A role of mitochondria in mast cell activation and possible involvement in auto-immunity

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ABSTRACT

Mast cells, derived from bone marrow, are key immune effector cells that cause allergic symptoms, regulate innate and acquired immunity, but are also involved in many autoimmune and inflammatory diseases. Mast cells participate in immune process by secreting granule-stored components, such as histamine, and *de novo* synthesized mediators, such as IL-6, 8 and 13 in response to allergic, neuropeptide and environmental triggers. Mast cells are well known to be activated by aggregation of high affinity receptors for the immunoglobulin E during which most mediators are released with detrimental pathophysiological effects. However, the regulation of secretion of granule and non-granule stored mediators is poorly understood.

We show that degranulation and secretion of granule-stored TNF but not non-granule stored *de novo* synthesis and release, of TNF from human cultured mast cells requires substantial mitochondrial-associated energy and calcium. We further show that degranulation by various triggers leads to rapid granule-stored TNF secretion and mitochondrial (mt) fission and translocation to sites of exocytosis. Extracellular calcium depletion prevents the mitochondrial translocation, while the calcium ionophore A23187 induces translocation, indicating the necessarily of calcium influx. The calcium-dependent calcineurin and Dynamin Related Protein1 (Drp1) which are critical for mitochondrial dynamics, are activated rapidly after SP stimulation. Reduction of Drp1 activity by its inhibitor MDIVI-1 and decrease of Drp1 expression using siRNA inhibit mitochondrial translocation, TNF secretion and degranulation. In addition, gene expression of calcineurin, Drp1 and SP is higher in skin biopsies from patients with Atopic Dermatitis (AD) as compared to biopsies from normal control. The results

presented here show that mitochondria could be a novel regulator of mast cell activation.

We also show that human mast cell degranulation leads to mitochondrial fission into small particles and mtDNA release extracellulary. Mitochondrial components are not normally found outside the cells. Mitochondria purified from cultured sarcoma and LAD2 cells stimulate mast cell degranulation and pro-inflammatory mediators' secretion. Increasing evidence indicates that autism may be associated with some immune dysregulation, and may have a neuroimmune component. We find that mtDNA (7s, Cytochrome B) is elevated in the serum of children with autism.

Mitochondria-based drug discovery may be used for the treatment of inflammatory and autoimmune diseases.

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CHAPTER I

Introduction

1.1 Abstract

Mast cells are well known for their role in allergic and anaphylactic reactions, as well as their involvement in acquired and innate immunity. Recent Increasing evidence now implicates mast cells in inflammatory diseases where they are activated by non-allergic triggers, such as neuropeptides and cytokines, often exerting synergistic effects as in the case of IL-33. Mast cells can also release pro-inflammatory mediators selectively without degranulation. In particular, IL-1 induces selective release of IL-6, while corticotropin-releasing hormone secreted under stress induces the release of vascular endothelial growth factor. How these differential mast cell responses are regulated is still unresolved. Preliminary evidence suggests that mitochondrial function and dynamics regulate mast cell degranulation, but not differential release. Many inflammatory diseases involve mast cells in cross-talk with T cells, such as atopic dermatitis, psoriasis and multiple sclerosis, which worsen by stress. findings also indicate that mast cells have immunomodulatory properties. Understanding selective release of mediators could explain how mast cells participate in numerous diverse biologic processes, and how they may exert both immunostimulatory and immunosuppressive actions. Unraveling selective mast cell secretion could also help develop unique mast cell inhibitors with novel therapeutic applications.

Abbreviations: AD, atopic dermatitis; BBB, blood-brain-barrier; Bcl10-Malt1, B cell lymphoma 10-mucosal-associated lymphoid tissue 1; BDNF, brain derived neurotrophic factor; CRH, corticotropin-releasing hormone; CRHR, corticotropin-releasing hormone receptor; Drp1, dynamin related protein 1; EAE, experimental allergic encephalomyelitis; FcεRI, high affinity surface receptors for IgE; GM-CSF, granulocyte-macrophage colony-stimulating factor; hCBMCs, human umbilical cord-derived mast cells; HPA, hypothalamic-pituitary-adrenal; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; MBP, myelin basic protein; MCP-1, monocyte chemoattractant protein-1; MS, multiple sclerosis; MMP, matrix metalloproteinase; NGF, nerve-growth factor; NK, neurokinin; NT, neurotensin; PACAP, pituitary adenylate cyclase activating polypeptide; PAF, platelet activating factors; PAR, protease activated receptors; PI3-K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome ten; RANTES, regulated upon activation, normal T cell expressed and secreted; RBL, rat basophil leukemia; SCF, stem cell factor; SF-1α, stromal cell-derived factor-1 alpha; SLPI, secretory leukocyte protease inhibitor; SP, substance P; TGFβ, transforming growth factor β; TLR, toll-like receptors; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; Ucn, urocortin; UCP2, uncoupling protein 2; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

1.2 Mast cells

1.2.1 Mast cell mediators

Mast cells derive from distinct precursors in the bone marrow or other hematopoietic tissues [1, 2]. They mature under the influence of local tissue microenvironmental conditions, through various cytokines such as stem cell factor (SCF) [2, 3] SCF enhances mast cell degranulation and cytokine production through cross-linking of their high affinity surface receptors for IgE (FcεRI), even though it does not induce degranulation on its own [4-7]. Other molecules that promote mast cell maturation include nerve growth factor (NGF) [8]. NGF which acts via tyrosine kinase receptors (TrkA, B, C), different from the c-kit activated by SCF [9]. Moreover, human mast cells express mRNA and protein for the Trk ligands NGF, brain derived neurotrophic factor (BDNF) and neurotrophin 3 [9], suggesting autocrine actions. Neurotrophin 3 was shown to promote maturation of fetal mouse skin mast cells [10] and human intestinal mast cells [11]. However, unlike NGF, which stimulates mast cell degranulation [12], neurotrophins do not. Mast cell chemoattractants include SCF, monocyte chemoattractant protein-1 (MCP-1) and the "regulated upon activation, normal T cell expressed and secreted" (RANTES) [13]. SP is a also potent chemoattractant for human basophils [14]. Depending on their location, stage of maturation, or species [15] mast cells express different types and levels of surface antigens and receptors, some of which are involved in activation and others in cell recognition [16].

In addition to IgE and antigen [5], immunoglobulin free light chains [17, 18], anaphylatoxins, hormones and neuropeptides [19, 20] can trigger mast cell secretion [21-23] (Table 2). The latter include substance (SP) [24], hemokinin [25], neurotensin (NT) [26], NGF [12, 27] which is released under stress [28], and pituitary adenylate cyclase activating polypeptide

(PACAP) [29, 30]. Skin mast cells are located close to sensory nerve endings and can be triggered by neuropeptides [21, 31], such as NT [26], NGF [12], SP [32], and PACAP [30], which can be released from dermal neurons. In fact, skin mast cells contain SP [33], while cultured mouse and human mast cells contain and secrete NGF [34]. Thymic stromal lymphopoietin (TSLP), released in response to inflammation, pathogens and trauma [35], also activates mast cells, but only when used together with interleukin (IL)-1 and tumor necrosis factor (TNF) [35, 36]. A number of additional immune and infectious triggers (e.g. stimulants of Toll-like receptors, TLR) can lead to selective release of mast cell mediators (See under "Selective release" below.

Once activated, mast cells secrete numerous vasoactive and pro-inflammatory mediators [37-42]. These include pre-formed molecules such as histamine, serotonin, TNF, kinins and proteases stored in secretory granules. Leukotrienes (LT), prostaglandins and platelet activated factor (PAF) are synthesized during mast cell activation from arachidonic acid liberated by the action of phospholipases. In addition, a number of cytokines (e.g. IL-1, 2, 5, 6, 8, 9, 13, TNF) and vascular endothelial growth factor (VEGF) [43] are synthesized de novo and released several hours after stimulation. VEGF is also released from normal human cultured mast cells selectively in response to corticotropin releasing hormone (CRH) [44]..

CRH is secreted from the hypothalamus under stress and regulates the hypothalamic-pituitary-axis (HPA) axis [45] through specific receptors [46]. These include CRHR-1 [47] and CRHR-2 [48], the latter being subdivided in CRHR-2α and CRHR-2β [49]. All CRHR are activated by urocortin (Ucn), a peptide with about 50% structural similarity to CRH [50]. Ucn II [51] and Ucn III [52] are potent selective CRHR-2 agonists. CRH

can also be secreted from immune cells [53] and mast cells [54]. CRH and related peptides released locally under stress may regulate mast cell function [55], and the brain-skin connection [56]. It was recently reported that CRH stimulates generation of mast cells from human hair follicle precursors [57].

Mature mast cells vary considerably in their cytokine [58] and proteolytic enzyme content, but their phenotypic expression is not fixed [59, 60]. Mast cells in the presence of SCF produce predominantly pro-inflammatory cytokines, whereas when used together SCF and IL-4, produce mostly Th2 cytokines [61]. For instance, human umbilical cord-derived mast cells (hCBMCs) primed with IL-4 or IL-5 before stimulation with IgE released more TNF, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF), compared to hCBMCs maintained in SCF alone. In contrast, IL-4 enhanced SCF-dependent mast cell proliferation and shifted IgE-stimulated response to Th2 cytokines such as IL-3, IL-5 and IL-13, but not IL-6 [62].

Mast cells play an important role in innate or acquired immunity [63], bacterial infections [64-66] as well as in autoimmunity [67]. Mast cells are also important for maturation of Th17 cells, recognized as key cells in autoimmune disorders [68]. For instance, mast cells in the presence of IL-6 and transforming growth factor β (TGFβ) are necessary for the production of Th-17 cells [69], while TNF and vasoactive intestinal peptide (VIP) drive IL-6-independent Th17 cell maturation [69-71]. A number of immune molecules also contribute to mast cell activation. Addition of compliment fragment 3a (C3a) led to increased degranulation of human mast cells stimulated by aggregated IgG [72]. Immunoglobulin-free light chains elicited immediate hypersensitivity-like reactions [18, 73], with subsequent T cell-mediated immune responses. The antibacterial peptides, human B-

defensins, can activate mast cells and induce degranulation [74]. In fact, mast cells interact with T cells [75, 76] and superactivate them through TNF, as shown with mouse [77], [78] and human [79, 80] mast cells. It was recently shown that T cells release "microparticles" that stimulate human mast cell degranulation and IL-8 release [81]. Mast cells, in turn, secrete heparin "microparticles" that contain and deliver TNF to lymph nodes [82].

In addition, mast cells which may be restricted to a subset highly expressing both FcεRI and MHC II [83] express MHC class I and II molecules, which allow them to function as antigen presenting cells[84-86]. . Basophils can act as Th2-inducing antigen-presenting cells [87, 88] and have emerged as key inducers of Th2 responses [89, 90]. Basophils also co-operate with dendritic cells for optimal Th2 responses [91]. Moreover, basophil activation by "autoreactive IgE" induces their "homing" to lymph nodes, where they promote Th2 cell differentiation and production of auto-reactive antibodies that contribute to lupus nephritis [92]. Interestingly, mast cells can act both as positive and negative modulators of immunity [93]. In addition, mast cells can coordinate the adaptive immune response by directing migration of dendritic and T cells to lymph nodes and secreting T cell-polarizing cytokines [94]. Such regulatory activities of mast cells may stem from selective release of immunomodulatory molecules that could have both autocrine and paracrine actions (Fig. 1.1).

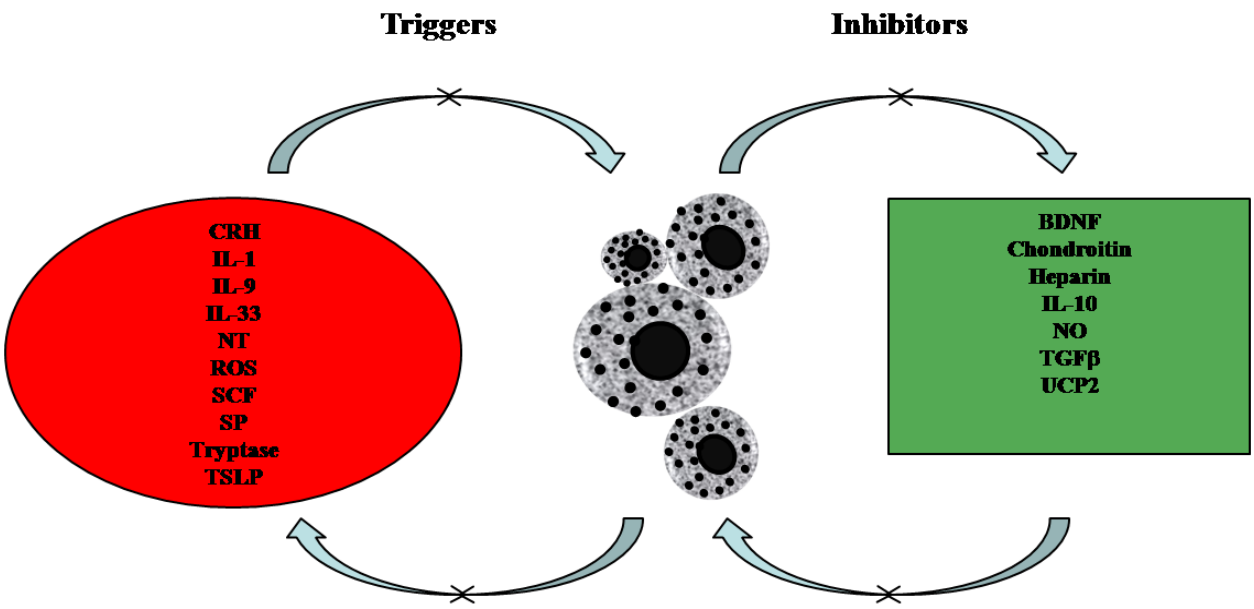

Figure 1.1. Schematic representation of mast cell autocrine triggers and modulators. Numerous molecules secreted by mast cells can have autocrine actions, some activating while others inhibit the mast cells.

BDNF, brain-derived neurotrophic factor; CRH, corticotropin-releasing hormone; IL, interleukin; NT, neurotensin; NO, nitic oxide; ROS, reactive oxygen species; SCF, stem cell factor; SP, substance P; TGFβ, transforming growth factor β; TSLP, thymic stromal lymphopoietin; UCP2, uncoupling protein 2.

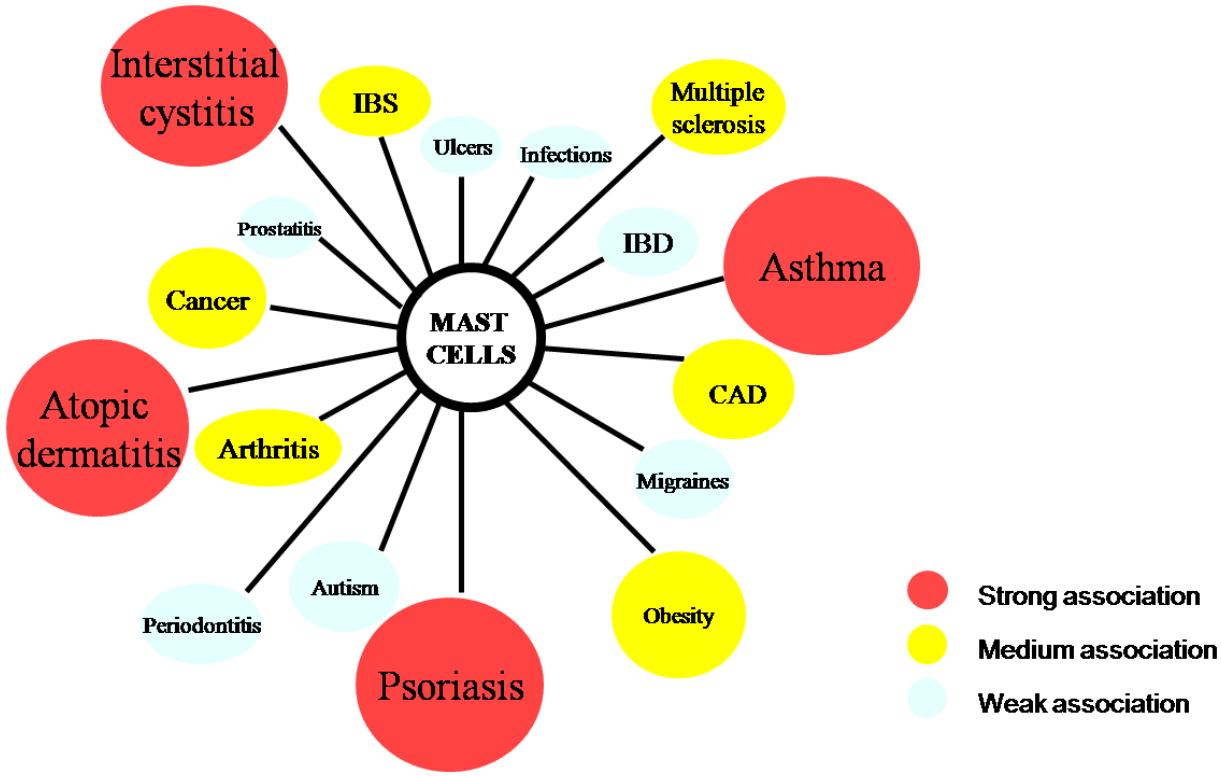

Figure 1.2. Mast cell involvement in inflammatory diseases. Increasing evidence indicates that mast cells are involved in many diseases. Colors indicate the strength of the association (red = strongest, white = weakest).

CAD, coronary artery disease; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome.

Mast cells also have the unusual ability to be triggered by certain molecules and then either activate them or degrade them. For instance, mast cells can act on precursor protein molecules and generate active peptides [95], such as histamine releasing peptides [96] and NT [97] from plasma. However, mast cells can also degrade NT [98] and limit its biologic effects [99]. Mast cells can also synthesize endothelia [100], but also release proteases that

degrade endothelin [64]. Finally, mast cells can be activated by snake toxins, but also degrade them [101]. Whether these actions will prove useful or detrimental obviously depends on the ability of mast cells ability to secrete specific mediators in a well-regulated fashion.

1.2.2 Inflammatory processes and role of selective release

Increasing evidence indicates that mast cells are critical for the pathogenesis of inflammatory diseases [19, 20], such as arthritis [102], atopic dermatitis, psoriasis [103, 104], and multiple sclerosis [105] (Fig. 1.2) . Gene array analysis of human mast cells activated by IgE showed overexpression of numerous, mostly inflammation-related, genes [106]. Proteases released from mast cells could act on plasma albumin to generate histamine-releasing peptides [96, 107] that would further propagate mast cell activation and inflammation. Proteases could also stimulate protease-activated receptors (PAR) inducing microleakage and widespread inflammation [108, 109]. However, unlike allergic reactions, mast cells are rarely seen to degranulate during inflammatory processes . The only way to explain mast cell involvement in non-allergic processes would be through “differential” or “selective” secretion of mediators without degranulation [110] (fig. 1.3) This ability could occur through different mechanisms..

Unlike the explosive secretion typical of allergic or anaphylactic reactions, mast cells can secrete the content of individual granules [111]. Mast cells can also secrete some granular contents

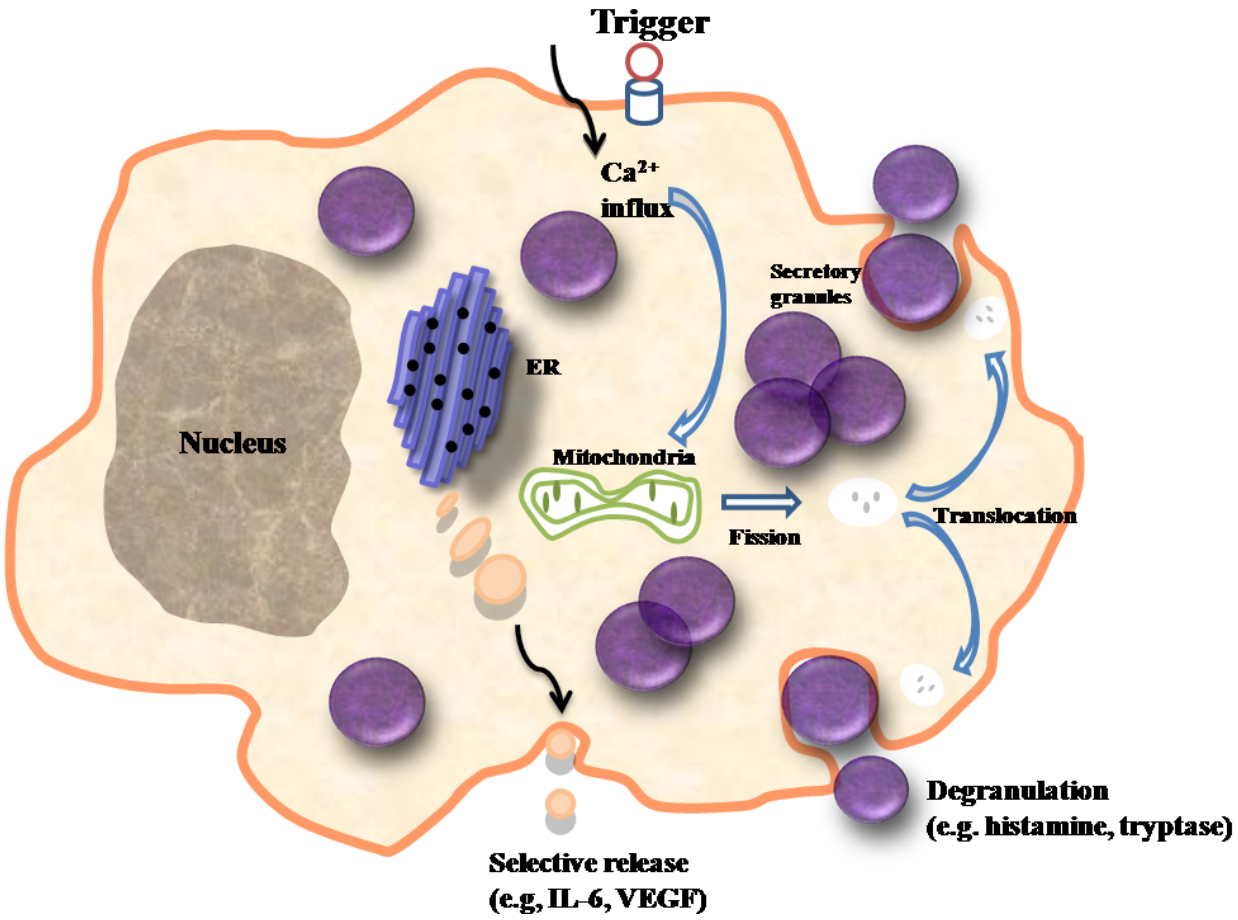

Figure 1.3. Schematic representation of physiological and environmental mast cell triggers, and the inhibitory effect of certain flavonoids, such as luteolin. Many of these triggers stimulate selective release of mediators such as IL-6, TNF or VEGF without degranulation but with *de novo* synthesize cytokines.

CRH, corticotropin releasing hormone; LPS, lipopolysaccharide; NT, neurotensin; PCBs, polychlorinated biphenols; PTH, parathyroid hormone; SP, substance P; VIP, vasoactive intestinal peptide.

through a process associated with ultrastructural alterations of their electron dense granular core indicative of secretion, but without evidence of degranulation [112], a process that has been termed ‘‘activation’’ [113], ‘‘intragranular activation’’ [114] or ‘‘piecemeal’’ degranulation [115] (Fig. 1. 4). Mast cells can also release specific mediators such as serotonin without histamine [116]. Selective release of serotonin was reported to take place through sequestration from secretory granules inside vesicles containing high affinity serotonin-binding proteins from which it was released [117]. A somewhat similar process was reported for eosinophils where it was shown that eotaxin stimulation induced movement of preformed IL-4 from granules into secretory vesicles from which it was released [118]. IL-1 stimulated human mast cells selectively release IL-6 without degranulation through vesicles (40–80nm) unrelated to the secretory granules (800–1000 nm) [119].

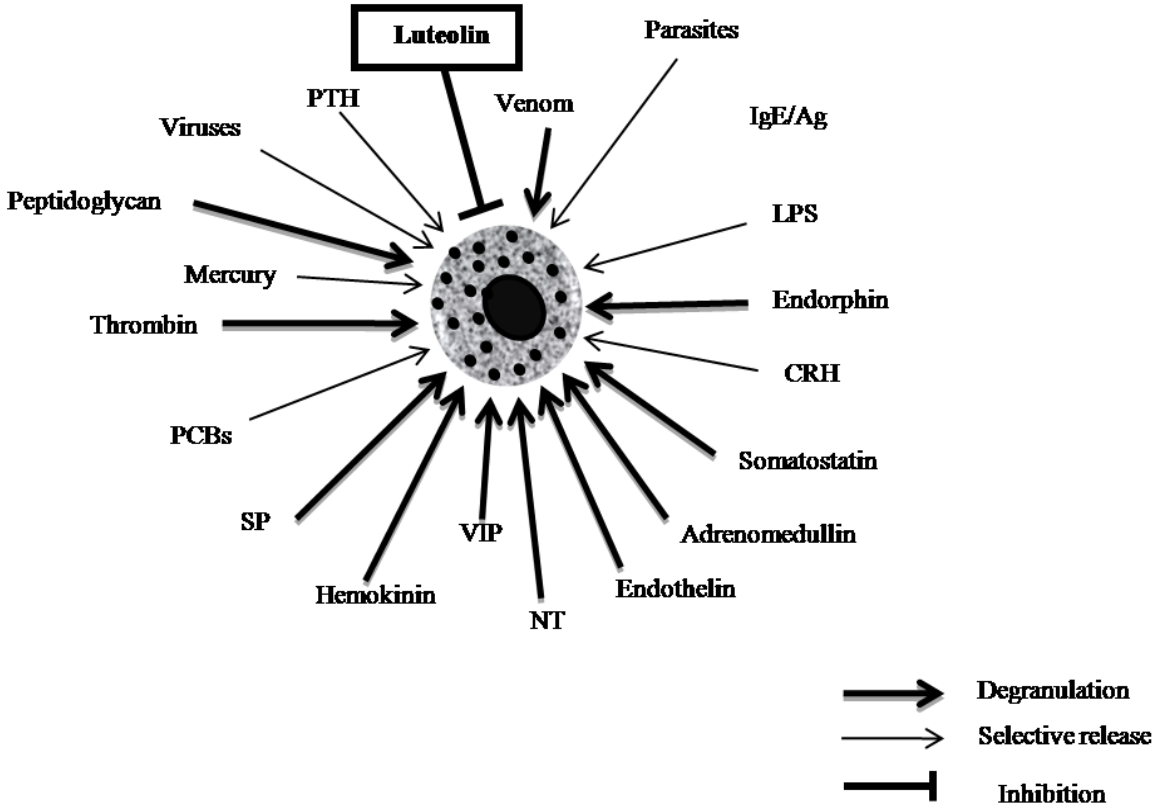

Figure 1.4. Schematic representation showing mast cell degranulation as compared to selective mediator release. During non-granule *de novo* synthesized cytokine release, vesicles much smaller than secretory granules transport mediators to the cell surface for exocytosis.

Others showed selective release of eicosanoids [120-122] or IL-6 in response to bacterial lipopolysaccharide (LPS), in the presence of the phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin, or triggered by SCF [123-125]. CRH [126] and PGE2 [127] induced selective VEGF release, while PGE2 also induced release of MCP-1 without degranulation [128]. Yet, PGE2 inhibited FcεRI-induced histamine release from human lung

mast cells [129]. Stromal cell-derived factor-1 alpha (SF-1α) selectively produced IL-8 from human mast cells also without degranulation [130]. Activation of human cultured mast cells by CD30 ligands led to release of the chemokines IL-8 and MCP-1 without histamine and without degranulation [131]. IL-33 induced IL-13 release independent of IgE stimulation [132].

TLR are critical in innate and acquired immunity [133, 134] . Mast cells activation via TLRs, leads to release of different cytokines [135]. For instance rodent mast cell TLR-4activation by LPS induces TNF release without degranulation. TLR-4 is also activated by extra domain A of fibronectin to release several cytokines, including TNF, in the same way as LPS [136]. Furthermore, LPS induces secretion of IL-5, IL-10 and IL-13, but not GM-CSF, IL-1 or LTC4. [137, 138] In contrast, staphylococcal peptidoglycan induces degranulation and histamine release through TLR-2 [137, 139]. TLR-2 and TLR-4 activation has a synergistic action with antigen in enhancing cytokine production from rodent mast cells [140]. Elsewhere, it was shown that TLR-2 activation produces IL-4, IL-6 and IL-13, but not IL-1, while LPS produces TNF, IL-1, IL-6 and IL-13, but not IL-4 or IL-5, again without degranulation [141].

TLR 3, 7 & 9 activation by poly-oligodeoxynucleotide and CpG induced release of TNF and IL-6 without degranulation from fetal rat skin-derived mast cells [142]. Human mast cells produce IL-6 through viral TLR-9 activation [143], while they produce interferon (IFN) following TLR-3 activation by double-stranded RNA [144].

1.2.3 Regulation of mast cell activation

FcεRI–induced mast cell degranulation involves calcium-dependent exocytosis, and SNAP-23 phosphorylation [145], but granule translocation to the surface is calcium-independent [146]. Mast cell activation by different triggers apparently engages different downstream pathways. FcεRI aggregation induces PI3K, ERK, JNK, NF-κB and PKC activation, although the PKCε isozyme may be redundant [147, 148]. PI3K inhibition by the "phosphatase and tensin homologue deleted on chromosome ten" (PTEN) or PTEN knockdowns induce constitutive cytokine production, without degranulation, that involves phosphorylation of AKT, p38/MAPK and JNK [149]. Secretion in response to compound 48/80 requires PLC, tyrosine kinase, p38/MAPK and PKC [150]. In contrast, IL-1 stimulation of selective IL-6 release is extracellular calcium-independent and involves p38/MAPK, but only PKCθ isozyme activation [151]. CRH mast cell activation of selective VEGF release is also extracellular calcium-independent, and involves only PKA and p38/MAPK activation [126].

Degranulation in response to FcεRI–aggregation was severely impaired in IL-2-inducible T cell kinase -/- mice [152]. FcεRI-induced mast cell activation in rat basophil leukemia (RBL) cells was inhibited by the Syk-tyrosine kinase inhibitor Piceatannol [153]. Suboptimal antigen challenge of human mast cells led to FcεRI-unresponsiveness that correlated with reduced Syk levels [154], apparently through actin assembly that blocked degranulation [155]. However, low antigen still permitted MCP-1 release suggesting yet another mechanism of differential release [156].

The Src family kinase Lyn is a negative regulator of allergic mast cell activation, but Lyn -/- mice had increased FcεRI expression, circulating histamine and eosinophilia [157]. Fyn deficient mast cells could not generate IL-6, TNF or MCP-1 during FcεRI

aggregation, but IL-13 production was intact, suggesting divergent regulatory pathways [158].

Adaptor complexes such as B cell lymphoma 10-mucosal-associated lymphoid tissue 1 (Bcl10-Malt1) permit FcεRI-dependent IL-6 and TNF release without degranulation [159]. Mice deficient in either Bcl10 or MALT1 proteins did not produce TNF or IL-6 upon FcεRI signaling: yet, degranulation and LT secretion was normal [160]. Neutralization of the inhibitory receptor IRp60 (CD300a) in human cord blood mast cells in mice led to increased mediator release [161]. In contrast, engagement of the myeloid cell inhibitory receptor CD200 in human mast cells inhibited FcεRI-induced activation [162]. Mast cells also express the inhibitory receptors cd300 and Siglec-8, as well as the death receptor TRAIL [163]. Two peptides derived from complement components C3a, C3a+ and C3a9 inhibited FcεRI-induced degranulation and TNF release [164]. IL-4 enhanced, whereas IFN-γ inhibited the FcεRI-mediated production of IL-8 and GM-CSF from human mast cells.

