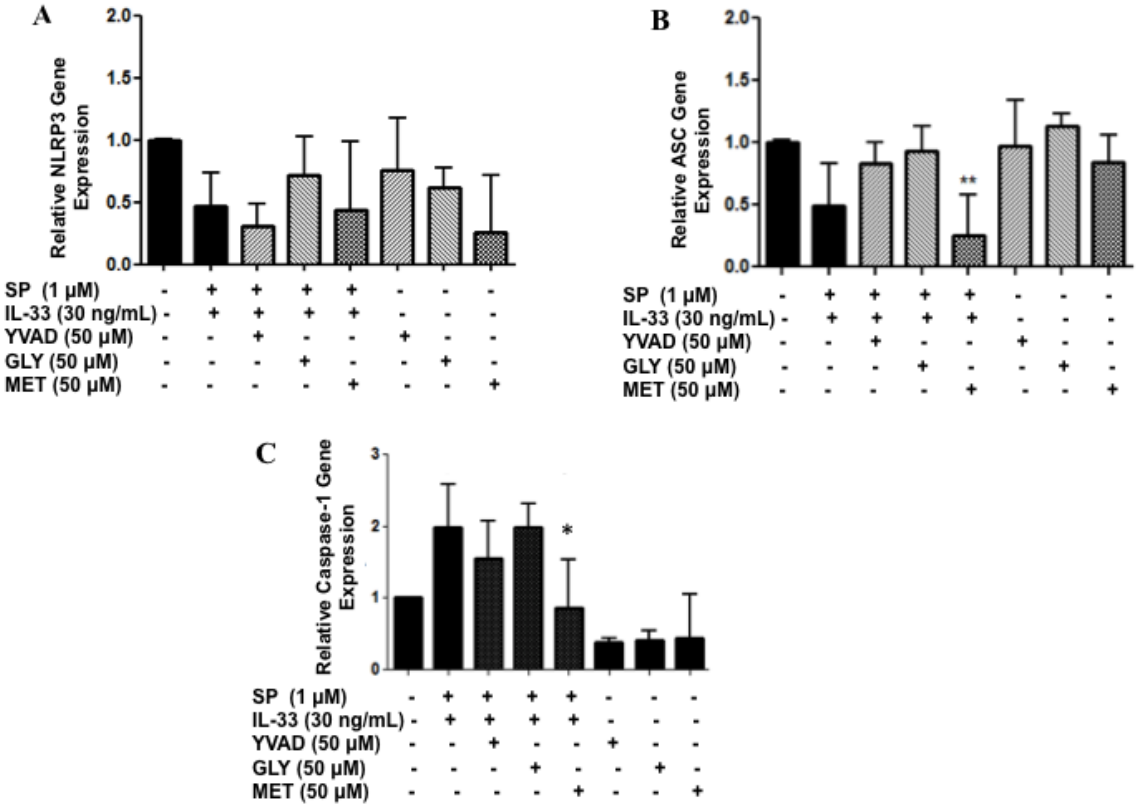

Figure 3.17. Methoxyluteolin and NLRP3 inflammasome inhibitors prevent IL-1β synthesis. (A) LAD2 cells (1×10^6 cells/well) were seeded in 12-well culture plate and pre-incubated with AC-YVAD-CMK (YVAD 50 μM), Glybenclamide (GLY 50 μM), methoxyluteolin (MET 50 μM) for 2 hrs and then stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. Cell lysates were collected after 24 hrs and protein levels of the NLRP3 inflammasome components (NLRP3, ASC, caspase-1), pro-IL-1β and active IL-1β (p17) were measured by Western blot, using β-actin as loading control (n=3).

Caspase-1 activity is reduced in the supernatant fluids after pre-incubation of stimulated LAD2 cells with the inflammasome inhibitors and significantly ($p<0.01$) inhibited by methoxyluteolin (Figure 3.18). However, inflammasome inhibitors and

methoxyluteolin do not reduce the percentage of active caspase-1-positive cells, but to a small extend increase it instead (Figure 3.19), as they do not change the protein expression of active caspase-1 (Figure 3.16).

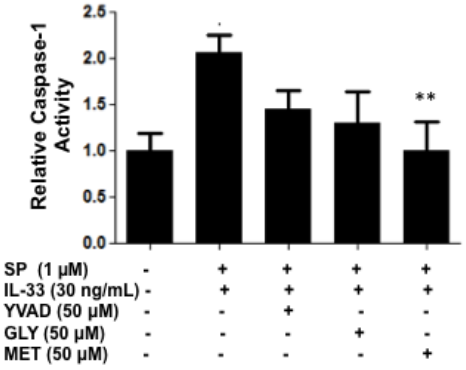

Figure 3.18. Methoxyluteolin decreases caspase-1 activity. LAD2 cells (0.5×10^5 cells/well) were pre-incubated with AC-YVAD-CMK (YVAD 50 µM), Glybenclamide (GLY 50 µM) or methoxyluteolin (MET 50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. After stimulation, 50 µM YVAD-AFC substrate was added to cells and incubated for 2 hr. Caspase-1 activity in the supernatant fluids was determined by measuring fluorescence at 405 nm. Fold-increase in caspase-1 activity was determined by comparing the values to the untreated control samples (n=3).

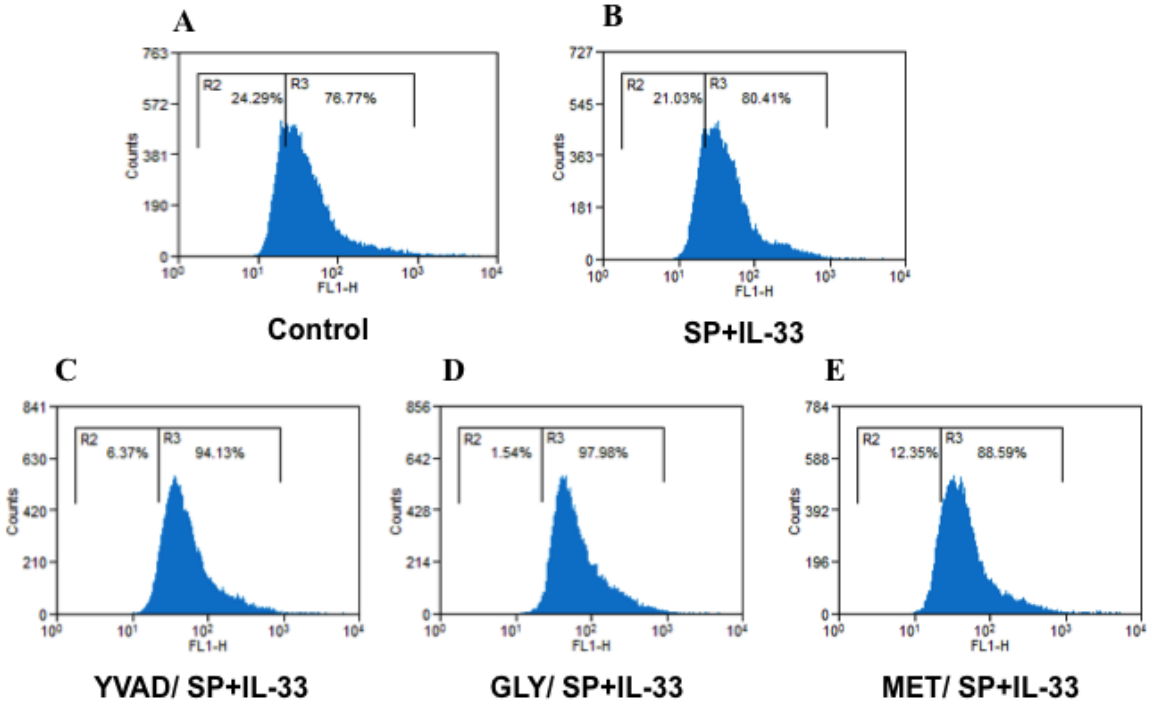

Figure 3.19. Inflammasome inhibitors and methoxyluteolin do not decrease the percentage of active caspase-1 expressing human mast cells. LAD2 cells (0.25×10^6 cells/well) were pre-incubated with AC-YVAD-CMK (YVAD 50 µM), Glybenclamide (GLY 50 µM) or methoxyluteolin (MET 50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. Stimulated cells were incubated with FLICA caspase-1 probe for 1 hr and active capsase-1 positive cells were assessed by flow cytometry (n=3).

3.4 Conclusion

This study identifies novel stimuli of IL-1β secretion from human mast cells that provide a new way of understanding the complexity of stress and inflammation on the example of neuropeptides, cytokines and mast cells interplay. The findings reported here are unique in showing that SP and IL-33 result in a significant synergistic increase of IL-1β gene expression, intracellular protein levels and secretion from human cultured mast cells. The ability of the SP and IL-33 alone, and most importantly when administered together, to stimulate IL-1β secretion from mast cells is even more striking due to the fact that none of the known triggers of the NLRP3 inflammasome (LPS, ATP, TNF, IFN-γ) alone or in combination, except for Nigericin, can stimulate IL-1β secretion from cultured human mast cells.

