




Fall 11-16-2015

The Mast Cell Receptor Mas-Related Gene X2 (MrgX2) in Health and Disease

Chizobam N. Idahosa

University of Pennsylvania, chizounaks@gmail.com

Follow this and additional works at: http://repository.upenn.edu/dental_theses

 Part of the [Dermatology Commons](#), [Medical Immunology Commons](#), [Oral Biology and Oral Pathology Commons](#), and the [Periodontics and Periodontology Commons](#)

Recommended Citation

Idahosa, Chizobam N., "The Mast Cell Receptor Mas-Related Gene X2 (MrgX2) in Health and Disease" (2015). *Dental Theses*. Paper 5.

The Mast Cell Receptor Mas-Related Gene X2 (MrgX2) in Health and Disease

Abstract

Mas-related gene X2 (MrgX2) is a receptor expressed on human mast cells (MCs) but its role in health and disease is unknown. We therefore utilized immunohistochemistry and immunofluorescence techniques to determine the expression of MrgX2 in normal and diseased human tissue. We also tested the feasibility of utilizing humanized mice as a model for *in vivo* functional studies on MrgX2. We analyzed control samples as well as skin, gingiva and lung samples from patients with atopic dermatitis, rosacea, chronic periodontitis and asthma and found that MCs in healthy and diseased skin, gingiva and lungs express MrgX2. The expression of MrgX2 was not significantly different in atopic dermatitis, rosacea and chronic periodontitis compared with controls. However, in rosacea, there was a significant reduction in tryptase expression by MCs suggestive of increased degranulation. There was a significant increase in MCs, MrgX2-positive cells as well as MrgX2-positive MCs in asthmatic lung tissue compared with healthy lung tissue. Finally, we found that human MCs, which develop in the lungs of humanized mice, express MrgX2. Therefore, expression of MrgX2 by MCs in healthy skin, gingiva and lung suggests that it plays an important role in host defense. Our finding that MCs that express MrgX2 are upregulated in chronic asthma suggests it participates in the pathogenesis of asthma. Humanized mice may be used as models for future *in vivo* studies on the role of MrgX2 in human disease conditions.

Degree Type

Thesis

Degree Name

MSOB (Master of Science in Oral Biology)

Primary Advisor

Hydar Ali, PhD

Keywords

MrgX2, mast cells, atopic dermatitis, rosacea, chronic periodontitis, asthma

Subject Categories

Dentistry | Dermatology | Medical Immunology | Oral Biology and Oral Pathology | Periodontics and Periodontology

**The Mast Cell Receptor
Mas-related gene X2 (MrgX2)
in Health and Disease**

Chizobam N. Idahosa, DDS

A thesis submitted in partial fulfillment of the requirements for the
Degree of Master of Science in Oral Biology

University of Pennsylvania School of Dental Medicine

November 2015

**The Mast Cell Receptor
Mas-related gene X2 (MrgX2)
in Health and Disease**

Chizobam N. Idahosa, DDS

Hydar Ali, PhD

Principal Thesis Advisor

Sunday Akintoye, BDS, DDS, MS

Thesis Committee Member

Faizan Alawi, DDS

Thesis Committee Member

Thomas Sollecito, DMD, FDS RCS

Thesis Committee Member



University of Pennsylvania Dental Medicine

The mast cell receptor Mas-related gene X2 (MrgX2)
in health and disease

Chizobam N. Idahosa, DDS

THESIS

Presented to the Faculty of Penn Dental Medicine in Fulfillment of the
Requirements for the Degree of Master of Science in Oral Biology

A blue ink signature of Hydar Ali, PhD, written in a cursive style.

Hydar Ali, PhD

A blue ink signature of Sunday Akintoye, BDS, DDS, MS, written in a cursive style.

Sunday Akintoye, BDS, DDS, MS

A blue ink signature of Faizan Alawi, DDS, written in a cursive style.

Faizan Alawi, DDS

A blue ink signature of Thomas Sollecito, DMD, FDS RCS, written in a cursive style.

Thomas Sollecito, DMD, FDS RCS

ACKNOWLEDGEMENTS

I am especially grateful to my mentor, Dr. Hydar Ali for accepting me into his lab as a student and for giving me the opportunity to learn under him. I am thankful for the guidance, motivation and thoughtful insight he graciously provided throughout this research project.

I would like to thank Dr. Jonathan Korostoff for his help throughout my masters education, especially with the IRB process.

I would also like to thank all the members of Dr. Ali's lab especially Dr. Kshitij Gupta and Dr. Hariharan Subramanian for their patience and for answering a minimum of ten thousand questions in the last year.

I am grateful to all the faculty and staff of the Oral Medicine Department, School of Dental Medicine, University of Pennsylvania particularly Dr. Eric Stoopler and Dr. Martin Greenberg for their encouragement and support.

And I will like to express my appreciation to the members of my thesis committee, Dr. Sunday Akintoye, Dr. Faizan Alawi and Dr. Thomas Sollecito for their encouragement and for providing valuable expertise during this project.

This work was supported in part by the Penn Skin Disease Research Center (NIAMS P30-AR057217)

DEDICATION

To God:

You are my savior, shepherd, stronghold, strength, shield and sustainer.

You are my strength and my shield; my heart trusts in you and I am helped.

Psalm 28:7

ABSTRACT

Background: Mast cells (MCs) are granule containing immune cells with varied functions in both health and disease. Mas-related gene X2 (MrgX2) expressed on human but not mouse MCs functions as a binding site for a wide variety of biologically relevant cationic peptides including human beta defensins (hBDs), LL-37, eosinophil-derived major basic protein (MBP) and substance P (SP) leading to degranulation. Atopic dermatitis is a cutaneous inflammatory disease that involves MCs, eosinophils and the neuropeptide SP. Rosacea is a chronic inflammatory cutaneous disease, which involves dysregulation of LL-37 and MCs and is associated with neurogenic inflammation mediated by neuropeptides such as SP. MCs, hBDs and LL-37 are involved in the pathogenesis of chronic periodontitis. Increased numbers of MCs are present in the bronchial smooth muscles of patients with asthma. Furthermore, neutrophils and eosinophils play important roles in the last phase of allergic asthma. However, possible roles of MrgX2 on host defense and inflammatory diseases have not been determined.

Objectives: We sought to determine if human skin, gingiva and lung MCs express MrgX2 and to test if its expression is upregulated in atopic dermatitis, rosacea, chronic periodontitis and asthma. We investigated the localization of MrgX2 before and after stimulation with a known ligand cortistatin and finally we tested the feasibility of utilizing humanized mice as a model for *in vivo* functional studies on MrgX2.

Methods: We analyzed skin samples from 3 patients with atopic dermatitis, 2 patients with rosacea and 3 control subjects. For the study on chronic periodontitis, we analyzed gingiva tissue from 3 patients with chronic periodontitis and 1 control subject. Lung tissue samples from 6 patients who died of asthma related complications and 6 control subjects that died of other causes as well as humanized mice lung tissue were also analyzed. Immunohistochemical analysis

was used to determine the presence of MCs in relation to structural cells in the tissues. Immunofluorescence double-staining method was used to detect the expression of MrgX2 by MCs. RBL-2H3 cells transiently expressing MrgX2 were used to investigate receptor localization.

Results: MCs in healthy and diseased skin, gingiva and lungs express MrgX2. The expression of MrgX2 was not significantly different in the skin of patients diagnosed with atopic dermatitis and rosacea compared with control subjects. We did not quantitate the expression of MrgX2 in chronic periodontitis due to limited sample size, however MCs in gingiva of chronic periodontitis patients express MrgX2. There was a significant increase in MCs, MrgX2-positive cells as well as MrgX2-positive MCs in asthmatic lung tissue compared with healthy lung tissue. Using RBL-2H3 cells, we found that MrgX2 was expressed on the plasma membrane as well as intracellularly and on stimulation, we found evidence of possible mobilization of the receptor. Finally, we found that human MCs, which develop in the lungs of humanized mice, express MrgX2.

Conclusions: Expression of MrgX2 by MCs in healthy skin, gingiva and lung suggests that it plays an important role in host defense by enabling crosstalk between MCs, hBDs expressed by epithelial cells and LL-37 expressed by neutrophils. Our finding that MCs that express MrgX2 are upregulated in chronic asthma suggests it participates in the pathogenesis of asthma. Given that hBD3, LL-37 and eosinophil-derived peptides are increased in periodontitis, rosacea and AD, respectively suggest that MrgX2 contributes to these diseases via increased MC degranulation. Finally, humanized mice may be used as models for future *in vivo* studies on the role of MrgX2 in human disease conditions.

TABLE OF CONTENT

AKNOWLEDGEMENT _____	4
DEDICATION _____	5
ABSTRACT _____	6
TABLE OF CONTENT _____	8
LIST OF ABBREVIATIONS _____	10
1 INTRODUCTION _____	12
1.1 Mast Cells _____	12
1.1.1. Biology of mast cells _____	12
1.1.2. Functions of mast cells _____	13
1.1.3. Mast cells and antimicrobial peptides _____	14
1.1.4. Mast cells and the nervous system _____	15
1.1.5. Mast cells and wound healing _____	16
1.1.6. Mast cells and homeostasis _____	17
1.1.7. Mast cell activation _____	17
1.2 Mas-related gene X2 (MrgX2) _____	18
1.2.1. What is MrgX2? _____	18
1.2.2. Receptor localization and internalization _____	20
1.3 Mast cells and MrgX2 in disease _____	21
1.3.1. Role of mast cells and MrgX2 in chronic urticarial _____	21
1.3.2. Role of mast cells in atopic dermatitis _____	22
1.3.3. Role of mast cells in rosacea _____	25
1.3.4. Role of mast cells in periodontal disease _____	28
1.3.5. Role of mast cells in asthma _____	30
1.4 Humanized mice as a model for <i>in vivo</i> studies on MrgX2 _____	32

2	RESEARCH AIMS	34
3	MATERIALS AND METHODS	34
3.1	Sample collection	34
3.2	Materials	35
3.3	Methods	36
3.3.1.	Immunohistochemistry	36
3.3.2.	Immunofluorescence	37
3.3.3.	Transfection of RBL-2H3 cells	38
3.3.4.	Degranulation	40
3.3.5.	Cell counting and analysis	40
3.3.6.	Statistical analysis	42
4	RESULTS	42
4.1	Expression of MrgX2 in atopic dermatitis and rosacea	42
4.2	Expression of MrgX2 in chronic periodontitis	45
4.3	Expression of MrgX2 in asthma	46
4.4	MrgX2 localization	47
4.5	Effect of LL-37 and SP-induced degranulation	48
4.6	Expression of MrgX2 in humanized mice lung	48
5	DISCUSSION	49
6	CONCLUSION	55
7	FIGURES	58
8	FIGURE LEGEND	76
9	BIBLIOGRAPHY	83

LIST OF ABBREVIATIONS

1. AD: Atopic dermatitis
2. AMPs: Antimicrobial peptides
3. CDC: Centers for Disease Control and Prevention
4. CGRP: Calcitonin gene-related peptide
5. CP: Chronic periodontitis
6. CST: Cortistatin
7. CTMCs: Connective tissue type mast cells
8. CU: Chronic urticaria
9. FGF: Fibroblast growth factor
10. GPCRs: G protein coupled receptors
11. GRKs: G protein coupled receptor kinases
12. H and E: Hematoxylin and eosin
13. HA: Hemagglutinin
14. hBDs: Human beta defensins
15. HSC: Hematopoietic stem cells
16. HSV: Herpes simplex virus
17. IF: Immunofluorescence
18. IHC: Immunohistochemistry
19. KLK5: Kallikerin 5
20. MBP: Major basic protein
21. MCDP: Mast cell degranulating peptide

- 22. MCP-1: Macrophage chemotactic protein-1
- 23. MCs: Mast cells
- 24. MMCs: Mucosal mast cells
- 25. Mrgprs: Mas-related G protein coupled receptors
- 26. Mrgs: Mas related genes
- 27. MrgX2: Mas-related gene X2
- 28. PACAP: Pituitary adenylate cyclase-activating peptide
- 29. PDGF: Platelet-derived growth factor
- 30. SCF: Stem cell factor
- 31. SP: Substance-P
- 32. TGF: Transforming growth factor
- 33. TLR2: Toll-like receptor 2
- 34. TNF: Tumor necrosis factor
- 35. VEGGF: Vascular endothelial-derived growth factor
- 36. VIP: Vasoactive intestinal polypeptide
- 37. WT: Wild type

1. INTRODUCTION

1.1 Mast cells

1.1.1. Biology of Mast cells

Mast cells (MCs) are multifunctional immune cells that are derived from hematopoietic stem cells (HSCs) in the bone marrow but differentiate locally in the tissues (1, 2). MCs are present in almost all vascularized tissue, being more abundant at sites that are exposed to external pathogens and allergens such as the skin and the mucosal surfaces of the gastro-intestinal and respiratory tracts (3, 4). They are usually found under the epithelium residing in the connective tissue adjacent to blood vessels and in close proximity to nerve fibers (4-8). The growth, development and maturation of MCs are regulated by the c-kit ligand, stem cell factor (SCF) as well as other factors including nerve growth factor, neurotrophin-3, IL-3 and the Th2 associated cytokines IL-4 and IL-9 (9, 10).