There appear to be some innate inhibitors of mast cell secretion (Fig 1.3). Chondroitin sulfate and heparin, the major constituents of mast cell granules, inhibit human mast cell secretion [165]. Nitric oxide (NO) blocks FcεRI-induced cytokine secretion through inhibition of Jun [166]. In contrast IL-10 appears to have divergent effects depending on the mast cell type and stimulus [167]. The natural chymase inhibitors alpha 1-antitrypsin and secretory leukocyte protease inhibitor (SLPI) inhibit histamine release from human cells [168].

Recent evidence indicates that mitochondria are involved in the regulation of mast cell degranulation. Mitochondrial uncoupling protein 2 (UCP2) inhibited mast cell activation [169]. Moreover, our recent results indicate that mast cell degranulation requires mitochondrial translocation to the cell surface [170] (Fig. 1.5). Inhibition or downregulation of Dynamin Related Protein 1 (Drp1), a cytoplasmic protein responsible for mitochondrial fission and translocation, blocks mast cell degranulation [171]. The involvement of mitochondria in mast cell regulation may also explain the ability of certain flavonoids to inhibit mast cell degranulation [172], since quercetin was shown to accumulate in mitochondria [173].

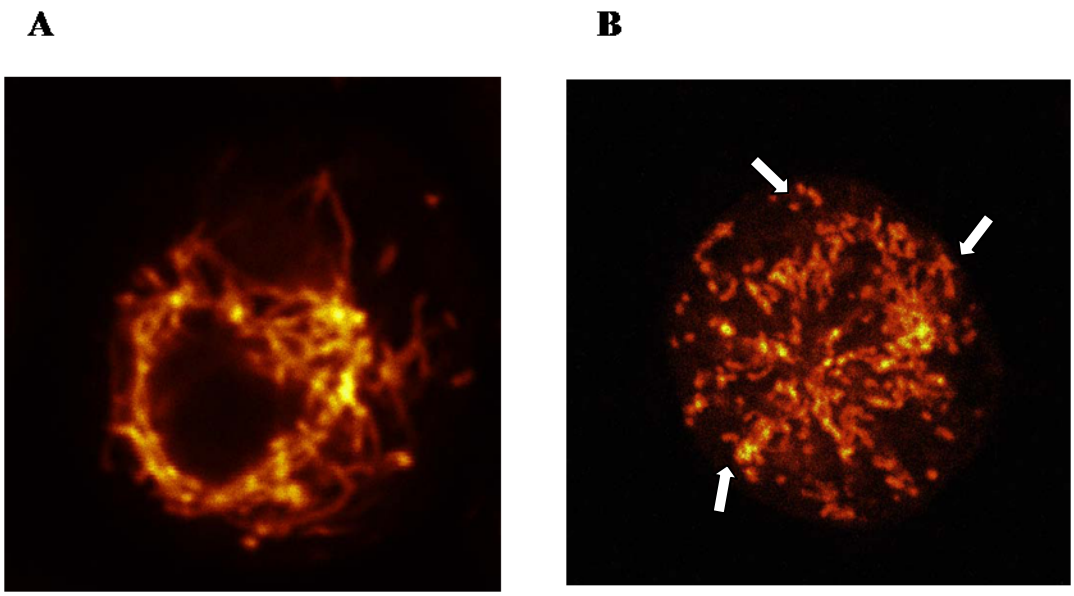

Figure 1.5. Two human cultured LAD2 mast cells, showing distribution of mitochondria stained with MitoTracker and photographed using confocal microscopy; (A) control in which mitochondria form a "net" around the nucleus and (B) after stimulation with SP (2 μM for 30 min at 37 °C) in which mitochondria are distributed throughout the cell.

(Magnification: ×1000). Arrows point to the areas with the highest concentration of MitoTracker (yellow color); thus the highest aggregation of mitochondria.

1.3 Atopic dermatitis and psoriasis

Skin mast cells may have important functions as "sensors" of environmental and emotional stress [56], possibly due to direct activation by stress-related CRH and related peptides [55]. Skin has its own equivalent of the HPA axis [174, 175]. CRH and CRHR mRNA are also expressed in human and rodent skin [176, 177]. CRH-like immunoreactivity is also present in the dorsal horn of the spinal cord and dorsal root ganglia, as well as in sympathetic ganglia [178, 179]. CRH administration in humans causes peripheral vasodilation and flushing reminiscent of mast cell activation [180]. Moreover, intradermal administration of CRH and Ucn activates skin mast cells and increases vascular permeability in rodents [181] and humans [182, 183], through activation of CRHR-1 [56]. Mast cell-related diseases, such as asthma, atopic dermatitis (AD), and psoriasis, are triggered or exacerbated by stress through mast cell activation [184, 185]. For instance, exercise was also shown to increase the responsiveness of skin mast cells to morphine only in patients with exercise-induced asthma [186]. Computer-induced stress enhanced allergen specific responses with concomitant increase in plasma SP levels in patients with AD [187]. Similar findings with increased plasma levels of SP, VIP and NGF, along with a switch to a Th2 cytokine pattern, was reported in patients with AD playing video games [188].

CRHR-2 expression was upregulated in stress-induced alopecia in humans [189], while CRHR-1 expression was increased in chronic urticaria [190]. Acute stress released CRH in the skin and increased local vascular permeability [191]. Acute stress also exacerbated skin delayed hypersensitivity reactions [192], and chronic contact dermatitis in rats, an effect that involved significantly increased mast cells in the dermis, and was dependent on CRHR-1 [193]. Acute restraint stress induced rat skin vascular permeability

[194], while was inhibited by a CRH receptor antagonist, and was absent in mast cell deficient mice [181, 195].

Psoriasis is also triggered or exacerbated by acute stress [103, 196-198]. Psoriasis is characterized by keratinocyte proliferation and inflammation, as well as mast cell accumulation and activation [104, 199]. Mast cells are increased in lesional psoriatic skin [103, 104]. Neuropeptides [200], especially SP [201], are involved in the pathogenesis of psoriasis. In particular, SP reactive fibers are localized close to mast cells [103, 202]. SP can stimulate mast cells [203, 204] and contributes to inflammation [205, 206]. SP-positive nerve fibers are more dense in psoriatic lesions and have an increased number of mast cell contacts compared to normal skin [202, 207, 208]. SP-positive nerve fibers and mast cell contacts are also increased by acute stress in mice [209], leading to dermal mast cell degranulation [197, 203, 210].

Psoriasis is associated with chronic inflammation and it often co-exists with inflammatory arthritis [211], in which IL-33 was recently implicated [212]. IL-33 is one of the newest members of the IL-1 family of inflammatory cytokines [213], and can mediate IgE-induced anaphylaxis in mice [214]. IL-33 also induces release of IL-6 from mouse bone marrow-derived cultured mast cells [215], and IL-8 from human umbilical cord blood-derived cultured mast cells (hCBMCs) [216]. We showed that IL-33 augments the action of SP-stimulated VEGF release from human mast cells; IL-33 gene expression is also increased in lesional skin from patients with psoriasis [217].

Keratinocytes also express neurokinin (NK) 2 receptors and can be stimulated by SP [218], to release IL-1 [219]. Keratinocyte proliferation is accelerated by PAF, which can be

secreted from mast cells [220], and stimulates human mast cells [221] and basophils. Mast cells may, therefore, be involved in the pathogenesis of psoriasis and other inflammatory skin diseases.

1.4 Mitochondria and immunity

Mitochondria are bacteria that evolved into a symbiotic relationship with eukaryotic cells and are responsible for cellular energy production. Mitochondria are organelles found in the cytoplasm of all eukaryotic cells that have multiple functions, including energy production, redox control and calcium homeostasis. Mitochondria are one of the main sources of ROS in the cell, and thus may be important in diseases such as cancer, diabetes, obesity, ischemia/reperfusion injury and neurodegenerative disorders. Mitochondria have both an inner and outer membrane. Energy is produced in the mitochondria by a process termed oxidative phosphorylation, which using uses a proton gradient across the inner mitochondrial membrane to drive ATP synthesis by F1F0-ATP synthase. Increasing evidence indicates that mitochondria are not static, but undergo dynamic fission-fusion reactions that permit them to travel on microfilament networks[222]. The ability to undergo translocation endows mitochondria with additional abilities, such as the buffering of intracellular calcium ions at surface “immunologic synapse” where T cells are presented with specific antigen[223].

1.4.1 Mitochondrial Structure and dynamics

The structure and dynamics of mitochondria connect tightly. Mitochondria are composed with two membranes, which is unique in animal cells. These two membranes divide mitochondria into five distinct compartments: the matrix, cristae, the innter membrane, the intermembrane region and outer membrane. The morphology and numbers of mitochondria vary dramatically according to the cell types: in hepatocytes which are metabolic active, mitochondria are connected as tubular and total number can reach up to 5,000. In T cells with smaller size, the number of mitochondria is only around 100 and they show ball-like shape [224]. Mitochondria are not static but keep on changing their morphology and distribution through fission-fusion balance. The steady-state morphology of the mitochondria network is a result of balanced fission, fusion and motility events

1.4.2 Components of the Mitochondrial Fission Machinery

Components of mitochondrial fission machinery were first identified through genetic screens in yeast. The cytoplasmic GTPase Dynamin Related Protein 1 (Drp1) is one of the most important regulators of both mitochondrial fission and translocation[225, 226]. Knockdown of Drp1 by RNAi leads to increased mitochondrial length and interconnectivity of mitochondria7, 32. Drp1 is found in the cytoplasm in an inactive form[227]. Multiple modifications contribute to localization and proper function of Drp1. Dephosphorylation at Ser-637 by calcineurin is critical to recruit Drp1 from the cytoplasm to the mitochondrial outer membrane[228] (Fig 1.6). Then, phosphorylation at Ser-616 controls its enzyme activity responsible for mitochondrial fission and translocation[229]. Mitochondrial dynamics and the function of Drp1 in mast cell degranulation and in inflammatory diseases have not been previously studied.

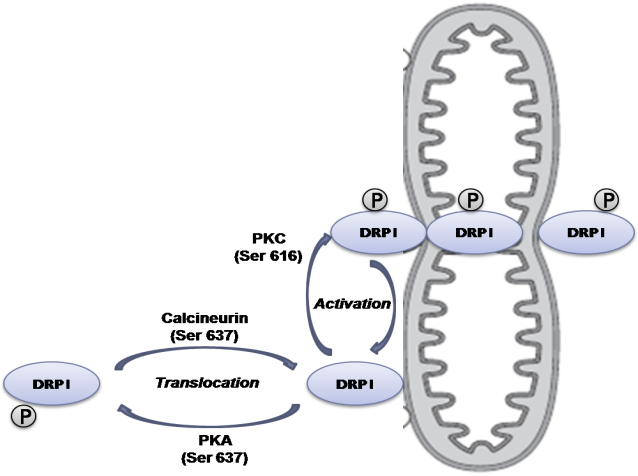

Figure 1.6 Scheme of mitochondrial fission and Drp1 activation**.** In resting condition, Drp1 locates in cytoplasma region with Ser637 phosphralated. Calcineurin dephosphralates Drp1 at Ser637 and recruits Drp1 to the outer membrane of mitochondria. PKC then activate Drp1 at Ser-616 site for its function.

1.4.3 Components of the Mitochondrial Fusion Machinery

The process of mitochondrial fusion involves the fusion of both inner and outer membranes. In human, mitochondrial fusion are regulated by mitofusin (Mfn 1&2) proteins which are located on the outer membranes and OPA1 of the inner membrane. Dysfunction of any of them will lead to mitochondrial hyperfission and fragmentation. Similarly, overexpression of Mfn or Opa1 in wild type cells results in highly fused and aggregated mitochondria. The molecular mechanism of Opa1 remains obscure,

however, its homology to dynamin family GTPases suggests it may be involved in controlling the structure and tubulation of the inner membrane and/or cristae remodeling.

1.4.4 Mitochondrial dynamics in immunity

Mitochondrial dynamics has been proved to be important for anti-viral reaction and pro-inflammatory production [230]. Immune cells detect virus infection through Toll-like Receptors (TLRs). These specific receptors activate Mitochondrial Anti-Viral Signaling Protein (MAVS) and intracellular adaptors MyD88 which then lead to the production of type I interferons and pro-inflammatory cytokines [231]. Fission of mitochondria has been shown to increase the antiviral signaling [230] and promote redistribution of MAVS [232]. SiRNA knock-down of Drp1 gene expression decrease such reaction. Similarly, MFNs have been found to inhibit mitochondrial antiviral immunity: over-expression of MFN2 silence the antiviral signaling induction [233].

T cells activation by Antigen-Presenting Cells (APC) is the most important connection between innate and acquired immunity. The formation of “immune synapses” between T cells and APCs and continually calcium influx are required for T cells activation [234]. Mitochondria play important role in calcium buffering to prevent inactivation of CRAC/ORAL1 calcium channel [235]. Recent evidence shows that mitochondrial fission and translocation to the “immune synapses” is necessary for such calcium buffering function [223].

Another important physiological process of T cells is chemotaxis. Upon activation, T cells need to circulate back to lymph node to achieve appropriate immunological reaction. Campello et al recently showed that mitochondrial

redistribution and accumulation at the uropode is required for T cells chemotaxis [226]. Depletion of mitochondrial ATP production can block T cell chemotaxis, indicating the necessary of local ATP production of mitochondria.

1.5 Conclusion

Mast cells clearly participate in the induction and/or propagation of certain inflammatory diseases, through selective release of mediators. The pharmacologic inhibition of this process would, therefore, have clear therapeutic potential. Luteolin formulations, alone or together with drugs that can also partially inhibit the release of pro-inflammatory mediators for skin inflammation and oral for brain inflammation are in the pipeline and hold promise.

Chapter II

Mitochondria participate in granule-stored but not non-granule stored *de novo* synthesized TNF secretion in human mast cells

Bodi Zhang, Konstantinos-Dionysios Alysandratos, Asimenia Angelidou, Shahrzad Asadi, Magdalini Vasiadi, Orian Shirihai, Theoharis Theoharides

BZ performed most of the experiments, analyzed the results and helped wrote the paper. KDA and AA helped to performed the in vitro stimulation experiment and analyzed the results. SA and MV helped prepare the cell cultures and performed computer searches. OS offered the Searhore machine to meausre energy consumption and made commnets on the manuscripts.TCT designed the study, organized the collection of human samples, transported the samples and supervised the analysis of the results and wrote the paper. All authors have read and approved the final version of the manuscript.

2.1 Introduction:

Mast cells are bone marrow-derived immune cells that can secrete pre-stored mediators such as histamine and tryptase through degranulation, as well as newly synthesized cytokines including interleukin-4 (IL-4) and IL-6, in response to allergic or neuropeptide triggers [63, 236]. In addition, mast cells uniquely store tumor necrosis factor (TNF) in their secretory granules [237, 238]. Stimulation of LAD2 [239] cells by SP induces degranulation of pre-stored TNF [240], while stimulation with LPS induces selective *de novo* synthesis and release of TNF without degranulation [137, 139], [241].

Other secretory cell types, like eosinophils, use distinct mechanisms for secretion; these include exocytosis of large storage granules, and release from small secretory vesicles [242]. Mast cells can also release mediators selectively without degranulation [110], first reported for release of serotonin without histamine [116], and later for IL-6 without histamine [119]. In both cases, this selective release involved small vesicles (80 nm) rather than the typical secretory granules (1000 nm) [117], [119]. This ability may allow mast cells to participate in a variety of distinct pathophysiological settings, in addition to allergy, such as innate and acquired immunity [93], inflammation [110], wound healing [243] and cancer growth [244]. However, little is known about what distinguishes rapid degranulation from delayed selective release.

Degranulation from rat peritoneal mast cells requires metabolic energy and calcium [245]. Mitochondria are the primary sources of energy production and have the ability to buffer calcium locally [246]. Mitochondria are dynamic organelles that that participate in many complicated cell functions through morphological and localization changes [247]. Recently, increasing evidence indicates the importance of mitochondria

dynamics in immune cell regulation. Local ATP production by mitochondria is required for T cells chemotaxis [226]. Moreover, there are close association between mitochondrial dynamics and intracellular calcium changes; for instance mitochondrial translocation is required for T cells “immune synapse” formation and sustainable calcium influx [223]. On the other hand, local intracellular calcium changes can also regulate mitochondrial dynamics and subcellular localization [248].

In this study, we show that SP-induced granule-stored TNF secretion, unlike newly synthesized selective TNF release by LPS, correlates with high mitochondrial energy consumption, intracellular calcium level as well as translocation.

2.2 Materials and methods:

Cell lines and reagents

LAD2 cells [239] (from Dr. A.S. Kirshenbaum and Dr. Metcalfe, NIH) were cultured in StemPro-34 Medium (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml recombinant human stem cell factor (rhSCF, from Biovitrum AB, Stockholm, Sweden) and 100 U/ml penicillin/streptomycin. Cells were grown in an incubator in 5% CO_2 and air at 37 ^{O}C. All cells were used during their logarithmic growth period.

TNF secretion assay

LAD2 cells were treated with SP (Sigma, St. Louis, MO) at 10 µM, in order to achieve strongest degranulation, or LPS at 10 ng/ml (Sigma) for 30 min, 6 hr and 24 hr. TNF release was measured by ELISA (R&D system, Minneapolis, MN) in the supernatant fluid. (n=3; * $p<0.05$ compared with control).

Quantitative PCR

Total RNA from cultured mast cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, CA) respectively, according to the manufacturer's instructions. Reverse transcription was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA). In order to measure TNF gene expression, quantitative real time PCR was performed using Taqman gene expression assays. The following probes obtained from Applied Biosystems were used: TNF: Hs00542477_m1. Samples were run at 30 cycles using Applied Biosystems 7300 Real-Time PCR System (Carlsbad, CA). Relative mRNA abundance was

determined from standard curves run with each experiment and TNF expressions were normalized to GAPDH (Hu, VIC TAMRA) endogenous control.

Intracellular calcium measurement

LAD2 cells were loaded with 5 μM fura-2 AM (Invitrogen, Carlsbad, CA) for 20 min, washed and then incubated for another 20 min. Cells were then treated with either SP (10 μM) or LPS (10 ng/ml). Fluorescence signals were acquired on Flexstation II (Bucher Biotech, Basel, Switzerland). Cytosolic calcium was calculated after subtraction of the background fluorescence by measuring the ratio of the two emission intensities (excitation at 340 nm and 380 nm, OD340/OD380). Each experiment was repeated three times independently.

Cell energy consumption measurement

LAD2 cell oxygen consumption rates were measured by Searhorse XF-24 Flux analyzer (Seahorse Bioscience Inc, North Billerica, MA). LAD2 cells were treated with SP (10 μM) or LPS (10 ng/ml). Energy consumption was inhibited by the mitochondrial ATP pump blocker oligomycin. Cells were incubated with 2 μM oligomycin for 20 min and then treated with SP (10 μM) or LPS (10 ng/ml). Experiments were conducted three times and results were similar.

Confocal microscopy

LAD2 cells were incubated with 20 nM MitoTracker deep red probe (Invitrogen, Carlsbad, CA) for 20 min and 50 nM LysoTracker DND (Invitrogen) for 30 min. Cells

were washed, moved to glass bottom culture dishes (MatTek, Ahsland, MA) and observed using a Leica TCS SP2 Confocal microscopy (Leica, Japan). Percentages of cells with mitochondrial translocation were counted from 100 randomly selected mast cells in each experiment by three independent operators. Confocal digital images were processed using the National Institute of Health ImageJ 1.32 and Adobe Photoshop 7.0 Programs.

Data Analysis

Statistical significance was determined by the Students t test using the SigmaPlot 9.0 (SPSS, Chicago, IL). Differences were considered significant if $p<0.05$.

2.3 Results

Time course of SP and LPS-induced TNF secretion and gene expression.

LAD2 cells secrete granule-stored TNF 30 min after SP stimulation (10 µM) (Fig. 2.1A). Incubation of mast cells with SP (10 µM) for 24 hr also induces *de novo* synthesized TNF. Stimulation with LPS (10 ng/ml) for 30 min has no effects on degranulation and granule-stored TNF secretion (Fig. 2.1A), but incubation for 24 hr induces selective *de novo* synthesis and release of TNF without degranulation (Fig. 2.1A). The gene expression level of TNF is significantly increased at 6 hr and 24 hr both after SP and LPS stimulation (Fig. 2.1B), indicating that SP and LPS induce *de novo* TNF synthesis. Under light microscopy, LAD2 cells stimulated by SP for 30 min show clear degranulation (Fig. 2.1C), while the SP-stimulated LAD2 cells show no sign of degranulation at 24 hr (Fig. 2.1D), further confirmed that *de novo* TNF secretion occurs without degranulation. SP induced degranulation does not have effects on cell viability at 10 µM (Fig. 2.2).

Intracellular calcium levels of SP and LPS-induced TNF secretion.

Mast cell degranulation requires calcium. SP triggers a rapid strong cytosolic calcium increase within 1 min that is not detected after LPS stimulation (Fig. 2.3A). After 50 min SP incubation, the cytosolic calcium level drops to the same level of control, and there is still no difference observed between LPS treated cells and control (Fig. 2.3B), indicating *de novo* TNF synthesis and secretion do not require intracellular calcium

changes. This is the first time to our knowledge showing that LPS stimulation does not appear to require any significant intracellular calcium increase.

Energy consumption during SP-stimulated granule-stored and LPS-induced de novo synthesized TNF release

Mast cell degranulation requires ATP [249]. To test if degranulation and *de novo* TNF secretion have different energy requirements, mitochondrial oxygen consumption was investigated during these two processes in LAD2 cells. SP induces a significant oxygen consumption spike, while there is almost no difference between LPS stimulated and control cells (Fig. 2.4A). In order to investigate if the mitochondrial energy production is required for degranulation, mitochondrial energy production was blocked by pre-treating LAD2 cells with the ATP synthase inhibitor oligomycin (2 μM) for 30 min. Oliygomycin treatment drops the metabolic baseline of both SP and LPS- treated cells to 30% of normal. In addition, the energy consumption spike (Fig. 2.4B) is inhibited. Oliygomycin treatment inhibits SP simulated granule-stored TNF secretion at 30 min (Fig. 2.4C). In contrast, LPS-induced TNF release at 24 hr is largely unaffected (Fig. 2.4D).

Degranulation, but not non-granule stored de novo synthesized cytokine secretion, induces mitochondrial translocation

Examination of mitochondrial location in live LAD2 cells by Confocal microscopy shows that mitochondria in resting mast cells are located around the nucleus as a "mitochondria pool" within the area indicated by the white dashed circle; very few mitochondria could be found close to the cell surface (Fig. 2.5A). This perinuclear mitochondrial localization is also observed in other immune cells, like T cells [226]. Since the average pH of mast cell granules is 5.5 [250], the lysosome dye LysoTracker [251] is used to stain secretory granules. After SP (10 μM) stimulation for 30 min at 37 C, mast cells undergo rapid degranulation as indicated by the content of numerous granules stained with LysoTracker outside the cell (Fig. 2.5B, left panel); during degranulation, many mitochondria appear to be smaller (Fig. 2.5B middle panel) and have translocated close to the cell surface region (Fig. 2.5B, right panel). This phenomenon is not observed in LPS stimulated mast cells (Fig. 2.5C).

Degranulation induced mitochondria translocation is reversible

Confocal images of SP-activated mast cells at 24 hr show that there is no evidence of degranulation (Fig. 2.6B). At 24 hr, it is obvious that mitochondria have translocated back to perinuclear region (Fig. 2.6B). Only about 20% of cells contain translocated mitochondria, which is no different from that of controls. This finding indicates degranulation-induced mitochondrial translocation is reversible. Just there is no mitochondrial translocation cause any 30 min after LPS stimulation (Fig. 2.6C) there is no translocation at 24 hr either; LPS-treated cells still show intact mitochondria located in the perinuclear region (Fig. 2.6C).

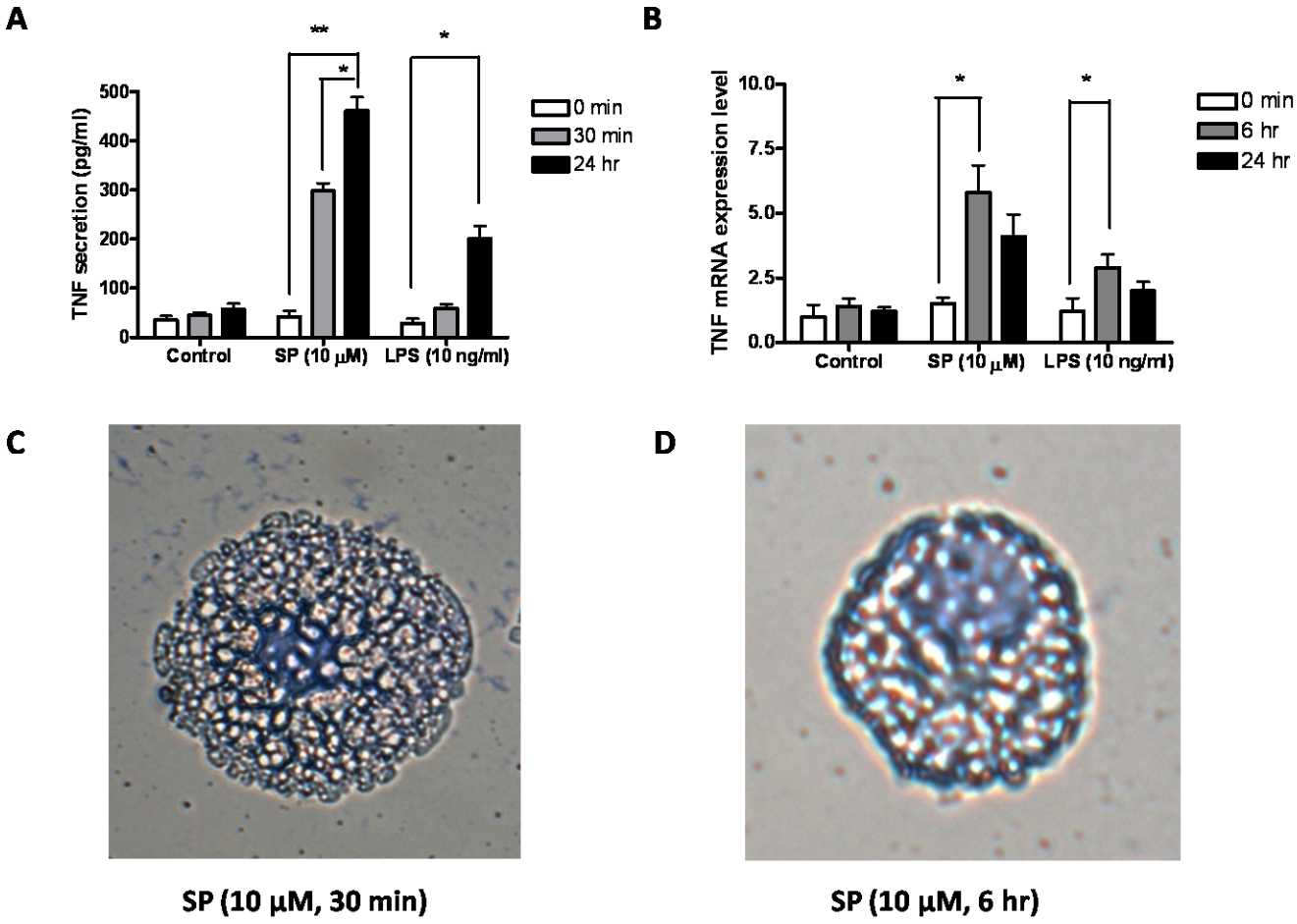

Figure 2.1. **SP and LPS-induced on TNF secretion mRNA expression and light microscopy of LAD2 cells.** LAD2 cells were treated with SP (10 μM) or LPS (10 ng/ml) for the time indicated. (A) TNF release was measured by ELISA (R&D system, Minneapolis, MN) in the supernatant fluid (n=3; * $P<0.05$, **$P<0.01$). (B) TNF mRNA expression was measured by Real Time PCR. (n=3; * compare with 30 min control, *$P<0.05$). (C, D) LAD2 cells were stained with Tuidine blue and were observed under

light microscopy. LAD2 cells were treated with SP (10 µM) for (C) 30 min and (D) 24 hr. Scale bars = 5 µm.

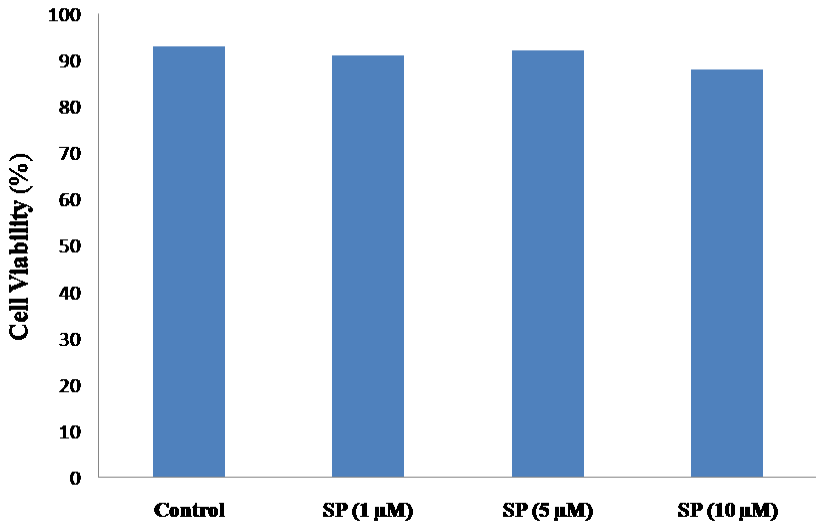

Figure 2.2. **Lack of loss of viability during human mast cell degranulation stimulated by SP.** LAD2 cells were stimulated with different concentration of SP for 30 min. Cell viability was tested using tryptan blue exclusion. (n=1)

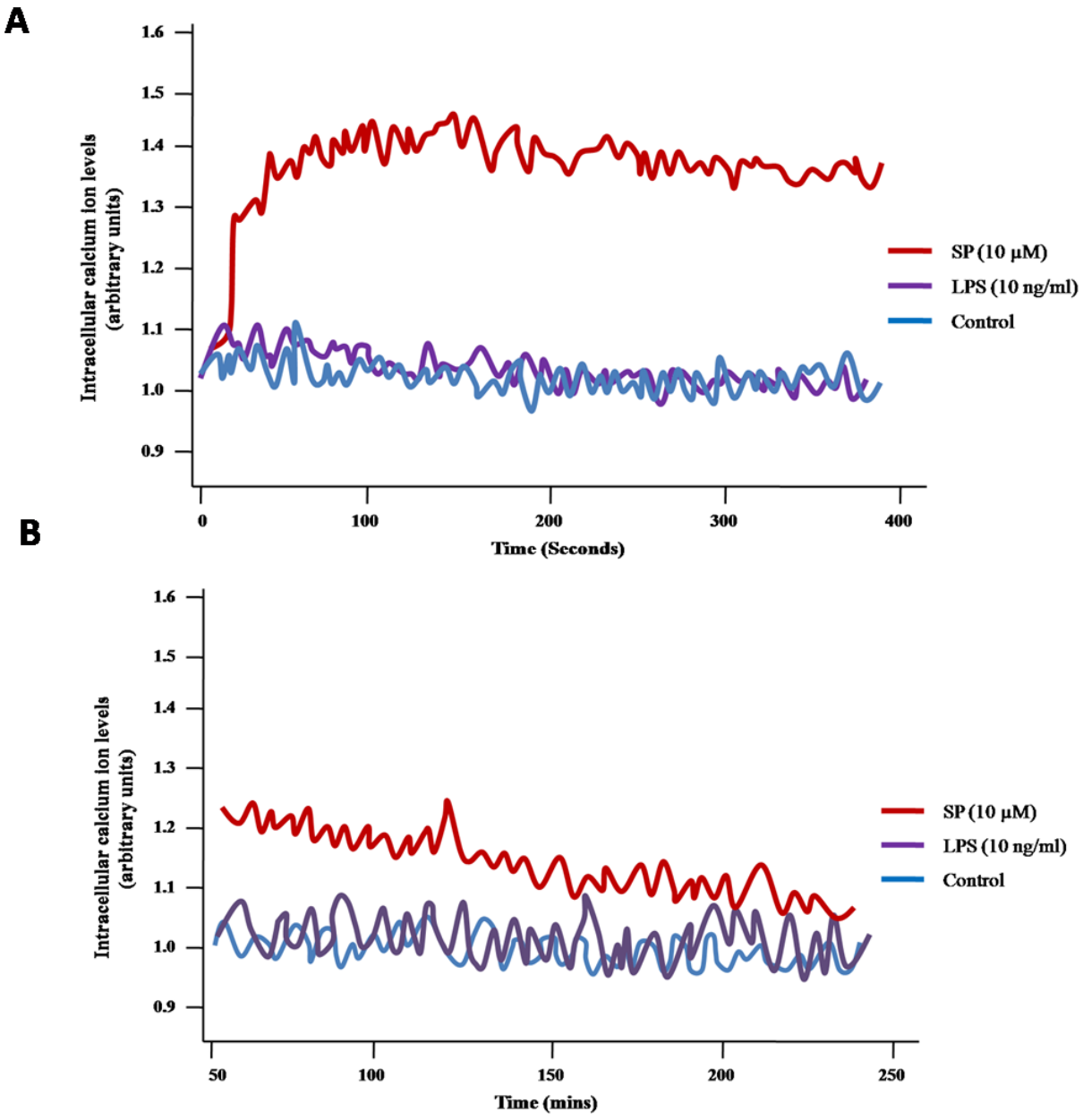

Figure 2.3. SP but not LPS stimulation on LAD2 cell induce cytosolic calcium increase. LAD2 cells were loaded with 5 µM fura-2 AM (Invitrogen, Carlsbad, CA) for 20 min at 37^{o}C, washed and then incubated for another 20 min. Cells were then treated with either SP (10 µM) or LPS (10 ng/ml) or together. Fluorescence signals were

acquired on Flexstation II. The figure is representative of three repeats with similar results.