The complex interactions between cytokines and neuropeptides could be regulated via interaction of their receptors. We show that pre-incubations with SP receptor (NK-1R) antagonists or IL-33 receptor (ST2) neutralizing anybody inhibit SP and IL-33-induced IL-1β secretion at different extend. A surprising finding is the ability of these antagonists to also inhibit IL-1β stimulation by IL-33 alone, implying some form of the interaction between IL-33 receptor, ST2, and SP receptor, NK-1R. It was previously shown that ST2 receptor can interact with c-Kit receptor on mast cells [195].

Human cultured mast cells show an alternative assembly of the NLRP3 inflammasome and regulate IL-1β secretion primarily via signal 1. The protein levels of all the NLRP3 components (NLRP3, ASC, capsase-1) and IL-1β have been detected in LAD2 cells; however, their active forms' presence is responsible for the alternative assembly. IL-33 alone triggers IL-1β gene expression and pro-IL-1β synthesis and the

combination of SP and IL-33 increases it even further, suggesting that this process is mostly regulated via transcriptional NF-κB activation (signal 1). The simulation of SP and IL-33 alone or in combination does not affect the gene and protein expression of NLRP3 and ASC proteins, suggesting that the oligomerization of the NLRP3 inflammasome (signal 2), where NLRP3, ASC and pro-caspase-1 complex together to release active caspase-1, is not essential for the secretion of IL-1β. Moreover, caspase-1 and IL-1β are detected in their active forms (p20 and p17) in unstimulated mast cells, which means that mast cells have pre-stored constitutively active caspase-1 and IL-1β and may not require the full assembly of NLRP3, ASC and pro-caspase-1 proteins to release active caspase-1 and subsequently cleave pro-IL-1β into active IL-1β. The only other similar reports are of human blood monocytes [219] and melanoma cells [220] constitutively expressing active caspase-1 and spontaneously secreting IL-1β [219]. However, unlike those studies, the human mast cells do not spontaneously release active IL-1β even though its active cleaved form (p17) is found in mast cell lysates. The presence of the active caspase-1 in unstimulated LAD2 cells was confirmed by cell sorting showing over 75% of the untreated control mast cells contain active caspase-1. We also found that caspase-1 activity was increased by 2-fold in the supernatant fluids of mast cells following stimulation with SP and IL-33. Active caspase-1 secretion along with IL-1β was previously shown in mouse macrophages and dendritic cells [221]. We speculate that mast cells pre-store caspase-1 and IL-1β in their active forms in order to respond rapidly to danger signals [212]. In contrast to the rapid response, the stimulation of SP and IL-33 over 24 hr triggers synthesis of new IL-1β and caspase-1 mainly regulated via signal 1.

Even though IL-1β neutralizing therapies are available [222], there are no available inhibitors that block IL-1β synthesis and secretion. Here we report that methoxyluteolin, a naturally occurring flavonoid inhibits IL-1β synthesis and secretion via signal 1. Luteolin (5,7,3',4'-tetrahydroxyflavone) a structural analog of methoxyluteolin, had been reported to inhibit the NLRP3 inflammasome and subsequent IL-1β secretion in endothelial cells, but the mechanism of NLRP3 inhibition was not explored [223]. Strikingly, in addition to preventing IL-1β secretion, methoxyluteolin significantly inhibits IL-1β and caspase-1 gene expression as well as pro-IL-1β and pro-caspase-1 protein expression, while also reducing caspase-1 activity in the supernatant fluids. These inhibitory effects could be explained by the ability of methoxyluteolin to inhibit the NF-κB transcription factor in LAD2 mast cells [165]. These novel findings indicate that methoxyluteolin might be targeting IL-1β synthesis and secretion and at the transcriptional level.

NLRP3 activation and IL-β secretion are key players in the pathogenesis of a number of inflammatory diseases: (a) genetically defined like familial Mediterranean fever, Muckle-Well syndrome, and CAPS, as well as (b) diseases that involve NLRP3 activation by danger signals such as gout, pseudogout, Alzheimer's disease, asbestosis, silicosis, and type 2 diabetes mellitus [138, 138, 224]. Moreover, NLRP3 inflammasome activation and IL-1β secretion, as well mast cells, have been implicated in rheumatoid arthritis [225], multiple sclerosis [226], psoriasis [110, 227, 228], and asthma [229].

In conclusion, SP and IL-33 are identified as novel stimuli of IL-1β synthesis secretion in human mast cells, which exhibit an alternative assembly of the NLRP3 inflammasome, and are inhibited by methoxyluteolin. Together these findings provide

new insights into the complex interplay among neuropeptides, cytokines and mast cells and expand our understanding of the regulation and inhibition of IL-1β secretion that could be new approaches for the development of anti-inflammatory therapies.

Chapter 4: Non-canonical Regulation of IL-1β Secretion via Caspase-8 in Human Mast Cells Stimulated by the Neuropeptide Substance P (SP) and the Cytokine Interleukin (IL)-33

4.1 Background

Interleukins are crucial cytokines in the signaling and regulation of immune and inflammatory reactions [109]. IL-1β and IL-33 play an important role in regulation of innate and adaptive immune systems [113, 111]. As introduced in the previous chapter IL-1β is present in the cytoplasm in a biologically inactive form. It is activated by the proteolytic cleavage of caspase-1, which is also present in the cytoplasm in pro-form, and it is activated by the multiprotein complex known as NLRP3 inflammasome [111]. IL-33 is a newly identified IL-1 family cytokine, which is synthesized in its pro-form and can also be processed by caspase-1. However, pro-IL-33 contains the nuclear localization sequence (NLS), and can induce production of Th2-associated cytokines [114]. In addition, IL-33 can stimulate activation of mast cells with further release of several mediators such as TNF, IL-6, PGD_2, and monocyte chemoattractant protein 1 (MCP-1)[114]. IL-33 also has synergistic effect with inflammatory neuropeptides such as SP stimulating the secretion of vascular endothelial growth factor (VEGF) [47]. As it was shown in the previous chapters, IL-33 and SP synergistically trigger IL-1β and TNF release form human mast cells, as well.

Due to our new interesting findings of alternative assembly of the NLRP3 inflammasome in human mast cells, we were interested to explore non-canonical regulation of IL-1β secretion. Recent studies on IL-1β processing and release show that caspase-8, a caspase better known for its role in apoptosis, can cleave IL-1β when triggered via dectin 1, a pathogen-recognition receptor (PPR) for antifungal immunity[230]. Also, caspase-8 can be activated to process IL-1β by TIR-domain-containing adapter-inducing interferon-β (TRIF) signaling, which is downstream of Toll-like Receptor

(TLR) 3 and TLR4 receptors [230]. Moreover, ripoptosome formation in response to genotoxic stress activates receptor-activating protein 3 (RIP3), which is required for pro-IL-1β processing by NLRP3, as well as by caspase-8[221]. Caspase-8 deficiency in mouse epidermal keratinocytes stimulates inflammatory skin disease[168], as well as assembly and function of NLRP3 inflammasome in mouse dendritic cells[167]. Thus, it is necessary to address the non-canonical processing of IL-1β by caspase-8 and its interactions with NLRP3 inflammasome in mast cells.

4.2 Materials and Methods

SP and E64 were purchased from Sigma-Aldrich (St Louis, MO). Recombinant human IL-33, ZVAD-fmk and IETD-fmk were obtained from R&D Systems (Minneapolis, MN). RNeasy Mini (Qiagen Inc., Valencia, CA) and iScript cDNA synthesis kits were purchased from BioRad (Hercules, CA). Taqman gene expression primers/assays for *NLRP3 (*Hs00918082_m1), *PYCARD (*Hs01547324_gH), *CASP1* (Hs00354836_m1), *IL1B* (Hs01555410_m1), *TNF* (Hs99999043_m1), and *GAPDH* endogenous control (4310884E) were purchased from Applied Biosystems (Foster City, CA). ELISA kits for IL-1β (DY201) and TNF (DY210) were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-human primary antibodies for NLRP3, pro-IL-1β, caspase-1, β-actin were purchased from Cell Signaling Technology (Danvers, MA) and mouse anti-human ASC was obtained from Santa Cruz Biotechnology (Dallas, TX). FLICA® Caspase-1 Activity Assay was purchased from ImmunoChemistry Technologies (Bloomington, MN) and Ac-YVAD-AFC was obtained from Santa Cruz Biotechnology (Dallas, TX).

Culture of human mast cells

LAD2 cells, derived from a human mast cell leukemia [192], were kindly supplied by Dr. A Kirshenbaum (NIH, Bethesda, MD) and were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human stem cell factor (rhSCF, Stemgen), kindly supplied by Swedish Orphan Biovitrum AB (Stockholm, Sweden). Cells were maintained at 37ºC in a humidified incubator at an atmosphere of 95% O_2/5% CO_2. LAD2 cells were doubling

within 2 weeks in the presence of 100 ng/mL of SCF showing slow proliferation rates. Cell viability was measured by Trypan blue exclusion [47] as well as by Propidium Iodide at all SP and IL-33 concentrations tested.