Human MCs are classified into two subtypes based on the composition of their secretory granules. Thus, MC_T, predominantly express tryptase while MC_{TC} express both tryptase and chymase (11). MC_{TC} are more prominent in skin and intestinal submucosa while MC_T are more prominent in the lung alveolar wall and intestinal mucosa (12). Mouse MCs are classified into connective tissue type MCs (CTMCs) and mucosal MCs (MMCs). However, MCs are heterogenous and changes in subtypes can be modulated by environmental factors and differences in stimuli (3, 13).

Upon activation, MCs release a diverse range of potent inflammatory mediators which can be classified into (1) pre-formed mediators including histamine, serotonin, renin and proteases such as tryptase, chymase and carboxypeptidase-A (2) newly synthesized lipid mediators such as leukotrienes, prostaglandins and (3) chemokines, growth factors and cytokines including tumor necrosis factor (TNF), IL-4, IL-5, IL-6, IL-13, macrophage chemotactic protein-1 (MCP-1), platelet-derived growth factor (PDGF), vascular endothelial-derived growth factor (VEDGF), transforming growth factor (TGF) and fibroblast growth factor (FGF) (2, 4, 8, 9).

1.1.2. Functions of MCs

The role of MCs in allergy and anaphylaxis has been extensively studied (8, 14, 15). However, MCs play an important role in inflammatory and autoimmune conditions, as well as in host defense against various pathogens including parasites, bacteria and viruses (16, 17). Because of their ubiquitous location in vascularized tissue, MCs are one of the first immune cells to interact with environmental antigens and pathogens (4). They also play a pivotal role in maintaining a healthy physiology by promoting innate immunity, angiogenesis and wound healing (18, 19). MCs are involved in regulating the differentiation of naïve T-cells, the development of B cells, as well as the migration, recruitment and activation of dendritic cells, T-cells, neutrophils, eosinophils and monocytes (20-22). MC-derived histamine and proteases potently induce vasodilatation and vascular permeability, which enhances the migration of leucocytes to sites of

inflammation, infection and tissue repair (2, 10). Other MC mediators such as leukotrienes, proteases and cytokines also act as chemotactic signals for neutrophils, basophils and eosinophils (23).

MCs also promote the initiation and generation of the adaptive immune response by expressing both major histocompatibility complex (MHC) class I and class II proteins via which they present antigens to T cells (24-26).

1.1.3. MCs and Antimicrobial peptides

Antimicrobial peptides (AMPs) such as defensins and cathelicidins are small positively charged amphipathic molecules, which are crucial in the clearance of microbial pathogens and thus play an important role in host defense. They interact with negatively charged phospholipid moieties on microbes resulting in membrane destabilization, disruption and killing of the offending pathogen (27). In addition to their anti-microbial properties, AMPs also modulate the immune system, angiogenesis and wound healing (28-30). In humans, defensins are characterized into α and β -defensin families depending on the position of cysteine residues that are involved in disulphide linkages (31). α -defensins are produced by polymorphonuclear leucocytes and intestinal paneth cells while human β - defensins (hBDs) are produced primarily by epithelial cells (30). Of the four members of the hBD family that have been characterized in detail (hBD1-4), hBD1 is expressed constitutively, while the rest are induced by bacteria, viruses and cytokines (32). LL-37 is produced by leucocytes as an inactive precursor

(hCAP18), which is enzymatically cleaved to release the active LL-37. Its gene expression is induced by infection, inflammation and 1,25-dihydroxyvitamin D3 (33).

MCs interact with AMPs. Both hBDs and LL-37 induce Ca^{2+} mobilization, chemotaxis and degranulation in MCs (34-37). Niyonsaba et al showed that epithelial cell derived hBD2 acts as a chemo-attractant for MCs (38). The same group also showed that in addition to its anti-microbial properties, LL-37 induces MC chemotaxis thereby participating in their recruitment to sites of inflammation (39). MC degranulation mediated by these AMPs results in the release of mediators such as histamine, which causes vasodilation and increased vascular permeability resulting in the recruitment of leucocytes and immune cells necessary for combating the microbial pathogens (40).

1.1.4. MCs and the nervous system

MCs are localized near nerve endings at several anatomical sites such as the skin, lungs, intestinal mucosa and central nervous system. They interact with neurons in a bi-directional manner to affect various physiological functions such as wound healing and stress response (41, 42). Histamine, serotonin and tryptase released from MCs have an impact on the activity of sensory neurons, and conversely, MCs are activated by neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P (SP) released by nerve terminals of sensory neurons (43).

The neuropeptide SP, released from nerve terminals in the central and peripheral nervous system has a key function in stress response, neurogenic inflammation and pain (43, 44). Human MCs have been shown to degranulate and release mediators in response to SP (45-47). Other neuropeptides, which activate MCs include vasoactive intestinal polypeptide (VIP) and neuropeptide Y (46, 48, 49).

The skin has a very rich supply of sensory nerve endings, which release SP and other neuropeptides leading to MC degranulation in response to the continuous exposure to physical as well as emotional stimuli. Therefore, the interaction between MC and neuropeptides results in the promotion of MC-driven inflammation and granulocyte infiltration (44, 50).

1.1.5. MCs and wound healing

MCs play pivotal roles in all stages of wound healing: the initial inflammatory response, revascularization and re-epithelialization of the damaged tissue, deposition of temporary connective tissue and finally remodeling of the matrix (23, 51). During wound repair, the MC mediators, histamine, heparin, cytokines (TNF, IL-6 and IL-8) and growth factors (PDGF, VEGF, TGF and FGF) modulate the stages of neovascularization thereby contributing to the regrowth of the endothelial cells (42, 52). MC mediators histamine, tryptase, TGF and the cytokines IL-1, IL-4 and TNF are able to stimulate chemotaxis, migration, phenotypic differentiation and activation of fibroblasts (23). MCs can also modulate keratinocyte proliferation and migration during the re-epithelialization

process of wound healing (53, 54) and at the final stages of the wound healing process, MCs influence the structural re-modeling of newly formed capillaries by releasing angiogenic factors (55).

1.1.6. MCs and homeostasis

MCs play a role in the control of tissue remodeling. They are involved in the regulation and maintenance of organs that undergo continuous growth and structural remodeling such as hair follicles and bones (56). Hair follicle cycling through periods of rest and growth is impaired in MC-deficient mice and MC-derived histamine and TNF have been implicated in regulating hair follicle transformation from resting to active hair growth (56, 57). MCs also contribute to bone remodeling. The amount of new bone matrix synthesized in MC-deficient mice is diminished compared to wild type (WT) mice suggesting that MCs and their mediators influence the recruitment of osteoblast and osteoclast progenitors during bone remodeling (58). MCs have also been reported to secrete osteopontin, a glycoprotein that is involved in immune responses and controls bone metabolism (59).

1.1.7. MC activation

MCs express the high affinity Fc receptor for IgE known as FcεRI on their cell surface. During IgE dependent immune responses, activation and degranulation of MCs occurs with crosslinking of the high affinity receptor by IgE/antigen complexes (3, 9). In addition to IgE mediated degranulation of MCs, multiple non-

IgE mediated mechanisms are also known to trigger MC secretion including activation via the G-protein coupled receptor, Mas-Related Gene X2 (MrgX2) expressed on MCs.

1.2 Mas-related gene X2 (MrgX2)

1.2.1. What is MrgX2?

The G protein coupled receptors (GPCRs) are one of the largest known family of transmembrane proteins. Mas-related G protein coupled receptors (Mrgprs) belong to the GPCR family and are divided into several subfamilies. In humans four Mrg genes, MrgX1 – MrgX4 are known, while in mice, the Mrg family comprises of 32 different Mrg coding genes (60). Mrgprs are also referred to as sensory neuron specific GPCRs because they were originally thought to be exclusively expressed in dorsal root ganglia (60, 61). However, it has now been shown that MrgX2 is expressed in LAD2 (a human MC line) and CD34⁺ cell derived MCs while human cord blood-derived MCs express both MrgX1 and MrgX2 (62-64). Also, a higher expression level of MrgX2 mRNA has been reported in skin-derived cultured MCs compared to lung-derived cultured MCs (65).

From prior studies on MCs, pertussis-sensitive G proteins have been shown to be activated by a wide range of endogenous and exogenous polycationic molecules collectively known as basic secretagogues (66). Neuropeptides such as SP and VIP are basic secretagogues, which play a role in the pathogenesis of

chronic urticaria, atopic dermatitis and other inflammatory skin conditions (67, 68).

MrgX2 has now been shown to function as the non-selective, low-affinity binding site for a wide range of basic secretagogues such as SP, cortistatin (CST), somatostatin, mast cell degranulating peptide (MCDP), neuropeptide Y, compound 48/80 and VIP (62, 64, 69, 70). Although these peptides are structurally unrelated, they are all amphipathic small peptides, which induce dose-dependent degranulation of human MCs via the activation of MrgX2 with an associated increase in the intracellular Ca^{2+} concentration (64, 70).

Subramanian et al also showed that hBDs and the cathelicidin LL-37 activate human MCs via MrgX2 (32, 63). In their study, LL-37 and the neuropeptide CST induced sustained Ca^{2+} mobilization in LAD2 cells and CD34⁺-cell-derived primary human MCs, which are known to endogenously express MrgX2. Substantial MC degranulation with associated increased intracellular Ca^{2+} mobilization was induced by hBD2, hBD3 and CST in a rodent MC line, RBL-2H3 stably expressing MrgX2 (32). Therefore, MrgX2 functions as a non-selective binding site that links these basic peptides to G proteins, leading to the secretion of histamine and other mediators by MCs (71).

To facilitate *in vivo* studies on the function, activation and downstream effects of MrgX2, it is important to identify the mouse counterpart of the human receptor.

There are significant differences between Mrg receptors in different species. Human and mouse Mrg receptors share only 45-65% amino acid sequence identity making it difficult to determine the mouse orthologue (64). In a study by Subramanian et al, they found that degranulation of murine MCs was resistant to activation by hBDs while human MCs were susceptible highlighting the possibility that there may be differences between the human and murine receptors (32). However, in a recent study by McNeil et al, they reported that basic secretagogues activated mouse MCs *in vitro* and *in vivo* through Mrgbrb2, which they concluded to be the mouse counterpart of the human MrgX2 receptor (72).

1.2.2. Receptor localization and internalization

Receptor desensitization and internalization are key regulatory mechanisms involved in GPCR signaling. Activated GPCRs are phosphorylated by a group of serine/threonine protein kinases known as G protein coupled receptor kinases (GRKs) with subsequent recruitment of β -arrestin. This results in desensitization and internalization of the receptors with resultant inhibition of function (73, 74). It has been reported that MrgX2 is resistant to agonist-induced receptor phosphorylation, desensitization and internalization, which is contrary to established reports of the behavior of most GPCRs such as C3aR, the receptor for the anaphylatoxin C3a (63, 75). In the study by Subramanian et al., MrgX2 was relatively resistant to phosphorylation after stimulation with CST and LL-37 in HEK-293T cells transfected with hemagglutinin (HA)-tagged MrgX2 compared to C3aR stimulation with the corresponding ligand, C3a. Using Ca^{2+} mobilization

assay, they demonstrated that MrgX2 did not undergo desensitization. Instead, sustained intracellular Ca^{2+} mobilization was noted in LAD2 cells after stimulation with CST and LL-37. Also, MrgX2 was not internalized in HMC-1 cells stably expressing HA-tagged MrgX2 upon stimulation with LL-37 in contrast to C3aR (63).