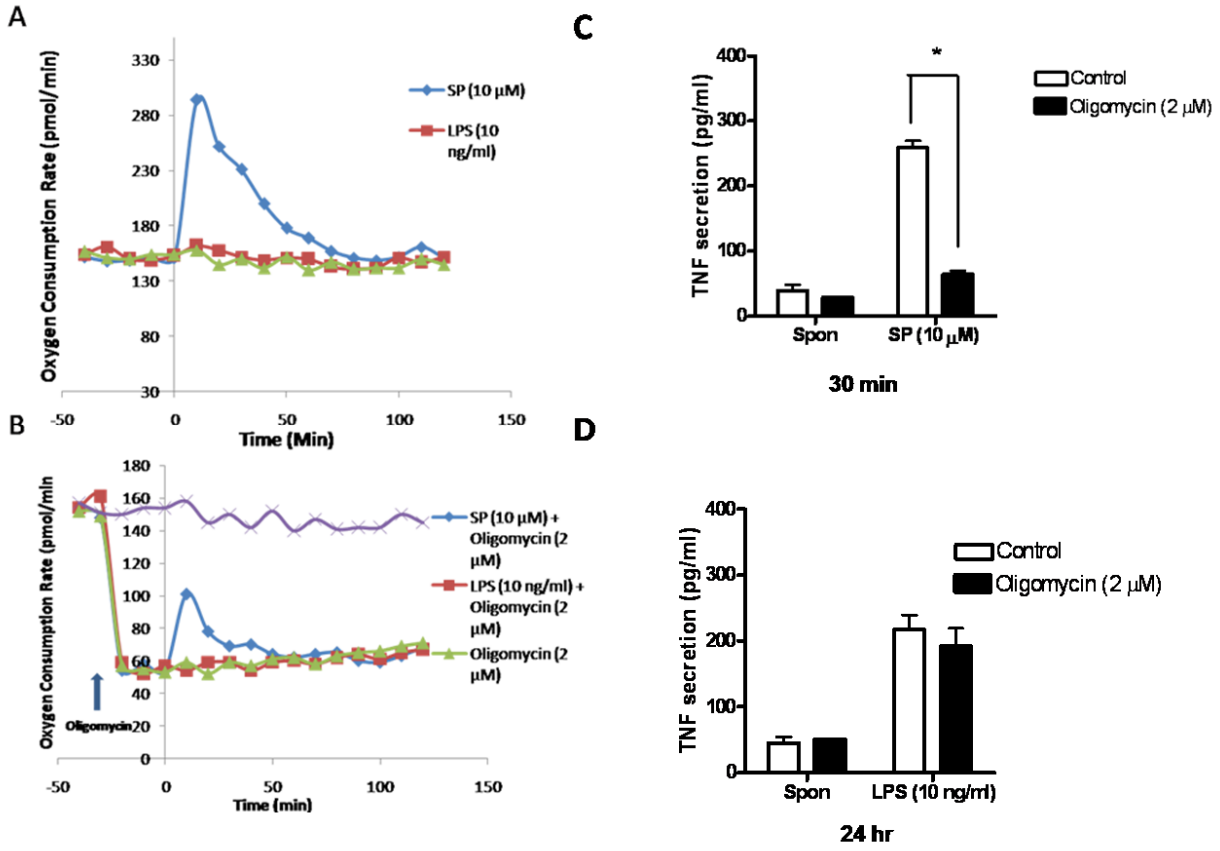

Figure 2.4. **SP but not LPS stimulation on LAD2 cell induces significant mitochondrial energy consumption**. (A, B) Cell oxygen consumption rates were measured by Searhorse XF-24 Flux analyzer (Seahorse Bioscience Inc, North Billerica, MA) in LAD2 cells treated with either SP (10 µM) or LPS (10 ng/ml) . Energy consumption was inhibited (B) by the mitochondrial ATP pump blocker oligomycin. Cells were incubated with oligomycin (2 µM) for 20 min and then treated with SP (10 µM) or LPS (10 ng/ml). Experiments were conducted three times and one representative

experiment is shown. (C) SP but not LPS induced TNF secretion was inhibited by oligomycin. TNF secretion was measured by ELISA as indicated before (n=3; *p<0.05).

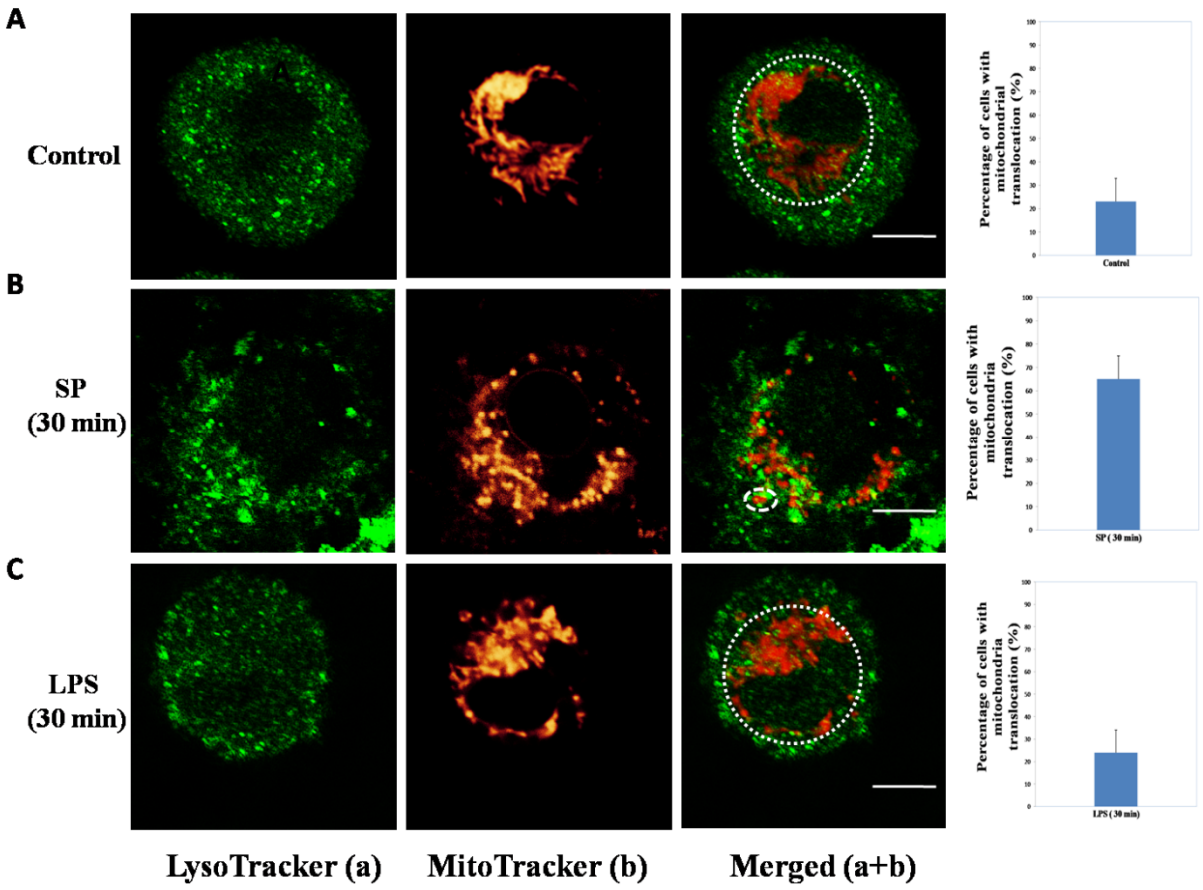

Figure 2.5. Degranulation, but not *de novo* TNF release, is associated with mitochondrial translocation. LAD2 cells were stained with MitoTracker deep red probe (20 nM) for 20 min and LysoTracker DND green probe (50 nM) for 30 min. The cells were harvested in glass bottom culture dishes and observed under the Leica TCS SP2 Confocal microscope. The left panels depict granules in green and the middle panels represent mitochondrial fluorescence. The right panels represent images merged from the

two previous panels. The graphs showed at the far right represent percentages of mast cells with mitochondrial translocation obtained from 100 randomly selected cells. (A) Control groups. (B) SP (10 µM, 30 min). (C) LPS (10 ng/ml, 30 min). The white dashed circles on the right panels show the "mitochondrial pool". Each experiment was repeated three times and was evaluated by three independent operators. Scale bars = 5 µm.

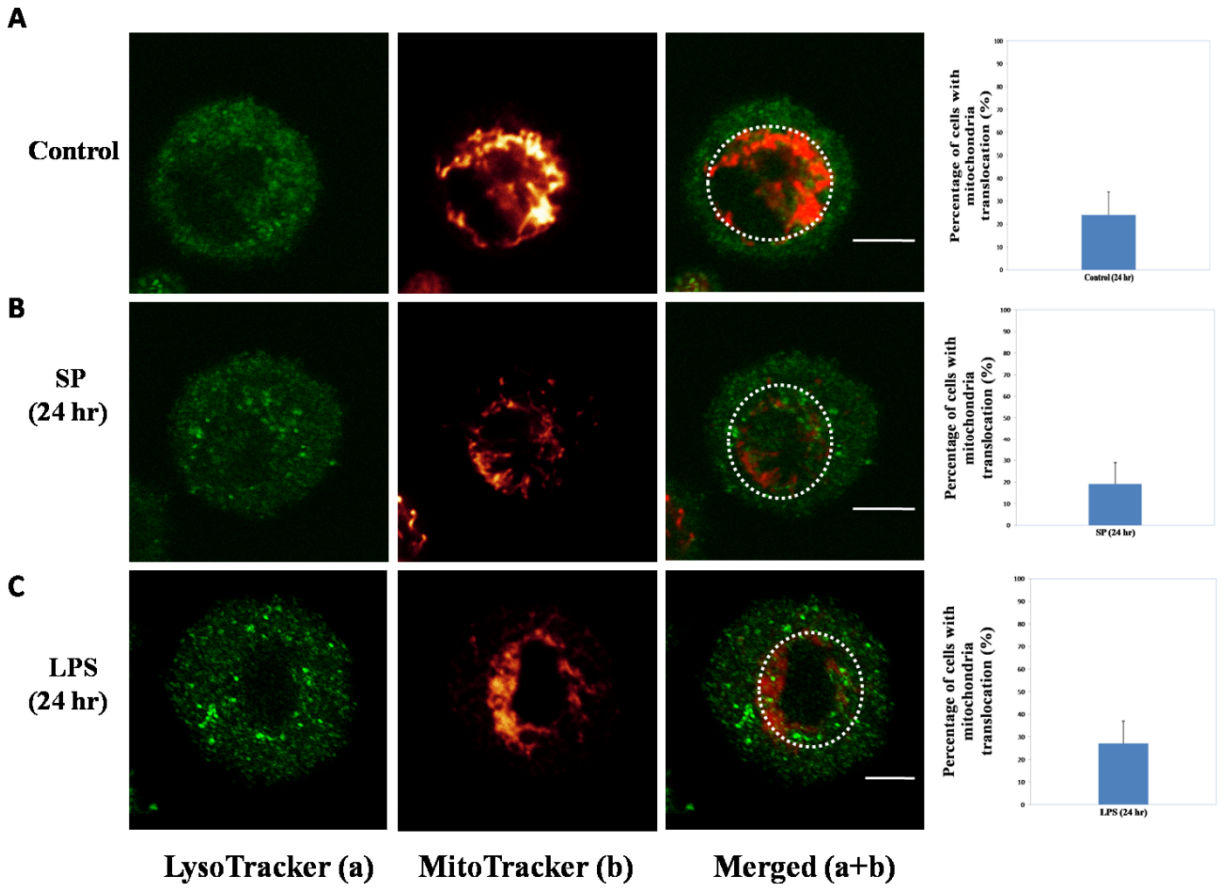

Figure 2.6. Degranulation-associated mitochondrial translocation is reversible. LAD2 cells were stained with MitoTracker deep red probe (20 nM) for 20 min and LysoTracker DND green probe (50 nM) for 30 min. The left panels depict granules in green and the middle panels represent mitochondrial fluorescence. The right panels represent images merged from the two previous panels. The graphs at the far right

represent percentages of mast cells with mitochondrial translocation from 100 randomly selected cells. (A) Control groups. (B) SP (10 μM, 24 hr). (C) LPS (10 ng/ml, 24 hr). Each experiment was repeated three times and were confirmed by three independent operators. Scale bars = 5 μm.

2.4 Discussion

The present findings show that TNF secretion from human mast cells can occur through distinct pathways with different energy and calcium requirements, as well as mitochondrial dynamics. The process of degranulation is much more energy-consuming than *de novo* synthesized TNF release. We used high SP concentration (10 µM) in order to induce maximum degranulation. Such high concentration had also previously been used to induce maximal LAD2 cell activation [240]. Moreover, only SP concentrations higher than 1 µM were reported to be able to enhance the rate of oxygen consumption of isolated cardiac cell mitochondria [252].Delayed (24 hr) TNF release could not be from continuous degranulation because morphological observations of SP-stimulated mast cells showed no degranulation after 24 hr.

One possible explanation for our findings is that mitochondria translocate close to the secretory granules in order to provide energy locally for the granules to fuse with the plasma membrane and undergo exocytosis. However, this is difficult to prove experimentally. Mitochondrial translocation may also be needed to maintain optimal local calcium levels necessary for exocytosis [245], most likely for the calcium dependent proteins involved in degranulation, such as the Soluble NSF Attachment Protein (SNARE) and the Vesicle-Associated Membrane Protein 8 (VAMP-8) [253]. It was previously shown that mitochondrial translocation was necessary to keep calcium channels open at the "immunological synapse" in activated T cells [223].

Unlike degranulation, mast cell selective release of *de novo* synthesized mediators [110] could involve vesicular traffic that requires negligible and hard to measure calcium

and energy levels. For instance, differential release of serotonin without degranulation involved small vesicles shuttling serotonin from secretory granules to the cell surface [254]. Similarly, IL-1 induced small vesicles containing *de novo* synthesized IL-6 [119]. Vesicular secretion may also be involved in the ability of corticotropin-releasing hormone (CRH) to induce selective release of vascular endothelial growth factor (VEGF) without degranulation [44].

The regulatory processes investigated here could help explain how human mast cells may participate in diverse biological processes. Mitochondrial dynamics may be necessary for a rapid mast cell response to an environmental trigger, as opposed to a delayed mediator release in other conditions, such as inflammation [255], innate and acquired immunity [63], or wound healing [243]. An implied importance of our findings could be the possibility of inhibiting mast cell mitochondrial translocation as anti-allergic therapy, while permitting *de novo* synthesis of molecules, such as VEGF that is useful in would healing. Better understanding of the mitochondrial dynamics involved in mast cell activation may permit individualized therapy for allergic and inflammatory disorders.

Chapter III

Human mast cell degranulation and granule-stored TNF secretion require mitochondrial translocation to exocytosis sites- relevance to atopic dermatitis

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BZ performed most of the experiments, analyzed the results and helped wrote the paper. KDA and AA helped to performed the in vitro stimulation experiment and analyzed the results. SA and MV helped prepare the cell cultures and performed computer searches. NS and DAD helped conduct the TNF secretion assay. ZW, AM, MV and BM helped for the western blot assay. AKK and DK provided human atopic dermatitis samples. SL helped designed the study and made critical commnets on the manuscripts.TCT designed the study, organized the collection of human samples, transported the samples and supervised the analysis of the results and wrote the paper. All authors have read and approved the final version of the manuscript.

3.1 Introduction

Mast cells are tissue immune cells deriving from hematopoietic precursors that can secrete pre-stored mediators such as histamine, tryptase and IL-4 through degranulation in response to allergic or neuropeptide triggers.[63, 236] In addition, mast cells uniquely store preformed TNF in their secretory granules,[237, 238] and constitute a major source of rapid (1-30 min) TNF secretion[256]. Stimulation of human mast cells by the pro-inflammatory peptide substance P (SP) induces degranulation and secretion of preformed TNF.[240] The pathophysiology of many inflammatory diseases also involves mast cells,[236] SP [257] and TNF.[258, 259]

In Chapter 2, we showed that mitochondria dynamics participate in mast cell degranulation but not non-granule stored mediator release. Thus, it will be critical to further investigate the underlying molecular mechanism. Regulation of mast cell stimulation following aggregation of the high affinity surface IgE receptor (FcεRI) has been studied extensively.[5, 6, 260] Activation by SP, however, is less well-understood and may involve either the full NK1 receptor, [261] or its truncated form [262] or the MRGX receptor. [263] However, the regulation of the steps involved in degranulation following stimulation regardless of triggers are still largely unknown. Mast cell degranulation requires intracellular calcium and metabolic energy.[264] Mitochondria are the primary energy-generating organelles in eukaryotic cells,[246] and also regulate intracellular calcium.[224] Mitochondria can accumulate in subcellular regions requiring high metabolic activity, such as growth cones of developing neurons,[265] or dendritic protrusions in spines and synapses.[266] Mitochondrial dynamics enable them to participate in various complex

cell behaviors, such as neuronal development [266] and insulin secretion.[267] In particular, translocation of mitochondria is required for T cell chemotaxis [226] and T cell activation by antigens.[223]

The intracellular pathways that participate in mitochondrial dynamics have been studied in several different biological systems [246]. In human cells, mitochondrial shape results from a regulated balance between fusion and fission events, tightly controlled by a growing family of proteins that include the dynamin-like GTPases Opa1 and mitofusin (Mfn) 1 and 2, which promote fusion, the cytosolic GTPase dynamin-related protein 1 (Drp1) and the outer mitochondrial membrane adaptor hFis1.

Drp1 is one of the most important regulators of both mitochondrial fission and translocation.[225, 226] Drp1 is found in the cytoplasm in an inactive form.[227] Multiple modifications contribute to localization and proper function of Drp1. Dephosphorylation at Ser-637 by calcium-activated calcineurin is critical to recruit Drp1 from the cytoplasm to the mitochondrial outer membrane. [228] Then, phosphorylation at Ser-616 controls its enzyme activity responsible for mitochondrial fission.[229] Mitochondrial dynamics and the function of Drp1 have not previously been studied in mast cell degranulation or in inflammatory diseases.

Here we show that human mast cell degranulation and secretion of preformed TNF require mitochondrial translocation to sites of exocytosis. This process is regulated by calcium-dependent calcineurin and Drp1 activation. We also show that calcineurin and Drp1 gene expression is increased in activated mast cells and in affected skin from patients with AD.

3.2 Materials and Methods:

Culture of Human Mast Cells

Human umbilical cord blood was collected following normal uncomplicated deliveries under Tufts Medical Center Boston, MA human Institutional Review Board (IRB) approval. Hematopoietic stem cells (CD34+) were isolated by positive selection of CD34+/AC133+ cells with magnetic cell sorting using an AC133+ cell isolation kit (Milltenyi Biotec, Auburn, CA, USA) as previously reported.[268] $CD34^+$ cells were suspended in AIM-V Medium (Gibco-BRL, Carlsbad, CA, USA), supplemented with 100 ng/ml recombinant human stem cell factor (rhSCF; kindly supplied by Biovitrum, Stockholm, Sweden) and 50 ng/ml IL-6 (Millipore, Billerica, MA, USA) for 12 to 16 weeks. The purity of hCBMCs was evaluated by immunocytochemical staining for tryptase as previously described.[268] Mast cells (100% purity) cultured over 12 weeks were used for the experiments. LAD2 cells (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, NIH) deriving from a human mast cell leukemia [239] were cultured in StemPro®-34 SFM medium (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL rhSCF.

Confocal Microscopy

HCBMCs and LAD2 mast cells were incubated with 20 nM MitoTracker deep red probe (Invitrogen, CA) for 20 min and 50 nM LysoTracker DND (Invitrogen) for 30 min. Cells were washed, moved to glass bottom culture dishes (MatTek, Ahsland, MA, USA) and observed using a Leica TCS SP2 Confocal microscope (Leica, Japan). Stimulation of the

hCBMCs was achieved as follows: 12–14-week-old hCBMCs were washed once in both Dulbecco's phosphate-buffered saline (DPBS) and human Tyrode buffer and resuspended in fresh medium containing rhSCF (100 ng/ml) alone, which was included in the stimulation medium in all experiments for optimal mast cell viability. HCBMCs were resuspended (10^6cells/ml) and passively sensitized by incubation with biotinylated human myeloma IgE (1 and 2 μg/ml/ 10^6 cells) (Chemicon, Billerica, MA, USA) for 48 h at 37ºC. Cells were then washed, resuspended in medium containing rhSCF alone and distributed to 96-well plates ($1x10^5$ cells per 200 μl) for stimulation with streptavidin (125 ng/ml; DAKO, Carpinteria, CA, USA) at 37ºC in 5% CO_2 for 30 min. LAD2 cells were stimulated with SP (2 μM for 30 min at 37ºC; Sigma, St Louis, MO, USA). The percentage of cells undergoing mitochondrial translocation was calculated following examination of 100 randomly selected mast cells in each experiment. Confocal digital images were processed using the NIH ImageJ 1.32 "mitochondria calculator" plug-in[269] and Adobe Photoshop 7.0 programs. The Z-stack mitochondrial density projection was done using ImageJ 1.32 from 30 different layers of the same cell.

β -Hexosaminidase Assay

β-hexosaminidase (beta-hex) release, as an index of mast cell degranulation, was assayed using a fluorometric assay. Briefly, hCBMCs and LAD2 cells ($0.5x10^5$/tube) were stimulated, supernatant fluids were collected and cell pellets were lysed with 1% Triton X-100. Supernatants and cell lysates were incubated in reaction buffer (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma) for 1h and then 0.2 M glycine was added to stop

the reaction. Absorbance was measured at 405 nm in an enzyme-linked immunosorbent assay reader, and the results are expressed as the percentage of β-hexosaminidase released over the total (n=3; *$p<0.05$ compared with control). MDIVI-1 (kindly supplied by Dr. O. Shrihai, Boston University, Boston, MA) was dissolved in DMSO (Sigma), which had no effect on mast cell activation or viability at the final concentration of 0.1%.

TNF Release Assay

LAD2 cells were treated with SP (1 or 2 μM, for 30 min) or pretreated with MDIVI-1 for 30 min prior to stimulation with SP. TNF release was measured by ELISA (R&D systems, Minneapolis, MN, USA) in the supernatant fluid or in lysates of unstimulated LAD2 cells.

Western Blot Analysis of total and phosphorylated Drp1

LAD2 cells were stimulated with SP (2 μM) for 30 min. The reaction was stopped by addition of ice-cold PBS. Cells were washed once with PBS and then lysed in buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.l% SDS, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM Na_3VO_4, and 50 mM NaF. Equal amounts of protein were electrophoresed on 4–12% polyacrylamide gels and then transferred to a 0.44-μm Polyvinylidene fluoride membrane (Invitrogen). After blocking with 5% BSA, membranes were probed with antibody against Drp1 or the Ser-616 phosphorylated form of Drp1 (Cell Signaling, Boston, MA, USA) at 1:1,000 dilution. B-actin was used as an internal control (Cell Signaling). For detection, the membranes were incubated with the appropriate secondary antibody conjugated to

horseradish peroxidase (Cell Signaling) at 1:1,000 dilution and the blots were visualized with enhanced chemiluminescence. Scanned densitometry and protein density calculation was performed by ImageJ.

Cytosolic Calcium Measurements

Cytosolic calcium was measured in LAD2 cells at 37 °C using Fura-2 (Invitrogen, CA, USA) as indicator. LAD2 cells suspended in Tyrode's buffer were loaded with 30 nM Fura-2 AM for 20 min to allow Fura-2 to enter the cells. After centrifugation to remove excess dye, cells were resuspended in Tyrode's buffer at a concentration of 10^6/ml and incubated for another 20 min. Cells were then transferred to 96-well plates (100 μl/well) and SP (2 μM) was added. Real-time Fura-2 fluorescence was read by MDC FlexStation II (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 340 nm/380 nm and emission wavelength of 510 nm. Results were analyzed according to the Invitrogen Fura-2 protocol and reported as the relative value of OD 340/380 nm as described previously.[217]

Drp1 siRNA Transfection

LAD2 cells were transfected by specific siRNA for Drp1 using the siRNA transfection kit (Santa Cruz, Santa Cruz, CA, USA). LAD2 cells were incubated with either pre-designed specific or scrambled (control) siRNA (sc-43732, Santa Cruz) in transfection medium for 5 h according to the protocol. Then, cells were resuspended in complete medium for 48h. Drp1 knock-down efficacy was tested by real-time PCR and Western blot analysis.

PCR and quantitative PCR

Total RNA from cultured mast cells and human skin biopsies was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and Trizol reagent (Invitrogen,) respectively, according to the manufacturer's instructions. Reverse transcription was performed with 300 ng of total RNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA, USA). In order to measure calcineurin, Drp1 and SP expression, quantitative real time PCR was performed using Taqman gene expression assays (Applied Biosystems, Carlsbad, CA, USA). The following probes obtained from Applied Biosystems were used: Hs 00174223_m1 PPP3CA for calcineurin, Hs 00247152_m1 DNM1L for Drp1 and Hs 00243225_m1 TAC1 for SP. Samples were run at 45 cycles using Applied Biosystems 7300 Real-Time PCR System. Relative mRNA abundance was determined from standard curves run with each experiment; calcineurin, Drp1 and TAC1 (used to identify SP) gene expressions were normalized to GAPDH (Hu, VIC TAMRA) endogenous control.

Patients and Biopsies

Two full depth punch skin biopsies (4 mm^3) were collected from patients (n=10; 6 females and 4 males; mean age 32.9±21.1 years) and controls (n=10; 6 females and 4 males; mean age 39.8±17.2 years) who had not received any medication for 15 days prior to the biopsy and were seen at the 1st and 2nd Departments of Dermatology of the University of Athens Medical School, Athens, Greece. The Scientific Affairs Committee and the Institution IRB approved this protocol. All participants gave their written

informed consent according to the Declaration of Helsinki Principles. One set of biopsies was immediately placed in RNase later solution (Ambion, Inc., Austin, TX, USA) and stored at -20°C, while the other was preserved for TEM as described below.

Transmission Electron Microscopy (TEM)

Human skin biopsies from control and AD patients were fixed in modified Karnovsky's fixative containing 0.2% paraformaldehyde, and 3% glutaraldehyde, and 0.5% tannic acid in 0.1 mM Na-cacodylate buffer. Sections (5 μm) were cut using a microtome and observed using a Philips-300 TEM. High quality glossy photographs of individual mast cells were evaluated by three independent operators for the number of mitochondria and their distance from the cell surface.

Data Analysis

Image analysis was performed blindly to the treatment conditions. For each experimental condition, Confocal cell images were randomly taken from different wells of the microscope plate and ImageJ software was used for Confocal image processing. TEM photomicrographs of mast cells were evaluated for mitochondria by three independent operators. All data are expressed as mean ± SD. Statistical significance was determined by the Student's *t*-test or the non-parametric Mann-Whitney *U* test for the patient samples, using the SigmaPlot 9.0 (SPSS, Chicago, IL, USA). Significant differences were considered if $p<0.05$.

3.3 Results

Mitochondrial translocation in degranulating mast cells

Observation with Confocal microscopy of unstimulated hCBMCs (Fig. 1A, upper panels), as well as those treated only with IgE for 30 min (Fig. 3.1A, middle panels), after staining with LysoTracker green (to identify secretory granules) and MitoTracker red probe (to identify the mitochondria), reveals that the overwhelming majority of mitochondria are connected in a net located in the perinuclear region (within the dash circles). LysoTracker identifies secretory granules because their pH is 5.5, similar to that of lysosomes[250]. In contrast, hCBMCs stimulated by IgE and streptavidin, show rapid (30 min) degranulation (Fig. 3.1A7) and mitochondrial translocation (Fig. 3.1A8). The merged images (Fig. 1A9) clearly show the presence of mitochondrial-associated fluorescence near the cell surface of a degranulating mast cell (arrows). Degranulation was also confirmed by β-hex release (Fig 3.1B). Similar results were obtained with human LAD2 mast cells stimulated by SP (2 μM, for 30 min) (Fig 3.2A and 2B). Intererstingly, the results of mitochondrial translocation from IgE-Streptavidin is consistent with the results obtained with SP and LPS in chapter 2: mitochondrial translocation is only associated with mast cell degranulation. Neither IgE nor LPS, the non-degranulation mast cell triggers can induce mitochondrial translocation.

This time, we also utilized light microscopy to visualize degranulation: we alternated objectives at the Confocal microscope and obtained images of mast cells using Differential Interference Contrast (DIC), clearly showing degranulation (Fig. 3.2A, middle lower panel). Just like stimulation with IgE and streptavidin, mast cell stimulation by SP (2 μM) at 37ºC results in rapid cell degranulation (noticeable at 30 min), while

mast cells stained with LysoTracker following stimulation with SP (2 μM) show extensive degranulation with numerous granule core heparin particles outside the domain of the cells that stained with LysoTracker (Fig. 3.3A5). There are also concomitant mitochondrial morphological changes, consistent with translocation throughout the cells (Fig. 3.3A6). Merging of LysoTracker and MitoTracker images shows mitochondria close to cell surface in the mast cell undergoing degranulation (Fig. 3.3A7). Secretion is also confirmed with β-hex (Fig. 2B) and TNF (Fig. 3.2C) release.