Mast Cell Treatments

LAD2 cells stimulated with SP (1 μM) and IL-33 (30 ng/mL) were treated with a pan-caspase inhibitors ZVAD (50 μM), caspase-8 inhibitor IETD (50 μM), and a combination of caspase-8 inhibitor IETD and cathepsin inhibitor E64 to identify the mechanism of NLRP3 activation. Treated cells were collected and analyzed by qPCR, Western Blot and ELISA to determine IL-1β and TNF gene expression, protein and cytokine levels, respectively. In addition, gene expression and protein levels of NLRP3 components (NLRP3, ASC, pro-caspase-1) were assayed using qPCR and Western Blot.

IL-1β and TNF assays

LAD2 cells (1×10^5 cells/well) were treated with SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with the same volume of culture media. Supernatant fluids were collected and assayed using IL-1β and TNF DuoSet ELISA kits (R&D systems). IL-1β DuoSet ELISA Kit had 6.3% cross-reactivity with precursor human recombinant IL-1β according to the manufacturer's instructions.

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

Mast cells were stimulated with the combination of SP (1 μM, 6 h) and IL-33 (30 ng/mL, 6 h). Total mRNA was extracted with an RNeasy Mini kit (Qiagen Inc.) in

accordance with the manufacturer's intructions. An iScript cDNA synthesis kit (BioRad) was used for reverse-transcription of each mRNA sample. Quantitative RT-PCR was performed using Taqman gene expression assays for IL-1β, TNF, NLRP3, ASC and caspase-1 (Applied Biosystems). Samples were run at 45 cycles using a real-time PCR system (7300, Applied Biosystems, Foster City, CA). Relative mRNA levels were determined from standard curves run with each experiment. The mRNA gene expressions were normalized to GAPDH endogenous control (Applied Biosystems).

Western blot analysis

LAD2 cells (1×10^6 cells) were pre-incubated with ZVAD (50 μM), IETD (50 μM) or IETD (50 μM) and E64 (50 μM) for 2 hrs and then stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. The reaction was stopped by addition of ice-cold phosphate buffered saline (PBS). Cells were washed once with PBS and then lysed using protein lysis radio-immuno precipitation (RIPA) buffer (Sigma-Aldrich) in the presence of protease and phosphotase inhibitor cocktails (Thermo Fisher Scientific Inc.). Total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific Inc.) method using bovine serum albumin (BSA) as standard. The total cellular proteins (20 μg aliquots) were separated using 4-20 % Mini Protean TGX gels (Biorad) under SDS denaturing conditions and electrotransfered onto PVDF membranes (Biorad). Blocking was carried out with 5% BSA in Tris-buffered saline containing 0.05 % Tween-20. The membranes were probed with the following primary antibodies at 1:1,000 dilutions: NLRP3, pro-IL-1β, caspase-1, β-actin (Cell Signaling Technology), and ASC (Santa Cruz Biotechnology). For the loading control β-actin was probed. For detection,

the membranes were incubated with the appropriate secondary HRP-conjugated antibody (Cell Signaling Technology) at 1:1,000 dilution and the blots were visualized with enhanced chemiluminescence using SuperSignal© West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.).

FLICA® Caspase-1 Activity Assay

LAD2 cells ($2.5x10^5$) were treated SP (1 μM) and IL-33 (30 ng/mL) for 24 h, centrifuged at 500 g for 5 min and washed in Apoptosis Wash Buffer (ImmunoChemistry Technologies). Cells were resuspended in 300 μL 1X FLICA-YVAD (ImmunoChemistry Technologies), fluorescent caspase-1 binding probe, and incubated for 1 hr at 37° C. Propidium Iodide (PI) staining was used as negative control. Following the incubation, unreacted substrate was removed by washing the cells with Apoptosis Wash Buffer. Finally, cells were resuspended in 300 μl of Apoptosis Wash Buffer for flow cytometry analysis. Active caspase-1 was determined using a FACSCalibur flow cytometer (BD Biosciences).

Caspase-1 Activity Assay

LAD2 cells (5×10^4) were stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 h in black 96-well plates (Corning Inc.). After stimulation, 50 μM YVAD-AFC was added to cells and incubated for 2 hr at 37°C (Ac-YVAD-AFC, Santa Cruz Biotechnology). Fluorescence was measured by a SpectroMax Gemini XS fluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 400 nm and an

emission wavelength of 505 nm. The medium background was subtracted from raw fluorescent values and then the values were normalized to control untreated cells.

Statistics

All experiments were performed in triplicate and were repeated for at least three times (n=3). Data are presented as mean ± SD. Results were analyzed using the unpaired, 2-tailed, Student's *t*-test. Significance of comparisons between conditions is denoted by * $p<0.05$, ** $p<0.01$ and *** $p<0.001$, **** $p<0.0001$, respectively.

4.3 Results

To investigate the role of capsase-8 on the activation of the NLRP3 inflammasome and IL-1β processing we used the caspase-8 specific inhibitor IETD-FMK (50 μM), the pan-caspase inhibitor ZVAD-FMK (50 μM), and the combination of caspase-8 inhibitor IETD-FMK and cathepsin inhibitor E64 (30 μM). Pre-incubation of LAD2 cells with ZVAD-FMK, IETD-FMK and the combination of IETD-FMK and E64 significantly ($p<0.05$) increase IL-1β intracellular protein (Figure 4.1A) when stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL). Only pre-treatment with IETD-FMK significantly ($p<0.01$) increases IL-1β secretion (Figure 4.1B). None of the inhibitors have a significant effect on TNF intracellular protein or secretion (Figure 4.1C-D), confirming that the effect of capsase-8 is specific to IL-1β processing and secretion. Pre-treatment with ZVAD-FMK significantly ($p<0.05$) increases IL-1β gene expression (Figure 4.2A) in SP and IL-33-stimulated LAD2 cells.

Next we investigated the effect of capsase-8 inhibition on the protein levels of the NLRP3 inflammasome components and IL-1β. LAD2 cells pre-incubated with ZVAD-FMK, IETD-FMK and the combination of IETD-FMK and E64 and then stimulated with SP (1 μM) and IL-33 (30 ng/mL) show increased protein levels of pro-caspase-1 and pro-IL-1β (Figure 4.3) but not the levels of active caspase-1 (p20) and cleaved IL-1β in stimulated LAD2 cells (Figure 4.3). The caspase-8 inhibitors also do not affect the gene expression of the NLRP3 inflammasome components (NLRP3, ASC, caspase-1) in LAD2 cells when stimulated with SP and IL-33 (Figure 4.4) and do not change the protein levels of NLRP3 but decrease the protein level of ASC (Figure 4.3).