Unlike most GPCRs, MrgX2 is expressed intracellularly. Fujisawa et al reported localization of MrgX2 in the cytoplasm of LAD2 cells and in the same study, they determined that MrgX2 was expressed intracellularly in human adult peripheral blood-derived cultured MCs (65).

1.3 MCs and MrgX2 in disease

Under healthy conditions, MC numbers are relatively constant. However, in inflammatory conditions and infection, their numbers are increased.

1.3.1. Role of MCs and MrgX2 in chronic urticaria

Chronic urticaria (CU) is defined as the presence of daily hives for at least six weeks (76). MCs are involved in the pathogenesis of CU through the release of various inflammatory mediators (77, 78). Neuropeptides are also involved in the pathogenesis of CU. The neuropeptide SP is upregulated in the serum of patients with chronic spontaneous urticaria (79) and a study has shown that intradermally injected SP in CU patients led to enhanced and longer lasting wheal reactions compared with controls (80). Urticaria is associated with

eosinophil infiltration and the deposition of eosinophil-derived major basic protein (MBP) (81). Recently, Fujisawa et al. reported that the number and percentage of MrgX2 positive MCs is increased in the skin of patients with severe chronic urticaria compared with control subjects (65). Since MBP is a cationic peptide, which has been reported to activate MCs (82), in the same study by Fujisawa et al., they investigated the possibility that MrgX2 is the receptor for MBP and found that MBP induced histamine release from human skin MCs through MrgX2 (65). Thus, it is likely that MrgX2 expressing MCs are also involved in the pathogenesis of other inflammatory disease conditions involving MCs including but not limited to atopic dermatitis, rosacea, periodontal disease and asthma.

1.3.2. Role of MCs in atopic dermatitis

Atopic dermatitis (AD), synonymous with atopic eczema is a chronic inflammatory relapsing disease of the skin, which often precedes other allergic disorders such as food allergy, asthma and allergic rhinitis (83, 84). It is the result of a complex interaction between genetic, immunological and environmental factors. The onset of AD is usually in early childhood, most commonly between 3 and 6 months of age. However a small percentage of patients develop their first symptoms in adulthood (85). It affects 15-20% of children and 1-3% of adults worldwide (86).

The diagnosis of AD is based on a constellation of clinical features, which include persistent pruritus, age-dependent eczematous eruptions, a chronic relapsing course, early age of onset, atopy and xerosis (85). Pruritus is often worse at night

resulting in sleep disturbance and impaired quality of life. The cheeks and scalp are typically affected in infants, while adolescents and adults usually present with lichenified plaques affecting the head, neck and flexures (87). Histologically, AD is characterized by spongiosis which is a non-specific histological pattern seen in various eczematous reactions.

There is a genetic predisposition to the development of AD. Approximately 70% of AD patients have a positive family history of atopy (88). Also, the rate of AD is 77% among monozygotic twins compared to 15% in dizygotic twins (89). Loss of function mutations in the filaggrin gene that result in dysfunction of the skin barrier are strongly associated with AD (90-92). Filaggrin is involved in maintaining the epidermal barrier and integrity. Hence filaggrin gene mutations result in impairment of the skin barrier function with resultant increase in transepidermal water loss and microbial infections (90). AD patients are therefore susceptible to colonization and infection with *Staphylococcus aureus* as well as herpes simplex virus (HSV) (93).

The expression levels of the AMPs, hBD2 and LL-37 are downregulated in AD lesions. Both AMPs are involved in the control of microbial infections and are very potent against the microbes of interest in AD. In addition, they have chemotactic properties that link the innate and adaptive immune responses and LL-37 also plays a role in maintaining normal epidermal permeability barrier function (94). Therefore in AD, the decreased levels of AMPs amplify the susceptibility to infection as well as the barrier dysfunction.

In acute lesions of AD, MCs are degranulated but not increased in number. However, the number of MCs in chronic AD lesions is significantly increased (95-98). The increased number of MCs could either be the result of recruitment from other sites or the proliferation of resident cells (99). Dense dermal infiltration of MCs was noted in an animal model of AD providing more evidence for their role in the pathogenesis of AD (100).

MCs may play a role in the pathogenesis of AD by contributing to Th2 polarization in skin lesions of AD patients through the production of IL-4 and IL-13. AD is characterized by an increased Th2 response (101) and MCs are a major source of IL-4 and IL-13 in AD (102, 103). Using an animal model, Spergel et al showed that IL-4 might play an important role in the inflammation and hypertrophy of the skin in AD (104). Other mediators of MC degranulation such as histamine and tryptase contribute to the development of pruritus and skin barrier defects in AD (90).

Inflammation induced by neuropeptides, which are released from sensory nerves in response to psychological stress as well as other physical stressors is known as neurogenic inflammation (105). MCs are able to maintain neurogenic inflammation in AD through activation by neuropeptides such as SP released by cutaneous nerves. Jarvikallio et al showed that dermal contacts between MCs and nerves were increased in number in both lesional and non-lesional skin of AD patients compared with normal controls. They also found that SP fibers were more frequent in lesional papillary dermis compared to nonlesional in AD (106).

On the same note, SP plasma level in AD patients is reportedly higher than in controls (107).

Eosinophilia is present in most patients with AD and correlates with disease activity (99). Eosinophil degranulation with significant dermal deposition of eosinophil-derived MBP in AD has also been demonstrated and also correlates with disease activity (108). Therefore, we hypothesized that MrgX2 is involved in the pathogenesis of AD and MCs may be activated by eosinophil-derived MBP as well as neuropeptides such as SP via MrgX2 resulting in the release of histamine and Th2 cytokines.

1.3.3. Role of MCs in rosacea

Rosacea is chronic inflammatory cutaneous disorder, which primarily involves the facial skin. It is a relatively common dermatological condition, which according to the National Rosacea Society affects approximately 16 million people in the United States. It affects women more frequently than men (109) with the onset of symptoms usually occurring between 30-50 years of age (110). A genetic component has been implicated in the occurrence of rosacea, as there is a higher incidence in fair-skinned Caucasians compared to those of other ethnicities (111, 112).

The clinical course of rosacea is characterized by intermittent episodes of exacerbation followed by remission. Patients present with variable symptomatology, which include, non-transient centrofacial erythema, flushing,

telangiectasia, stinging, burning sensation, dryness, scaling, papules, pustules, phymatous growths and red watery eyes (113, 114). Based on these clinical features, rosacea has been classified into four subtypes:

(a) Erythematotelangiectatic rosacea (ETR) characterized by prolonged flushing, persistent erythema and telangiectasia (b) Papulopustular rosacea, which is characterized by papules and pustules in addition to the features of ETR (c) Phymatous rosacea characterized by skin thickening and irregular nodularities mostly affecting the nose and (d) ocular rosacea characterized by watery bloodshot eyes, burning and stinging sensation in the eyes (110, 115). Exposure to ultraviolet light radiation, stress, extremes of temperature and the consumption of hot beverages, alcohol and spicy foods are some of the known triggers for rosacea exacerbations (116, 117).

The pathophysiology of rosacea has not been fully elucidated, however it has been shown to involve dysregulation of the immune, vascular and nervous systems. Expression levels of the AMP LL-37 has been shown to be upregulated in patients with rosacea (118, 119). LL-37 has antimicrobial, pro-inflammatory and angiogenic properties and is induced in keratinocytes by the activation of the Vitamin D pathway via UV light (120). Likewise, in lesional rosacea skin, there is increased expression of the epidermal trypsin-like serine protease enzyme, kallikerin 5 (KLK5), which is responsible for selectively cleaving the inactive precursor protein (hCAP18) to form the biologically active LL-37 (119, 121, 122). Also found in rosacea skin are abnormal peptide fragments of both LL-37 and

KLK5, which are more pro-inflammatory and differ from those found in healthy skin (119). The expression of toll-like receptor 2 (TLR2), which is an innate immune system pattern recognition receptor is increased in rosacea. TLR2 is possibly activated by environmental factors and structural molecules on microbes such as chitin released from Demodex mites, which are commensal mite species ubiquitous to human skin. Elevated TLR2 expression in rosacea results in increased production of KLK5, which cleaves hCAP18 leading to the production of LL-37 (123, 124). Thus LL-37 plays a central role in the pathogenesis of rosacea.

MCs have been shown to be involved in the pathogenesis of rosacea. Studies have revealed significantly greater number of MCs in rosacea lesions compared to non-lesional skin (125, 126). In addition to keratinocytes and neutrophils, LL-37 is also produced from MCs (127). In a study by Muto et al, MC deficient mice did not develop inflammation following LL-37 injection whereas WT mice developed rosacea-like inflammation (128). In the same study they also showed that activation of MCs by LL-37 led to the secretion of MC proteases. MMP-9 is secreted by activated MCs and is upregulated in rosacea (128, 129). MMP-9 cleaves the precursor of KLK5 into the active form of KLK5 also leading to the generation of LL-37. Therefore both LL-37 and MCs play critical roles in the pathogenesis of rosacea.

Neurogenic inflammation mediated by multiple neuropeptides such as SP, VIP and pituitary adenylate cyclase-activating peptide (PACAP) has been associated with rosacea flare-ups. MCs release their contents upon activation by neuropeptides. In an immunohistochemistry study by Wollina et al, there was a denser distribution of the VIP receptor positive cells in rosacea lesions compared to controls showing the VIP may contribute to the pathophysiology of rosacea (130). Increased expression of SP has also been reported in rosacea lesions (131). Likewise, tryptase mRNA expression was significantly increased by PACAP stimulation in WT mice compared to MC deficient mice (128). Thus, the findings that (a) MC numbers are increased in rosacea (b) expression levels of LL-37 is upregulated in rosacea (c) neurogenic inflammation mediated by neuropeptides is associated with rosacea flare-ups and (d) both LL-37 and neuropeptides are MrgX2 ligands suggest that MrgX2 expressed in MCs contribute the pathogenesis of rosacea. However, it is unknown if MrgX2 is expressed in skin of rosacea patients.

1.3.4. Role of MCs in periodontal diseases

The periodontium consists of the gingiva, periodontal ligament, cementum and alveolar bone. Periodontal disease refers to a group of inflammatory conditions that affect these structures. Gingivitis is the form of periodontal disease, which involves the gingiva and is not associated with destruction of the periodontal ligament or alveolar resorption while periodontitis involves destruction of the attachment apparatus. Chronic periodontitis (CP) is an infectious disease, which

results in progressive attachment and bone loss as a consequence of inflammation within the supporting tissues of the teeth.

Dental plaque is considered the principal etiological factor for periodontal disease as a result of colonization of the gingival sulcus by microorganisms within the dental plaque, which promote an inflammatory response in the periodontal tissues. However, the pathogenesis of periodontal disease involves a complex multifactorial interaction between plaque microorganisms and the host response. Various social, behavioral, systemic and genetic factors can modify the susceptibility of the host to periodontal disease. Both inflammatory and immunologically mediated pathways are upregulated in the presence of bacterial plaque resulting in the production of pro-inflammatory mediators by cells of the immune system, which are subsequently responsible for causing the damage seen in periodontal disease.

MCs are thought to be involved in the pathogenesis of periodontal disease. Studies by Huang et al showed a correlation between MC density, degree of degranulation and the severity of human periodontitis (132). Other studies have also documented an increase in the number of MCs in the gingiva of patients with periodontal disease compared with clinically healthy gingival tissue (133, 134). Activated MCs release histamine, proteases, cytokines and other mediators, which perpetuate the immune response and contribute to the healing process as well as the destructive process.