Cell viability measured in LAD2 cells stimulated by SP (1, 5 or 10 μM) for 30 min is >90%, indicating that mitochondrial translocation is not the result of apoptosis or necrosis. Although SP at 10 μM can induce maximum degranulation, concentration at 2 μM is already enough to trigger observable effects both on degranulation and mitochondrial translocation. Thus, all experiments conducted below are under SP 2 μM condition. TNF secretion during 30 min following stimulation with SP is preformed and derives from secretory granules because it is well-known that *de novo* TNF synthesis requires 6-24 h. Moreover, lysed, unstimulated LAD2 cells contain substantial amount of preformed TNF as shown by ELISA (Fig. 3.4).

The number of mitochondria located close to the cell surface area is further calculated using the ImageJ Software "mitochondria calculator" plug-in as reported previously[269] (Fig. 3.3A4, 3A8). Mitochondrial translocation was observed in approximately 60% of LAD2 cells stimulated with SP (2 μM) as compared to 20% of control cells (Fig. 3.3B). The cells undergoing degranulation are the ones presenting with mitochondrial translocation according to our observations in the experiments described above. The number of mitochondria close to the cell surface is significantly increased

after SP stimulation (2μM) compared to the control cells (Fig. 3.3C). Human cultured mast cells do not attach to the culture flask; in order to minimize the effects of multiple layers, images shown were chosen to represent the layer with the maximum nuclear diameter.

To further confirm that our results are not distorted by multilayer effects, we used stacked images (30 per cell) from a single mast cell, taken at 0.3 μm spacing. Mitochondrial fluorescence density from all images is then projected onto the Z stack for distribution analysis. Before stimulation, high mitochondrial density is visualized only around the nuclear region (dash circle) (Fig. 3.5A). After SP stimulation (2 μM), mitochondrial fluorescence is detected throughout the cell (Fig. 3.5B), and especially close to the cell surface, occupying 80% of the total cell area (Fig. 3.5C).

Mitochondrial translocation is present in degranulated human skin mast cells from AD skin biopsies

Images of skin mast cells from control and AD patients were obtained and examined using TEM as described previously.[181] Mitochondria of control human skin mast cells are clustered around the cell nucleus and appear large and intact (Fig. 3.6A). In contrast, mitochondria of degranulated mast cells from AD patients are much smaller in size and are located close to secretory granules undergoing exocytosis (Fig. 3.6B). Analysis of the number of mitochondria and their distance from the cell surface shows that the average number of mitochondria (Fig. 3.6C) and the percentage of those close to the cell surface (Fig. 3.6D) are both significantly higher in degranulated mast cells compared to controls.

Mast cell degranulation and preformed TNF secretion require Drp1-dependent mitochondrial translocation

In order to address the possibility that mitochondrial translocation to the cell surface may simply be an effect secondary to cell shape changes during degranulation, we investigated the role of Drp1, a protein necessary for mitochondrial fission, in mast cell degranulation. Pretreatment of LAD2 mast cells for 30 min with the Drp1 inhibitor MDIVI-1 (40 μM at 37 ºC) [270] inhibits mitochondrial translocation in response to SP (2 μM for 30 min) (Fig. 3.7A) by 60% (Fig. 3.7B). Moreover, pretreatment of LAD2 cells with MDIVI-1 significantly inhibits SP-triggered preformed TNF secretion by 75% (Fig. 3.7C) and β-hex release by 55% (Fig. 3.7D) , compared to cells only stimulated by SP. In order to minimize the "off-target" effects of the small molecule inhibitor MDIVI-1, Drp1 mRNA was also reduced in LAD2 cells by transfecting cells with specific Drp1 siRNA. After siRNA treatment for 6h, Drp1 mRNA levels in LAD2 cells drop to 30% of normal, as measured by real-time PCR (Fig. 3.8A), while Drp1 protein levels measured by Western blot analysis are reduced to 20% of normal, 48h after siRNA treatment (Fig.3.8B). At 48h after siRNA treatment, LAD2 cells were stimulated by SP (2 μM) and β-hex secretion was 60% less, compared to siRNA-untreated, SP-stimulated cells (Fig. 3.7E). The lack of complete inhibition in these experiments may be because Drp1 expression and function were not blocked 100%.

Mitochondrial translocation and Drp1 function are dependent on extracellular calcium influx

Absence of extracellular calcium prevents SP-triggered intracellular calcium increase (Fig. 3.9A) and consequent degranulation (Fig. 3.9B). Instead, LAD2 cells stimulated by the calcium ionophore A23187 show mitochondrial translocation (Fig. 3.10), suggesting that an increase in calcium alone is sufficient to induce mitochondrial translocation. Intracellular calcium increase is, therefore, critical for mast cell degranulation and mitochondrial translocation. Consequently, we investigated the effect of intracellular calcium on Drp1 activation and Drp1-dependent mitochondrial translocation. We show that LAD2 cells stimulated by SP (2 μM) for 6h also produce significant Drp1 mRNA increase (Fig. 3.11A). Drp1 dephosphorylation at Ser-637 is regulated by calcineurin [271] and is required for Drp1 translocation to the mitochondrial outer membrane. We also show that 6h after SP stimulation, there is significant increase of calcineurin activity in LAD2 cells (Fig. 3.11B), an effect diminished in the absence of extracellular calcium (Fig. 3.11B). Drp1 requires phosphorylation at Ser-616 in order to be activated and promote mitochondrial fission. We examined Drp1 activation after SP stimulation (2 μM) using Western blot analysis. Upon stimulation with SP (2 μM), increased Drp1 phosphorylation is detected within 30 min using Ser-616 phospho-specific antibodies (Fig. 3.9C) and is statistically significant compared to controls (Fig. 3.9D). However, this effect is not observed in cells cultured in extracellular calcium-free medium (Fig. 3.9C), indicating that calcium influx is required for measurable Drp1 activation. We did not perform the effects of total Drp1 level after SP treatment in 30 min. However, according to the previous siRNA gene-knock assay, degrading existing Drp1 took up to 48 hr. Drp1 is not a fast turn-over proteins and there should be no effects of SP on total Drp1 level.

Calcineurin, Drp1 and SP gene expression in AD

Analysis of skin biopsies from AD and normal control subjects reveals that gene expression of Drp1 (Fig. 3.12A) is significantly increased in AD patients (n=10) as compared to controls (n=10). The expression of calcineurin is also significantly increased (Fig. 3.12B) in AD patients (n=9), as compared to controls (n=10).

Given that SP stimulates human mast cells, we investigated whether SP is expressed in lesional skin from AD patients. Gene expression of SP is also significantly increased in affected skin biopsies from AD patients (n=10) as compared to controls (n=10) (Fig. 3.13).

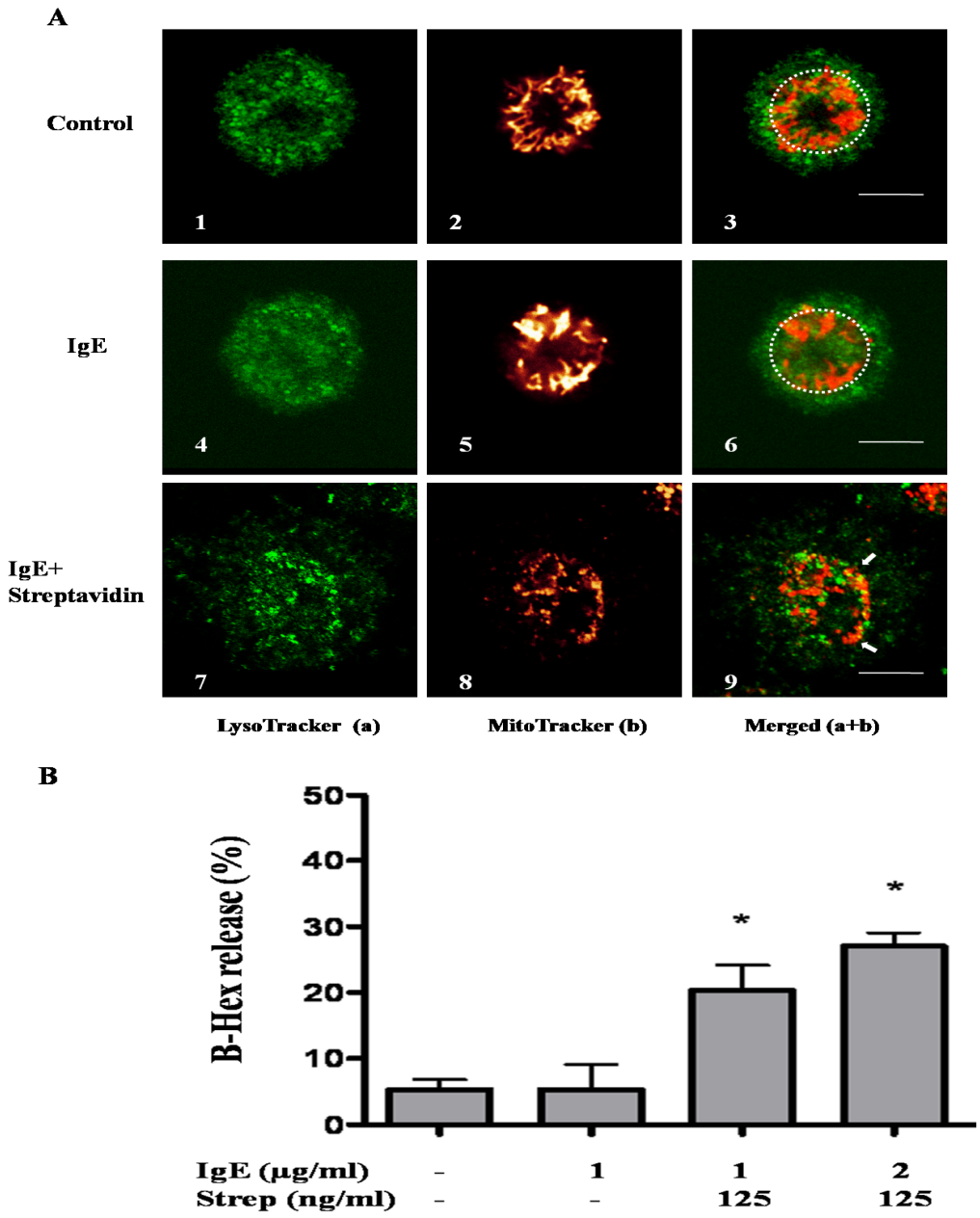


Figure 3.1. IgE+ Streptavidin triggerd hCBMCs mitochondrial translocation and degranulation. (A) Mitochondrial distribution in resting (upper panels), IgE-incubated (middle panels) and IgE+streptavidin-stimulated (bottom panels) cells. (B) β-Hex release from hCBMCs treated with IgE or IgE+Streptavidin for 30 min. (n=3; *$p<0.05$, compared to control). Bars equal to 5 µm

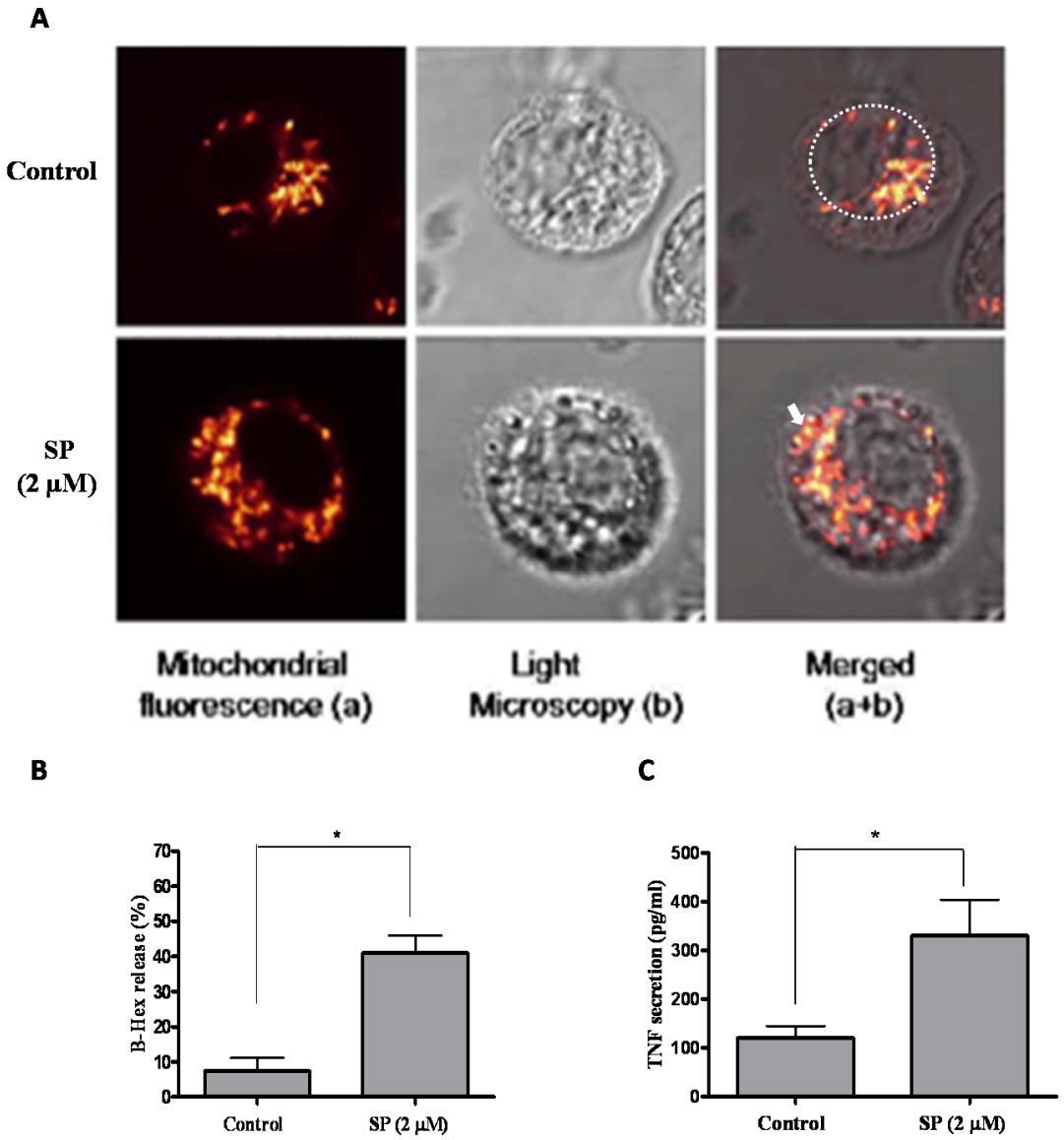


Figure 3.2. SP induced LAD2 mast cells mitochondrial translocation observed by DIC, and degranulation and TNF secretion. (A) Mitochondrial distribution of control (upper panels) and degranulated (bottom panels) mast cells stimulated with SP (2 µM) for 30 min. Mast cell degranulation by SP was confirmed by β-hex release (B) and TNF secretion (C). (n=3, *: $p<0.05$ compared to control).

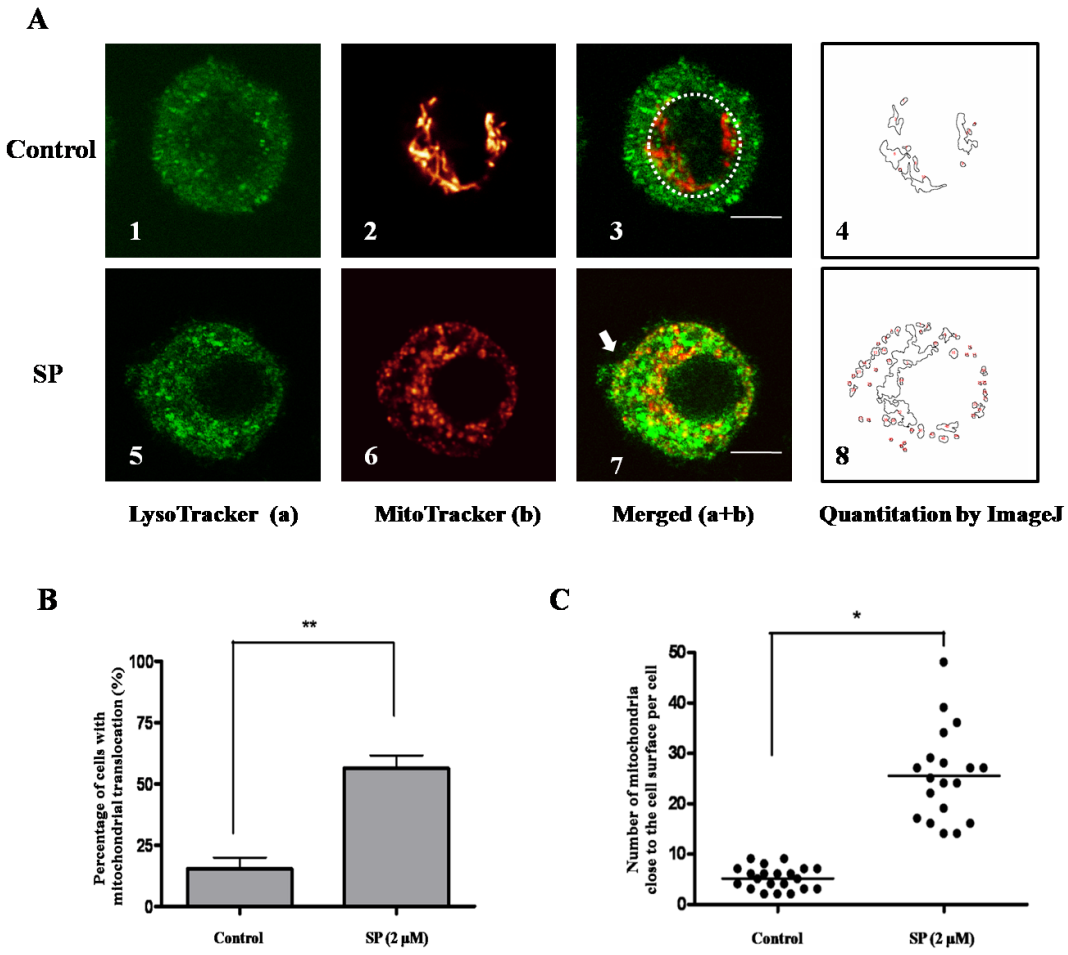


Figure 3.3. Mitochondrial experienced fission and translocation during human LAD2 cells degranulation. (A) Control (n=19, upper panels) and degranulated (n=20, bottom panels) mast cells (SP 2 µM, 30 min). (B) Percentage of translocated mitochondria in resting and stimulated cells (n=3). (C) Number of mitochondria per cell within 1 µm from the cell surface. Cells were randomly selected for analysis (*$p<0.05$, **$p<0.01$). Bars equal to 5 µm.

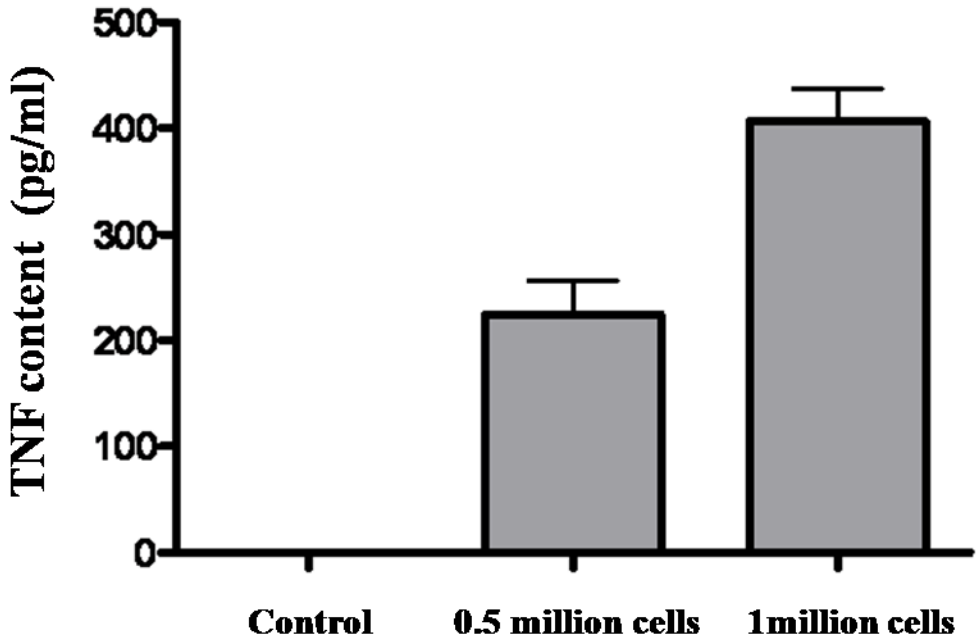


Figure 3.4, Preformed TNF in unstimulated LAD2 mast cells was detected by ELISA.

Unstimulated LAD2 cells were lysed and sonicated to release all intracellular components. TNF concentration was determined by ELISA. (n=3)

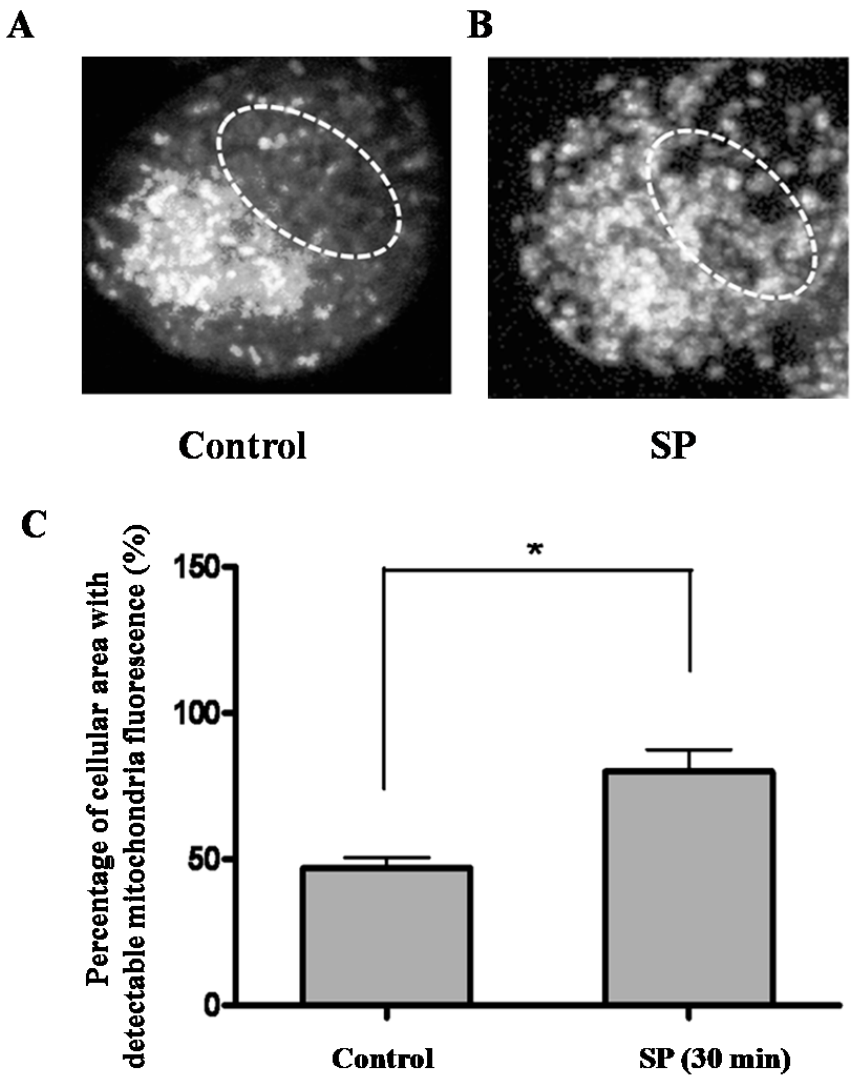


Figure 3.5. Z-stack mitochondrial fluorescence projection showing mitochondrial translocation to the cell surface in human LAD2 mast cells after SP stimulation.

Images (30) of different layers from the same cell are projected to the Z-stack. Representative images (of more than 20 cells studied) of control and a degranulated LAD2 cell (SP 2 µM, 30 min) are shown on the left and right panels, respectively. White dashed lines represent the nuclear region identified from corresponding light microscopic images. Magnification bar: 1000x. (C) The percentage of the cellular area with

detectable mitochondrial fluorescence indicating mitochondrial distribution was calculated from 20 cells (n=3; *p<0.05 compared to control).

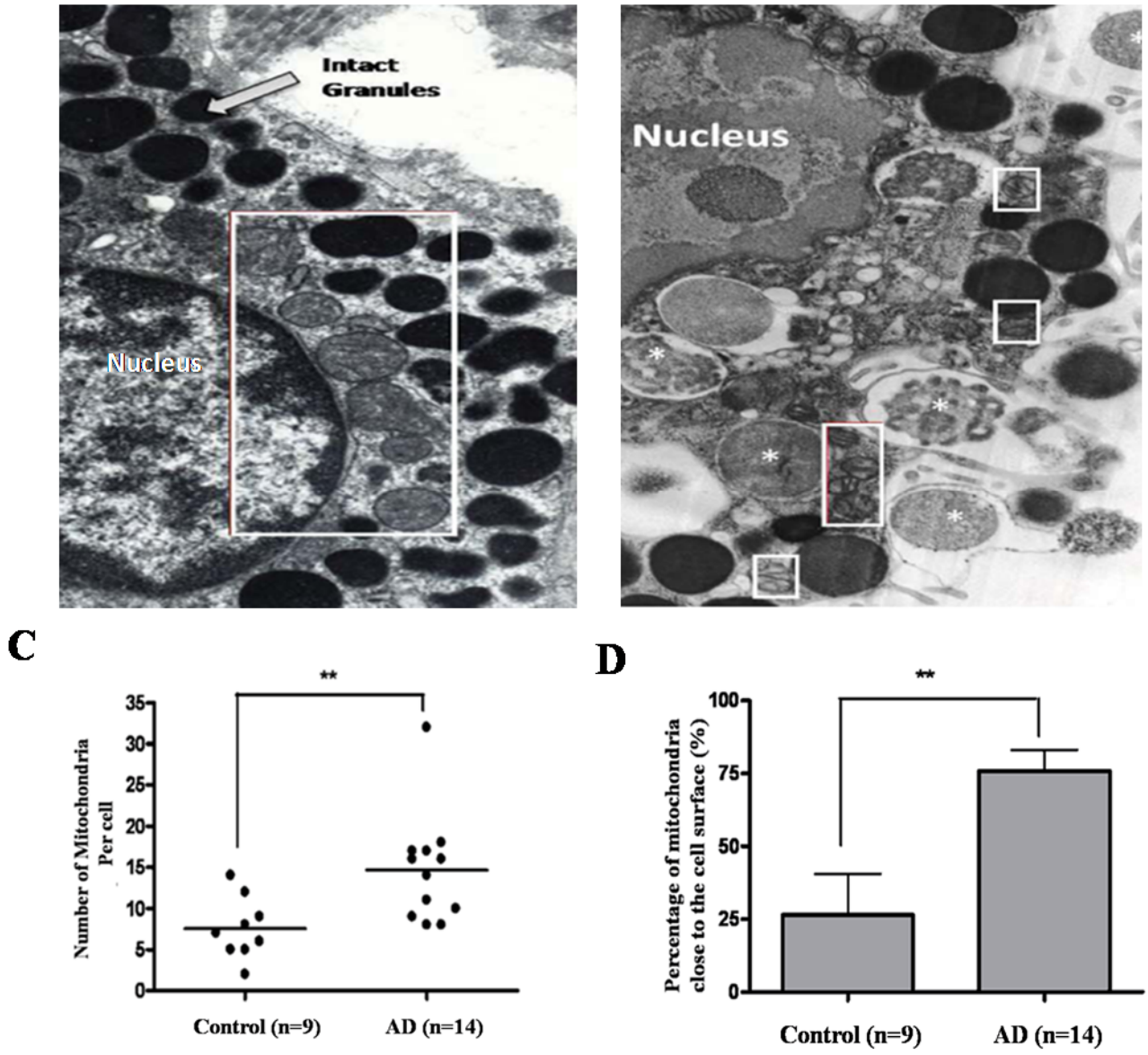


Figure 3.6. Electron photomicrographs showing mitochondria translocated to the cell surface in human skin mast cells.

Human skin mast cells from (A) normal control and (B) lesional skin from AD patients (Magnification: 13,800x). Mitochondria are shown within white rectangles. White asterisks represent secreted granular material. (C) Number of mitochondria and (D) percentage of mitochondria close to the cell surface in each cell were semi-quantitated from high magnification (11-16,000x) glossy prints by three independent evaluators. ; (n=11; *p<0.05, **p<0.01 compared to control).

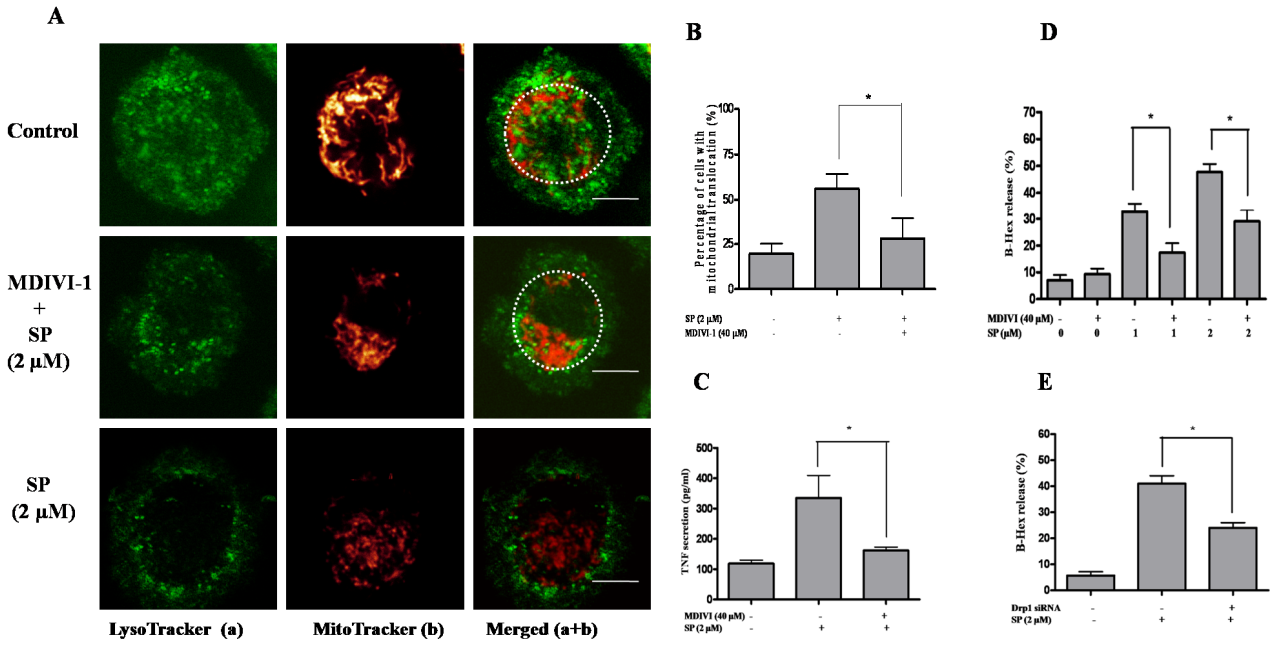


Figure 3.7. The mitochondrial fission blocker MDIVI-1 and Drp1 siRNA inhibit mast cell degranulation and granule-stored TNF secretion.