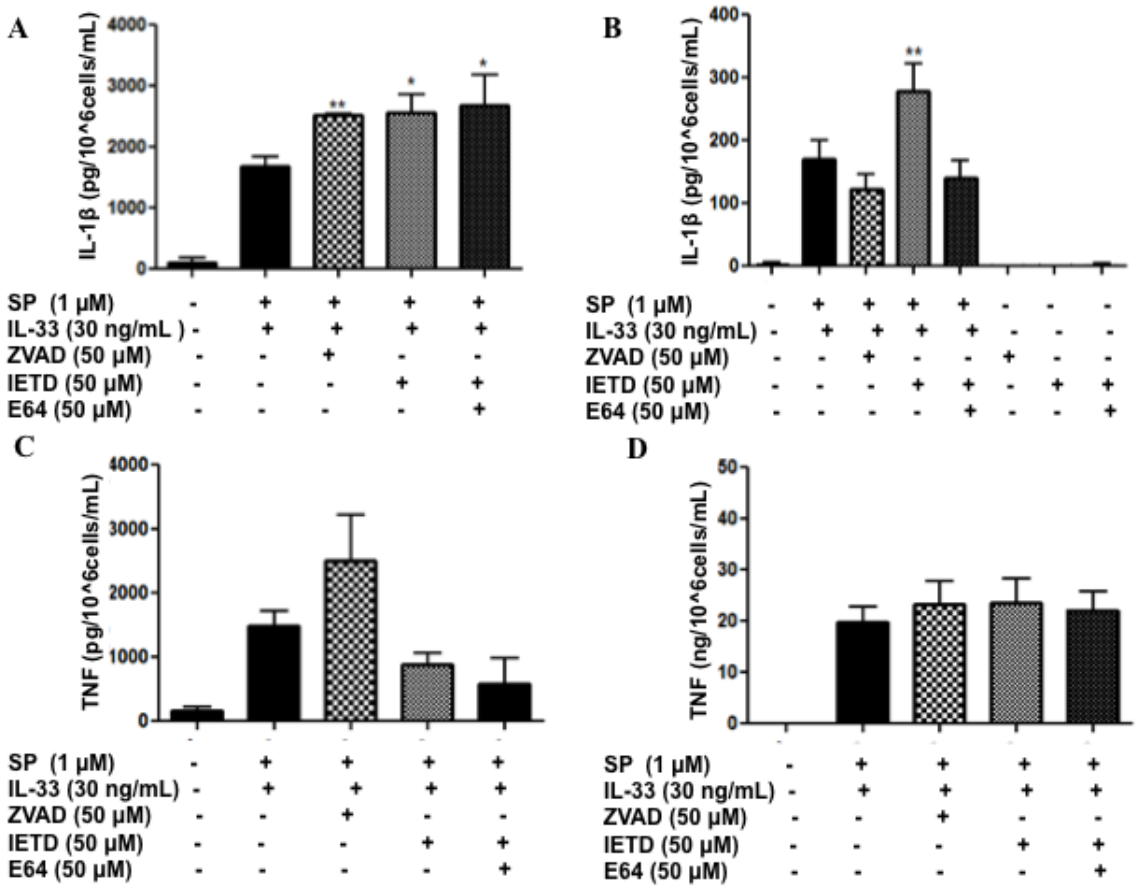

Figure 4.1. Caspase-8 specific inhibitor increases IL-1β secretion. (A-D) LAD2 cells (1×10^5 cells per well) were seeded in 96-well culture plate and pre-incubated with AC-ZVAD-FMK (ZVAD 50 μM), AC-IETD-FMK (IETD 50 μM), and E64 (50 μM) and IETD (50 μM) for 2 hrs and then stimulated with the combination of SP (1μM) and IL-33 (30 ng/mL) for 24 hr. Control cells were treated with 0.1% DMSO, the highest concentration corresponding to that of 50 μM. Collected lysates (A, C) and supernatant fluids (B, D) were assayed using ELISA for IL-1β and TNF, as a negative control. (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

Also, caspase-1 activity in the supernatant fluids of SP and IL-33-stimulated LAD2 cells is not affected by the ZVAD-FMK, IETD-FMK and/or the combination of IETD-FMK and E64 (Figure 4.6). However, the capsase-8 inhibitors increase the percentage of active caspase-1-positive cells from approximately 80% to 92% (Figure 4.7).

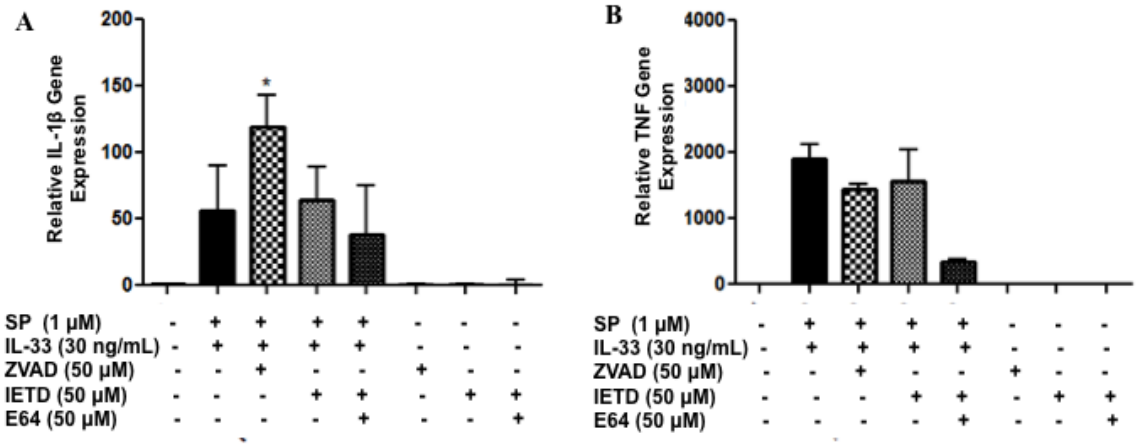

Figure 4.2. Capsase-8 specific inhibitor does not affect IL-1β gene expression. LAD2 cells (1×10^6 cells per well) were seeded in 12-well culture plate and pre-incubated with AC-ZVAD-FMK (ZVAD 50 μM), AC-IETD-FMK (IETD 50 μM), and E64 (50 μM) and IETD (50 μM) for 2 hrs and then stimulated with the combination of SP (1μM) and IL-33 (30 ng/mL) for 6 hr. IL-1β and TNF, as negative control, gene expression levels were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

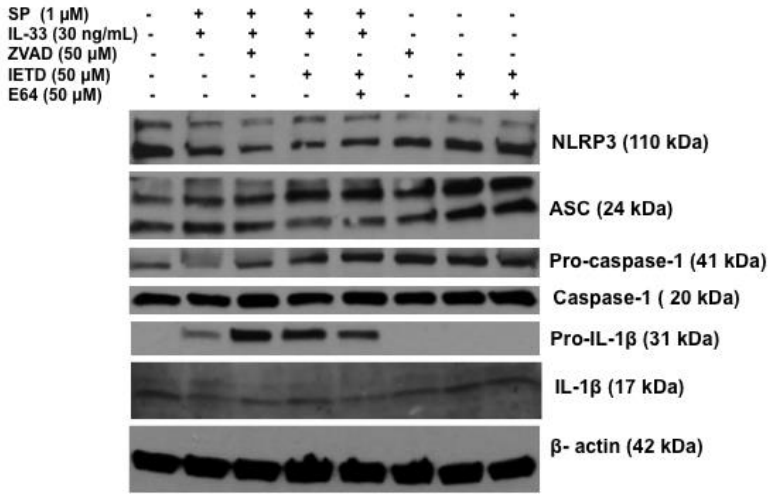

Figure 4.3. Caspase-8 specific inhibitor increases IL-1β synthesis. LAD2 cells (1×10^6 cells/well) were seeded in 12-well culture plate and pre-incubated with AC-ZVAD-FMK (ZVAD 50 μM), AC-IETD-FMK (IETD 50 μM), and E64 (50 μM) and IETD (50 μM) for 2 hrs and then stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 hr. Cell lysates were collected after 24 hrs and protein levels of the NLRP3 inflammasome components (NLRP3, ASC, caspase-1), pro-IL-1β and active IL-1β (p17) were measured by Western blot, using β-actin as loading control (n=3).

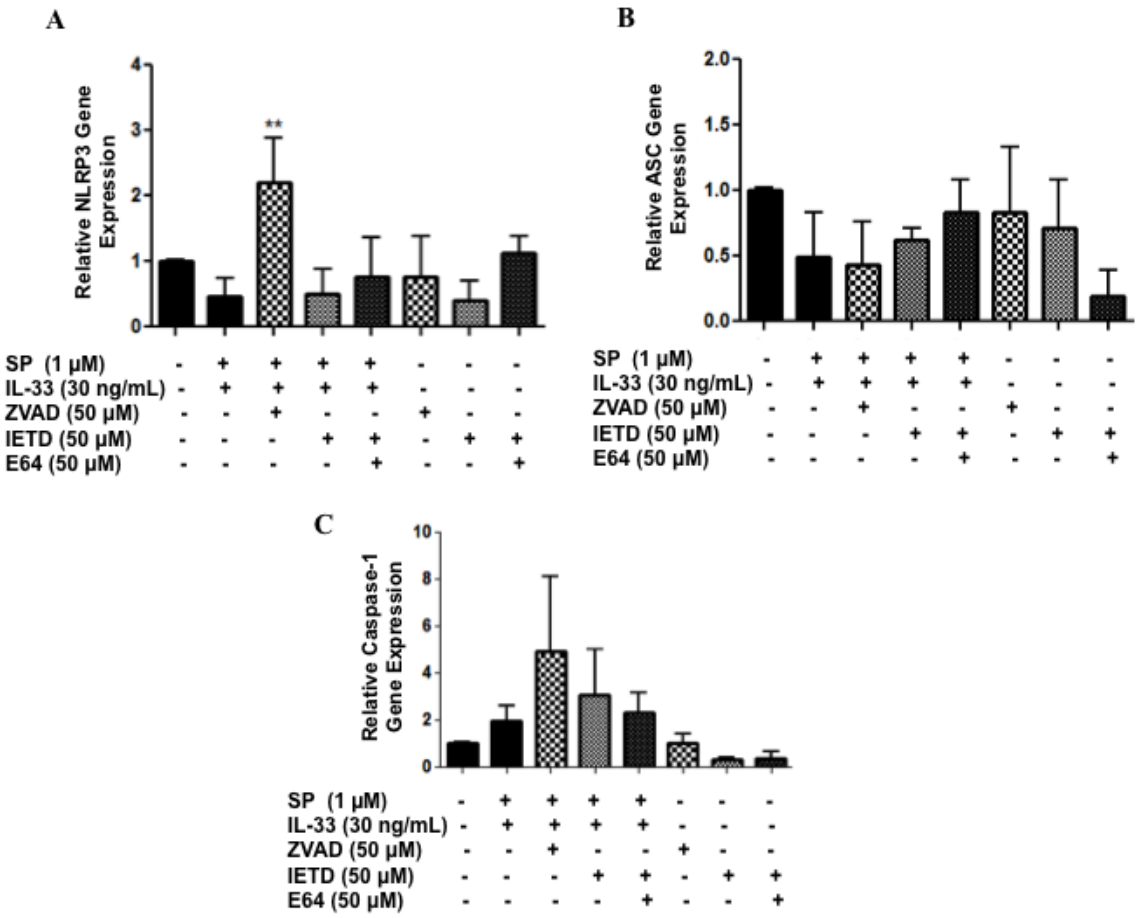

Figure 4.4. Caspase-8 Specific Inhibitor does not affect the gene expression of the NLRP3 inflammasome components. LAD2 cells (1×10^6 cells/well) were seeded in 12-well culture plate and pre-incubated AC-ZVAD-FMK (ZVAD 50 µM), AC-IETD-FMK (IETD 50 µM), and E64 (50 µM) and IETD (50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 6 hr. The gene expression of the NLRP3 components, **(A)** NLRP3, **(B)** ASC and **(C)** caspase-1 gene expression were measured by qRT-PCR and normalized to human GAPDH endogenous control (n=3, * $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