The AMPs such as hBDs, which are produced by epithelial cells activate human MCs via MrgX2 (32). A study by Lui J. et al reported significantly higher basal hBD3 expression in keratinocytes from periodontitis patients compared with healthy controls (135). LL-37, a cathelicidin derived from neutrophils activates human MCs via MrgX2. Expression of LL-37 is increased in gingival crevicular fluid of patients with chronic periodontitis compared with healthy subjects (136, 137). These findings suggest that AMPs may play facilitate the immunological response in chronic periodontitis through the activation of MCs via MrgX2. However, the possibility that human gingival tissue MCs express MrgX2 has not been tested.

1.3.5. Role of MCs in asthma

Asthma is a multifaceted airway inflammatory disease characterized by reversible airflow obstruction, airway inflammation, persistent airway hyper-responsiveness and airway remodeling which results in recurring incidents of wheezing, breathlessness, chest tightness and coughing (138-140). According to the Centers for Disease Control and Prevention (CDC), asthma affects 7.3% of Americans with the burden of disease resulting in reduced quality of life, hospitalizations and deaths.

The etiology of asthma involves a complex interplay between genetic and environmental factors. Allergic or extrinsic asthma, which is the most common form of asthma, is triggered by inhaled allergens such as pollen, dust, mites and

animal dander. Sensitization occurs upon initial exposure to an allergen and on subsequent exposure to the same allergen, the clinical symptoms of asthma occur. During sensitization, inhaled allergen is intercepted by dendritic cells, which function as antigen presenting cells. The antigen is processed by dendritic cells and subsequently presented to antigen-specific T-cells, which secrete cytokines that stimulate B cells to undergo class switching to IgE-producing B cells. The Fc portion of the circulating IgE then binds to MCs and basophils via the high affinity Fc receptor for IgE known as FcεRI on their cell surfaces. Upon re-exposure to the sensitizing allergen, activation and degranulation of MCs occurs with crosslinking of the high affinity receptor by IgE/antigen complexes resulting in the release of inflammatory mediators, which initiate an immediate acute phase reaction manifested as bronchospasm (140, 141).

MC_{TC} are more prominent in skin while normal human lung MCs are predominantly of the MC_T phenotype with the minority being MC_{TC}. These MC_T are found in the alveolar wall and epithelium of the lung while MC_{TC} are preferentially located in the bronchial smooth muscles and glandular areas (142). However, markedly increased numbers of MC_{TC} are present in the bronchial smooth muscles of patients with severe asthma (143-146). It has been shown that normal skin MCs which are typically MC_{TC} express GPCRs for MrgX2 while normal lung MCs do not express these receptors (147). These findings suggest that MrgX2 expressed in asthmatic lung MC_{TC} could participate in the pathogenesis of asthma.

Human rhinovirus (HRV) and Respiratory syncytial virus (RSV) which exacerbate asthma symptoms in children (148) are associated with increased induction of hBDs in bronchial epithelial cells (149-152). Therefore, it is possible that these hBDs activate MC_{TC} in the bronchial smooth muscles of asthmatic patients via MrgX2 leading to the exacerbation of their symptoms. Neutrophils and eosinophils are known to play important roles in the late phase of allergic asthma (153). Subramanian et al., showed that neutrophil-derived AMP, LL-37 activate human MCs via MrgX2 (63). Furthermore, Fujisawa et al. showed that cationic peptides released from activated eosinophils cause degranulation in MC_{TC}-type MCs via MrgX2 (65). These findings raise the interesting possibility that MrgX2 expressed in lung MC_{TC} contributes to the pathogenesis /exacerbation of asthma. However, it is unknown if human lung MCs express MrgX2 and if their expression is modulated during asthma.

1.4 Humanized mice as a model for in vivo studies on MrgX2

Studies have shown that MrgX2 is activated by small cationic peptides such as neuropeptides and AMPs. However, no *in vivo* functional studies have been carried out primarily due to the fact that MrgX2 is not expressed in mice. Also, studies by Subramanian et al showed that known MrgX2 ligands do not activate murine MCs (32, 63). Therefore, new models need to be developed to study the biology of human MrgX2 *in vivo*.

One of the most exciting recent advances in translational research has been the

development and utilization of humanized mice to study the role of human lymphoid and myeloid cells *in vivo* (154-158). It is well documented that the receptor for the murine SCF, c-kit is important for the development of murine MCs. Kambe et al., (159) provided the first demonstration that engraftment of human HSCs, (CD34⁺ cells) into severely immune deficient mice leads to the development of some human MCs in mouse lung and skin indicating that mouse endogenous SCF is able to support human MC development to a certain extent. However, functional characterization of these MCs has not been performed. More recently, a new model has been developed in which an immune-deficient mouse (NOD, SCID, IL2 receptor γ -null; NSG) expresses three transgenes; human SCF, GM-CSF and IL-3, each driven by a human cytomegalovirus promoter/enhancer and constitutively produce 2 - 4 ng/ml of these human cytokines in the serum (NSG-SGM3) (160, 161). Given that SCF and IL-3 are important growth factors for human MCs, we predicted that these mice will serve as “incubators” for the differentiation of human CD34⁺ cells into MCs. Therefore one of the aims of this study was to do an initial assessment of the feasibility of utilizing humanized mice as a model for *in vivo* functional studies on MrgX2, as well as mouse models to determine the role of MrgX2 in human disease conditions involving MCs.

2. RESEARCH AIMS

Objectives

There are three objectives of the present study:

- 1) To determine if human skin, gingival and lung MCs express MrgX2 and to test if its expression is upregulated in human disease conditions such as AD, rosacea, chronic periodontal disease and asthma.

- 2) To investigate the localization of MrgX2. To determine if the receptor is localized to the plasma membrane, cytoplasm or both and to further evaluate if the localization changes on stimulation.

- 3) To test if human MCs that develop in humanized mice express MrgX2.

3. MATERIALS AND METHODS

3.1 Sample Collection

The study protocols for the expression of MrgX2 by human skin and gingival tissues were approved by the Institutional Review Board (IRB) of the University of Pennsylvania. The IRB protocol number for the gingival study is 822486 and for the skin study, it is 808225. The gingiva study was conducted using pieces of otherwise discarded gum tissue that were removed during the normal course of periodontal surgery on patients at the Graduate Periodontics Clinics, School of Dental Medicine, University of Pennsylvania. Written informed consent was

obtained from all patients prior to collection of the gingiva samples. The discarded tissue were collected in an anonymous fashion without any patient identifiers and transported to the Department of Pathology in 10% formalin where they were processed and cut into tissue blocks. Skin samples used in this study were archival tissue samples of patients provided by the Penn Skin Disease Research Center while the human lung samples were provided by Dr. Reynold Panettieri (Airway Biology Program, School of Medicine, University of Pennsylvania).

3.2 Materials

All cell culture reagents were purchased from Invitrogen (Gaithersburg, MD). Amaxa transfection kit (Kit V) was purchased from Lonza (Gaithersburg, MD). Cortitastin (CST) – 14 was obtained from American Peptide (Vista, CA). LL-37 was from Anaspec (Freemont, CA) and SP was from American Peptide Company (Sunnyvale, CA). Rabbit anti-Mrgx2, catalog #: NB110-75035 was purchased from Novus Biologicals (Littleton, CO). Mouse anti-human tryptase, catalog #: sc-33676 was purchased from Santa Cruz (Dallas, TX). Anti-HA mouse monoclonal antibody, catalog #: 11583816001 was purchased from Roche Diagnostics (Mannheim, Germany). Donkey anti-mouse secondary antibody, Alexa Fluor 488, catalog #: A21202 was purchased from ThermoFisher Scientific (Grand Island, NY). Donkey anti-rabbit secondary antibody, Alexa Fluor 594, catalog #: A21207 was purchased from ThermoFisher Scientific (Grand Island, NY). 4',6-diamidino-2-phenylindole (DAPI) was obtained from Invitrogen

(Grand Island, New York). ProLong Gold antifade reagent was purchased from Invitrogen (Grand Island, NY). Target retrieval solution was purchased from Dako (Carpinteria, CA). Mouse IgG Vectastain ABC kit, catalog #: PK-4002 was purchased from Vector Laboratories (Burlingame, CA). 3,3'-diaminobenzidine (DAB) peroxidase substrate kit, catalog #: SK-4100 was purchased from Vector Laboratories (Burlingame, CA).

3.3 Methods

3.3.1. Immunohistochemistry

Immunohistochemistry (IHC) experiments were performed on human normal and diseased skin, gingiva and lung tissue as well as humanized mice lung samples to show the presence of MCs in the samples. Diseased tissue samples were from patients diagnosed with AD, rosacea, CP and asthma. Tryptase was used as the MC marker for all experiments. Tissue samples were fixed in 10% formalin for 24 hours and subsequently embedded in paraffin and sectioned to 5 μ m thickness on charged slides (2 sections per slide). The slides were heated to 55°C overnight, subsequently deparaffinized in HistoClear and hydrated in a series of graded ethanol incubations. Antigen retrieval was performed by heat treatment using 1X antigen retrieval solution in a water bath at 95°C for 1 hour. The slides were placed in a humidified chamber and peroxidase quenching was achieved with 0.3% H₂O₂ blocking solution for 10 minutes at room temperature followed by 2 washes in PBS. The slides were incubated for 1 hour at room temperature with blocking serum using normal horse serum followed by

incubation with primary antibody (anti-human tryptase) at a dilution of 1:750 overnight at 4°C in a humidified chamber. For negative control, serum blocking reagent was used in place of primary antibody on the first section of all slides. The sections were then washed 3 times with PBS/0.1% Tween and incubated with diluted secondary antibody, biotinylated horse anti mouse IgG for 30 minutes at room temperature. Following incubation with secondary antibody, they were washed 3 times in PBS/0.1% Tween and incubated for 30 minutes with Vectastain ABC reagent. Subsequently, they were washed 3 times in PBS/0.1% Tween and incubated in peroxidase substrate solution using DAB peroxidase substrate kit until desired color intensity was observed. The slides were rinsed under running tap water for 5 minutes, counterstained in Mayer's hematoxylin solution for 30 seconds and rinsed again in running tap water until excess solution was removed. Rehydration of the tissue was done using increasing graded alcohol series followed by Histoclear solution. The slides were allowed to air dry before mounting with Permount mounting medium. Images were captured on a Nikon Eclipse microscope with an Olympus digital microscope camera using 4X, 10X and 20X objectives.

3.3.2. Immunofluorescence

Tissue Samples

Immunofluorescence (IF) experiments were performed on the same tissue samples as IHC experiments. Tissue preparation, sectioning and deparaffinization were performed as described above for IHC. The tissue

samples were subsequently incubated with blocking buffer [5% normal donkey serum, 0.3% of Triton X-100 in phosphate buffered saline (PBS)] for 1 hour at room temperature. Incubation with primary antibody (anti-human tryptase) was carried out in antibody dilution buffer (PBS with 1% bovine serum albumin and 0.3% Triton X-100) overnight at 4°C at a dilution of 1:500. For negative control, antibody dilution buffer was used in place of primary antibody on the first section of all slides. The sections were then washed 3 times with PBS and incubated with secondary antibody, Alexafluor 488-conjugated donkey anti-mouse (1:400) in conjunction with DAPI (1:4000) for 1 hour at room temperature in the dark. Tissue sections were washed 3 times with PBS and incubated overnight at 4°C with anti-human MrgX2 primary antibody at a dilution of 1:500. The sections were again washed 3 times with PBS and incubated in the dark at room temperature with secondary antibody, Alexa flour 594-conjugated donkey anti-rabbit at a dilution of 1:400. The tissues were washed 3 times in PBS and mounted with ProLong Gold antifade reagent. Images were captured on a Nikon Eclipse microscope with an Olympus digital microscope camera using 20X and 40X objectives.

3.3.3. Transfection of RBL-2H3 cells

RBL-2H3 cells were transfected with plasmids encoding HA-tagged MrgX2 using the Amaxa nucleofactor device and Amaxa kit V, according to the manufacturer's protocol (32, 62). Following transfection, a proportion of the transfected RBL-2H3

cells were stimulated with CST for 5 minutes and used in immunofluorescence experiments to evaluate the effect of stimulation on the localization of MrgX2.