LAD2 cells were treated with MDIVI-1 (40 µM) for 15 min at 37ºC. (A) Mitochondrial morphology before or after stimulation with SP (2 µM, 30 min, 37ºC). Left panels represent granules, middle panels show mitochondria and the right panels represent

images merged from the two previous panels. The images shown are representative of more than 40 cells studied. (B) β-hexosaminidase (β-Hex) and (C) TNF secretion. (D) β-Hex release from LAD2 cells treated with Drp1 siRNA before stimulation by SP (2 μM) for 30 min (n=3; *p<0.05, compared to control).

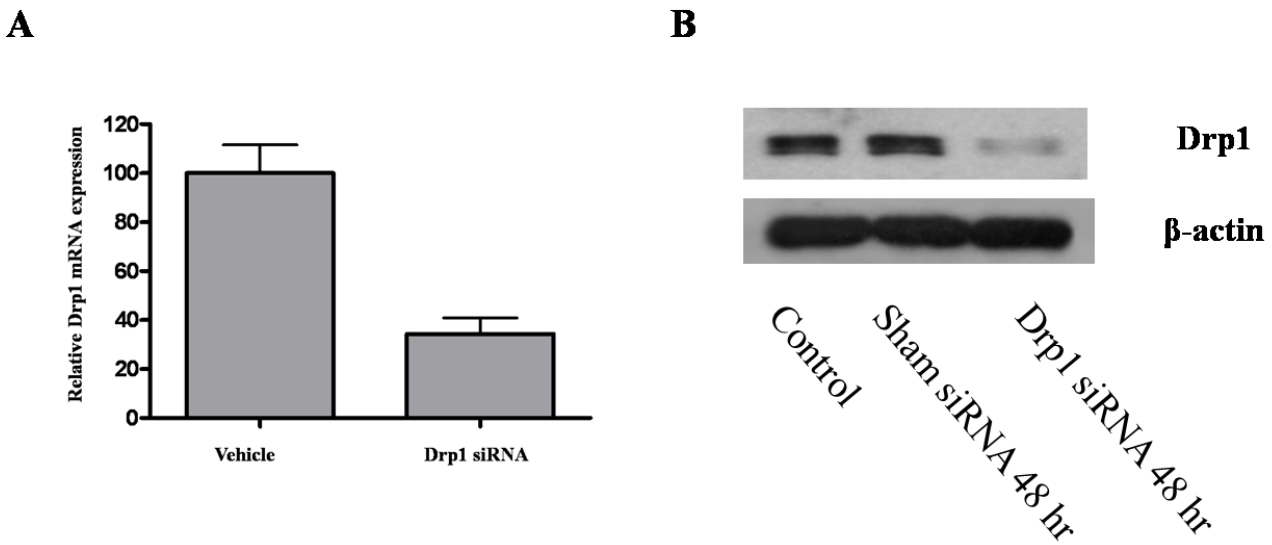


Figure 3.8. S**iRNA treatment of LAD2 cells decreased both Drp1 (A) mRNA and (B) protein level.**

LAD2 cells were transfected with Drp1 siRNA for 5 h and incubated in growth medium for 6 h. (A) Relative quantities of mRNA expression were measured by quantitative RT-PCR and normalized to GAPDH. TaqMan PCR was performed with cDNA reverse transcribed from 300 ng RNA from each sample. Experiment was repeated three times. (B) Western Blot analysis of the Drp1 protein 48 h after siRNA treatment. The picture was representive for 3 repeats that show similar results.

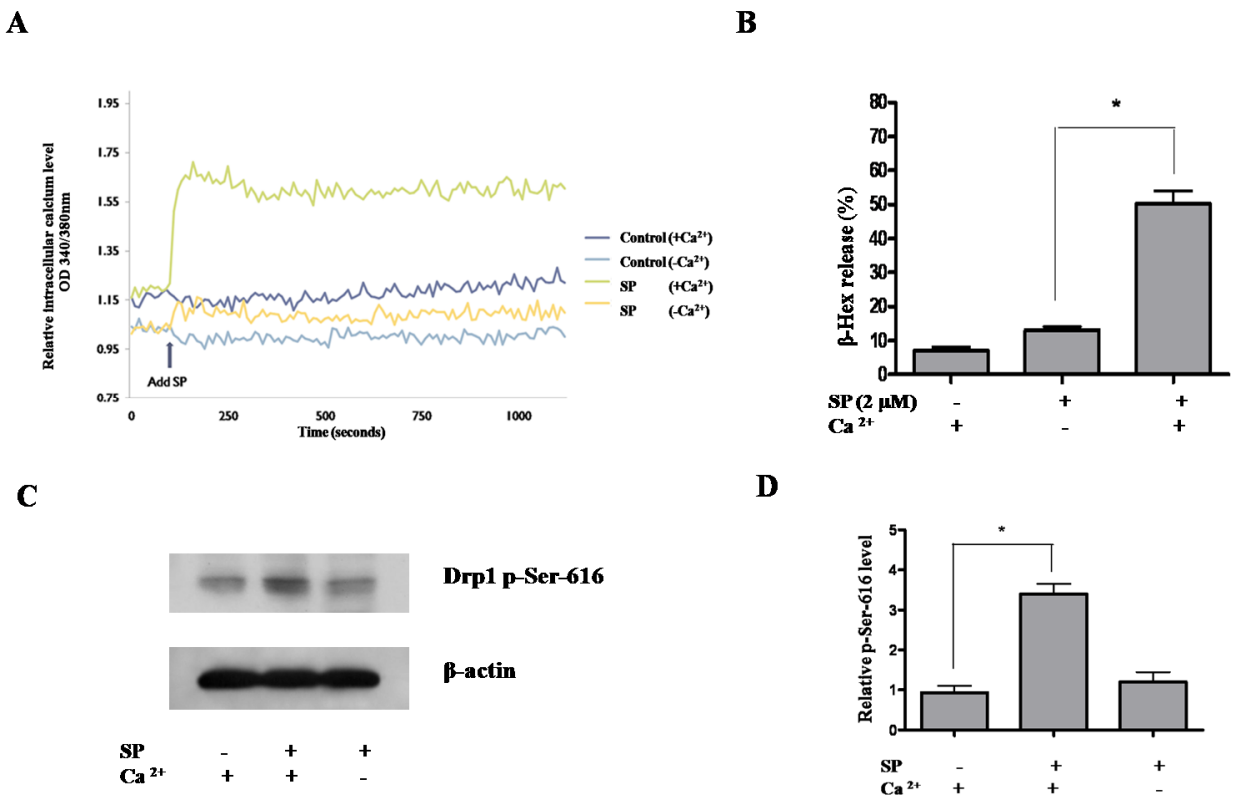


Figure 3.9. Intracellular calcium increase and Drp1 phosphorylation at Ser-616 during human LAD2 mast cell degranulation.

(A)After SP (2 μM) 30 min stimulation for LAD2 cell intracellular calcium level was measured by Fura-2 and is reported as OD 340/380nm. (B) LAD2 cell degranulation with or without extracellular calcium, as measured by β-Hex release. (C) Western blot of Drp1 phosphorylation at Ser-616 after SP (2 μM) stimulation either with or without extracellular calcium. (D) Drp1 protein density was normalized against control B-actin. (n=3; *p<0.05 compared to control).

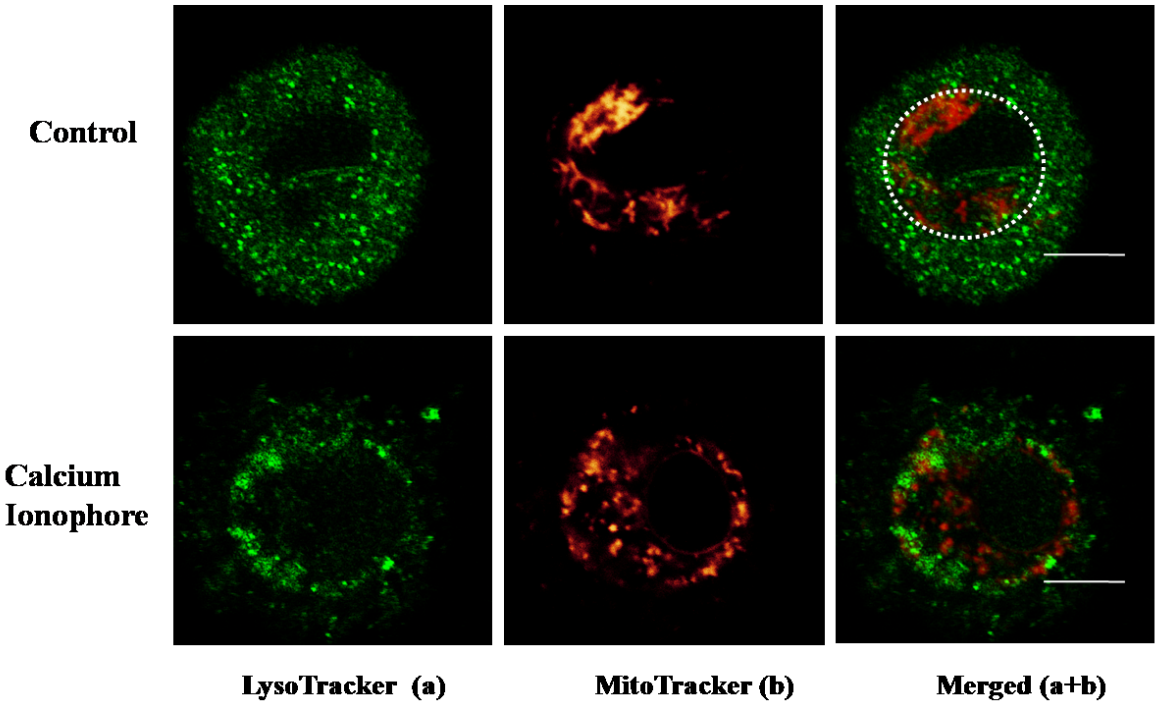


Figure 3.10. **Calcium ionophore A23187 induced LAD2 mitochondrial translocation.** LAD2 cells were either unstimulated (upper panels) or stimulated with calcium ionophore A23187 (1 μM) for 30 min (upper panels).

The left panels show secretory granules in green, the middle panels represent red mitochondrial fluorescence and the right panels are merged images of the two previous panels. Experiments were repeated 3 times.

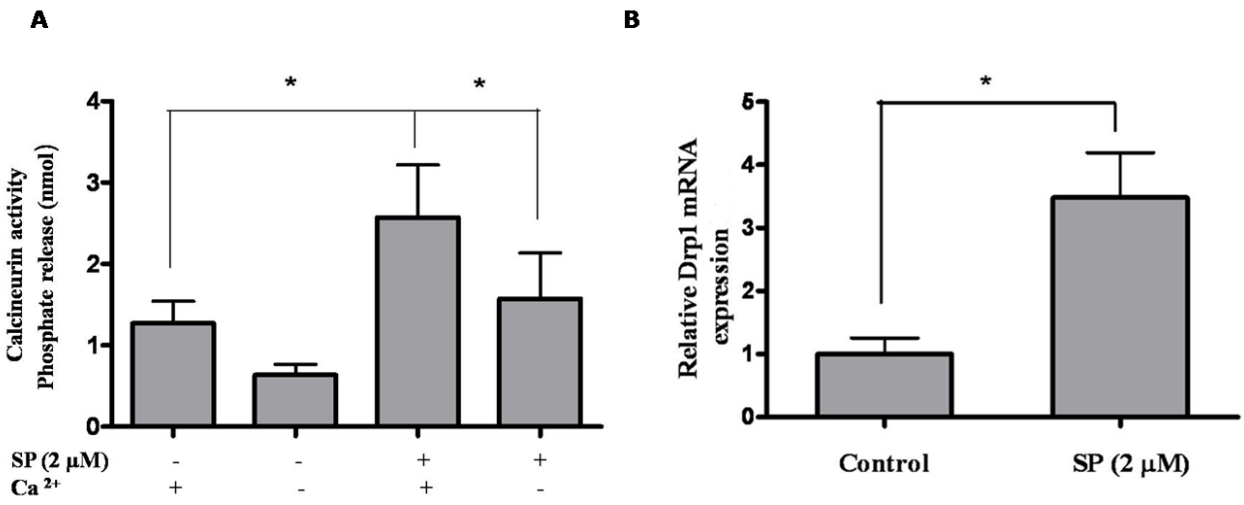


Figure 3.11. **Gene expression of (A) calcineurin, and (B) Drp1 following SP (2 µM) stimulation for 4 h of LAD2 cells were increased compared with control.**

(A) Calcineurin activity in SP-stimulated LAD2 cells is extracellular calcium dependent. LAD2 cells were stimulated by SP (2 µM) for 30 min in either calcium or calcium free medium. Calcineurin activity was measured as phosphate release following the protocol provided. (B) Drp1 gene expression in SP-stimulated LAD2 cells LAD2 cells were stimulated by SP (2 µM) for 4h. Relative quantities of Drp1 mRNA expression were measured by quantitative RT-PCR and normalized to GAPDH. (n=3, *$p < 0.05$ compared to control).

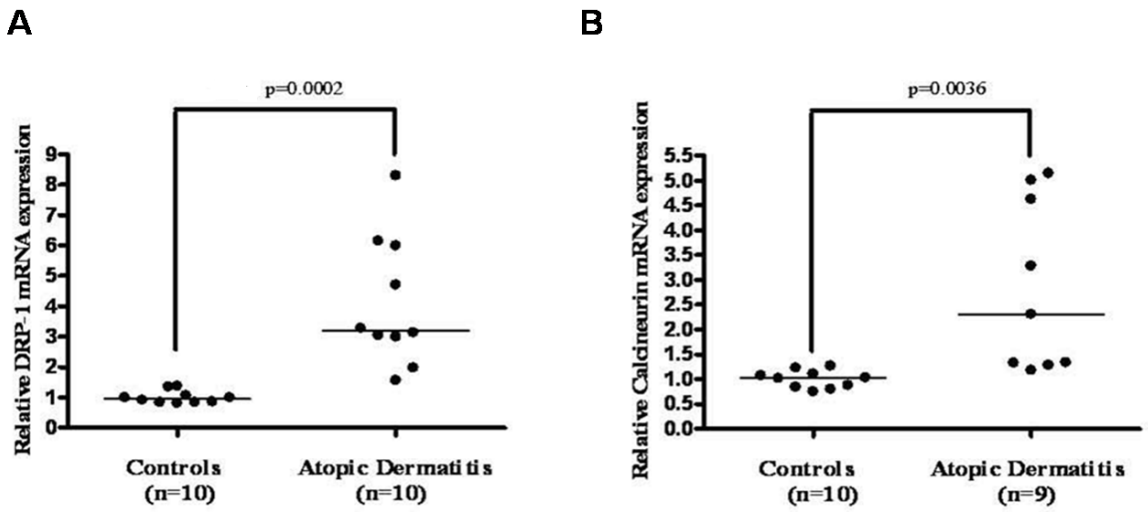


Figure 3.12. Calcineurin and Drp1 gene expression was increased in skin from AD patients as compared to healthy controls.

Gene expression of (A) calcineurin (controls n=10; patients n=9) and (B) Drp1 (controls n=10; patients n=10). The calcineurin patient samples was one less because the cDNA was exhausted. Relative quantities of mRNA expression were measured by quantitative RT-PCR and normalized to GAPDH (*$p<0.05$ compared to control, horizontal bars indicate the means).

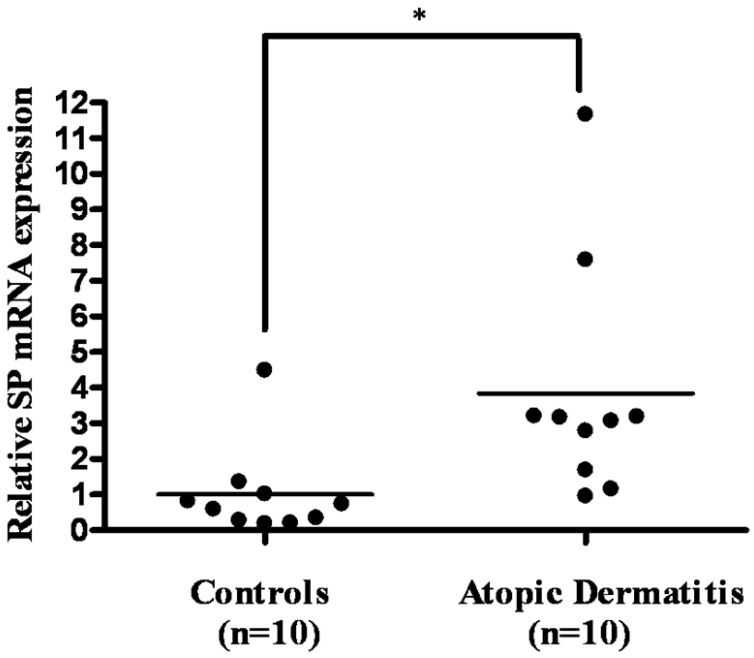


Figure 3.13. **Increased TAC1 gene expression in lesional skin from AD patients as compared to controls ($p<0.05$)**

Gene expression of TAC-1 (controls n=10; patients n=10). Relative quantities of mRNA expression were measured by quantitative RT-PCR and normalized to GAPDH. (*$p < 0.05$ compared to control; horizontal bars indicate the means).

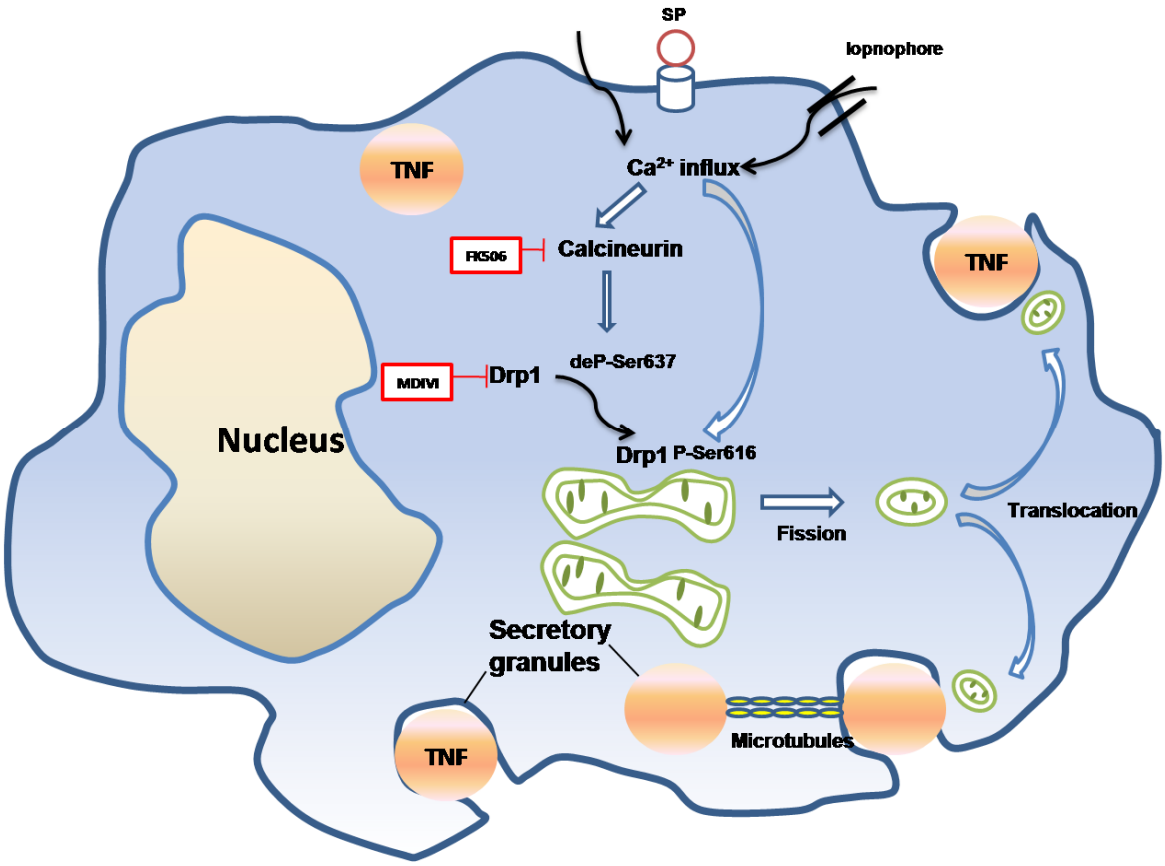


Figure 3.14. Diagrammatic representation of the proposed intracellular steps involved in mitochondrial fission and translocation during human mast cell degranulation.

3.4 Discussion

Here we show that stimulation of hCBMCs by IgE and antigen, as well as LAD2 stimulation by the pro-inflammatory peptide SP, leads to mitochondrial translocation to the cell surface during degranulation, as documented by Confocal microscopy.. SP (1μM) has previously been shown to induce secretion of TNF from LAD2 cells and isolated human skin mast cells.[256]. This TNF is preformed , which is evidenced by (a) its rapid (30 min) secretion, as compared to *de novo* synthesized TNF release that occurs much later (6-24 h), and (b) its presence in unstimulated LAD2 cells confirmed by ELISA. Moreover, SP (1 μM) was able to enhance the rate of oxygen consumption of isolated rat cardiac cell mitochondria.[252] The fact that the Drp1 inhibitor MDIVI-1 and Drp1 siRNA treatment reduce SP-triggered β-hexosaminidase and TNF release support the requirement of Drp1 and mitochondrial translocation in mast cell degranulation. The fact that neither of these treatments completely blocks mitochondrial translocation and mast cell degranulation may be because Drp1 function is not completely suppressed by either inhibitory approach. SiRNA and small molecule inhibiton block Drp1 function from two different aspects: protein amount and protein function. It will be worth to use both methods together to get a better inhibition effect.

Previous studies have shown that mitochondria undergo fission during insulin secretion from storage vesicles in activated β-pancreatic cells.[267] Also, mitochondria have been reported to accumulate at the uropod of migrating T cells.[226] Mitochondrial translocation may, therefore, be necessary to provide energy ***locally*** suggested for ATP-producing organelles and ATP-consuming cellular structures [272]. Another possibility would be that translocated mitochondria might buffer calcium ions locally. For instance,

our present findings show that increased intracellular calcium in stimulated LAD2 cells is necessary for (a) mitochondrial translocation, (b) calcineurin activation, which leads to dephosphorylation of Drp1 at Ser-637 stimulating recruitment of Drp1 to the mitochondrial surface, [271] and (c) Drp1 activation through Ser-616 phosphorylation leading to increased mitochondrial fission.[273] (Fig. S7). The reason for some increased calcineurin activity, even in the absence of extracellular calcium, may be that the cells can still utilize intracellular calcium released from ER or mitochondria. Local calcium increase also activates a cytoplasmic GTPase called Miro, which promotes mitochondrial translocation through Drp1.[274] It was also recently shown that during T cell activation, mitochondria translocate to the "immunological synapse" where they buffer local calcium in order to permit calcium channels to remain open.[223]

We do not presently know if all mitochondria undergo fission and translocation during mast cell degranulation. Some mitochondria may remain perinuclear to provide energy for general cellular needs, while others translocate on demand. Previous papers have reported that mitochondria are heterogeneous in terms of their localization, structure and function.[275]

Finally, we show that gene expression of calcineurin, Drp1 and SP is increased in skin biopsies from AD patients. AD is characterized by skin inflammation[276] and involves both T cells and mast cells.[277] It is of interest that one of the most effective treatments of AD is the calcineurin inhibitor tacrolimus (FK506),[278] which was also reported to prevent pruritus in a mouse model of AD.[279] FK506 also inhibits IgE/anti-IgE-induced secretion of histamine from secretory granules in rat mast cells.[280] Additionally, FK506 depletes SP from peripheral sensory neurons [279,281] which may be

relevant to our present finding of increased SP gene expression in affected AD skin. Mast cells can also serve as antigen-presenting cells [83] and can super-stimulate activated T cells through both TNF and cell-to-cell contact in both mouse [83] and human cells.[80] Inhibition of pre-stored TNF secretion from mast cells may help explain why anti-TNF therapy is often useful in severe cases of AD.[282-284]. Since in lesional skin of AD patients, there are many lymphocytes infiltration, both the cell components and tissue structure of lesional skin will be different from healthy control. Thus, it is possible that the increase of Drp1 mRNA level is not because of increased activity of mitochondrial dynamics, but the difference of cell types and numbers. This problem can be solved using laser micro capture to collect single cell from skin samples and compare the gene expression using single cell PCR.

The present findings document that human mast cell degranulation and preformed TNF secretion require mitochondrial translocation close to sites of exocytosis. This is the first time that mitochondrial dynamics are shown to regulate mast cell degranulation. Mitochondrial translocation constitutes a novel regulatory process that could be targeted for the development of effective anti-allergic and anti-inflammatory drugs.

Chapter IV

Mast cell degranulation results in secretion of mitochondrial components with autocrine and paracrine immune effects

Bodi Zhang, Shahrzad Asadi, Zuyi Weng, Theoharis Theoharides

BZ, performed most of the experiments, analyzed the results and helped wrote the paper. SA and KW helped prepare the cell cultures, performed the in vitro stimulation experiment and analyzed the results. TCT designed the study, organized the collection of human samples, transported the samples and supervised the analysis of the results and wrote the paper. All authors have read and approved the final version of the manuscript.

4.1 Introduction:

Mast cells are bone marrow-derived immune cells that secrete pre-stored mediators, such as histamine and tryptase through degranulation in response to allergic or peptide triggers [63, 236]. Interestingly, mast cells are the only cell type that stores pre-formed TNF in secretory granules [237, 238]. However, secretion of pro-inflammatory mediators is often not sufficient to explain the inflammatory nature of certain diseases, such as psoriasis characterized by hyperproliferation of keratinocytes and skin inflammation. Psoriasis is also associated with increased skin level of the peptide Substance P, TNF and VEGF. Moreover, SP and IL-33 seems to have synergistic action in inducing VEGF release from mast cells in psoriasis.

Mast cell degranulation requires intracellular calcium and energy production, mitochondrial dynamics. Mitochondria are the primary energy-generating organelles in eukaryotic cells [246], that participate in multiple intracellular processes [224]. Such functions were recently shown to require mitochondrial translocation [228]. Mitochondrial shape changes and translocation were also recently shown to be involved in T cell activation and chemotaxis [177] [177]. However, no mitochondrial components have been identified extracellularlay, except during apoptosis and necrosis.

4.2 Material and Methods

fMLF, protease inhibitor cocktail TNF and DMSO were purchased from Sigma. Mitotracker deep Red and LysoTracker DND were purchased from Molecular Probes. IL-8, TNF, IL-2 and VEGF ELISA kits were purchased from R&D system. Mt-7s and MT-CytB taqman probes and Taqman master mix were purchased from Applied Biosystem. Mitochondria Isolation kit were purchased from Pierece Scientific.

Mitochondrial isolation from clinical material

The mitochondria Isolation Kit for cells (Pierce) was used to isolate mitochondria from patients' serum and cell lines. Mitochondria were isolated under sterile conditions at 4°C following protocol. Mitochondria were then sonicated to release all inner components. MtDNA and protein concentration were determined by Nano Drop 2000.

Preparation of mtDNA

Isolated mitochondrial pellets from human LAD2 mast cells (6×10^7 cells) were suspended in 1ml of PBS. Mitochondrial DNA was extracted from the isolated mitochondria of various tissues using DNeasy Blood & Tissue kit (Qiagen). mtDNA were prepared under sterile conditions. Mitochondrial DNA concentration was determined by Nano Drop 2000. No protein contamination was found and nuclear DNA was not detectable in 45 cycles by quantitative PCR.

Culture of Human Mast Cells and Keratinocyte cell lines

LAD2 cells (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health) derived from a human mast cell leukemia were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor (rhSCF; kindly supplied by Amgen). Human umbilical cord blood was collected at Tufts Medical Center. Hematopoietic stem cells (CD34+) were isolated by positive selection of CD34+/AC133+ cells by magnetic cell sorting using an AC133+ cell isolation kit (Milltenyi Biotec) as previously reported (79). Hacat human keratinocytes (kindly supplied by Dr. Solominsky UT) were cultured in DMDM medium (Invitrogen, CA) with 10% FBS.

Confocal microscopy

LAD2, hCBMCs and RBL-2H3 cells were incubated with 20 nM MitoTracker deep red probe (Invitrogen, Carlsbad, CA) for 20 min and 50 nM LysoTracker DND probe for 30 min. Cells were washed, moved to glass bottom culture dishes (MatTek, Ahsland, MA) and observed using a Leica TCS SP2 Confocal microscopy (Leica, Japan). Percentages of cells with mitochondria redistribution were counted from 100 randomly selected mast cells in each experiment. Confocal digital images were processed using the National Institute of Health ImageJ 1.32 and Adobe Photoshop 7.0 Programs.

Electron microscopy

hCBMCs and rat skin mast cells were fixed in modified Karnovsky's fixative containing 0.2% paraformaldehyde and 3% glutaraldehyde, and 0.5% tannic acid in 0.1 mM

Nacacodylate buffer. Samples were photographed using a Philips-300 transmission electron microscopy. Magnification n=6,300 or n =7,000. Images were selected with largest nucleus area to avoid the selection bias of different layers.

β -Hexosaminidase assay

β-hexosaminidase release, as an index of mast cell degranulation, was assayed using a fluorometric assay as previously reported. Briefly, β-hexosaminidase activity in the supernatant fluid and cell lysates (LAD2 cells, 0.5X105/tube, were lysed with 1% Triton X-100 to measure residual cell-associated β-hexosaminidase) were incubated with substrate solution (p-nitrophenyl-N-acetyl-β-D-glucosaminide from Sigma, St Louis, MO) in 0.1 M NaOH/0.2 M glycine. Absorbance was measured at 405 nm in an enzymelinked immunosorbent assay reader, and the results are expressed as the percentage of β- hexosaminidase released over the total. (n=3; *$p<0.05$).

Cytokine release assay

LAD2 and Hacat cells were treated with indicated components for 24 hr. TNF, IL-8, VEGF and IL-2 release were measured ELISA (R&D systems, Minneapolis, MN) in the supernatant fluid. (n=3; *$p<0.05$ compared with control).

Quantitative PCR

Total DNA from mast cells' supernatant were isolated using RNeasy Mini Kit (Qiagen, CA) according to the manufacturer's instructions. In order to measure Mt-7s and Mt-CytB release level, quantitative real time PCR was performed using Taqman gene

expression assays. The following probes obtained from Applied Biosystems were used, Hs 00247152_m1 DNM1L, Hs 01047019_m1 OPA1and Hs 00174223_m1 PPP3CA. The cycling conditions consisted of 1 cycle of 50ºC for 2 min, 1 cycle of 95ºC for 10 min, 40 cycles of 95ºC for 15 s, 1 cycle of 60ºC for 1 min, 1 cycle of 95ºC for 15 s, 1 cycle of 60ºC for 30 s and 1 cycle of 95ºC for 15 s. GAPDH were used as internal control to test DNA contamination. Samples that produced no PCR products after 45 cycles were considered 'undetectable' and the Ct number set to 40 for statistical purposes.

Data Analysis

Image Analysis was performed blind to the treatment conditions. For each experimental condition, 20 or 30 confocal cell images were randomly taken from different wells of the microscope viral. ImageJ software are used for image process. EM images were observed and classified by three different operators. All data are expressed as Mean+ SD, as indicated in the figure legends. Statistical significance was determined by the Student's t-test using the SigmaPlot 9.0 (SPSS, Chicago, IL). Significant differences were considered if $p<0.05$.