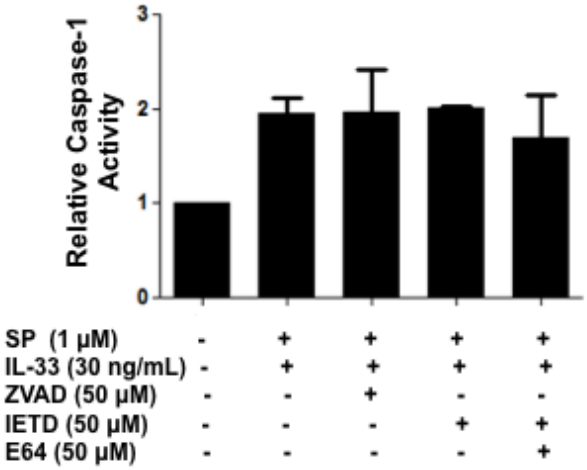

Figure 4.5. Caspase-8 specific inhibitor does not change caspase-1 activity. LAD2 cells (0.5×10^5 cells/well) were pre-incubated with AC-ZVAD-FMK (ZVAD 50 µM), AC-IETD-FMK (IETD 50 µM), and E64 (50 µM) and IETD (50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. After stimulation, 50 µM YVAD-AFC substrate was added to cells and incubated for 2 hr. Caspase-1 activity in the supernatant fluids was determined by measuring fluorescence at 405 nm. Fold-increase in caspase-1 activity was determined by comparing the values to the untreated control samples (n=3).

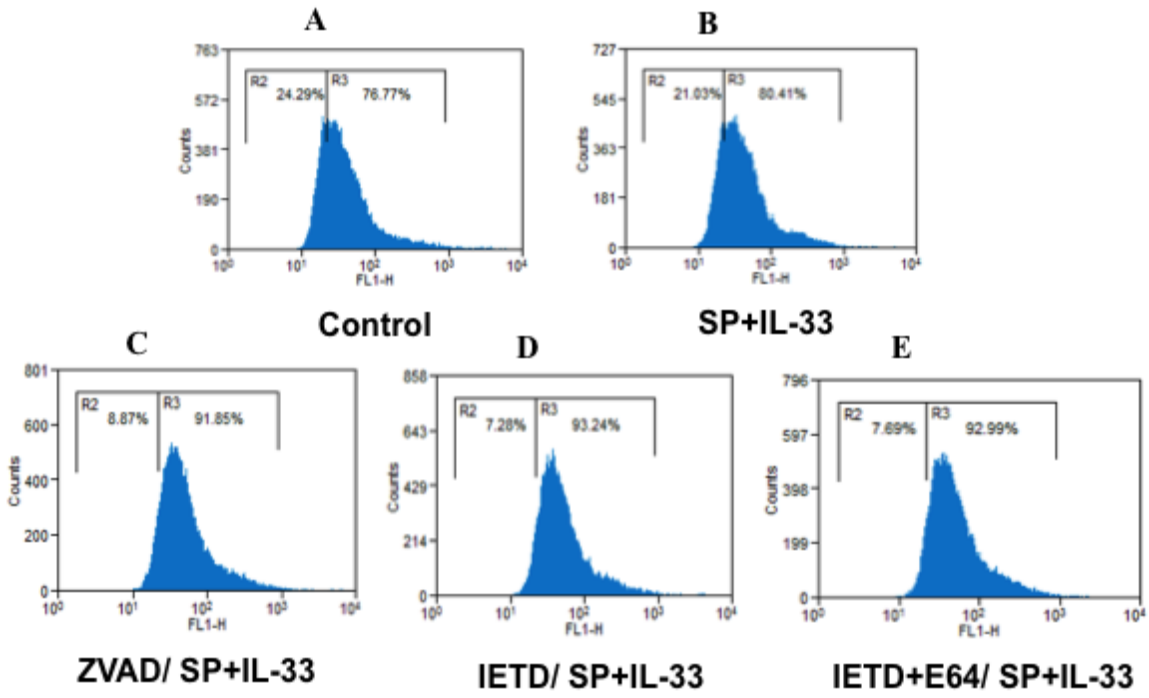

Figure 4.6. Capsase-8 inhibitor increases the percentage of active caspase-1 expressing human mast cells. LAD2 cells (0.25×10^6 cells/well) were pre-incubated with AC-ZVAD-FMK (ZVAD 50 µM), AC-IETD-FMK (IETD 50 µM), and E64 (50 µM) and IETD (50 µM) and then stimulated with SP (1 µM) and IL-33 (30 ng/mL) for 24 hr. Stimulated cells were incubated with FLICA caspase-1 probe for 1 hr and active capsase-1 positive cells were assessed by flow cytometry (n=3).

4.4 Conclusion

Capsase-8 may have multiple ways of negatively regulating IL-1β secretion in human mast cells. Selective caspase-8 inhibition in LAD2 cells results in significant increase of IL-1β intracellular protein and secretion, but does not have an effect on the intracellular protein and release of TNF. Also caspase-8 inhibition increases pro-IL-1β and pro-caspase-1 protein levels. Therefore, the presence of capsase-8 negatively regulates protein levels and secretion of IL-1β by preventing translation of pro-capsase-1 and pro-IL-1β. This suggests that caspase-8 affects the NLRP3 inflammasome activation via signal 1. There is evidence that caspase-8 promotes the assembly and function of NLRP3 inflammasome in mouse dendritic cells [167]. Moreover, caspase-8 deficiency in mouse epidermal keratinocytes stimulates inflammatory skin disease [168].

Also capsase-8 may alter the assembly of the NLRP3 inflammasome and subsequently reduce IL-1β secretion in human mast cells. The interactions of caspase-8 and NLRP3 inflammasome have never been studied in mast cells and specifically in terms of inflammatory disease pathogenesis. Western blot analysis showed that all caspase inhibition and selective caspase-8 inhibition in stimulated mast cells increased pro-IL-1β and pro-caspase-1 protein levels, but had no effect on NLRP3 and active caspase-1 (p20) and active IL-1β (p17) protein levels. This again indicates that caspase-8 inhibition effects signal 1 of the NLRP3 inflammasome activation pathway. Interestingly, selective inhibition of caspase-8 results in deceased levels of ASC protein, implying that capsase-8 presence may affect levels of ASC protein, which is a component of NLRP3 inflammasome that is required for the NLRP3 inflammasome oligomerization and activation to release active caspase-1 (signal 2). Thus, these results suggest that caspase-8

may regulate signal 2 of the NLRP3 inflammasome activation, as well. Caspase-8 may alter the assembly of the NLRP3 inflammasome by affecting levels of ASC protein. It has been reported that NLRP3 inflammasome can be negatively regulated via phosphorylation of ASC protein by IKK-α that restricts phosphorylated ASC protein to the nucleus [231]. This negative regulation could be disturbed during selective caspase-8 inhibition, since we observe the decrease of ASC protein bands, thus allowing ASC protein to be released into the cytoplasm to associate with NLRP3 protein and pro-caspase-1 to activate signal 2 of the inflammasome signaling and subsequently increase IL-1β secretion.

Additionally, capsase-8 may regulate constitutively active caspase-1 because caspase-8 inhibition results in the increased percentage of the casapase-1 positive mast cells but does not affect the activity of the capsase-1 per se. This finding suggests that mast cells could have developed a way of negatively regulating the presence of the constitutively active caspase-1 by the presence of other caspases. Other reports have shown that caspase-1 can be negatively regulated by the CARD-only proteins (COPs) and pseudo substrates such as Flightless-I [232]. During COPs negative regulation, proteins ICEBERG and pseudo-ICE (COP1) interact the caspase-1 via CARD domain and prevent its association with ASC protein [233]. During pseudo substrate regulation, NLRP3 protein associates with the leucine-rich repeat Fli-I-interacting protein 2 (LRRFIP2) that recruits pseudo substrate Flightless-I to capsase-1 and inhibits capsase-1 activation [234] and subsequent IL-1β secretion. Mast cells may employ similar mechanisms where capsase-8 or other molecules could act as negative regulators.