Immunofluorescence on RBL-2H3 cells expressing HA-tagged MrgX2

Stimulated and unstimulated RBL-2H3 cells transiently expressing HA-tagged MrgX2 were counted and diluted in PBS. Cytospin slides were labeled and prepared by attaching plastic cytopsin funnel and slide to cytopsin clip. 100µl of the diluted cells were pipetted into each section of the cytopsin funnel. The slides were centrifuged at 450rpm for 5 min, left to air dry, then fixed in 100% ice-cold methanol for 10 min, followed by rinsing in PBS. The cells were permeabilized with 0.5% saponin in PBS for 10 min, washed 3 times in PBS, blocked with 5% donkey serum for 1 hour at room temperature and washed again in 3 changes of PBS. The cells were incubated overnight at 4°C with anti-HA mouse monoclonal antibody diluted in antibody dilution buffer at a dilution of 1:500. The cells were washed 3 times in PBS and incubated with secondary antibody, Alexafluor 488-conjugated donkey anti mouse (1:400) in conjunction with DAPI (1:4000) for 1 hour at room temperature in the dark. This was followed by 3 times washing in PBS and mounting with ProLong Gold antifade reagent. Images were captured on a Nikon Eclipse microscope with an Olympus digital microscope camera using 20X objective.

3.3.4. Degranulation

RBL-2H3 cells (5×10^4) stably expressing MrgX2 were seeded into 96-well plates in a total volume of 50 μ l HEPES buffer containing 0.1% BSA and exposed to the peptides LL-37 and SP at 5 μ M and 10 μ M. For total β -hexosaminidase release, unstimulated cells were lysed in 50 μ l of 0.1% Triton X-100. Aliquots (20 μ l) of supernatant or cell lysate were incubated with 20 μ l of 1mM p-nitrophenyl-N-acetyl- β -D-glucosamine for 1.5 hours at 37 $^{\circ}$ C. Reaction was stopped by adding 250 μ l of a 0.1 M Na₂CO₃/0.1 M NaHCO₃ buffer and absorbance was measured at 405nm (32, 63).

3.3.5. Cell Counting and Analysis

Expression of MrgX2 by MCs in human skin

Skin samples from 3 patients diagnosed with AD, 2 patients diagnosed with rosacea and 3 control patients were analyzed by IF to evaluate the differences in number of skin MCs as well as differences in the expression of MrgX2 by skin MCs. Pictures were taken from 5 randomly selected visual fields in each skin sample at a magnification of 40X. The image exposure, size and dimensions were exactly the same for all analyzed images. MCs were identified using tryptase antibody and were stained green. MrgX2 positive cells were red in color. The number of MCs and were counted in the 5 visual fields and the average of the 5 fields was taken as the final count of MCs for that sample. Differences in the expression of MrgX2 by MCs were also evaluated. Intensity of tryptase expression by MCs as well as intensity of MrgX2 expression in the same MCs

was evaluated using Metamorph image analysis software. Using colocalized images, the intensity of the green tryptase staining by MCs as well as red MrgX2 staining in MrgX2-positive MCs was measured. Image exposure was exactly the same for all analyzed images. The intensity values were normalized by the area of the MCs and an average intensity value was calculated for each skin sample analyzed.

Expression of MrgX2 by MCs in human gingiva

Gingiva tissue samples from 3 patients diagnosed with CP and 1 control patient who did not have CP were evaluated qualitatively for the expression of MrgX2 by gingiva MCs due to small sample size.

Expression of MrgX2 by MCs in human lung

Lung tissue samples from 6 patients who died from complications of asthma and 6 control subjects who died from other causes were analyzed by IF to evaluate the differences in number of lung MCs, MrgX2-expressing cells, as well as MrgX2-positive MCs. Pictures were taken from 3 randomly selected visual fields in each lung sample at a magnification of 20X. The image size and dimensions were exactly the same for all analyzed images. MCs were identified using tryptase antibody and were stained green. MrgX2-positive cells were red in color. The number of MCs, MrgX2 positive cells, as well as MrgX2-positive MCs was counted in the 3 visual fields and the average of the 3 fields was taken as the final count for that sample.

Expression of MrgX2 in humanized mouse lung MCs

Immune-deficient mice transgenic for human cytokines (NSG-SGM3) mice (3 -4 weeks old) were preconditioned with busulfan (Sigma, 30 mg/kg, I.V.) or sublethally irradiated (150 cGy). The following day, purified human cord blood-derived CD34⁺ cells (2×10^5 cells) were injected via tail vein. After 12 weeks mice were euthanized and lung preparations were used for IHC and IF studies to determine if human MCs develop in these mice and if they express MrgX2.

3.3.6. Statistical analysis

GraphPad Prism scientific software was used for statistical analysis. Differences in the number of skin MCs as well as differences in the expression of MrgX2 by skin MCs were evaluated by using the unpaired student t-test. Differences in the numbers of lung MCs, MrgX2-positive cells and MrgX2-positive MCs in the lungs were also evaluated by using the unpaired student t-test.

4. RESULTS

4.1 Expression of MrgX2 in skin samples of patients with AD and Rosacea.

AD is an inflammatory disease that involves MCs and eosinophils (96, 108). Previous studies have shown that MrgX2 is expression in human skin MCs and their activation by eosinophil-derived peptides contributes to the pathogenesis of another inflammatory skin disease; CU (65). Our first goal was therefore to determine if MCs from patients with AD also express MrgX2. For all studies

described here and in the sections below with human tissue samples, we utilized two approaches to study MCs and MrgX2. First, we used IHC with an anti-tryptase antibody in combination with hematoxylin and eosin (H & E) to determine the presence of MCs in relation to structural cells in skin. Second, we utilized a three-color IF approach to determine the expression of MrgX2 (red), MCs (green) in the context of the nucleus (blue). Using IHC, we found that MCs are present in the dermal layers in both healthy (Fig. 1A and B) and diseased (Fig. 1C and D) skin tissue compared to the epidermis. Using IF, we found that MrgX2 is expressed in normal and AD skin samples (Fig. 2B and D). Furthermore, staining of the same sample with anti-tryptase antibody showed that the same cells are MCs. When the MrgX2 and tryptase panels were merged, it clearly showed that MCs in normal and AD skin samples express MrgX2. However, there was a greater intensity of colocalization in the diseased samples compared to the normal samples which could indicate that MrgX2 expression by MCs is more active in AD compared to healthy skin (Fig. 2B and D). Using IF, we quantitated the number of MCs and found that the number of MCs (tryptase-positive green cells) was not significantly different in skin tissues from control subjects compared with AD patients (Fig. 3). The intensity of tryptase-positive green cells was also not significantly different in skin tissues from control subjects compared with AD patients (Fig. 4A). The expression of MrgX2 by MrgX2-positive MCs was also evaluated and there was no significant difference in the intensity of the red staining by MrgX2-positive MCs between the 2 groups (Fig. 4B). Of note is the fact that the control skin samples in this study were taken

from scar tissue. MCs play key roles in wound healing. Therefore, it is possible that the MCs in the control samples were also increased in number and activity resulting in similar results between the two groups.

MCs and LL-37 are key players in the pathogenesis of rosacea (118, 125). Neurogenic inflammation is associated with rosacea flare-ups. Prior studies have shown that LL-37 and neuropeptides such as SP activate human MCs via MrgX2 (63, 64). Therefore we sought to determine if MCs from patients diagnosed with rosacea express MrgX2. Similar to AD, MCs are present in the dermal layers in both healthy (Fig. 5A and B) and diseased (Fig. 5C and D) skin tissue compared to the epidermis. As shown in Fig. 6D, MCs in the skin of rosacea patients express MrgX2. There is also a greater intensity of colocalization between green cells (MCs) and red cells (MrgX2) in the diseased skin sample (Fig. 6D) compared to the normal skin (Fig. 6B) possibly indicating greater activity of MrgX2 in MCs in rosacea skin compared to healthy skin. Using IF for quantitation, we found that the number of skin MCs was not significantly different in skin samples of control subjects compared with rosacea patients (Fig. 7). Although there was a significant reduction in the intensity of tryptase-positive green cells in skin tissues from rosacea patients compared with controls ($P=0.0260$) (Fig. 8A), the expression of MrgX2 by tryptase-positive MCs was evaluated and there was no significant difference in the intensity of the red staining by MrgX2-positive MCs between the 2 groups (Fig. 8B). Tryptase is a protease found in MCs, which is released on degranulation. MC degranulation

plays a direct role in the pathogenesis of rosacea (115). Taken together, these results indicate that although the MC numbers are not significantly different between the groups, there is an increased degranulation of MCs in rosacea resulting in a significant reduction of the intensity of green tryptase-positive MCs in rosacea skin. Furthermore, although the intensity of green tryptase-positive MCs is reduced in rosacea, the expression of MrgX2 by MrgX2-positive MCs was not significantly different between the groups indicating that there is a higher activity of MrgX2 in MCs in rosacea skin compared to healthy skin.

4.2 Expression of MrgX2 in normal gingiva and gingiva from patients with CP

MCs and AMPs are involved in the pathogenesis of periodontal disease (132, 136). Since AMPs have been shown to activate MCs via MrgX2, we sought to determine if MrgX2 is expressed by gingival MCs. IHC and IF analysis were performed on gingiva tissue samples from three patients diagnosed with CP as well as a control subject not diagnosed with CP. On IHC, most MCs were observed in the lamina propria and submucosa in both healthy (Fig. 9A and B) and diseased (Fig. 9C and D) gingiva tissue compared to the epithelium. However, gingiva samples from patients with CP showed increased number of MCs in the epithelium compared to the healthy gingiva indicating that MCs migrate into the epithelium in disease. With IF, we found that Mrgx2 is expressed in healthy and CP gingiva samples (Fig. 10B and D). Furthermore, as shown in Fig. 10B and D, MCs are present in the human gingival tissue and these cells

express MrgX2. Due to small sample size (3 CP patients and 1 control), we did not do a quantitative analysis. However, as noted in the skin samples, there was also a more intense colocalization in the CP gingiva compared to normal gingiva.

4.3 Expression of MrgX2 in normal and asthmatic lung

MC_{TC} are markedly increased in the bronchial smooth muscle during asthma (144, 146). IHC and IF analysis were performed on lung tissue from patients who died from asthma related complications as well as control samples of lung tissue from subjects who died from other causes. IHC was performed on lung tissue from both groups using human anti-tryptase antibody. As shown in Fig. 11, more MCs are observed in the lung smooth muscle of patients who died from asthma complications (Fig. 11C and D) compared with control subjects (Fig. 11A and B).

Although it well established that MCs are present in human lung tissue, it is unknown if these cells express MrgX2. Furthermore, outside the dorsal root ganglia, MCs are the only cells that have been shown to express MrgX2 (60, 62, 63). To determine if human lung tissue express MrgX2 and if they are found in MCs, IF study was performed using anti-MrgX2 and anti-tryptase antibodies. As shown in Fig. 12, MrgX2 (red) and MCs (green) could be detected in normal (Fig. 12B) and diseased (Fig. 12 D) human lung tissue. Merging of the images revealed that these MCs express MrgX2. The number of lung MCs was significantly greater in lung tissue from patients who died from asthma complications compared with individuals who died from other causes (P= 0.0178)

(Fig. 13A). The number of MrgX2- positive cells was also significantly greater in lung tissue from patients who died from asthma complications compared with control subjects ($P= 0.0211$) (Fig 13B). Furthermore, there were MrgX2-positive cells that were not tryptase-positive MCs as shown in Fig. 12D. There were also a few MCs, which were not MrgX2-positive but this was not significant between the two groups. Next, we compared the number of MrgX2-positive MCs between the groups and found a significant increase in the number of MrgX2-positive MCs in lung samples of patients who died of asthma complications compared to controls ($P= 0.0176$) (Fig. 14). These results indicate that MrgX2 is involved in the pathogenesis of asthma.