4.3 Results

Mast cell degranulation is associated with extracellular mitochondrial component secretion

LAD2 cells are mitochondria are stained with MitoTracker. The average pH of mast cell granules is about 5.5 [177]. Thus LysoTracker is a good indicator for mast cell secretory granules that are also considered to act as lysosomes. Examination of mitochondrial morphology of non-fixed LAD2 cells by Confocal microscopy shows that they contain a number of mitochondria located around the nucleus (Fig. 4.1A). Degranulation by SP (2 μM) leads to significant mitochondrial fission and translocation first observed at 15 min with many mitochondria appearing much smaller and translocate throughout the cells (Fig. 4.1B) as shown in our previous work. Surprisingly, many mitoTracker signals are detected outside the cells (Fig. 4.1B, white rectangle). Larger scale confocal images (Fig. 4.1C) show that such extracellular mitochondria signals can be found around large proportion of cells, while the viability of the cells is about 99%. Since MitoTracker is specifically retained in mitochondria and the signal is not change due to the health of mitochondria, it is very likely that mitochondria are secreted by mast cell during degranulation.

Human skin mast cells release mitochondria components

Under Transmission Electron Microscopy (TEM)[181], mitochondria of unstimulated human skin mast cells are located around the cell nucleus and are appear

intact (Fig. 4.2A). After SP stimulation, mitochondria are mostly found along the cell surface and sometimes they can be seen out side mast cells (Fig 4.2B arrow).

SP-stimulated mast cell secretion is associated with mitochondrial DNA (mtDNA) release

During degranulation, mitochondria translocate to the cell surface close to granules undergoing exocytosis. Supernatant fluid from LAD2 cells stimulated with SP (2 μM) for 60 min contains mitochondrial DNA (mtDNA, MT-7s), detected by real time PCR, which is increased 200 times compared with that of control (Fig. 4.3). In contrast, cytosolic enzymes, such GAPDH genes are not detected, indicating the detection of mtDNA is not due to cell death or supernatant contamination by cells.

Mitochondria components act as autocrine stimuli to mast cells to trigger degranulation and cytokine release

Many of the mitochondrial components are with immunogenicity as they are the ligands of many TLRs. Mitochondria were isolated from either human sarcoma cells or LAD2 cells. The mitochondria are then sonicated to release all inner components. Mitochondria components with mtDNA concentration larger than 0.1 μg/ml were able to trigger mast cell degranulation (Fig. 4.4A). Interestingly, sonicated mast cells have much stronger ability to trigger degranulation than intact mitochondria (Fig. 4.4A). Then we tested the ability of mitochondria components to stimulate *de novo* cytokine synthesis from LAD2 cells. The supernatant fluids of LAD2 cells incubated with mitochondria

components for 24 hours show significant amount of IL-8 and TNF production (Fig 4.4B, C).

Mitochondrial components act as paracrine stimuli to human keratinocytes to trigger cytokines release

Hacat human Keratinocytes are incubated with sonicated mitochondria isolated from LAD2 mast cells for 24 hrs. We find that IL-8 and VEGF, two of the most important cytokines from Keratinocytes are significant elevated in supernatant (Fig. 4.5A, B).

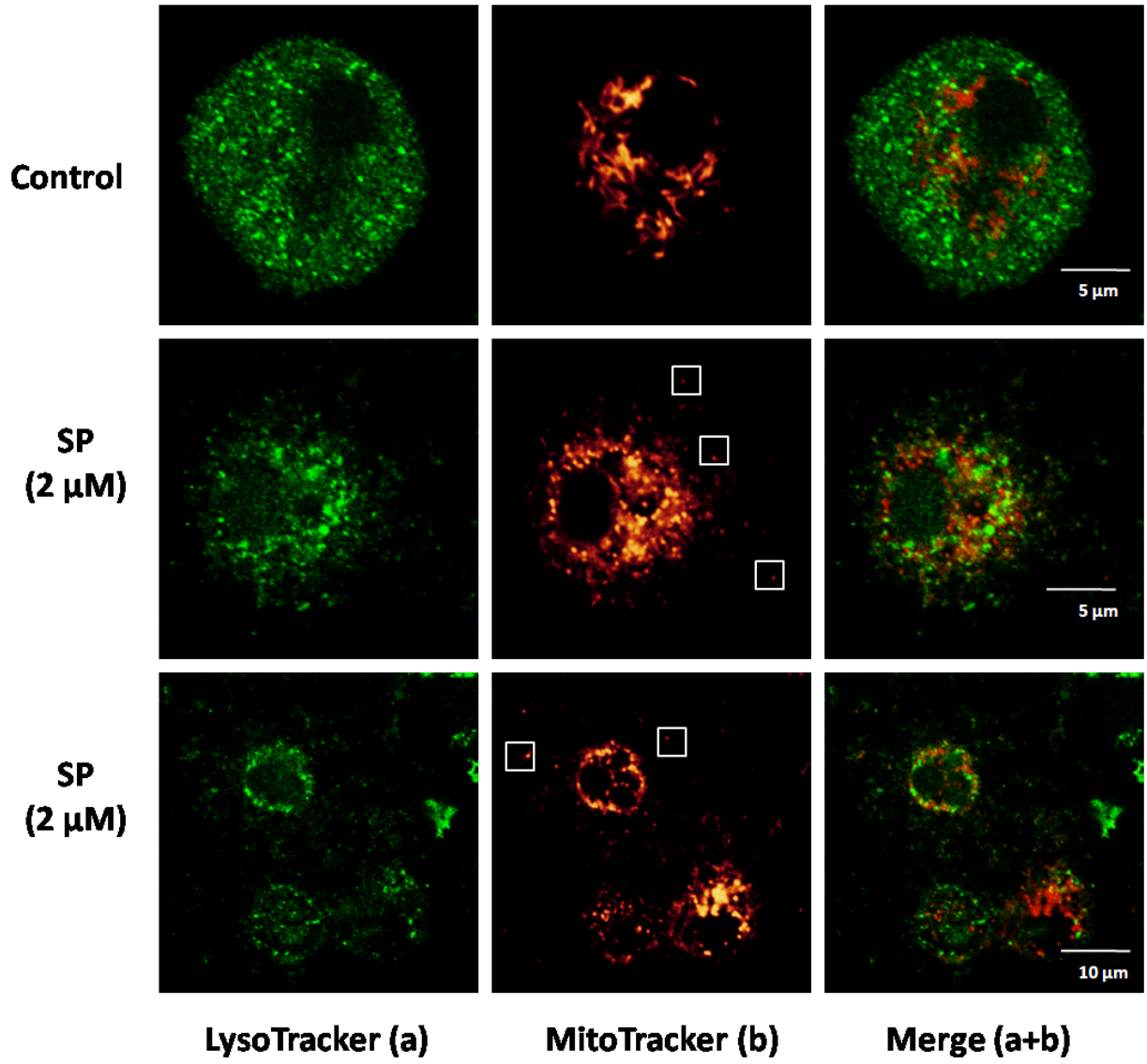


Figure 4.1. Human mast cells release mitochondria components during degranulation observed by Confocal microscopy. Mitochondrial distribution in resting (upper panels), SP stimulated (middle and bottom panels) cells. (n=50). Mast cells were stained by LysoTracker (green) and MitoTracker (red). The white rectangles in the middle panels indicate extracellularly released mitochondria.

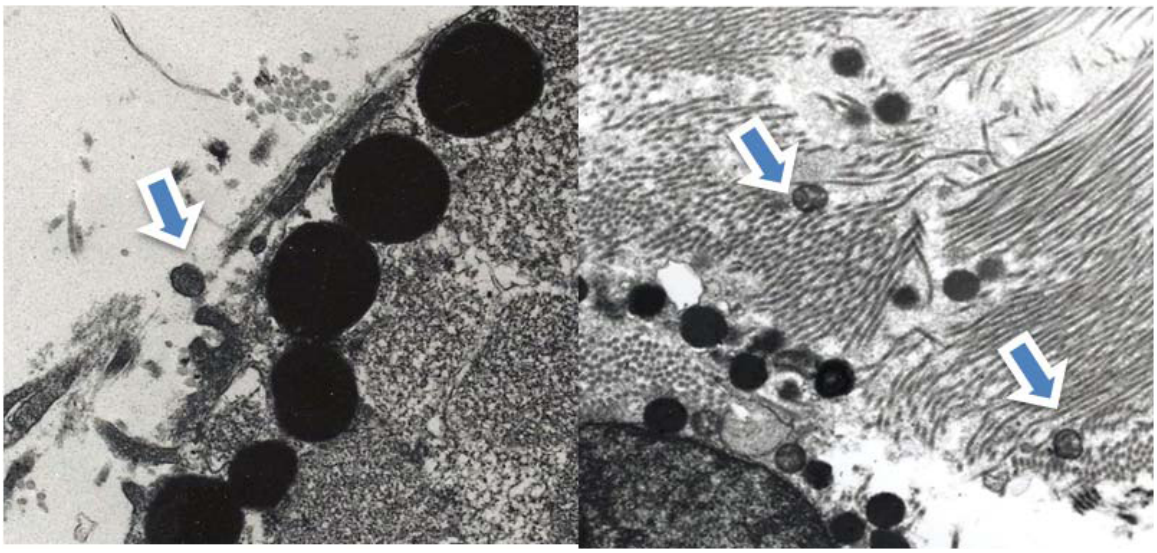


Figure 4.2 Electron photomicrographs showing mitochondria released extracellularly in activated human skin mast cells

Activated human skin mast cells (Magnification: 13,800x). Mitochondria are shown within white rectangles. Arrows indicate extracellular mitochondria.

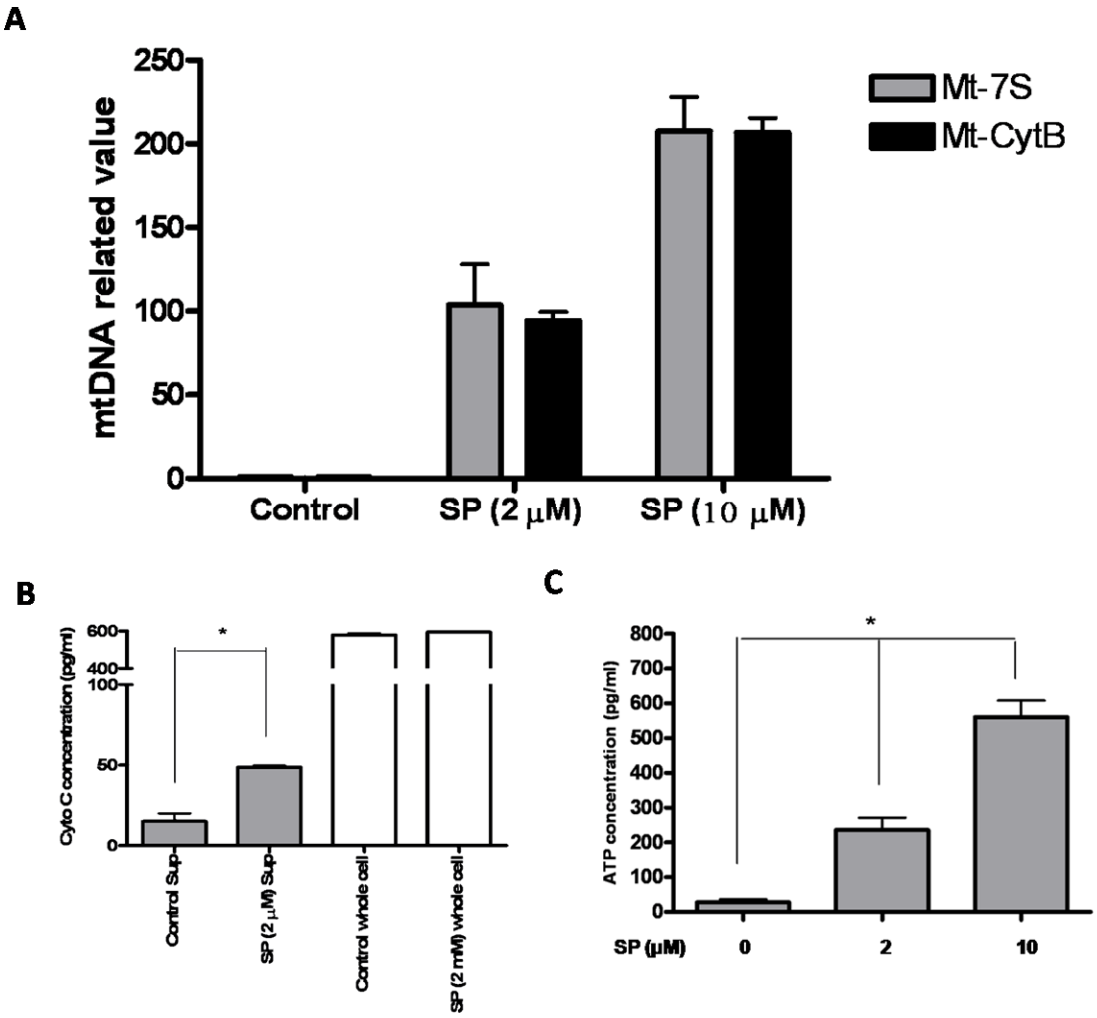
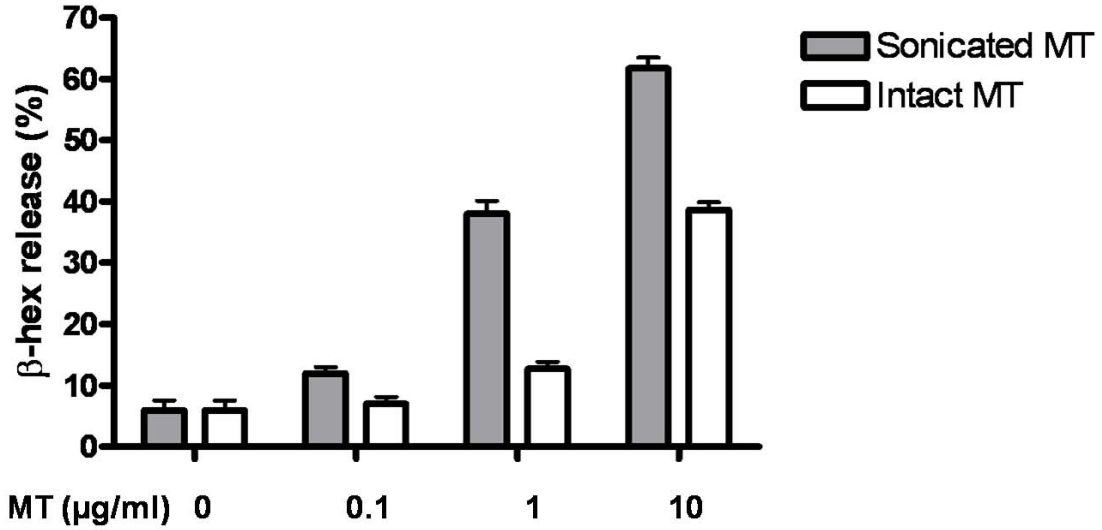
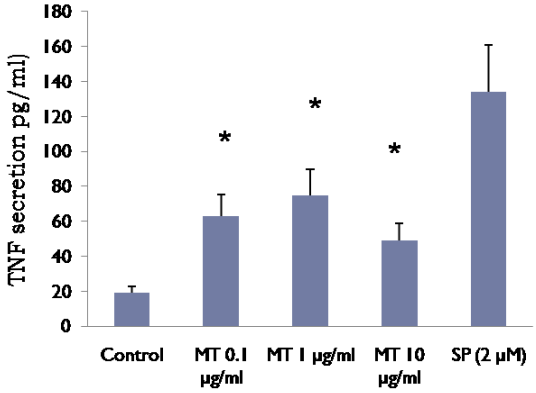


Figure 4.3. mtDNA, CytC and ATP were detected in supernatant of degranulating mast cells. Mitochondrial DNA (A), Cyt C (B) and ATP (C) detected in the supernatant fluid from SP LAD2 cells. LAD2 cells were stimulated with SP (2 and 10 μM) for 2 hr. (n=3, *p<0.01, **p<0.0001).

A


β-hex release (%)
70
60
50
40
30
20
10
0
MT (µg/ml) 0
0.1
1
10
Sonicated MT
Intact MT

B


TNF secretion pg/ml)
180
160
140
120
100
80
60
40
20
0
*
*
*
Control
MT 0.1 µg/ml
MT 1 µg/ml
MT 10 µg/ml
SP (2 µM)

C

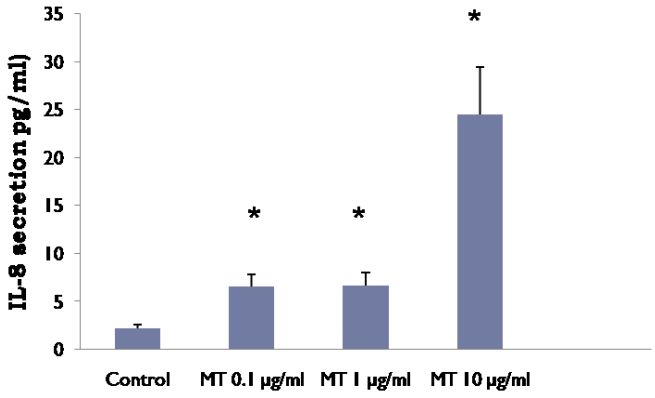

IL-8 secretion pg/ml)
35
30
25
20
15
10
5
0
*
*
*
Control
MT 0.1 µg/ml
MT 1 µg/ml
MT 10 µg/ml

Figure 4.4. Mitochondria triggered human mast cells degranulation, IL-8 and TNF release. Beta-hex (A) release was detected after 30 min of incubation. TNF (B) and IL-8 (C) were measured after 24 hr incubation. (n=3, *p<0.01)

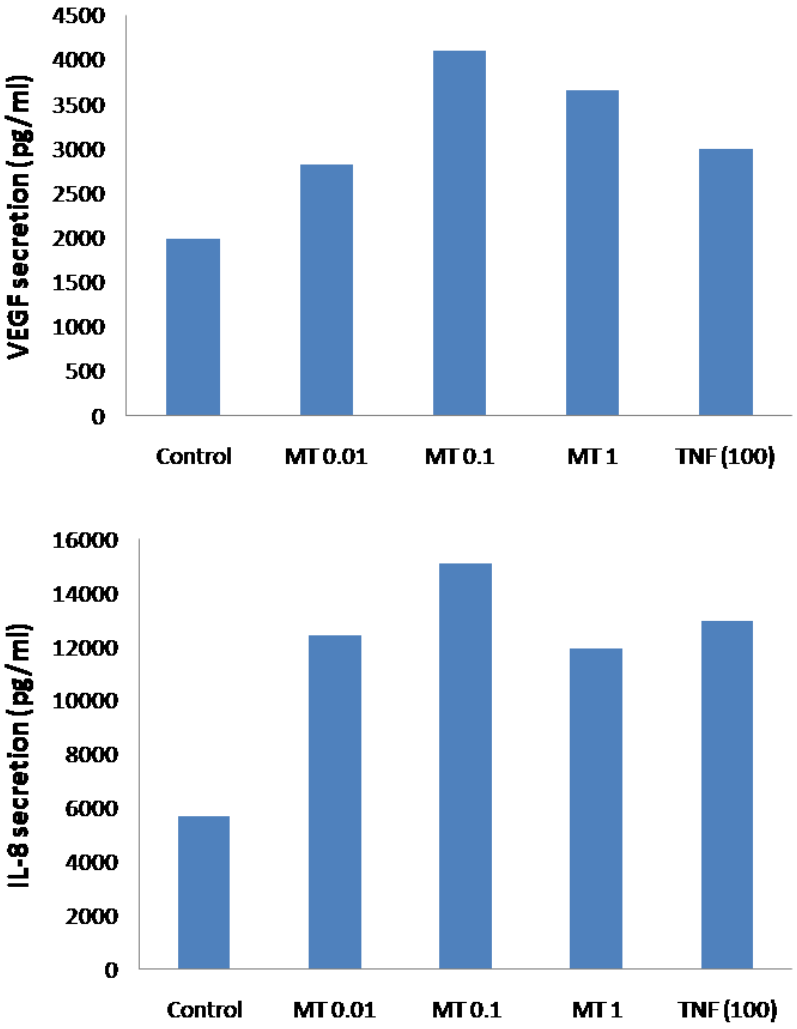


Figure 4.5. Sonicated mitochondria activated human Hacat cells to release IL-8 and VEGF. VEGF (A) and IL-8 (B) were measured after 24 hr incubation. (n=1)

4.4 **Discussion**

Here we show that mast cell activation leads to extracellular secretion of mitochondrial components, including mtDNA and ATP. We also show that mitochondria can stimulate both mast cells and HACAT cultured keratinocytes. Finally, we show that mtDNA can be detected in the blood of patients with severe psoriasis. Mast cells are abundant in connective tissues, especially in skin. Secretion of cytokines from mast cells contributes to many skin inflammatory diseases. We suspect that mitochondria secreted from mast cells can be also important immunological triggers. The fact that mitochondrial components can stimulate Hacat cells implies that they may be involved in the pathogenesis of psoriasis, which is characterized by keratinocyte hyperproliferation. Mast cells have been implicated in psoriasis and we recently show that SP and IL-33 are important in psoriasis.

In one instance, mitochondria were reported be secreted from TNF-activated apoptotic cells. Another paper reported relevance of mtDNA inside exosomes. They are membrane enclosed macro-particles that can carry nucleus acids and other biological molecules from cell to cell. Interestingly, after sonication, mitochondrial lysis is more potent to activate mast cell degranulation compared to intact mitochondria. We also show that ATP from mitochondria can activate mast cell degranulation. There are also many other components within mitochondria can activate cells. The concentration we used to activate mast cell activation is 1 μg/ml. This amount equals to DAMPs release from 0.1% liver injury systemically.

The only other evidence to our knowledge of extracellular mitochondrial presence is a recent report where they purified mitochondrial components from blood from patients with trauma-induced sepsis [285]. Extracellular mtDNA could activate TLR9 receptors as shown for human peripheral polymorphonuclear leukocytes [285], leading to inflammation. Such novel extracellular signaling could have pathological importance in our understanding of inflammatory diseases and autoimmunity. Anti-mtDNA could serve as a diagnostic assay, while neutralizing mtDNA sequences could be used as novel therapeutic approaches for psoriasis and other inflammatory diseases.

In conclusion, the present findings point to a unique and heretofore unrecognized function of mitochondria in mast cell activation that may serve as a novel process to be targeted for the development of new and effective anti-allergic and anti-inflammatory medications.

Chapter V

Mitochondrial DNA and anti-mitochondrial antibodies in serum of autistic children

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BZ performed the experiments and analyzed the results. AA and KDA performed the in vitro stimulation experiment, analyzed the results and helped write the paper. MV and SA helped prepare the samples and performed computer searches. KF and KS collected all the autistic samples and reviewed the results. AT provided all the normal controls. LL and DK supervised the collection of the human samples. TCT designed the study, organized the collection of human samples, transported the samples and supervised the analysis of the results and wrote the paper. All authors have read and approved the final version of the manuscript.

5.1 Introduction

Autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by varying degrees of dysfunctional communication and social abilities, repetitive and stereotypic behaviors, as well as attention, cognitive, learning and sensory deficits [286]. The prevalence of ASD has increased impressively during the last two decades with the most current estimates being about 1/100 children [287]. In spite of numerous clues regarding the possible underlying pathophysiology there are major disagreement among scholars as to the significance of such clues for either the pathogenesis or diagnosis of autism [286]. Moreover, there are no reliable biomarkers or effective treatment of the core symptoms [288], [247].

A number of papers have suggested that ASD may be associated with some immune dysfunction in the patients [289], or the mother during gestation [290],[291]. However, these papers do not provide support of direct evidence. Additional evidence suggests that ASD may have a neuroimmune component [292], In particular, it was recently shown that the peptide neurotensin (NT) is significantly increased in young children with autistic disorder [293]. A number of studies reporting mitochondrial (mt) dysfunction in autism have focused on altered energy metabolism [294], and concluded that it may involve a subset of children with autism [295]. Mitochondria are the primary energy-generating organelles in eukaryotic cells, and they participate in multiple intracellular processes, including calcium buffering [246]. However, mitochondria evolved from bacteria that became

symbiotic with eukaryotic cells and are typically prevented from being released extracellularly by autophagy [296].

We hypothesized that mitochondrial components, such as mtDNA may be released extracellularly early in life and induce an “autoimmune” response that may contribute to the pathogenesis of autism.

5.2 Materials and Methods

We investigated a homogeneous group of young Caucasian children with the same endophenotype. Subjects were diagnosed with autistic disorder using the ADI-R and ADOS-G scales, which have been validated in the Greek population [297]. There were no apparent clinical differences, such as gastrointestinal problems, as reported by the parents, or mitochondrial dysfunction, as indirectly suggested by normal plasma lactate/ pyruvate ratio, that may have allowed separation of the autistic patients in subgroups.

Blood was obtained in the morning at least 2 hours after breakfast to minimize any diurnal or postprandial effects. Serum from patients and controls was aliquoted and frozen at -80^{o}C until assayed. All samples were labeled only with a code number, as well as the age and sex of the respective subject. Patients were selected from the Second Department of Psychiatry at Attikon General Hospital, University of Athens Medical School (Athens, Greece), and an NIH-approved site for biological samples. Parents signed an appropriate consent form according to the Helsinki Principles. All children met ICD-10 criteria for autistic disorder. The exclusion criteria included: (1) any medical condition likely to be etiological for ASD (e.g. Rett syndrome, focal epilepsy, fragile X syndrome or tuberous sclerosis); (2) any neurologic disorder involving pathology above the brain stem, other than uncomplicated non-focal epilepsy; (3) contemporaneous evidence, or unequivocal retrospective evidence, of probable neonatal brain damage; (4) any genetic syndrome involving the CNS, even if the link with autism is uncertain; (5) clinically significant visual or auditory impairment, even after correction; (6) any circumstances that might possibly account for the picture of autism (e.g. severe nutritional or psychological deprivation); (7) active treatment with pharmacological or

other agents; (8) mastocytosis (including urticaria pigmentosa); (9) history of upper airway diseases; (10) history of inflammatory diseases; and (11) history of allergies. The controls were normally developing, healthy children, unrelated to the autistic subjects, and were seen for routine health visits at the Pediatric Department of the Institute of Social Health Insurance, Thessaloniki, Greece. There were no identifiers except for age and sex. All autistic and control samples were collected over a period of six month by trained health providers. Serum was prepared immediately and stored in -80 ^{o}C. All autism and control samples were then transported by the senior author on dry ice to Boston for analysis. Previous work has shown that samples are stable at this temperature. Moreover, DNA is known to be fairly stable and can be stored for months even at -20 ^{o}C .

Anti-mt antibody Type 2 (AMA-M2) was detected using a commercial EIA Kit (DRG International, Germany) [298]. Total DNA was extracted from serum samples using Qiagen DNA Micro extraction kit (Qiagen, CA). Mitochondrial specific DNA for Cytochrome B (mt-CytB) and 7S (mt-7S) was detected and quantified by Real time PCR using Taqman assay (Mt-7S: Hs02596861_s1; Mt-CYB: Hs02596867_s1; GAPDH: Hu, VIC, TAMRA, Applied Biosystems, CA).[285]. GAPDH DNA was used to exclude any genomic "contamination" [285]. Total DNA was isolated from supernatant fluids of cultured LAD2 cells using the same method.

Culture of LAD2 mast cells

LAD2 cells (NIH, Bethesda, MD, USA) were cultured in StemPro-34 SFM Medium (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml recombinant human

stem cell factor (rhSCF, from Biovitrum, Sweden) and 1% U/ml penicillin/streptomycin. Cells were grown in an incubator in 5% CO_2 and air environment at 37°C. All cells were used during their logarithmic growth period.

Statistical analysis

The results are presented as scattergrams, with the horizontal lines indicating the means. The ASD group was compared to the control using unpaired, unequal, 2-tailed, Student's *t*-test, as well as the non-parametric Mann-Whitney *U* test. Any correlation between independent variables (mt-CytB DNA and mt-7S DNA, as well as mt-CytB DNA and AMA-M2 protein amount) was investigated using linear regression analysis. Significance of comparisons between healthy subjects and subjects with ASD is denoted by $p<0.05$.

5. 3 Results

We tested serum samples from autistic patients for mtDNA (n=20; 16 males and 4 females; mean age 3.0 ± 0.4 years) and AMA-M2 antibodies (n=14; 11 males and 3 females; mean age 3.0 ± 0.4 years) compared to controls (n=12; 11 males and 1 female; mean age 3 ± 1.2 years). The number of patients analyzed for AMA-M2 was smaller only because of the lack of availability of the EIA kit that would have allowed us to assay the rest of the patients.

We show that serum from young autistic children contains amount of mtDNA is significant higher for mt-CytB (p=0.0002) and for mt-7S (p=0.006) (Fig. 5.1A and B). Linear regression shows an excellent correlation (R^2=0.89) between mt-CytB and 7S (Fig. 5.1C). No presence of GAPDH DNA was detected indicating there was no genomic DNA release.

Serum of autistic patients also contains AMA-M2 antibodies (p=0.001) compared to unrelated, normal controls (Fig. 5.2A). However, there was no correlation between mt-CytB DNA and AMA-M2 level (Fig. 5.2B).

It is obvious from the scattergrams that the values corresponding to the autistic patients seem to segregate in 2 groups, indicating the possible presence of different endophenotypes. However, nothing in the clinical presentation of these children allowed for easy separation of the subjects in subgroups.

We further hypothesized that NT may be able to trigger release of extracellular mt components from human mast cells. Stimulation of human cultured LAD2 cells with NT

(1, 5 and 10 μM, for 1h at 37^{o}C) resulted in significant release of CytB and 7S mtDNA in the supernatant fluid (Fig. 5.3).

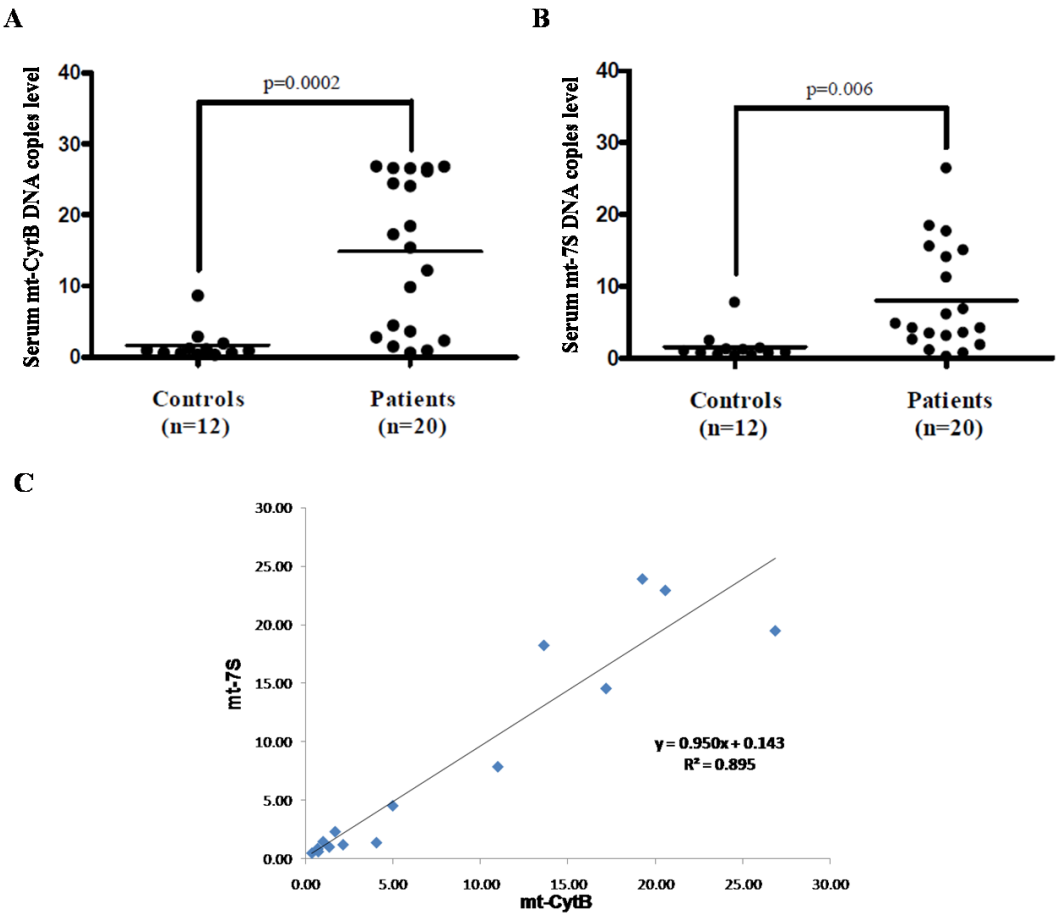

Figure 5.1. Serum levels of (A) mt DNA Cytochrome B (CytB) and (B) mt DNA 7S in autistic patients (n=20; 11 males and 3 females; mean age 3.0 ± 0.4 years) and controls (n=12; 11 males and 1 female; mean age 3 ± 1.2 years). Genomic DNA GAPDH was undetectable, excluding the possibility of cell contamination. The horizontal lines indicate the means.(C) Linear regression analysis showing strong correlation between CytB and 7S.