Overall, our study of the alternative regulation of IL-1β secretion from human mast cells showed that these immune cells may employ different mechanisms of NLRP3 inflammasome activation and regulation to secrete IL-1β. Mast cells are hemopoietically-derived mast cells, which could travel through blood vessels to the tissues, thus their characteristics in the release of potent pro-inflammatory cytokines would be different in comparison to other immune cells. For example, blood monocytes also express constitutively active caspase-1[219], which could release rapidly IL-1β under conditions of an infection. The same way tissue immune cells would require multiple triggers to stimulate IL-1β release since its active form's uncontrolled release could have detrimental effects on the surrounding tissue. Mast cells are unique immune cells since they have abilities to both (1) traverse through blood and (2) become a resident in a tissue. Therefore, it would be beneficial for these cells to express constitutively active caspase-1, as blood monocytes do, to rapidly respond and release IL-1β when needed and at the same time have additional steps of regulation of this process, as the tissue immune cells have, in order to prevent the spontaneous release of IL-1β. The findings from this study provide new insights into the mechanism of IL-1β secretion from human mast cells and could be useful for the development of novel anti-inflammatory therapies.

Chapter 5: Discussion

5.1 Interplay of stress, inflammation and mast cells

Elevated levels of cytokines often characterize inflammatory responses and the complex interplay among neuropeptides and cytokines is seldom considered. The findings presented here on the synergistic interactions between the neuropeptide SP and the cytokine IL-33 are relevant to the understanding of the molecular mechanisms in a number of inflammatory diseases. For example, it is known that stress alters the stressor-specific patters of proinflammatory cytokine and chemokine expression in CNS [235] that could contribute to neuroinflammation. Stress is a known to exacerbate inflammation [236]. The role of stress and inflammation goes beyond the brain. It was previously shown that patients with rheumatoid arthritis show higher stress-induced levels of IL-1β [237] and patients with psoriasis show a heightened cortisol response to stress [238]. Many hypothesize that impaired innervations and neuropeptides are imbalanced in psoriatic skin [239].

SP may account for the temporal stress onset, clinical symmetry of lesions and the histopathologic features of psoriasis [240], and may induce typical and atypical cytokine secretion from T-cells [241]. It also is interesting that psoriasis worsens by acute stress [242, 243]. SP can stimulate TNF, IL-6 and IL-1β from keratinocytes [244], [245] evoking a storing pro-inflammatory response. SP is a known activator of mast cells [246] and the number of contacts between tryptase-positive mast cells and sensory nerves are increased in skin disease such as psoriasis and atopic dermatitis [247]. Mast cells are also increased in lesional psoriatic skin and there appears to be an association among sensory nerves, mast cell numbers and stress[93, 248]. IL-33 gene expression and protein [38] are increased in lesional skin of patients with psoriasis who report pain [249]. We have previously shown

that SP and IL-33 synergistically stimulate cultured human mast cells to release vascular endothelial growth factor (VEGF) [38].

Mast cells are now recognized as being critical in inflammatory processes [196] and are implicated in inflammatory diseases that include psoriasis [199], rheumatoid arthritis [197], inflammatory bowel disease [198], multiple sclerosis [200], mastocytosis [250, 251] and fibromyalgia syndrome [201]. Independent reports have shown that mast cells, SP, IL-33, IL-1β and TNF contribute to the inflammatory processes in these diseases [71, 188, 201-204, 237]. For instance, increased SP plasma levels correlate with mast cell load in mastocytosis patients [50]. IL-33 gene expression is increased in lesional in psoriatic skin and anti-TNF therapy is shown to reduce it [252]. Additionally, treatment with the TNF inhibitor, etanercept, reduces serum SP levels in rheumatoid arthritis patients [51]. Our findings provide new insights into how SP, IL-33, IL-1β, TNF and mast cells may interact in these inflammatory diseases. The combined enhancing effect of SP and IL-33 on synthesis and secretion of IL-1β and TNF from human mast cells could be a major contributor to inflammatory processes that occur in the pathological conditions mentioned, particularly inflammatory skin disease.

It is important to mention that a major challenge in the studies of mast cell pathophysiology is the source of primary mast cells. Ordinarily, mature human mast cells could come from either clean margins of breast mastectomies, extract tissue from fat reduction, or circumcisions. However, these cells vary enormously in their phenotypic characteristics due to the age of the donor, site of the skin used, the degree of inflammation, or the adipocytokines presence. The primary mast cells that we used are isolated from umbilical cord blood, collected from healthy donors after uncomplicated

deliveries. The drawback of these hCBMC cells is that their secretory granules do not appear to have the morphology of mature adult human mast cells. SP had previously been shown to stimulate small amounts of TNF from rat peritoneal mast cells [174, 205] and cultured human skin mast cells [175, 176]. One major difference between these publications may be the fact that human skin mast cells that respond to SP are derived from adult skin [175], while those cells that did not respond to SP were purified form circumcisions [194]. In addition, it was reported that SP stimulates TNF release from LAD2 cells, but not from purified human skin mast cells [194]. In fact, there was a great variability in the allergic response to IgE among mast cells derived from circumcisions [183 206]. Therefore, the studies of human mast cell physiology and pathology are complex due to the absence of a "true" human mast cell model. This model would be difficult to create because of a natural variability of "normal" human mast cells depending on their localization in the body.

5.2 Implications of Neuropeptides, Cytokines and Mast Cell Crosstalk in Psoriasis and Beyond

Psoriasis is characterized by increased epidermal vascularization, keratinocyte hyperproliferation and inflammation [94]. Neuropeptides [170], especially SP, were reported to be involved in the pathogenesis of inflammatory skin disease, such as psoriasis [49, 253]. SP has also been shown to stimulate mast cells to secrete histamine [37], TNF [174-176] both relevant to skin disease. SP-positive nerve fibers and mast cell contacts are also increased by acute stress in mice, leading to dermal mast cell degranulation [242, 254, 255]. IL-33 and has been implicated in the pathogenesis of psoriasis via keratinocyte and mast cell

activation [96]. Also IL-33 was reported to be elevated in the serum of generalized psoriasis patients and correlated with high serum TNF [188].

There have been several reports observing synergistic effects between peptides and cytokines. For example, SP together with IL-β or TNF synergistically increase IL-6 and PGE_2 secretion from cultured human spinal cord astrocytes [256] and IL-33 had been reported to also enhance allergic responses [184] and allergic bronchoconstriction via activation of mast cells in mice [185]. Also, SP and lipoteichoic-acid (LTA) synergistically increase production of IL-8, MCP-1, TNF and IL-6 in cultured human mast cells [257]. Moreover, SP and IL-4 accentuate IgE synthesis [258]. Interestingly, stem cell factor (SCF) and IL-4 make mast cell more responsive to SP stimulation [259] and fibronectin together with TGF-β synergistically induce SP production in fibrosis [260]. Other examples of enhancing effects include bradykinin and IL-1β synergistically increasing secretion of PGE_2 and cyclooxygenase 2 (COX-2) expression in human synoviocytes [261]; moreover, high-mobility group box 1 (HMGB1) alarmin together with LPS synergistically induce Th1 response (TNF, IFN-ϒ expression) in peripheral blood mononuclear cells [262]. These findings indicate that synergistic actions of peptides and cytokines are an important part of inflammatory responses, particularly when studying pathological conditions. Therefore, elucidation of the molecular mechanisms behind these synergistic actions could prove beneficial for successful anti-inflammatory therapy development**.**

The results presented here indicate novel complex interactions between the peptide SP and the cytokine IL-33 administered in combination that lead to an impressive increase in TNF and IL-1β synthesis and secretion in cultured human mast cells. These

responses depend on the activation of both NK-1, the receptor for the peptide SP, and ST2, the receptor for the cytokine IL-33.

We found it interesting that interfering with the NK-1 receptor function results in the decrease of the ST2 receptor activation and decrease in the ST2 receptor expression results in the decrease NK-1 receptor signaling. Treatment with 100X more of the NK-1 antagonists does not completely inhibit the robust TNF secretion, suggesting that another mechanism is contributing to this effect. NK-1 inhibition was previously reported to only partially inhibit SP stimulation of skin-derived human mast cells [194], supporting the idea of another pathway that might be participating in this response.

The complex interactions between cytokines and neuropeptides could be regulated via interaction of their receptors. We show that pre-incubations with SP receptor (NK-1R) antagonists or IL-33 receptor (ST2) neutralizing anybody inhibit SP and IL-33-induced IL-1β and TNF secretion to a different extent. Furthermore, the NK-1 antagonists inhibit IL-1β and TNF secretion in mast cells stimulated by IL-33 alone, while the ST2 siRNA also inhibits SP-stimulated TNF, indicating that there may be some receptor-receptor interaction between NK-1 and ST2. It was previously reported that the cross-activation of c-kit, the receptor for stem cell factor, undergoes a complex formation with ST2 and its co-receptor, the IL-1R accessory protein (IL-RAcP), in murine mast cells [195]. An analogous structural association could occur among NK-1, ST2 and IL-RAcP by a direct contact. The results of co-immunoprecipitation experiments show that an antibody specific to NK-1 recovers both ST2 and IL-1RacP proteins. The expression of IL-1RacP is markedly increased after the stimulation with the combination of SP and IL-33, suggesting that this protein participates in complexing NK-1 and ST2 receptors.