4.4 MrgX2 localization

Most GPCRs are expressed on the plasma membrane and are internalized after stimulation (162). However, it has been reported that MrgX2 is expressed on the plasma membrane as well as intracellularly (65). Furthermore, MrgX2 is resistant to agonist-induced receptor phosphorylation, desensitization and internalization (63). This study evaluated the localization of MrgX2 before and after stimulation with the basic secretagogue CST. IF experiments were performed on RBL-2H3 cells transiently expressing HA- tagged MrgX2 using anti-HA antibody for the detection of MrgX2.

As shown in Figure 15, MrgX2 is present on the plasma membrane as well as the cytoplasm. On stimulation with CST, the receptor remained on the plasma

membrane as well as in the cytoplasm but with a more peri-nuclear and punctate appearance.

4.5 Effects of LL-37 and SP-induced degranulation on RBL-2H3 cells stably expressing MrgX2

Excessive production of LL-37 as well as dysregulation of MC activity have been implicated in the pathogenesis of rosacea (118, 125). In both rosacea and AD, MCs are able to maintain neurogenic inflammation through activation by neuropeptides such as SP (131, 163). MrgX2 has been shown to function as a binding site for a wide range of unrelated basic peptides including SP and LL-37, leading to the degranulation of MCs (63, 64). Therefore, our goal was to show that ectopic expression of MrgX2 in RBL-2H3 cells makes these cells responsive to LL-37 and SP resulting in degranulation. It should be noted that without transfection of the corresponding cDNA encoding the receptor, RBL-2H3 cells do not endogenously express MrgX2 and are unresponsive to LL-37 and SP-induced degranulation. As shown in Fig. 16, stimulation by LL-37 and SP induced dose-dependent degranulation in RBL-2H3 cells stably expressing MrgX2.

4.6 Expression of MrgX2 in humanized mouse lung tissues

Very few MCs are present in the mouse bronchioles and lung parenchyma. However, engraftment of human HSCs into severely immune deficient mice transgenic for human cytokines (humanized mice) leads to the development of

human MCs in mouse tissues (159). The possibility that these mice develop human MCs in mouse lung and if they express MrgX2 have not been determined. First, we used NSG-SGM3 mice preconditioned with busulfan before engraftment of human HSCs. Our IHC study with tryptase antibody demonstrated that human MCs develop in the lung of humanized mice (Fig. 17A). We used IF analysis to determine if these MCs express MrgX2. As shown in Fig. 17B, MrgX2 is expressed in the lung of humanized mice and this receptor is expressed on human MCs. Similar results were obtained with NSG-SGM3 preconditioned by irradiation before engraftment of human HSCs (Fig 18 A and B).

5. DISCUSSION

Mrg receptors belong to the GPCR family. In humans, four MrgX genes, MrgX1 – MrgX4 have been identified while mice have 32 Mrg coding genes (60). As a result, human and mice Mrg receptors share only 45-65% amino acid sequence identity (64). Studies have shown that a wide variety of biologically relevant cationic peptides including neuropeptides and AMPs induce dose-dependent degranulation of MCs via MrgX2 (32, 63, 64). To the best of our knowledge, expression of MrgX2 has been reported only in dorsal root ganglia and MCs (64, 65, 69). Given the recent report that expression of MrgX2 on MCs is upregulated in skin of patients with severe chronic urticaria, we hypothesized that MrgX2 is also expressed on human lung and gingival MCs. We further hypothesized that its expression will be increased in AD, rosacea, CP and asthma.

MCs are granule containing immune cells with varied functions in both health and disease. They are more abundant at sites that are continuously exposed to the external environment and are usually located beneath the epithelium adjacent to blood vessels and in proximity to nerve fibers (4). In the physiological state, MCs are key players in promoting innate immunity and host defense against various pathogens (6, 56). They are also involved in maintaining normal homeostasis as well as angiogenesis and wound healing (5, 44). In the adaptive immune response, MCs play a role in regulating the development of B cells as well as the differentiation of naïve T cells (20, 21). MCs interact with AMPs and neuropeptides. hBDs and LL-37 induce Ca^{2+} mobilization, chemotaxis and degranulation in MCs resulting in the release of mediators (32). Neuropeptides such as VIP, CGRP and SP released by nerve terminals also activate MCs leading to degranulation.

Under physiological conditions the skin and gingiva are constantly exposed to pathogens. hBDs and LL-37 are potent AMPs produced by activated epithelial cells and neutrophils respectively (30). They exhibit antimicrobial properties against a wide variety of microorganisms. hBDs are produced by epithelial cells in response to exposure to infectious agents which they kill by binding to negatively charged phospholipid residues on their membrane thereby forming pores (164). Since hBDs activate MCs via MrgX2, this raises the interesting possibility that upon exposure to pathogens, epithelial cells produce hBDs, which activate MCs resulting in the secretion of MC-mediators. MC degranulation and

release of mediators such as histamine leads to increased vascular permeability and promotes the recruitment of leucocytes, which contribute to microbial killing by phagocytosis (165). Furthermore, the recruited neutrophils release LL-37, which also activates MCs via MrgX2 thereby contributing to host defense through activation of more MCs and recruitment of more neutrophils. Therefore, it appears that MrgX2 expressed on MCs facilitates vascular permeability and neutrophil recruitment and plays a central role in orchestrating host defense by functioning as an important link between the epithelium and neutrophils. Interestingly, AMPs and MCs are both involved in modulating the immune system, angiogenesis and wound healing (23, 166, 167). The skin, which is highly innervated, is also continuously exposed to external stimuli during the physiological state. The neuropeptide SP released by nerve endings in response to physical and emotional stimuli activates MCs via MrgX2 with the resultant effect of increased vascular permeability and granulocyte infiltration.

MCs play important roles in host defense and wound healing but their inappropriate activation leads to allergic and inflammatory disease. MCs are known to be involved in chronic inflammatory conditions, which involve AMPs and neuropeptides. Therefore, in disease conditions such as AD, rosacea, CP and asthma MC activation leads to undesirable clinical manifestations.

This study has demonstrated that MCs in both healthy and diseased skin express MrgX2. In the skin of patients diagnosed with AD, the number of MCs was not

significantly different compared to control subjects. Also, there was no difference in the expression of MrgX2 by MrgX2-positive MCs between the groups. The results may be explained by the fact that in healthy conditions, MC_{TC}, which are known to express MrgX2 are prominent in the skin and so do not need to be recruited to the skin in AD. It is more likely generation of ligands such as SP and MBP secreted from recruited eosinophils contribute to the symptoms associated with AD via the activation of MrgX2 expressed in MCs. It is noteworthy that the control skin samples used in our study were taken from scar tissue and MCs are known to play a very important role in wound healing. This was done because we did not have access to normal human skin samples. It is therefore possible that the control we used had elevated number of MCs making it difficult to assess the MC numbers in control and AD samples. Nevertheless, the presence of MrgX2 in MCs of skin samples taken from AD patients suggests that MrgX2 contributes to the pathogenesis of AD.

We also evaluated the expression of MrgX2 by MCs in the skin of patients with rosacea, which is a chronic inflammatory skin disorder that involves MCs and found that these MCs express MrgX2. Furthermore, we compared the number of MCs in rosacea skin with control skin and found no significant difference between the groups. However, there was a significant reduction in the expression of tryptase-positive MCs in the rosacea group, which could indicate that the reduced expression of tryptase by MCs in the rosacea group is due to degranulation of MCs. In spite of the reduced tryptase expression by MCs in the

rosacea group, there was no significant difference in the expression of MrgX2 by MrgX2-positive MCs between the groups signifying greater activity of MrgX2 in MCs in rosacea skin compared to control. LL-37 plays a central role in the pathogenesis of rosacea (118). Furthermore, increased expression of SP has also been reported in rosacea lesions (131). Therefore, we propose that MC activation and degranulation by LL-37 and SP via MrgX2 contribute to the pathogenesis of rosacea.

The expression of MrgX2 by gingival MCs from healthy and periodontally involved gingiva was assessed showing that MCs from both groups express MrgX2. We did not evaluate differences in MC numbers due to very small sample size. Studies have reported increased numbers of MCs in the gingiva of patients with periodontal disease compared with controls and a significant correlation between MC density, degree of degranulation and periodontitis severity has been reported (132). Expression of LL-37 is increased in gingival crevicular fluid of patients with periodontitis and a significantly higher basal hBD3 expression in keratinocytes from periodontitis patients compared to controls has been reported (135, 136). Our finding that MrgX2 is expressed in healthy and diseased gingival MCs, suggests that gingival MrgX2-positive MCs contribute to host defense in the oral cavity via activation by AMPs. It is also possible that MrgX2 also contributes to CP due elevated hBD3/LL-37 and increased number of MrgX2-expressing MCs.

We demonstrated that MCs in healthy and asthmatic lung express MrgX2. The number of MCs, MrgX2-positive cells as well as MrgX2-positive MCs were significantly increased in lung tissue from individuals who died from asthma patients compared to individuals who died from other causes. Also, an increased number of MCs were present in the lung smooth muscle of asthma patients compared with controls. This finding is consistent with previous reports that the number of MC_{TC} is increased in bronchial smooth muscles of patients with asthma compared with controls (144, 146). It is noteworthy that MC_{TC} express GPCRs for MrgX2 (147). Eosinophils and neutrophils play important roles in the last phase of asthma (153). Eosinophil-derived MBP as well as neutrophil-derived LL-37 activate MCs via MrgX2 (63, 65). In children, asthma is exacerbated by HRV and RSV, which are both associated with increased induction of hBDs in bronchial epithelial cells (148). Therefore it appears that MC_{TC}, which are recruited to the lungs of asthmatics, are activated by hBDs, LL-37 and MBP via MrgX2 leading to release of mediators, which cause bronchospasm and exacerbates the asthma symptoms.

Unlike most GPCRs that are expressed on MCs, MrgX2 is located at both plasma membrane and intracellular sites (65, 162). Using RBL-2H3 cells transiently transfected with HA-tagged MrgX2, we also obtained similar results, which indicate that MrgX2 ligands can activate MCs via interaction with the receptor at the plasma membrane and intracellular sites. Prior studies have shown that MrgX2 is resistant to agonist-induced receptor phosphorylation,

desensitization and internalization (63). Although MrgX2 remained on the plasma membrane and intracellularly on stimulation with CST in this study, there was a more punctate and peri-nuclear appearance indicating that there may be some mobilization of the receptor. More studies are ongoing to identify the exact intracellular location of the receptor before and after stimulation.

MrgX2 is expressed in human but not in mouse MCs, thus making it difficult to target the receptor for in vivo studies. Humanized mice have recently been used to study the role of human MCs in vivo. However, the possibility that these MCs express MrgX2 has not been previously tested. We found that as for asthmatic human lung MCs, lung of humanized mice develop human MCs that express MrgX2. Preliminary studies performed by others in Dr. Ali's lab have shown that human MCs develop in the skin and gingiva of humanized mice. Therefore, humanized mice may be used as models to study the role of MrgX2 on host defense and chronic inflammatory diseases such as AD, rosacea, periodontitis and asthma.

6. CONCLUSIONS

MCs have various functions in both health and disease. Prior studies have shown that a wide range of structurally unrelated small cationic peptides including AMPs and neuropeptides induce dose-dependent degranulation of MCs via MrgX2 (32, 63, 64). This study has shown that MrgX2 is expressed in healthy human skin, gingiva and lung tissue. This suggests that activation of MrgX2 on MCs by AMPs

generated by the epithelium (hBDs) and neutrophil (LL-37) plays a pivotal role in host defense. Currently, around 500 - 600 AMPs are in clinical development for the treatment of microbial infection; the mechanisms of their action include both antimicrobial activity and the modulation of the immune system (168). However, a major limitation of this strategy is that chemical synthesis of these AMPs is prohibitively expensive and post-synthesis modifications (cyclization, disulfide bonds, folding) are inadequate for optimal antimicrobial activity. Gupta et al., recently demonstrated that AMPs such as retrocyclin and protegrin purified from transgenic plant chloroplasts kill microbes directly and induce MC degranulation via MrgX2 (162). Thus, harnessing this dual feature of plant generated AMPs could facilitate their advancement to the clinic for the treatment of periodontal and cutaneous microbial infection overcoming major hurdles in current production systems.