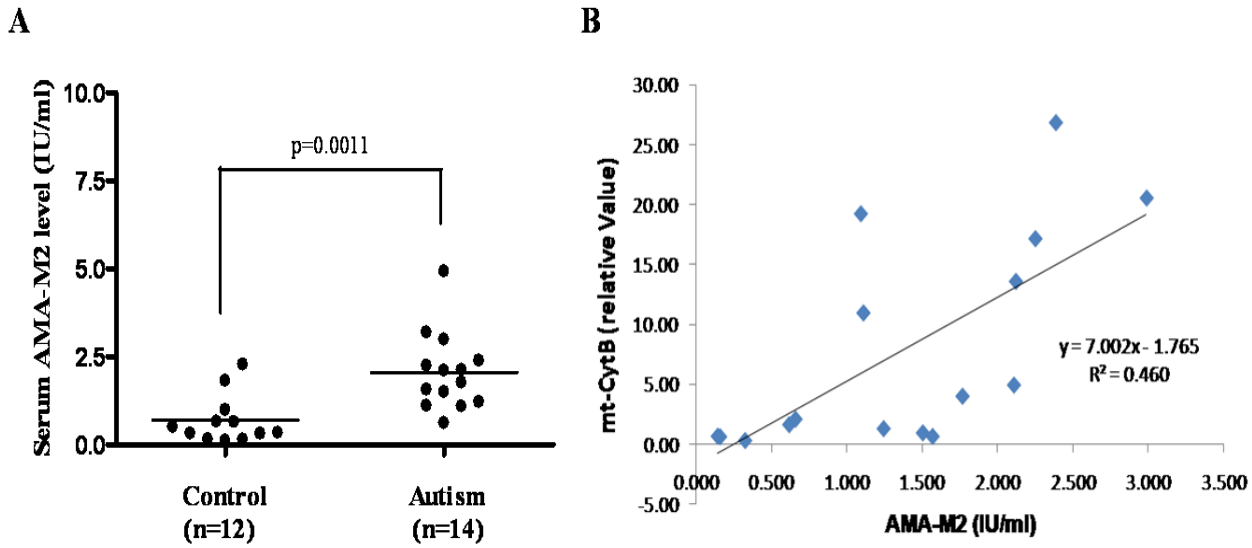

Figure 5.2. Serum levels of anti-mt antibodies type 2 (AMA-M2) in autistic children (n=14; 11 males and 3 females; mean age 3.0 ± 0.4 years) and controls (n=12; 11 males and 1 female; mean age 3 ± 1.2 years). The horizontal lines indicate the means. AMA-M2 level were measured in International Unit (IU)/ml. (B) Linear regression analysis showing no correlation between mtDNA and AMA-M2.

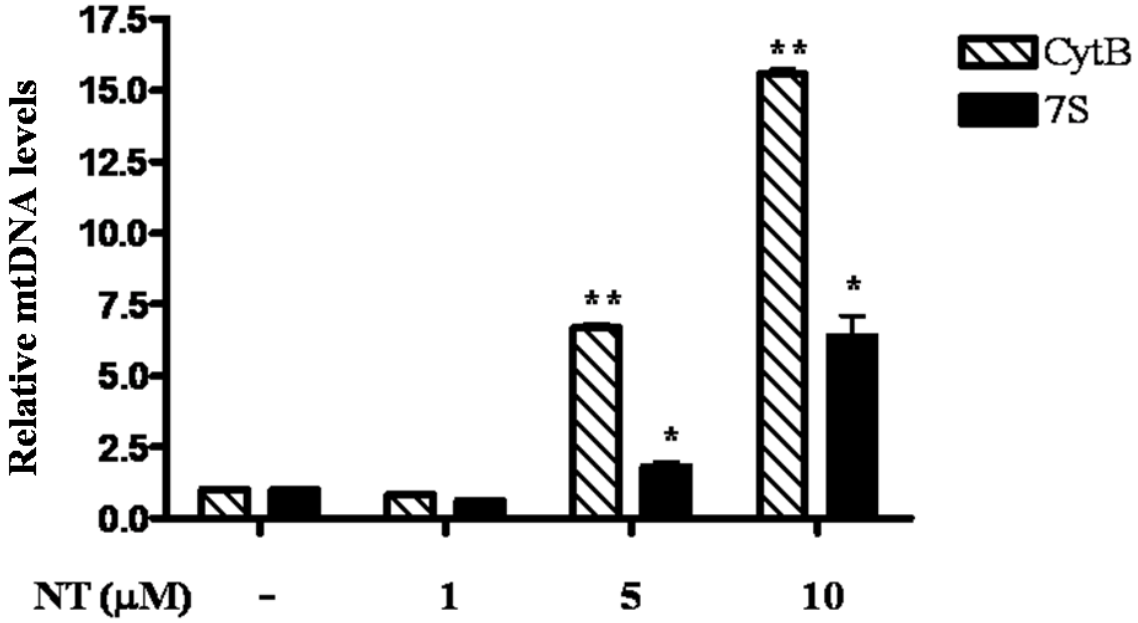

Figure 5.3. Mitochondrial DNA detected in the supernatant fluid from NT-stimulated LAD2 cells. LAD2 cells were stimulated with NT (1, 5, 10 μM) for 1 hr. Mitochondrial specific DNA mt-7S and mt-CytB were detected and quantified by Real time PCR using Taqman assay (Applied Biosystems, CA). Genomic DNA GAPDH was undetectable, excluding the possibility of cell contamination (*$p<0.01$, **$p<0.0001$).

5.4 Discussion

Here we report the presence of extracellular mtDNA and AMA-M2 in young children with autism. Serum mtDNA has never been associated with any neuropsychiatric disease. Moreover, AMA-M2 has been clinically detected only in primary biliary cirrhosis [298]. Consequently, our findings are unique even through there are no available data for what serum levels of either mtDNA or AMA-M2 may constitute an index of a pathological process. However, mitochondria evolved from bacteria that became symbiotic with eukaryotic cells and are typically prevented from being released extracellularly by autophagy [296]. Consequently, mtDNA released extracellularly would be extremely immunogenic. It was recently reported that damage-associated mitochondrial patterns (DAMPs), which contain mtDNA, are present in the blood of patients with trauma-induced sepsis in the absence of any apparent infection and are able to activate (TLR9) on human peripheral polymorphonuclear leukocytes leading to release of IL-8 [285].

The presence of extacellular mtDNA in children with autism suggests that it may be one source of "auto-immune" triggers, and may potential explain some aspects of immune dysregulation reported in autistic patients. For instance, mtDNA (or other extracellular mitochondrial components not measured in this study) could activate TLRs on immune or glial cells to release pro-inflammatory cytokines, such as IL-6, IL-8 or TNF, high gene expression of which was reported in brains of autistic children [299].

Our present results do not imply any mitochondrial dysfunction. Moreover, we can not state definitively that no subject had any mitochondrial dysfunction, since such confirmation requires extensive clinical and laboratory evaluation [300],[275] which was not performed in this case. Mitochondrial dysfunction has been reported in a subset of children with autism [295], [259], but apparently is not linked to altered energy metabolism [294]. Such subsets of ASD children with mitochondrial dysfunction may be more vulnerable to regression following a febrile episode [301].

The source of the extracellular mtDNA and other mitochondrial components in the serum of autistic patients is not presently known. There is no reason to suspect that these derive from apoptotic or necrotic cells because no GAPDH DNA was detected. Moreover, there is no apparent cell damage, at least outside the brain, in autism. One possibility is that mt components are secreted from immune cells, as was recently reported for activated neutrophils [302]. Another possibility could be activated tissue mast cells which are plentiful in the gut, are also a rich source of the NT [303], where it is known to induce mucosal permeability [304]. Our current findings showing that NT can trigger mtDNA release from human mast cells is supported by our previous report that NT, found both in the brain and the gut was elevated in autistic children [293]

The present results may be limited only to autistic disorder and only to the young age of the subjects studied. Nevertheless, these results suggest that serum mt components may induce autoimmune responses, as previously reported for TLR9 activation on human peripheral polymorphonuclear leukocytes [285], and may help with early diagnosis of at least a subgroup of autistic patients.

Chapter VII

Discussion and Future Direction

The overall goal of our research is to find understand the role of mast cells in auto-immune diseases and find a way to regulate mast cell activation. However, we still lack effective tools for such aim. Current treatments, such as anti-histamine, can only block single mediators, but have little power on many other pro-inflammatory molecules also released by mast cells. For the first time, we have shown that mitochondria dynamics and function is important regulator of mast cell secretion. Drp1, the

Although we have identified mitochondria as an important regulator of mast cell function and immune response, there are still many more questions that have been raised by these studies. Thus, we discuss below some of the next steps to be taken.

The mitochondria dynamics study in both chapters 2 and 3 leaves many questions unanswered. The first one is what direct driven force is for such mitochondria movement. Previous researches done by other groups show multiply possibilities. T cells activation and formation of immune synapses leads to initial local calcium burst, which become driven force of mitochondrial translocation to the sites of calcium increases. However, other researches suggests chemotaix induced mitochondrial translocation is not intracellular calcium dependent. It is important to revisit this question because it may lead to new targets that can be controlled to "turn off" mast cell degranulation.

Our experiment on calcium ionphone stimulation of mast cells indicates that influx of calcium is enough to drive mitochondrial translocation. Thus we hypothesize local intracellular calcium increase is necessary for mitochondrial translocation. There are

many experiments could be designed to prove our hypothesis. Firstly, intracellular calcium staining with calcium indicators such as Fura-2 can be used to monitor local calcium changes. Since mast cell degranulation associated calcium increases is very fast (within 10 seconds), high speed micro-seconds level Confocal must be used to capture such changes. Meanwhile, if our hypothesis is right, "partially degranulated" on part of a mast cell will lead to mitochondrial translocation only to the site of degranulation, but not toward all cell surface area. This can be released by bead-conjugated mast cell degranulation assay. Calcium ionophore will be conjugated to plastic beads. Degranulation can only be induced at sites of mast cell-beads direct contact.

The series of experiments we performed did not attempt to conclusively show a mechanism of why mitochondria translocate to sites of degranulation. Previous studies done by other groups shown that the aim of mitochondrial translocation in both neuron and T cells is to provide energy locally. Although we have the evidence that mitochondria energy, production inhibitor Olygomycin blocks mast cell degranulation, more studies should be done to examine whether this is the case.

The function of Drp1 should further examined in diseases. Our results in Chapter III show that mRNA expression level of Drp1 increases in atopic dermatitis patients skin biopsy samples. However, it holds more questions. Firstly, mRNA level is not always equivalent to protein levels. Secondly, the Drp1 real time PCR was conducted based on total RNA from whole tissue samples, which makes it impossible to indicate which cell types have elevated Drp1 level. Increased Drp1 may have a very different effect in mast

cells versus keratinocytes. Therefore, Drp1 protein expression should be examined, preferably using double immunohistochemistry to examine both expression level and specific cell types. Moreover, genetic association studies should be performed looking at the incidence of atopic dermatitis in people with different Drp1 polymorphisms. Because these diseases are polygenic and certain Drp1 polymorphisms are quite common, the subjects should also be genotpyed for other commom polymorphisms known to increase development of atopic dermatitis in order to look at gene interactions.

Mitochondria are bacteria that became symbiotic with eukaryotic cells and are responsible for cellular energy production. Mitochondrial components are not normally found outside the cells because they are extremely immunogenic. Mitochondrial health is kept in check by autophagy when damaged or "spent out" mitochondria are destroyed internally [296]. It is also intriguing that a bacterial wall component capable of binding penicillins, LACTB, was recently shown to be expressed by mitochondria [305], and may activate TLR-2. In fact, mast cells respond to bacterial peptidoglycan and lipopolysaccharide by activating TLR-2 [141] and TLR-4 [141], respectively, as well as by viral nucleic acid sequences by activating TLR-3 or 9 [110]. Glioblastoma cells and astrocytes were shown to release microvesicular exosomes containing mtDNA [306]. Exosome are nanovesicles [307] that transfer eukaryotic mRNA and microRNA, as well as DNA [308]. Neutrophils were also shown to release mitochondria locally in "extracellular traps" [302]. extracellular mt or individual components (peptidioglycan, DNA) maybe released (a) by live activated cells as whole mt particles and free mtDNA in exosome; (b) by apoptotic or necrotic cells (DMAPs).

We show in Chapter V that purified mitochondria can activate mast cells and keratinocytes to release pro-inflammatory cytokines. This reveals a whole class of immune triggers. However, there are many more works can be done based on this discovery.

Mitochondria are composed with proteins, lipids and mtDNA, many of which have been shown to be immunogenic. It will be important to identify which components are critical for mast cells activation. Proteins, mtDNA and other components will be isolated from mitochondria and used to stimulate mast cell separately. Meanwhile, antagonist toward different Pattern Recognition Receptors will also be used to identify the corresponding receptors on mast cells. Understanding the molecular signaling transduction pathways under mitochondria stimulation is critical for target-based inhibition and drug discovery.

The results of mast cell pro-inflammatory secretion by mitochondria indicates the potential that mitochondria can also activate other cell types, since PRRs are widely expressed in majority cell types. Thus, other cell types directly related with auto-immune diseases, such as T cells, dendritic cells and Polymorphnucleucytes would also be tested for activations.

Mitochondria are structurally stable and with double membranes. This characteristic allows them to be transported into a longer distance compared with traditional cell

cytokine release and activate broader types of immune cells. Therefore, it will be very important to trace the transportation of mitochondria in vivo. Lymph node is one of the most important immune organs for antigen-presentation and acquired immune activation. Mice model can be used to exam the presence of mitochondria in lymph node upon mast cell activation. Mice mast cells will be first stained with live cell mitochondria dye MitoTracker as we used before. These cells will be then injected into mice and activated by C48/80. Later, we will collect peritoneal lymph node from the host mice and exam stained mitochondria by fluoresce microscopy.

Here we show that in children with autism, serum level of mtDNA and AMA-M2 level are increased compared with control. Although it is not clear the origination of such mitochondria, due to the fact that there is no large number of cell death in autistic children, these mtDNA may come from live blood cells, more particularly, immune cells. Thus, extracellular mitochondria may also be appear in many auto-immune diseases and can even be one important pathogenesis reason.

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Additional works related to mast cells and psoriasis

IL-33 Augments SP-Induced VEGF Secretion from Human Mast Cells and is Increased in Psoriatic Skin

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BZ, DKe and MT performed most of the experiments, analyzed the results and helped wrote the paper. KDA and AA helped to performed the in vitro stimulation experiment and analyzed the results. SA and MV helped prepare the cell cultures and performed computer searches. DKa provided psoriasis samples. EP provied umbilicord blood. SL helped designed the study and made critical commnets on the manuscripts. PT helped designed the study and analyzed the data. TCT designed the study, organized the collection of human samples, transported the samples and supervised the analysis of the results and wrote the paper. All authors have read and approved the final version of the manuscript.

Short title: SP and IL-33 stimulate mast cell VEGF

Key words: IL-8, IL-33, inflammation, mast cells, psoriasis, substance P, vascular endothelial growth factor

Abbreviations: CBMCs, cord blood-derived cultured mast cells; CRH, corticotropin-releasing hormone; ERK, extracellular-signal regulated kinase; CXCL8 (IL-8), interleukin-8; HDC, histidine decarboxylase; IL-33, interleukin 33; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NK, neurokinin; PKC, protein kinase C; RPMC, rat peritoneal mast cell; SP, substance P; VEGF, vascular endothelial growth factor; TAC1.

1 Abstract

The peptide substance P (SP) has been implicated in inflammatory conditions, such as psoriasis, where mast cells and vascular endothelial growth factor (VEGF) are increased. A relationship between SP and VEGF has not been well studied, nor has any interaction with the pro-inflammatory cytokines, especially interleukin 33 (IL-33). Here we report that SP (0.1-10 μM) induces gene expression and secretion of VEGF from human LAD2 mast cells and human umbilical cord-derived cultured mast cells (hCBMCs). This effect is significantly increased by co-administration of IL-33 (5-100 ng/ml) in both cell types. The effect of SP on VEGF release is inhibited by treatment with the NK-1 receptor antagonist 733,060. SP rapidly increases cytosolic calcium, and so does IL-33 to a smaller extent; the addition of IL-33 augments the calcium increase. SP-induced VEGF production involves calcium-dependent protein kinase C (PKC) isoforms, as well as the ERK and JNK mitogen activated protein kinases (MAPKs). Gene-expression of IL-33 and histidine decarboxylase (HDC), an indicator of mast cell presence/activation, is significantly increased in affected and unaffected (at least 15 cm away from the lesion) psoriatic skin, as compared to normal control skin. Immunocytochemistry indicates that IL-33 is associated with endothelial cells in both the unaffected and affected sites, but is stronger and also associated with immune cells in the affected site. These results imply that functional interactions among SP, IL-33 and mast cells leading to VEGF release contribute to inflammatory conditions, such as the psoriasis, a non-allergic hyperproliferative skin inflammatory disorder with a neurogenic component.

2 Introduction

Substance P (SP) is an 11 amino acid peptide that mediates inflammation [205, 206], partially through mast cell activation [203, 204]**.** Neuropeptides [200], especially SP, could be involved in the pathogenesis of inflammatory skin disorders, such as psoriasis [201, 309], characterized by increased epidermal vascularization, keratinocytes hyperproliferation and inflammation [310]. SP-positive nerve fibers are more dense in psoriatic lesions and have an increased number of mast cell contacts compared to normal skin [202, 207, 208, 311]. Mast cells are also increased in lesional psoriatic skin (14) and there appears to be an association among sensory nerves, mast cell numbers and stress [103, 104]. SP-positive nerve fibers and mast cell contacts are also increased by acute stress in mice, leading to dermal mast cell degranulation [197, 203, 210]. It also is interesting that psoriasis is worsens by acute stress [197, 198].

Psoriatic plaques contain increased levels of vascular endothelial growth factor (VEGF) compared to normal skin [312-314]. VEGF is a major pro-angiogenic factor involved in many inflammatory diseases [315]. The VEGF 121 isoform is particularly increase in psoriatic plaques (23) and VEGF is also increased systematically in severe psoriasis [316, 317]. Genetic studies have shown that several different VEGF polymorphisms are associated with an increased risk of developing psoriasis [318, 319]. Mast cells can secrete VEGF in response to IgE [43, 320], and to corticotropin-releasing hormone (CRH) [44], secreted under stress. Epidermal overexpression of VEGF in transgenic mice leads to a phenotype nearly identical to that of psoriasis [321].

Given the fact that psoriasis involves skin inflammation, and that it is often

present with arthritis (psoriatic arthritis) [211], we were intrigued by the finding that IL-33 exacerbates antigen-induced arthritis in mice by activating mast cells [212]. IL-33 is one of the newest members of the IL-1 family of inflammatory cytokines [213], and was recently shown to mediate IgE-induced anaphylaxis in mice [214]. IL-33 also induces release of IL-6 from mouse bone marrow derived cultured mast cells (BMCMCs) [132], and IL-8 from human umbilical cord blood derived cultured mast cells (hCBMCs) [216].

Mast cells are found in large numbers around blood vessels in the skin where they participate in allergic and inflammatory reactions through release of multiple mediators with potent vasodilatory, inflammatory and nociceptive properties [20 322]. For example, CRH increases vascular permeability through release of histamine[181], which also stimulates cutaneous sensory nerves [323] contributing to pruritus. Skin mast cells may have important functions as "sensors" of environmental and emotional stress [324].

In the present study, we show for the first time that SP stimulates human mast cells to secrete VEGF, and this action is augmented by IL-33. Furthermore, we show that IL-33 mRNA expression is increased along with histidine decarboxylase (HDC), an indicator of mast cell presence/activation, in psoriatic skin.

3 Materials and Methods

Reagents

SP, IL-1, IL-33 and L-703,606 were purchased from Sigma (St. Louis, MO). PD98059, SP600125, SB203580, Bisindolylmaleimide I and Gö6976 were purchased from Calbiochem (La Jolla, CA). Cortistatin-17 was purchased from Phoenix Pharmaceuticals (Burlingame, CA). All MAPK antibodies were purchased from Cell Signaling Technology (Danvers, MA). L-733,060 was purchased from Sigma-Aldrich, St. Louis, MO).

Culture of human mast cells

LAD2 cells (kindly supplied by Dr. A.S. Kirshenbaum, National Institutes of Health, Bethesda, MD) derive from a human mast cell leukemia [239] were cultured in StemPro-34 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin/streptomycin and 100 ng/ml recombinant human stem cell factor (rhSCF; kindly supplied by Amgen, Thousand Oaks, CA). The ability of SP to induce VEGF release was also investigated in normal human cultured mast cells to confirm that any findings with LAD2 cells were not due to their leukemic origin. Human umbilical cord blood was collected at Tufts Medical Center (Boston, MA). Hematopoietic stem cells ($CD34^+$) were isolated by positive selection of $CD34^+/AC133^+$ cells by magnetic cell sorting (MACS) using an $AC133^+$ cell isolation kit (Milltenyi Biotec, Auburn, CA) as reported [268]. These cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; GIBCO BRL), containing 200 ng/ml recombinant human stem cell factor (rhSCF) from

Amgen, (Thousand Oaks, CA), 50 ng/ml IL-6 (Millipore, Temecula, CA) for 12 to 16 weeks. IL-4 (20 ng/ml) was used for 2 weeks prior to the experiment. All cells were maintained at 37ºC in a humidified incubator at 5% CO_2.

VEGF release assay

Mast cells (1 x 10^5 cells/250 µl) were distributed in 96-well microtiter assay plates in triplicate and stimulated in complete culture medium with the indicated concentrations of SP. For inhibition studies, inhibitors were added to the media 30 min prior to stimulation. VEGF was determined in cell-free supernatants with a commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's directions. VEGF secretion data are expressed as pg/10^6 cells.

Cytosolic calcium measurements

Cytosolic calcium was measured in LAD2 cells at 37 °C using Fura-2 as indicator. LAD2 cells were loaded in Tyrode's buffer with 1 mM Fura-2 AM (Invitrogen) for 20 min to allow Fura-2 to enter the cells. After centrifugation to remove excess dye, the cells were resuspended in Tyrode's buffer (with or without 1 mM calcium) at a concentration of 1 million cells/ml and incubated for another 20 min. Cells were then transferred to 96 plates with 100 µl per well. SP (1 □M) was added to cells for the time indicated. Fura-2 fluorescence was read by MDC FlexStation II (Molecular Devices Corporation, Sunnyvale, CA) at excitation wavelength of 340 nm/380 nm and emission wavelength of 510 nm. Results were processed according to Invitrogen's Fura-2 protocol.

Patients and biopsies

All skin biopsies requiring two stitches were collected from subjects (patients and controls) who had not received any medication for 15 days prior to the biopsy and were seen at the 2nd Department of Dermatology of the Attikon General Hospital, Athens University Medical School, Athens, Greece. The Medical Ethics Committee of Attikon Hospital Institution's Human Investigation Review Board (HIRB) approved this protocol. All participants gave their written informed consent according to the Declaration of Helsinki Principles. Patients had moderate chronic plaque psoriasis with Psoriasis Area and Severity Index (PASI) scores 5-16. Patients were free from any other medical problems. All biopsies (patients and controls) were obtained from non-exposed skin (back and gluteal). Samples of unaffected skin of psoriasis patients were obtained from sites at least 15 cm away from the affected lesional areas. All biopsies were immediately placed in RNAlater solution (Ambion, Inc., Austin, Texas, USA) and stored at -20˚C.

PCR and quantitative PCR

Total RNA from skin biopsies or cultured mast cells was isolated using Trizol (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed with 200 ng of total RNA using the iScript cDNA synthesis kit (Ambion, Austin, TX).

PCR was performed with GoTaq Green master mix (Bio-Rad, Hercules, CA) and 400 nM of the following primers; β-actin forward, 5'- TGTGATGGTGGGAATGGGTCAG-3' and β-actin reverse, 5'- TTTGATGTCACGCACGATTTCC-3', which amplifies a 511 bp

fragment. The cycling conditions consisted of 35 cycles of 95°C for 15 s and 60°C for 1 min. Amplified DNA was run on a 3% agarose gel and visualized with ethidium bromide.

Quantitative PCR was performed in triplicate with an Applied Biosystems (Foster City, CA) 7300 Real-Time PCR System using iTaq SYBR Green Supermix (Ambion) and 400 nM of each primer. The following probes obtained from Applied Biosystems, were used: IL-33 (Assay ID: Hs00369211_m1); HDC (Assay ID: Hs00157914_m1), and TAC1 (tachykinin precursor 1, precursor of substance K, substance P, neurokinin 1, neurokinin 2, neuromedin L, neurokinin alpha, neuropeptide K, neuropeptide gamma) (Assay ID: Hs00243227_m1). Human GAPD (GAPDH) Endogenous Control (VIC/TAMRA Probe, Primer Limited), (Part Number: 4310884E). The cycling conditions consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 1 cycle of 60 °C for 1 min, 1 cycle of 95 °C for 15s, 1 cycle of 60 °C of 30 sec and 1 cycle of 95 for 15 s. Relative mRNA abundance was determined from standard curves run with each experiment, and IL-33, HDC and TAC1 expression was normalized to GAPDH Endogenous Control.

IL-33 immunocytochemistry

Skin biopsies from patients with psoriasis and control subjects were immediately placed in Tissue Freezing Medium (TBS, Cat #H-TFM) and stored at -80 C. Five sections (5 □m) were cut using a cryostat and fixed with cold acetone for 10 min.

Immunocytochemical staining was performed using the LSAB+ system kit (DAKO, Glostrup, Denmark). Incubation with the primary antibody (mouse monoclonal-anti-human-IL-33 antibody, at 1:100 dilution, Abcam, Cambridge, MA) was performed for 30 min; secondary antibody was proved in the DAKO kit and was also used for 30 min, followed by appropriate washes.. As negative control, the primary antibody was omitted. Faramount, Aqueous Mounting Medium (DAKO, Denmark) was used for aqueous mounting. Sections were examined by three different investigators and photographs were taken using an upright Olympus BH2 (Rorth Reading, MA) microscope and a digital camera attached to a computer for image storage and analysis.

Statistical analysis

Data are expressed as the mean ± SD. Statistical significance between experimental samples and controls was calculated using the Student's t-test. $P<0.05$ was considered statistically significant.

4 Results

SP stimulates VEGF mRNA expression and protein production in human mast cells

To examine the effect of SP on VEGF secretion, LAD2 cells were treated with SP (0.01- 10 μM) for 24 hr. Stimulation with SP caused a concentration-dependent production and release of VEGF with a maximum of 546 pg/10^6 cells at 10 μM, a 2.7-fold induction (Fig. 1A). In order to investigate if this effect was limited to the leukemic human LAD2 mast cells, we repeated the experiments using hCBMCs (Fig. 1B). SP stimulation caused a maximum release from hCBMCs of 445 pg/10^6 cells at 10 μM. VEGF release in response to 1 μM SP was time-dependent over 48h (Fig. 1C). This slow VEGF secretion suggests *de novo* synthesis. We next examined whether the VEGF mRNA transcript is induced by SP using quantitative PCR. VEGF mRNA was increased after SP (1 μM) stimulation with a maximum ~12-fold increase occurring at 6 hr (Fig. 1D). Elevation of VEGF mRNA was sustained for at least 24 hr. Together, these data show that SP induces both VEGF mRNA and protein synthesis in human mast cells.

IL-33 augments the effect of SP on VEGF release from human mast cells

We next examined if IL-33 could induce VEGF release from LAD2 mast cells. IL-33 alone (5-100 ng/ml) did not induce VEGF release (Fig. 2A). However, the addition of IL-33 (100 ng/ml) to SP (1 μM) augmented VEGF production by 1.5-fold as compared to that induced by SP alone (Fig. 2A). IL-33 alone (5-100 ng/ml) over 6 hours increased VEGF gene expression up to 4.5-fold (Fig. 2B). The addition of IL-33 (100 ng/ml) to SP (0.1 or 1 μM) further increased VEGF mRNA expression (Fig. 2B). In order to ensure

that these findings were not limited to the use of the LAD2 leukemic human mast cells, we repeated the experiments using hCBMCs, except that these cells require higher amount of SP for stimulation. IL-33 (100 ng/ml) again did not induce any VEGF release on its own, but augmented (Fig 2C) VEGF release induced by SP (5 μM).

Given that IL-33 belongs to the IL-1 cytokine family, we also tested if IL-1 could induce VEGF release and whether it could augment the release due to SP. While IL-33 (100 ng/ml) had no effect, IL-1 (10 ng/ml) induced significant VEGF release (Fig. 2C); addition of IL-1 (10 ng/ml) to SP (5 μM) augmented the effect of SP (Fig. 2C, $p<0.05$). Use of IL-32, a cytokine that does not belong to the IL-1 family had no effect (results not shown).

NK-1 receptor antagonist inhibit SP-induced VEGF release

In order to determine whether SP-induced VEGF release is mediated through the NK-1 receptor, we pre-incubated LAD2 cells with the NK-1 receptor antagonist L-733,060 (10 μM) for 30 min prior to and then during stimulation with the SP (1 μM). Treatment of LAD2 cells with L-733,060 (10 μM) completely blocked SP-induced VEGF release (Fig. 3). In fact, this antagonist also significantly reduced basal VEGF secretion (Fig. 3).

IL-33 augments cytosolic calcium ions levels increased by SP

In order to examine the possible mechanism of action through which IL-33 enhances the ability of SP to increase VEGF release, we measured their effects on intracellular calcium ion levels. SP (1 μM) significantly increased cytosolic calcium, while IL-33 (100 ng/ml) had a similar but smaller increase (Fig. 4). Addition of IL-33 to SP augmented the cytosolic calcium increase due to SP (Fig. 4). Neither SP nor IL-33 or their combination had any effect in the absence of extracellular calcium (not shown).

IL-33 and HDC mRNA expression is increased in psoriatic skin

IL-33 and HDC, an indicator of mast cell activation, mRNA expression was studied in psoriatic affected skin, and unaffected skin obtained from at least 15 cm away from the lesion, as well as normal skin from healthy controls.