For future directions, it would be crucial to determine the functionality of NK-1, ST2 and IL-1RacP complex. Using Froster resonance energy transfer (FRET) [263] we can identify the proximity of the receptors and observe it before, during and after stimulation with SP and IL-33 in live cultured human mast cells. From our findings, we concluded that only SP and IL-33 show synergistic effect on IL-1β secretion. However, it would be interesting to explore other potential interactions of ST2 receptor and its co-receptor IL-1RacP. Mast cells express the most of ST2 receptor compared to other cells types, while IL-1RacP is expressed ubiquitously [264]. Additional molecular studies on cytokine receptor interactions could prove relevant for future pharmacological studies and drug development.

5.3 Regulation of IL-1β secretion from Human Mast Cells

NLRP3 activation and IL-β secretion are key players in the pathogenesis of a number of inflammatory diseases: (a) genetically defined like familial Mediterranean fever, Muckle-Well syndrome, and CAPS, as well as (b) diseases that involve NLRP3 activation by danger signals such as gout, pseudogout, Alzheimer's disease, asbestosis, silicosis, and type 2 diabetes mellitus [138, 138, 224]. Inflammasomes have been associated with an array of auto-inflammatory and autoimmune diseases [224]. Moreover, NLRP3 inflammasome activation and IL-1β secretion, as well mast cells, have been implicated in rheumatoid arthritis [225], multiple sclerosis [226], psoriasis [110, 227, 228], and asthma [229]. It has been reported that microbiota and mast cells initiate cellular events that lead to abnormal IL-1β production and skin inflammation in mice with CASPs-associated Nlrp3 mutation

[228]. Several studies showed that caspase-1 gene expression and activity are increased in lesional psoriatic epidermis [265] [266] [267].

Our findings identify novel stimuli of IL-1β secretion from human mast cells that provide a new way of understanding the complexity of stress and inflammation, especially interplay among neuropeptides, cytokines and mast cells. The findings reported here are unique in showing that SP and IL-33 result in a significant synergistic increase of IL-1β gene expression, intracellular protein levels and secretion from human cultured mast cells. The ability of the SP and IL-33 alone, and most importantly when administered together, to stimulate IL-1β secretion from mast cells is even more striking due to the fact that none of the known triggers of the NLRP3 inflammasome alone or in combination, except for Nigericin, can stimulate IL-1β secretion from cultured human mast cells.

Nevertheless, it was previously shown that activated cultured human mast cells show increased caspase-1 activity [268] and secrete IL-1β when stimulated with mitochondrial DNA[269]. It is interesting to mention that SP increases IL-1 receptor expression as well as NLRP3 and capsase-1 gene and protein in mouse skin [270]. Our findings show that cultured human mast cells exhibit an alternative assembly of the NLRP3 inflammasome and regulate IL-1β secretion primarily via signal 1. The protein levels of all the NLRP3 components (NLRP3, ASC, capsase-1) and IL-1β have been detected in LAD2 cells; however, the presence of their active forms is responsible for the alternative assembly. IL-33 alone triggers IL-1β gene expression and pro-IL-1β synthesis and the combination of SP and IL-33 increases it even further, suggesting that this process is mostly regulated via transcriptional NF-κB activation (signal 1). The

simulation of SP and IL-33 alone or in combination does not affect the gene and protein expression of NLRP3 and ASC proteins, suggesting that the oligomerization of the NLRP3 inflammasome (signal 2), where NLRP3, ASC and pro-caspase-1 complex together to release active caspase-1, is not essential for the secretion of IL-1β. Moreover, caspase-1 and IL-1β are detected in their active forms (p20 and p17) in unstimulated mast cells, which means that mast cells have pre-stored constitutively active caspase-1 and IL-1β and may not require the full assembly of NLRP3, ASC and pro-caspase-1 proteins to release active caspase-1 and subsequently cleave pro-IL-1β into active IL-1β. The only other similar reports are of human blood monocytes [219] and melanoma cells [220] constitutively expressing active caspase-1 and spontaneously secreting IL-1β [219]. However, unlike those studies, human mast cells do not spontaneously release active IL-1β even though its active cleaved form (p17) is found in mast cell lysates. The presence of the active caspase-1 in unstimulated LAD2 cells was confirmed by cell sorting showing over 75% of the untreated control mast cells contain active caspase-1. We also found that caspase-1 activity was increased by 2-fold in the supernatant fluids of mast cells following stimulation with SP and IL-33. Active caspase-1 secretion along with IL-1β was previously shown in mouse macrophages and dendritic cells [221]. We speculate that mast cells pre-store caspase-1 and IL-1β in their active forms in order to respond rapidly to danger signals [212].

In contrast to the rapid response, the stimulation of SP and IL-33 over 24 hr triggers synthesis of new IL-1β and caspase-1 mainly regulated via signal 1. However, the cleavage of newly synthesized pro-IL-1β is still a requirement for its subsequent secretion and this process largely depends on NLRP3 inflammasome activation (signal

2). There are several accepted theories that identify molecular mechanism that stimulate signal 2: ROS generation, change in potassium and calcium ions and/or lysosomal damage [112]. It has been reported that SP induces intracellular calcium ion mobilization and degranulation of mast cells [165] [271] and stimulates ROS [272] and the mitochondrial electron transport chain [273]. These effects of SP can regulate NLRP3 activation too; hence we observed both pro-IL-1β intercellular protein production and IL-1β secretion.

5.4. Regulation of NLRP3 inflammasome and IL-1β by caspase-8

It has been also reported that IL-1β can be processed independently of NLRP3 activation. Additionally, it was shown that alum-induced stimulation of IL-1β does not require NLRP3 inflammasome and can be cleaved by cathepsins S [274] and serine protease granzyme A can process IL-1β independently of capsase-1 [275]. Moreover, NLRP3 and ASC may form a non-canonical platform for capsase-8 activation, a protease mainly involved in apoptotic signaling, in mitochondria during apoptosis of epithelial cells [276]**.** It was reported that caspase-8 and receptor interacting protein 3 (RIP3) are crucial for NF-κB and inflammasome activation during bacterial infection with Yersinia pestis [277] and endogenous caspase-8 can cleave pro-IL-1β [278]. It is known that in dendritic cells caspase-8 expression prevents the onset of systemic auto-inflammation [279] and epidermal caspase-8 expression stimulates wound healing [280].

Our findings indicate that capsase-8 may have multiple ways of negatively regulating IL-1β secretion in human mast cells. Selective caspase-8 inhibition in LAD2 cells results in significant increase of IL-1β intracellular protein and secretion but does not have an effect on the intracellular protein and release of TNF. Also caspase-8

inhibition increases pro-IL-1β and pro-caspase-1 protein levels. Therefore, the presence of capsase-8 negatively regulates protein levels and secretion of IL-1β by preventing translation of pro-capsase-1 and pro-IL-1β. This suggests that caspase-8 affects the NLRP3 inflammasome activation via signal 1. There is evidence that caspase-8 promotes the assembly and function of NLRP3 inflammasome in mouse dendritic cells [167]. Moreover, caspase-8 deficiency in mouse epidermal keratinocytes stimulates inflammatory skin disease [168].

Also our findings indicate that capsase-8 may alter of the assembly of the NLRP3 inflammasome and subsequently reduce IL-1β secretion in human mast cells. The interactions of caspase-8 and NLRP3 inflammasome have never been studied in mast cells and specifically in terms of inflammatory disease pathogenesis. Western blot analysis showed that all caspase inhibition and selective caspase-8 inhibition in stimulated mast cells increased of pro-IL-1β and pro-caspase-1 protein levels but had no effect on NLRP3 and active caspase-1 (p20) and active IL-1β (p17) protein levels. This again indicates that caspase-8 inhibition effects signal 1 of the NLRP3 inflammasome activation pathway. Interestingly, selective inhibition of caspase-8 results in deceased levels of ASC protein, implying that capsase-8 presence may affect levels of ASC protein, which is a component of NLRP3 inflammasome that is required for the NLRP3 inflammasome oligomerization and activation to release active caspase-1 (signal 2). It has been reported that site-specific dephosphorylation, as well as microtubule dynamics control Pyrin inflammasome activation [281]. Thus, these results suggest that caspase-8 may regulate signal 2 of the NLRP3 inflammasome activation as well. Caspase-8 may alter the assembly of the NLRP3 inflammasome by affecting levels of ASC protein. It has been

reported that NLRP3 inflammasome can be negatively regulated via phosphorylation of ASC protein by IKK-α that restricts phosphorylated ASC protein to the nucleus [231]. This negative regulation could be disturbed during selective caspase-8 inhibition, since we observe the decrease of ASC protein bands, thus allowing ASC protein to be released into the cytoplasm to associate with NLRP3 protein and pro-caspase-1 to activate signal 2 of the inflammasome signaling and subsequently increase IL-1β secretion.