This study has demonstrated the presence of MrgX2-positive MCs in the skin of patients with AD and rosacea as well as gingiva tissue from CP patients. Given that eosinophil-derived peptides, LL-37 and hBDs are increased in AD, rosacea and CP respectively suggest that MrgX2 contributes to these diseases via increased MC degranulation. Our finding that MCs that express MrgX2 are upregulated in chronic asthma suggests it participates in the pathogenesis of asthma and may be potentially targeted for its treatment. Finally, we found that human MCs, which develop in the lungs of humanized mice, express MrgX2.

Therefore humanized mice may be used as models for future *in vivo* studies on the role of MrgX2 on host defense and chronic inflammatory diseases.

7. FIGURES

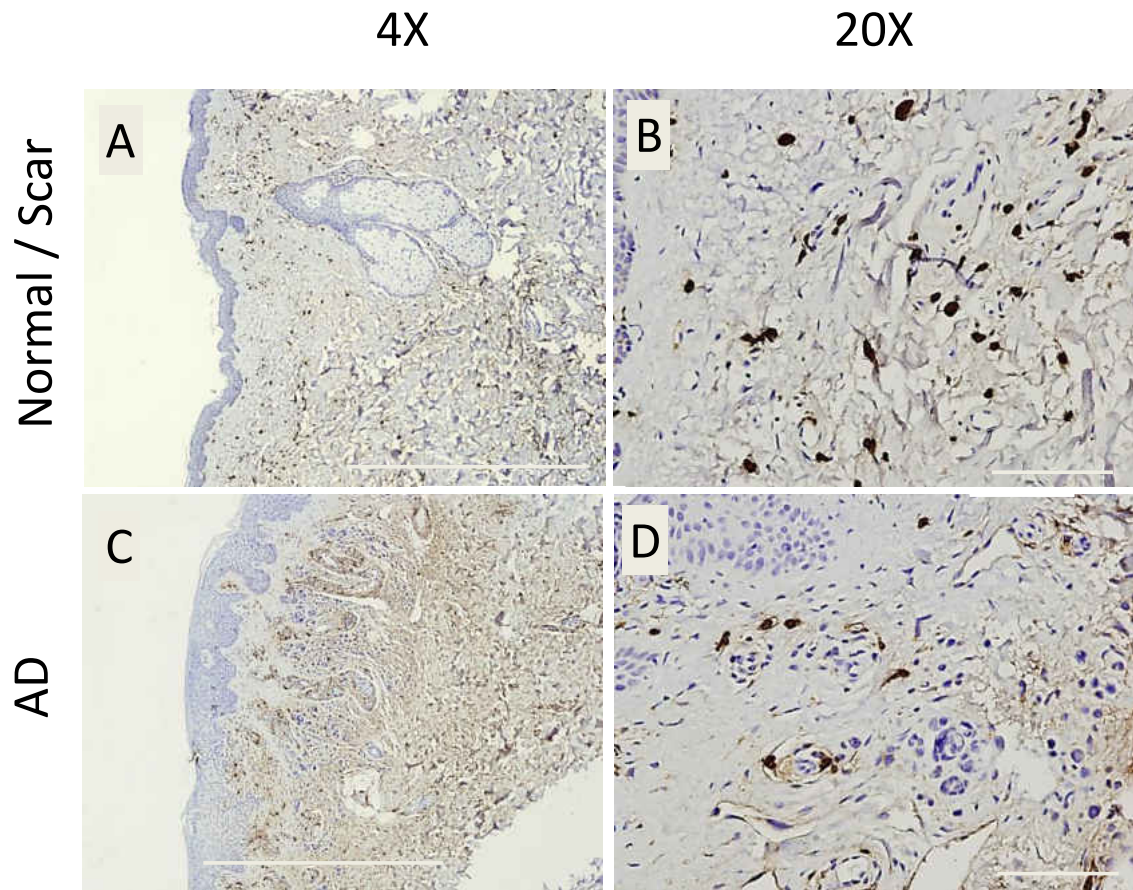


Figure 1

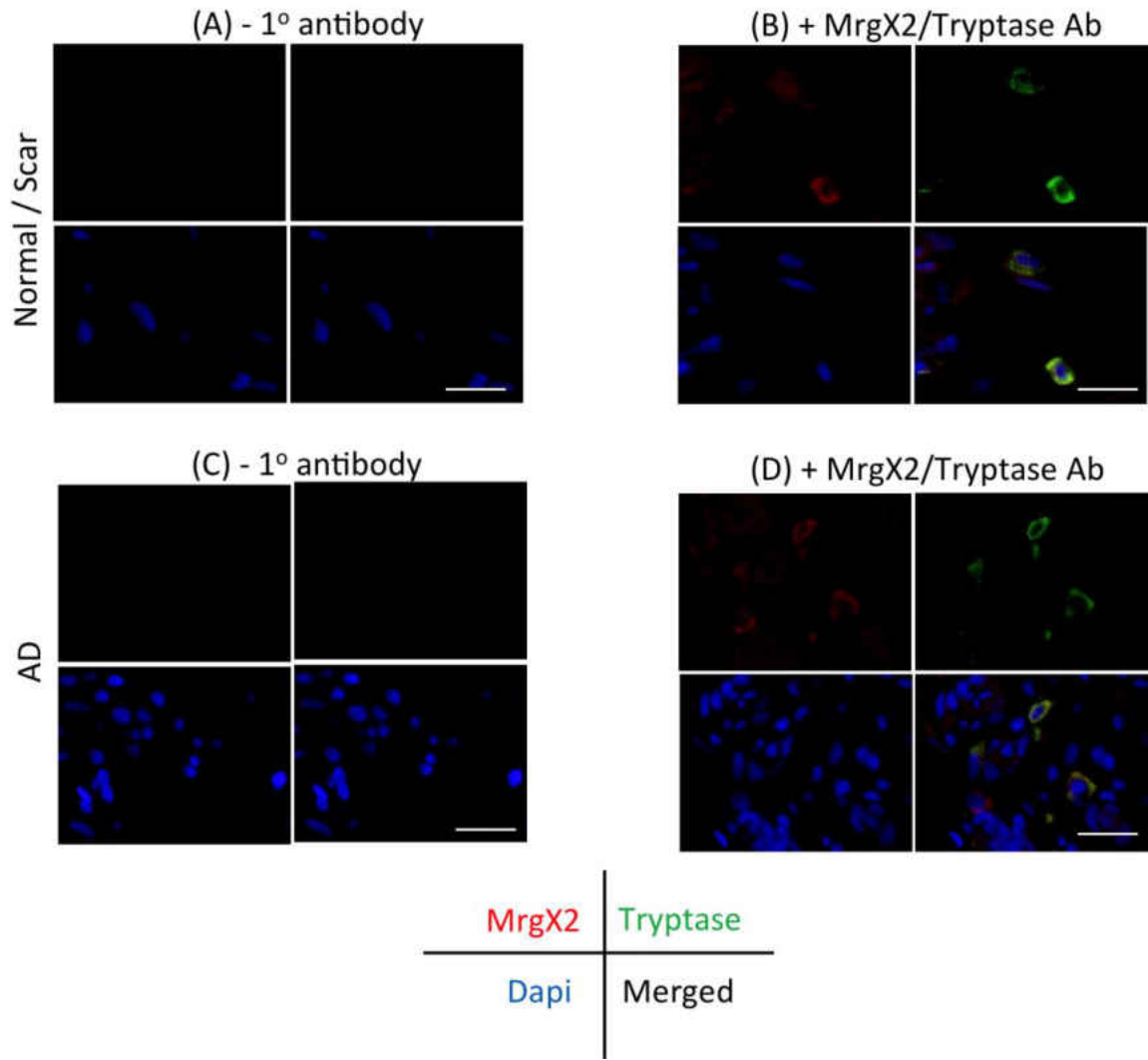


Figure 2

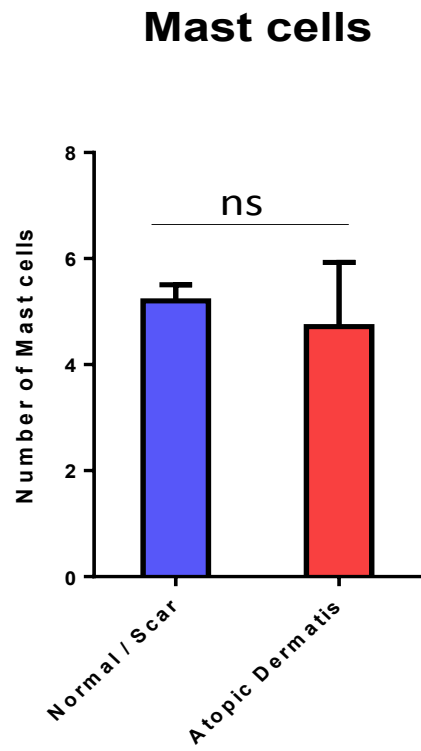
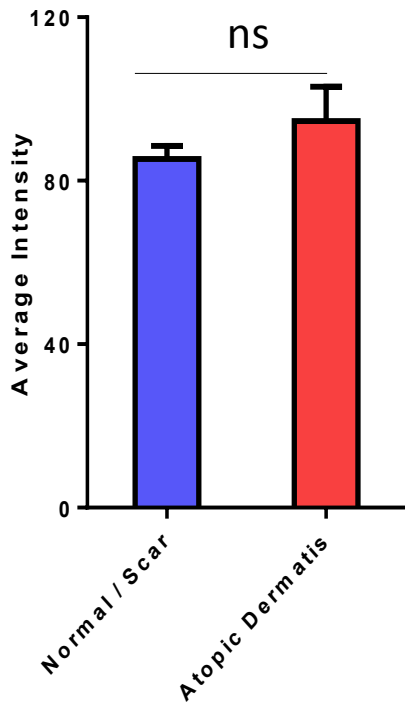