Analysis of skin biopsies from psoriatic lesional, unaffected, and control normal skin reveals that IL-33 gene expression is significantly higher in lesional psoriatic skin than normal control skin (Fig. 5A). Expression of HDC mRNA, investigated as an index of mast cell presence/activation, is also significantly increased in lesional psoriatic skin (Fig. 5B). Interestingly, both IL-33 and HDC gene expression is also significantly increased in unaffected psoriatic skin as compared to control skin (Fig. 5). These results suggest that IL-33 and HDC gene expression is not associated with lymphocyte infiltration or keratinocyte proliferation in the psoriatic plaque. SP mRNA expression is increased in the unaffected skin but is lower in the affected skin (Fig. 5C).

IL-33 immunocytochemistry

Investigation of IL-33 protein expression by immunocytochemistry showed that IL-33 was strongly associated with blood vessels, infiltrating inflammatory cells and sweat glands in the affected psoriatic skin areas (Fig. 6A). In contrast, IL-33 was weakly associated with blood vessels and sweat glands in unaffected psoriatic areas (Fig. 6B). There was no apparent association with mast cells.

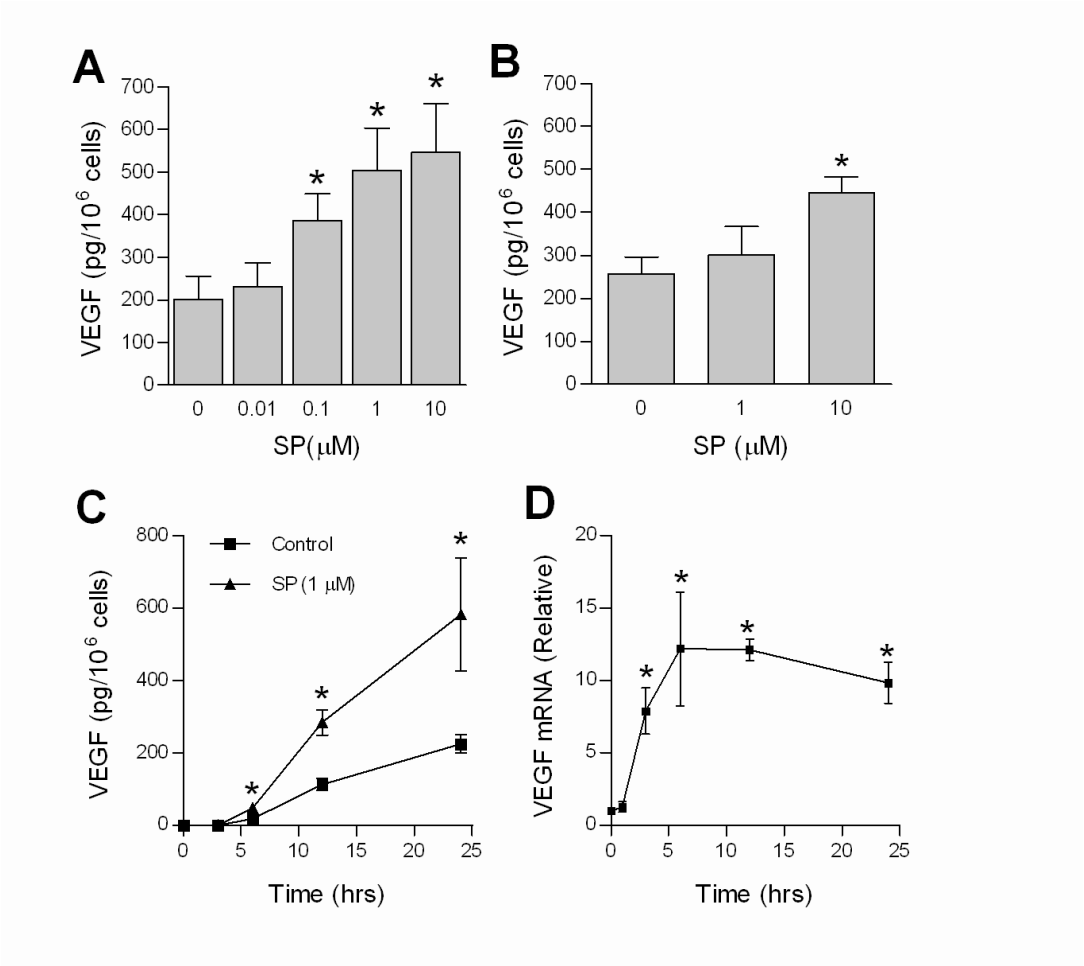

Figure 1. SP stimulates VEGF production in human mast cells. LAD2 cells (A) and hCBMCs (B) were stimulated with the indicated concentration of SP (0-10 μM) for 24 h and supernatant VEGF was measured by ELISA. (C) Time-dependent secretion of

VEGF. Cells were stimulated with SP (1 μM) or vehicle for the indicated times and supernatant VEGF was measured by ELISA. (D) SP induces VEGF mRNA. LAD2 cells were stimulated with SP (1 μM) for the indicated times, RNA was extracted and relative VEGF mRNA levels were determined by real-time PCR. Data are the mean ± SD of 3 separate experiments performed in triplicate (*$P < 0.05$ versus unstimulated cells).

Figure 2. IL-33 augments SP in inducing (A) VEGF protein secretion from LAD2 cells, and (B) VEGF mRNA expression from LAD2 cells, or (C) VEGF protein secretion from hCBMCs. Cells were treated for 6 hours with with IL-33 (100 ng/ml) or IL-1 (10 ng/ml) alone or together with SP as shown (n=3, *p<0.05)

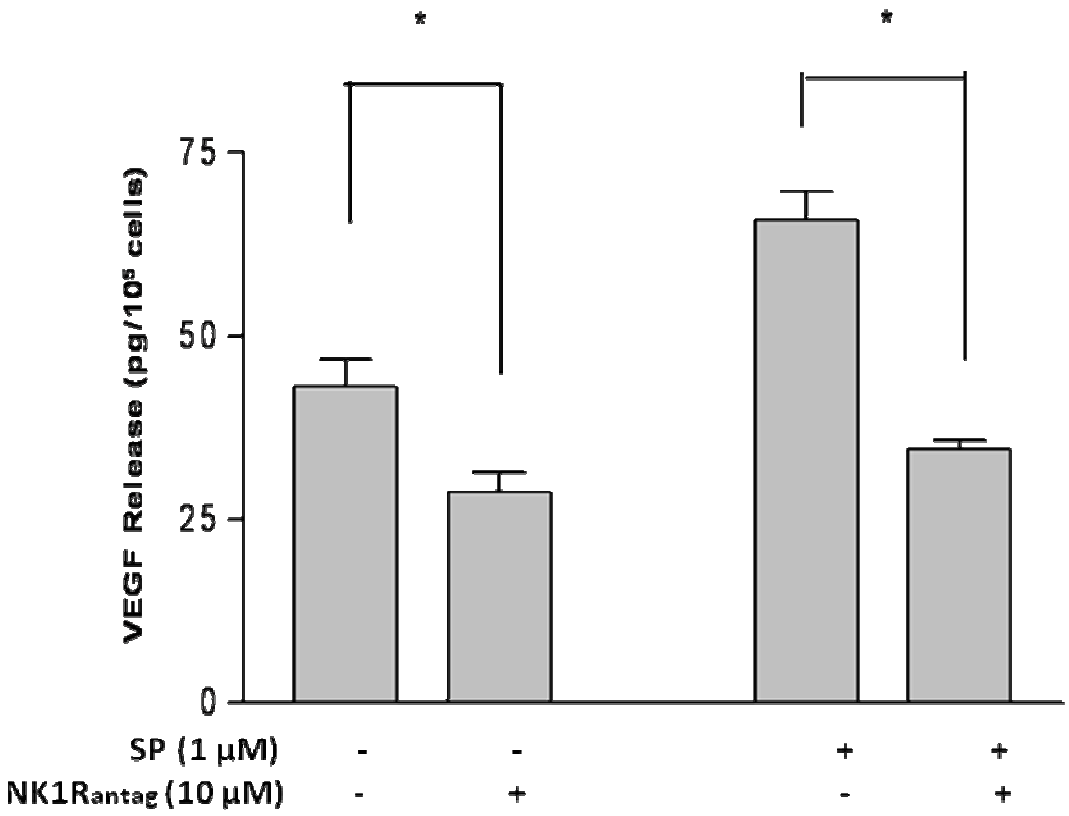

Figure 3. An NK-1 receptor antagonist inhibits SP-induced VEGF release from LAD2 cells. LAD2 cells were pre-treated with the NK-1 receptor antagonists (NK1RAntag) L-733,060 (10 μM) for 30 min and were then retained throughout stimulation with SP (1 μM) for 24 h. VEGF was measured in the supernatant fluid by ELISA. (n=3, *p<0.05).

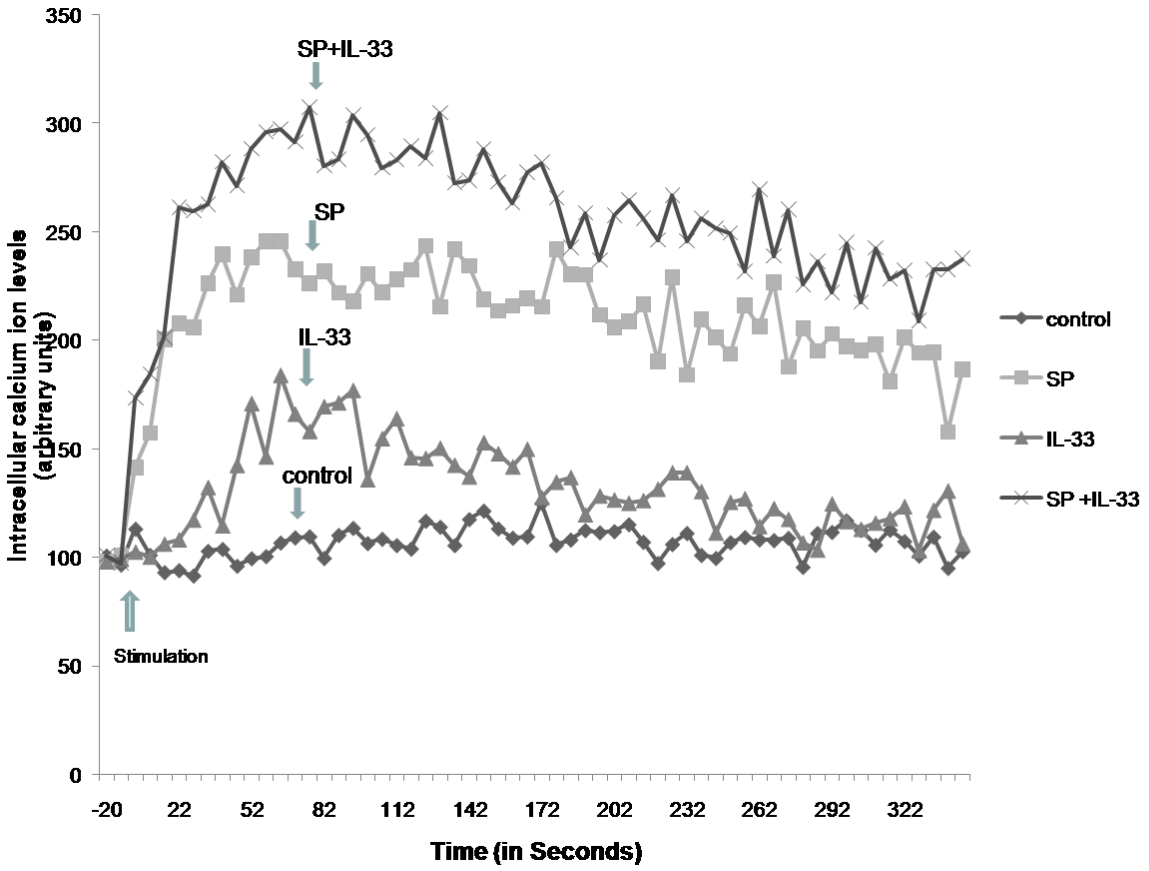

Figure 4. Effect of SP and IL-33 on LAD2 cytosolic calcium levels. Cytosolic calcium was measured in LAD2 cells using Fura-2 AM (1 mM, Invitrogen). Cells were stimulated with IL-33 (100 ng/ml) or SP (1 □M) or both for the time indicated. Results were processed according to Invitrogen's Fura-2 protocol. Stimulation was carried out in the presence of extracellular calcium (1 mM). One representative of three equivalent experiments is shown.

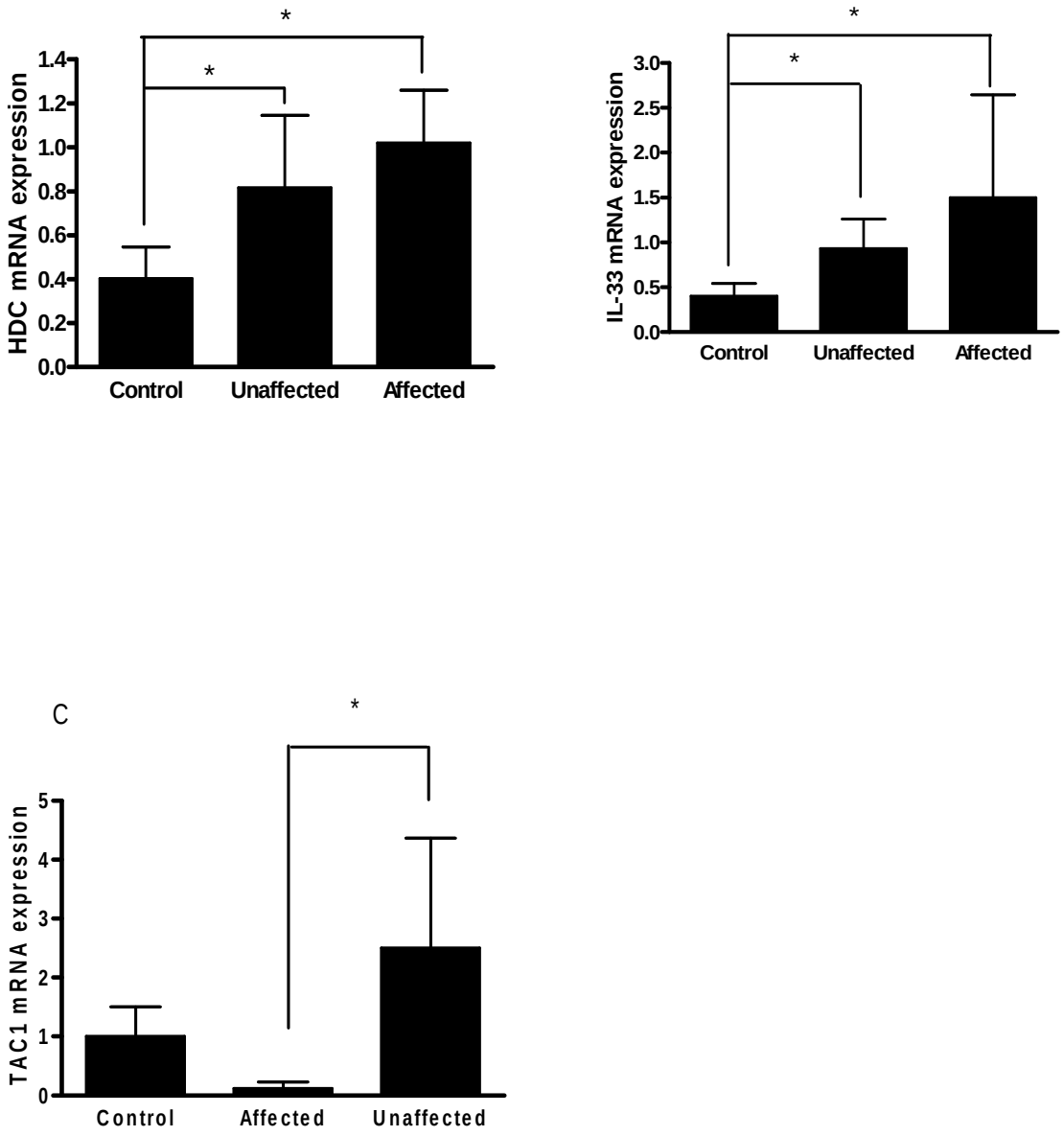

Figure 5. Increased gene expression in psoriatic affected (lesional) skin, psoriatic unaffected (at least 15 cm away from the lesion) skin, and normal skin from healthy controls. (A) IL-33 (control n=7; unaffected, n=6; affected n=9), (B) HDC (control n=5; unaffected n=6; affected n=5) and (C) TACI. Relative quantities of mRNA expression were measured by quantitative RT-PCR and normalized to 18S. TaqMan was performed

with cDNA reverse transcribed from 100 ng RNA from each sample. The number of samples were fewer for HDC because the amount of cDNA from some samples had been exhausted (*P<0.05 versus control).

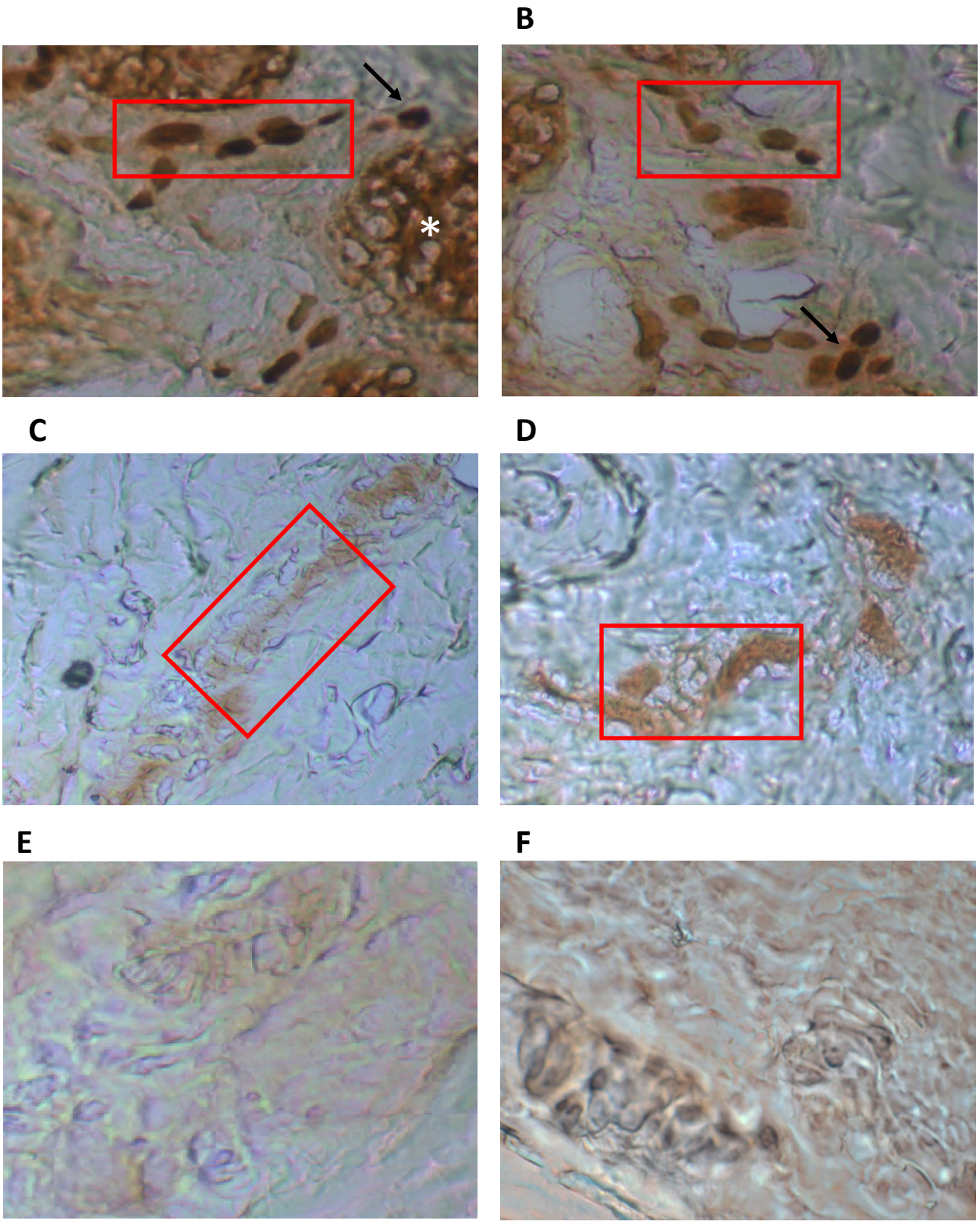
B
C
D
E
F

Figure 6. Photomicrographs of skin biopsies from patients with psoriasis (A, B) lesional, affected skin; (C, D) unaffected skin; (E, F) control without primary antibody. Immunocytochemical staining was performed using the LSAB+ system kit (DAKO, Glostrup, Denmark). Incubation with the primary antibody (mouse monoclonal-anti-human-IL-33 antibody, at 1:100 dilution, Abcam, Cambridge, MA) was performed for 30 min; secondary antibody was proved in the DAKO kit and was also used for 30 min, followed by appropriate washes. Magnification= x200; Red rectangle indicates blood vessel; asterisk indicates sweat gland; solid arrow indicates inflammatory cells.

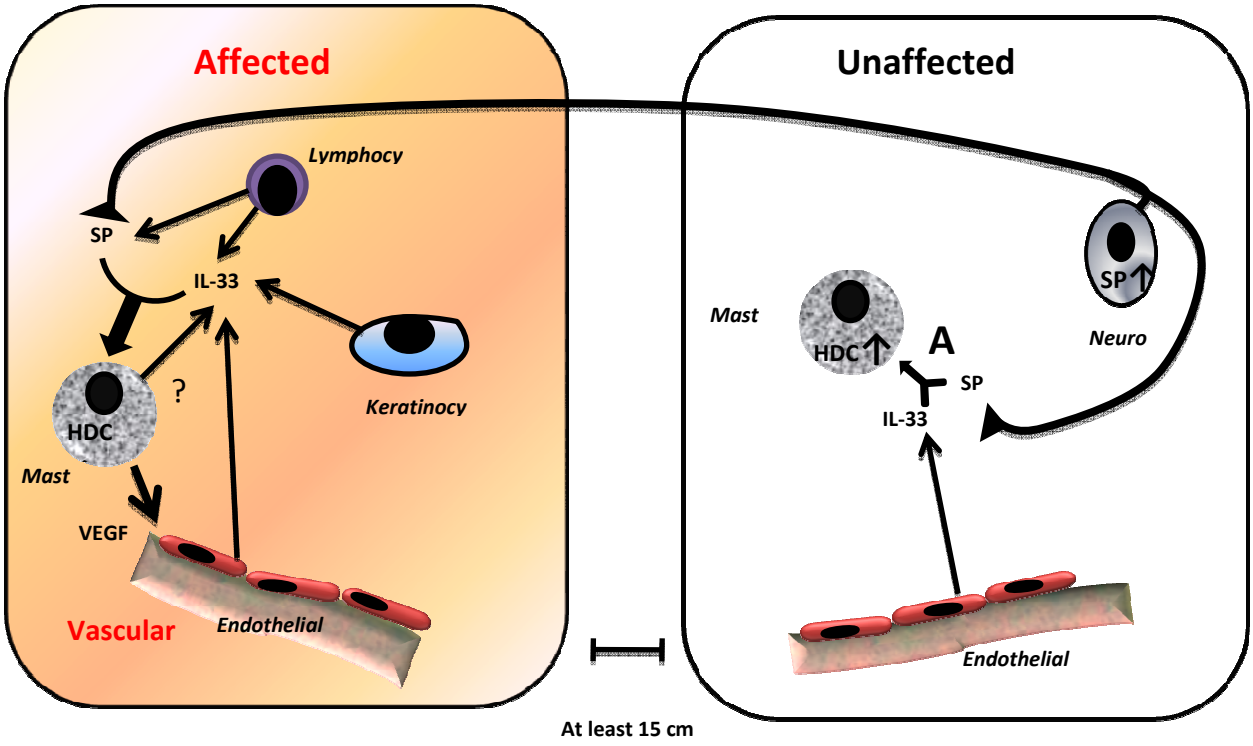

Figure 7. Diagrammatic representation of the proposed interrelationships in the unaffected and affected skin from psoriasis patients. SP synthesized in a neuron in the unaffected skin is transported and released from terminals in the affected psoriatic skin. Increased gene expression of IL-33 in the unaffected areas may derive either from endothelial, epithelial cells, and/or from increased number of mast cells as evidenced by elevated HDC expression. In the affected skin, increased IL-33 possibly coming from infiltrating lymphocytes, proliferating keratinocytes and endothelial cells can augment SP-induced mast cell release of VEGF, leading to increased vascular permeability and inflammation.

5 Discussion

We report here for the first time that IL-33 augments the SP-induced VEGF mRNA expression and VEGF protein secretion both from leukemic and normal human mast cells. IL-33 cannot induce VEGF secretion on its own. IL-33 is the newest inflammatory member of the IL-1 cytokine family [213], and we show here that IL-1 can also induce VEGF secretion from mast cells, as well as augment the effect of SP. IL-1 had previously been shown to induce VEGF secretion from inflammatory cells [325].

Here we also show for the first time that gene expression of IL-33 is increased in both affected and unaffected psoriatic skin. Gene expression of HDC, indicating increased mast cell presence/activation, is also increased in both affected and unaffected psoriatic skin, as reported previously [326].. Moreover, IL-33 in unaffected skin is weakly associated with blood vessels, while it is localized strongly with blood vessels and infiltrating inflammatory cells in the lesional affected skin. IL-33 had previously been reported to be expressed by endothelial cells [327]. These results indicate that the inflammatory process may be initiated in "unaffected" skin areas where IL-33 is initially secreted by endothelial cells and augments other non-allergic triggers, such as SP, to stimulate the mast cells. In this context, any participation of IgE is not relevant, because psoriasis is not an allergic condition, unlike atopic dermatitis which involves allergic inflammation, and where IL-33 expression was recently reported to be increased in lesional areas [214].

The receptor for IL-33 is mostly expressed on mast cells and Th2 cells, for which it acts as a chemoattractant and trigger [328]. It was recently shown that IL-33-mediated mouse anaphylaxis occurred only in the presence of IgE [214]. In contrast, IL-33 induced release of pro-inflammatory cytokines from mouse mast cells [215], especially IL-6 without degranulation from BMCMCs [132]. It also enhanced IL-8 production from hCBMCs by IgE/anti-IgE stimulation, but without histamine release [216]. IL-33 was also shown to augment the affect of IgE and Stem Cell Factor (SCF) on activating mast cells and basophils [329].

The non-peptide NK-1 receptor antagonist L-733,060 [330] blocked VEGF secretion from LAD2 cells by 100%, and also reduced basal VEGF release implying some autocrine activation. LAD2 mast cells (47) and skin mast cells (46) had previously been reported to express NK1 receptors. The NK-1 receptor is also expressed on rat basophilic leukemia (RBL) cells [331], activation of which by neuritis occurred via SP [261]. In contrast, murine bone-marrow-derived mast cells did not release histamine in response to SP, but they did produce prostaglandin D_2 and leukotrience C_4 [332]. Degranulation, as compared to de novo synthesis of selected mediators may involve direct activation of G proteins [333, 334], as shown for SP [335] and the bee venom peptide mastoparan [336, 337]. NK-1 receptor-independent activation of mast cells may involve activation of the MrgX2 receptor [263].

SP induces rapid cytosolic calcium increase in LAD2 cells, and addition of IL-33 further increases these levels, but to a lesser extent than what was recently reported for IL-33 addition to IgE-sensitized mouse mast cells [214]. Nevertheless, this augmentation of cytosolic calcium ion levels may be sufficient to lead to the synergistic VEGF release. IL-33 may also induce downstream signaling steps, such as p38 activation which was not appeared in our studies. For instance, IL-1 (same cytokine family as IL-33) increased p38 activation and VEGF release from human vascular smooth muscle cells [338]. Moreover, SP induced P38 phosphorylation independent of ERK and JNK associated with IL-6 release from human dental pulp fibroblasts [339].

SP-induced calcium increase in human skin mast cells subsequently activates calcium PKC isoforms [340]. In this report, PKC is involved, but not mandatory for VEGF induction. We also show that SP stimulates phosphorylation of both ERK and JNK MAP kinases, which can be activated by PKC-dependent and-independent mechanisms [341, 342]. Activation of these MAP kinases leads to activation of the AP-1 transcription factor, a heterodimer of c-Fos and c-Jun [343-345]. The VEGF promoter has several AP-1 binding sites that increase transcription [346], a possible explanation for the increased VEGF mRNA abundance in SP-stimulated cells. Induction of VEGF by hyperbaric oxygen in human umbilical vein endothelial cells also depended on AP-1 activation by ERK and JNK [347].

Mast cells are often located close to SP-positive neuronal processes (70, 71, [348, 349]. Mast cell-neuronal interactions may be involved in the pathophysiology of psoriasis,

and participate in the exacerbation of symptoms by stress [103, 309, 350]. The fact that SP mRNA is increased in the unaffected skin, but not in the affected areas, suggests that SP is synthesized in unaffected areas and secreted from nerve terminals in the affected site (Fig. 10). Increased HDC mRNA expression in the unaffected area indicates increased mast cell presence. These mast cells in the unaffected skin may be activated by IL-33 released from endothelial and epithelial cells [327], acting together with IgE [351]. Preliminary results (not shown) indicate that unstimulated LAD2 cells and hCBMCs express mRNA for IL-33, but not SP, suggesting that IL-33 may have autocrine effects on mast cells. Activated mast cells would then release histamine or interleukins that could activate neurons to synthesize more SP (Fig. 10). In the affected psoriatic skin, other possible sources of IL-33 may include infiltrating lymphocytes, proliferating keratinocytes, as well as endothelial cells from new vessels (Fig. 10). IL-33 would augment the effect of SP on mast cells to release VEGF, thus increasing vascular permeability and contributing to inflammation.

SP-positive nerve fibers were shown to be denser in psoriatic skin [207, 208, 311], and to have increased number of mast cell contacts compared to normal skin [202, 349]. Use of biotinylated SP suggested NK-1 expression may be increased in keratinocytes from psoriatic plaques [352]. Another study showed mast cells express the NK-1 receptor in both affected and unaffected psoriatic skin [201]. NK-1 is also important in stress-induced murine skin mast cell activation [203, 210], and in the development of atopic dermatitis in mice [353]. In fact, stress increases SP-positive nerve fibers and mast cell contacts in mice [209], while an NK-1 receptor antagonist inhibits stress-induced mast cell degranulation in

mice [354]. SP also induces mast cell-dependent leukocyte infiltration, thus amplifying the initial inflammatory response [24]. SP may contribute to the pruritus associated with psoriasis [355]. However, the effect of SP is apparently localized to the skin since plasma SP levels did not differ between psoriasis patients and controls [356].

The ability of IL-33 to augment the effect of SP on inducing mast cell release of VEGF is certainly relevant since angiogenesis [315] is at the core of psoriasis pathogenesis [312]. VEGF levels are increased in psoriatic plaques compared to normal skin [313, 314], especially the VEGF 121 isoform, which causes vascular permeability [316, 317]. Moreover, the higher VEGF expression correlates with the clinical severity of psoriasis [357, 358]. Genetic studies have shown that several different VEGF polymorphisms are associated with an increased risk of developing psoriasis [318, 319]. Moreover, transgenic delivery of VEGF in mouse skin can lead to an inflammatory state resembling psoriasis [321].

The present results is the first indication that interactions among SP, IL-33 and mast cells may be important in inflammatory diseases where there is excessive angiogenesis, such as psoriasis. They may also represent novel therapeutic

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