Additionally, capsase-8 may regulate constitutively active caspase-1 because caspase-8 inhibition results in the increased percentage of the casapase-1 positive mast cells but does not affect the activity of the capsase-1 *per se*. This finding suggests that mast cells could have developed a way of negatively regulating the presence of the constitutively active caspase-1 by the presence of other caspases. The reports have shown that caspase-1 can be negatively regulated by the CARD-only proteins (COPs) and pseudo substrates such as Flightless-I [232]. During COPs negative regulation, proteins ICEBERG and pseudo-ICE (COP1) interact the caspase-1 via CARD domain and prevent its association with ASC protein [233]. During pseudo substrate regulation, NLRP3 protein associates with the leucine-rich repeat Fli-I-interacting protein 2 (LRRFIP2) that recruits pseudo substrate Flightless-I to capsase-1 and inhibits capsase-1 activation [234] and subsequent IL-1β secretion. Mast cells may employ similar mechanism where capsase-8 or other molecules could act as negative regulators.

Overall, our study of the alternative regulation of IL-1β secretion from human mast cells showed that these immune cells may employ different mechanism of the NLRP3 inflammasome activation and regulation to secrete IL-1β. Mast cells are hemopoietically-derived mast cells, which could travel through blood vessels to the

tissue, thus its characteristics in the release of potent pro-inflammatory cytokines would be different in comparison to other immune cells. For example, blood monocytes also express constitutively active caspase-1[219], which could release rapidly IL-1β under conditions of an infection. The same way tissue immune cells would require multiple triggers to stimulate IL-1β release since its active form's uncontrolled release could have detrimental effects on the surrounding tissue. Mast cells are unique immune cells since they have abilities to both (1) traverse through blood and (2) become a resident in a tissue. Therefore, it would be beneficial for these cells to express constitutively active caspase-1, as blood monocytes do, to rapidly respond and release IL-1β when needed and at the same time have additional steps of regulation of this process, as the tissue immune cells have, in order to prevent the spontaneous release of IL-1β. The findings from this study provide new insights into the mechanism of IL-1β secretion from human mast cells and could be useful for the development of novel anti-inflammatory therapies.

For future studies, it would be crucial to investigate IL-1β and TNF secretion and contribution to psoriasis pathogenesis. Since our data provides new insights into how human mast cells release IL-1β and TNF it would be beneficial to study (1) the mechanism of neuropeptide and cytokine synergism and (2) NLRP3 activation and IL-1β secretion from human primary keratinocytes as well as from murine skin keratinocytes isolated from a psoriatic mouse model developed in either normal C57BL/6J or IL-33 knockout mice following IL-33 and SP stimulation.

We could use imiquimod-induced psoriasis-like mouse model. Psoriasis-like skin inflammation could be induced in C57BL/6 or IL-33 knockout mice using 5% imiquimod

(IMQ) cream application for six consecutive days, as described previously [282]. Following the induction of psoriasis-like lesions, NK-1 and ST2 receptors, IL-1β and TNF cytokines and NLRP3 inflammasome components protein levels, cytokine release (where applicable) and mRNA expression would be assessed to investigate the interplay among them *in vivo*.

In these studies we may find out whether SP and IL-33 synergism is only human mast cells-specific or can be observed in other cell types and species such as murine keratinocytes. Additionally, these experiments would help identify whether constitutively active capsase-1 is expressed in murine skin mast cells.

5.5 Methoxyluteolin as Anti-Inflammatory Therapy

The findings of this study provide new understanding of physiological and pathological interactions between peptides, cytokines and mast cells that may lead to discovery of novel pharmacological approaches for development of anti-inflammatory therapies. We used a naturally occurring molecule, methoxyluteolin, to explore its inhibitory effects on mast cells. Even though TNF and IL-1β neutralizing therapies are available [222], there are no available inhibitors that block synthesis and secretion of these pro-inflammatory cytokines.

Flavonoids show strong antioxidant and antimicrobial properties [154, 283]. For example, the flavonoid quercetin can induce the expression of glutathione S-transferase and increase its activity [284] and can inhibit different stages in the replication cycle of viruses[285]. Increasing evidence from *in vitro* and *in vivo* studies suggests that flavonoids also directly interfere with signaling cascades involved in inflammatory and immune

responses[286]. A number of studies have shown inhibitory actions of flavonoids on cytokine production and activation of B cells, T cells and neutrophils [154]. Quercetin, luteolin (5,7,3',4'-tetrahydroxyflavone) , and methoxyluteolin inhibit mast cell activation and degranulation [287] and also inhibit cytokine release from human mast cells by blocking NF-κB activation [165, 288-290].

Here we report that methoxyluteolin, a naturally occurring flavonoid, inhibits IL-1β synthesis. Luteolin has been reported to inhibit the NLRP3 inflammasome and subsequent IL-1β secretion in endothelial cells, but the mechanism of NLRP3 inhibition was not explored [223]. Strikingly, in addition to preventing IL-1β and TNF secretion, methoxyluteolin significantly inhibits caspase-1 gene expression as well as pro-caspase-1 protein expression, while also reducing caspase-1 activity in the supernatant fluids.

Methoxyluteolin inhibits the enhancing effect of SP and IL-33 on IL-1β and TNF gene expression and secretion from human mast cells, indicating that this flavonoid must be acting at some step of signaling pathway following the receptor activation. We showed that methoxyluteolin is a more potent inhibitor than luteolin of human mast cells [165] and human keratinocytes [191] and therapeutic doses can be achieved *in vivo*. Flavonoids [159], and in particular luteolin [291] and methoxyluteolin [163] are considered safe. The time course of the key signaling steps of the stimulation with the combination of IL-33 and SP leads to JNK kinase activation within the first 5 minutes, while IKβ-α becomes active within the first hour and both remain active for the next 4 hours of stimulation. Previously, SP/NK-1 receptor signaling has been shown to activate JNK kinase [47], while IL-33/ST2 receptor activation has been shown to signal via NK-κB [207]. One of the ways methoxyluteolin may inhibit IL-1β and TNF gene expression and secretion is through the

inhibition of IKβ-α phosphorylation. SP was previously reported to stimulate NF-κB in murine umbilical cord blood mast cells [205] at 10-100 μM and in hCBMCs at 1 μM [165]. Upon phosphorylation, IKβ-α releases NF-κB, which translocates from the cytoplasm into the nucleus and regulates gene expression of various inflammatory mediators including IL-1β and TNF [208]. IL-33 could contribute to IL-1β and TNF gene expression and secretion stimulated by the combination of IL-33 and SP via translocation into the nucleus, where it could possibly activate some transcription factors, particularly AP-1 [56], an affect that may be inhibited by methoxyluteolin. We had previously shown that both luteolin and methoxyluteolin inhibited NF-κB and also decreased NF-κB p65 DNA-binding activity in the nuclear extract of human mast cells [209]. Both flavonoids also decreased mRNA expression of two genes encoding different subunits in the NF-κB protein complex, NFKB1 (encoding NF-κB p50 subunit) and RELA (encoding NF-κB p65 subunit) [209].

Our results have important clinical implications for the understanding of the complex interplay among SP, IL-33 and mast cells in inflammatory processes. The impressive enhancing effect of IL-33 and SP combination on IL-1β and TNF gene expression and secretion in human mast cells may be a key step in the pathogenesis of inflammatory diseases. The potent inhibitory effect of methoxyluteolin suggests that it could be developed as systemic or topical anti-inflammatory treatment. The magnitude of TNF synthesis and secretion, due to interactions between SP and IL-33, suggests new therapeutic approaches through the use of SP and IL-33 receptor antagonists as well as methoxyluteolin.

Methoxyluteolin is lipid-soluble and could be developed into topical formulations that easily penetrate the skin. For future studies, it would be essential to investigate the anti-inflammatory effects and the ability to inhibit IL-1β and TNF by methoxyluteolin in a psoriatic mouse model developed in either normal C57BL/6J or IL-33 knockout mice following IL-33 and SP stimulation. The findings from these studies can provide *in vivo* data on efficacy and safety of methoxyluteolin in animals that can be used for the subsequent development of the topical formulations for the use in humans.

Overall, our studies show that the cytokine IL-33 administered in combination with the pro-inflammatory peptide SP cause a marked increase of IL-1β and TNF synthesis and secretion from cultured human mast cells. These responses are mediated via the activation of the SP receptor, NK-1, and the IL-33 receptor, ST2, and can be inhibited by the natural flavonoid methoxyluteolin. Our findings reveal novel interactions that increase the understanding of inflammation and offer new directions for the development of anti-inflammatory drugs.

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