Figure 3

(A) Mast cells



(B) MrgX2

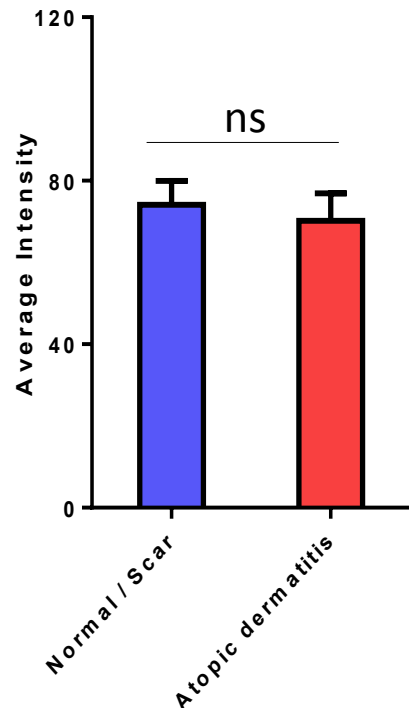


Figure 4

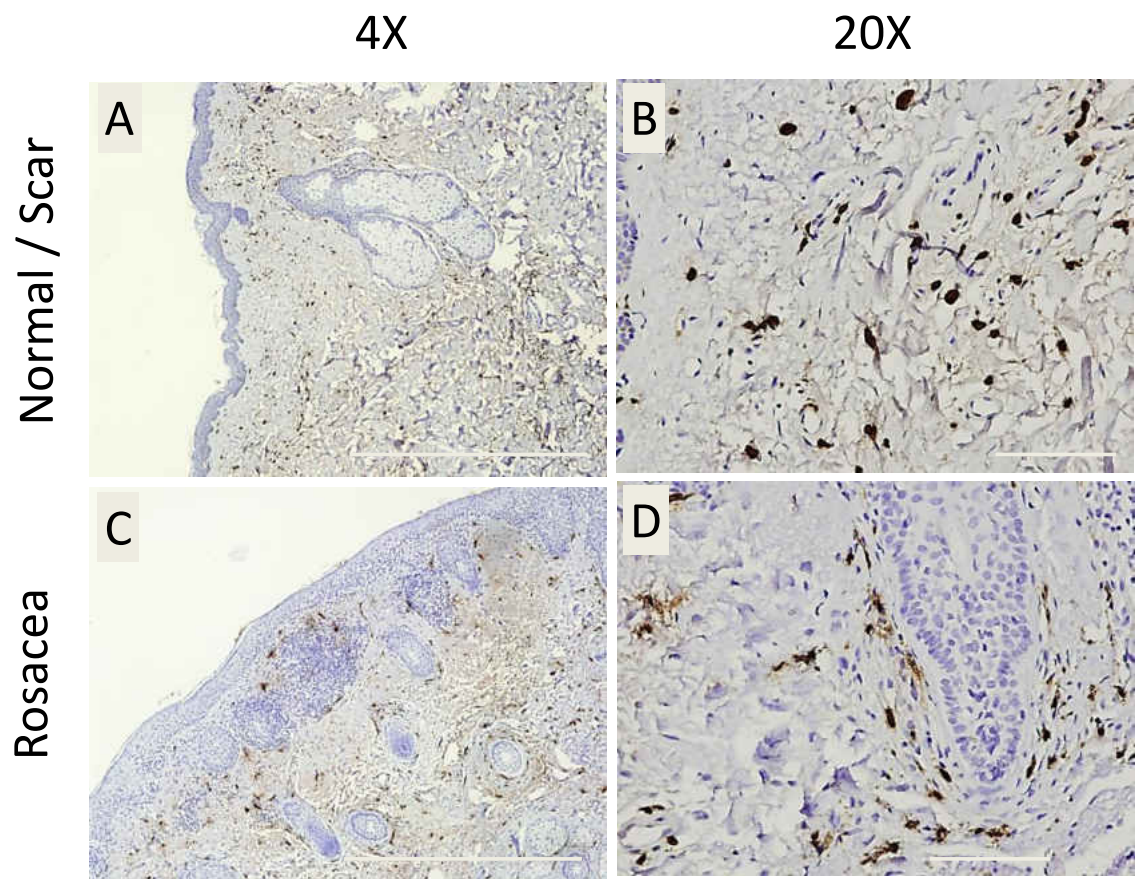


Figure 5

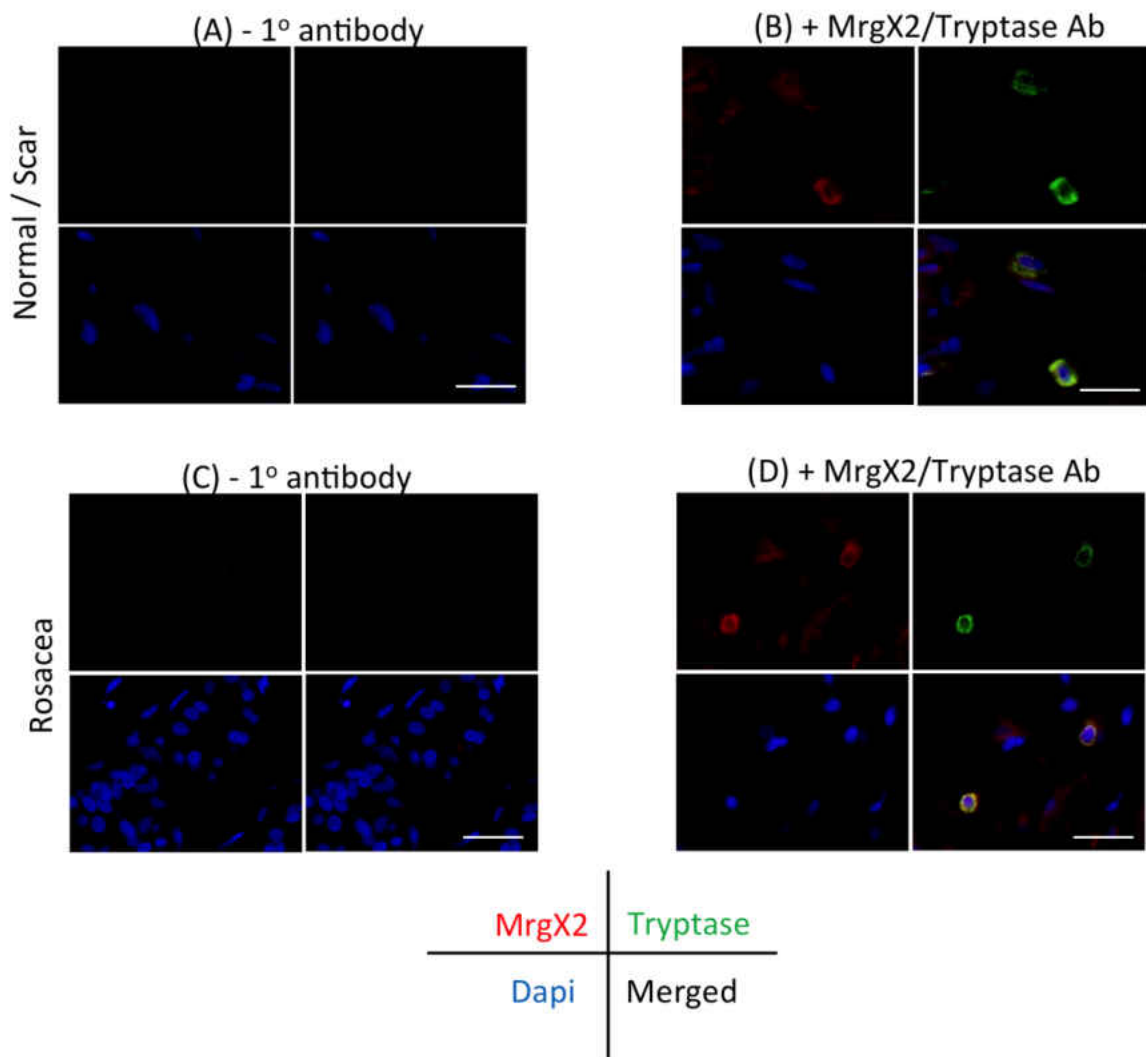


Figure 6

Mast cells

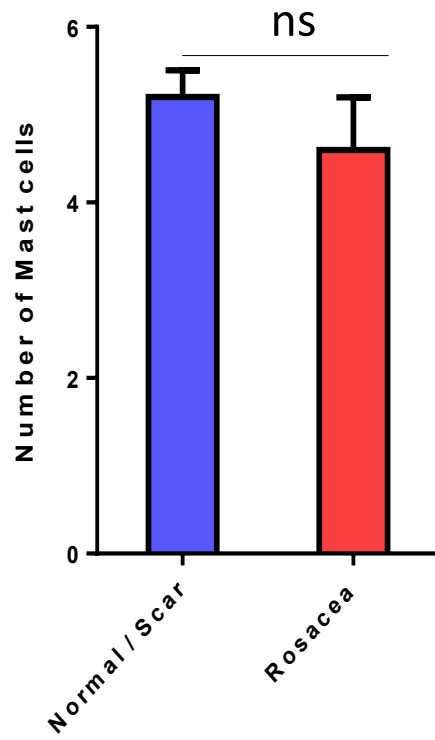
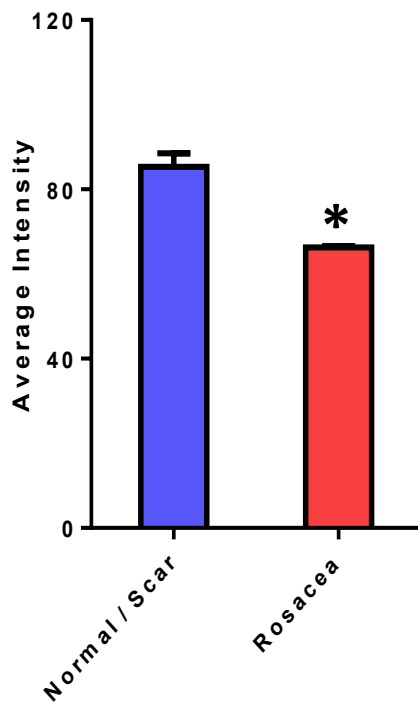


Figure 7

(A) Tryptase



(B) MrgX2

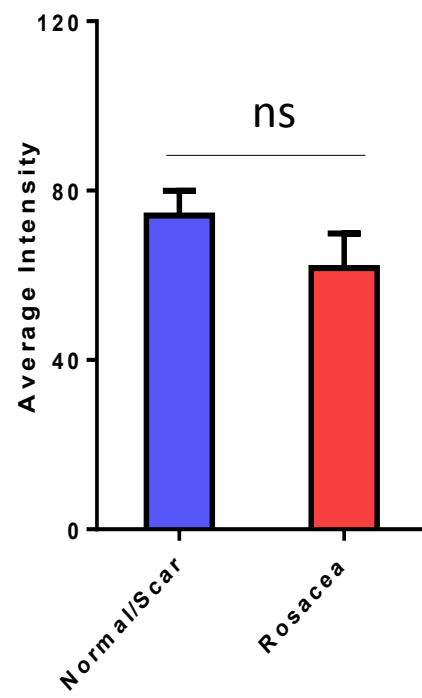


Figure 8

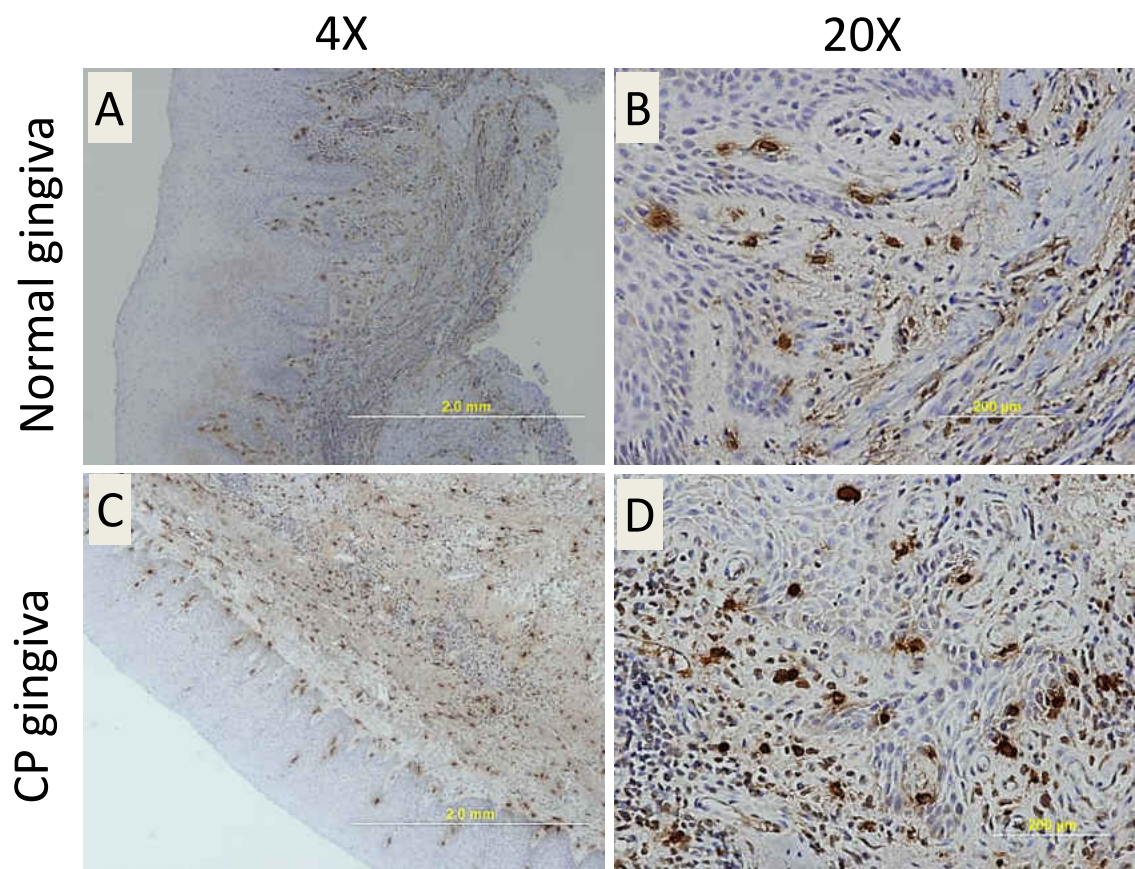


Figure 9

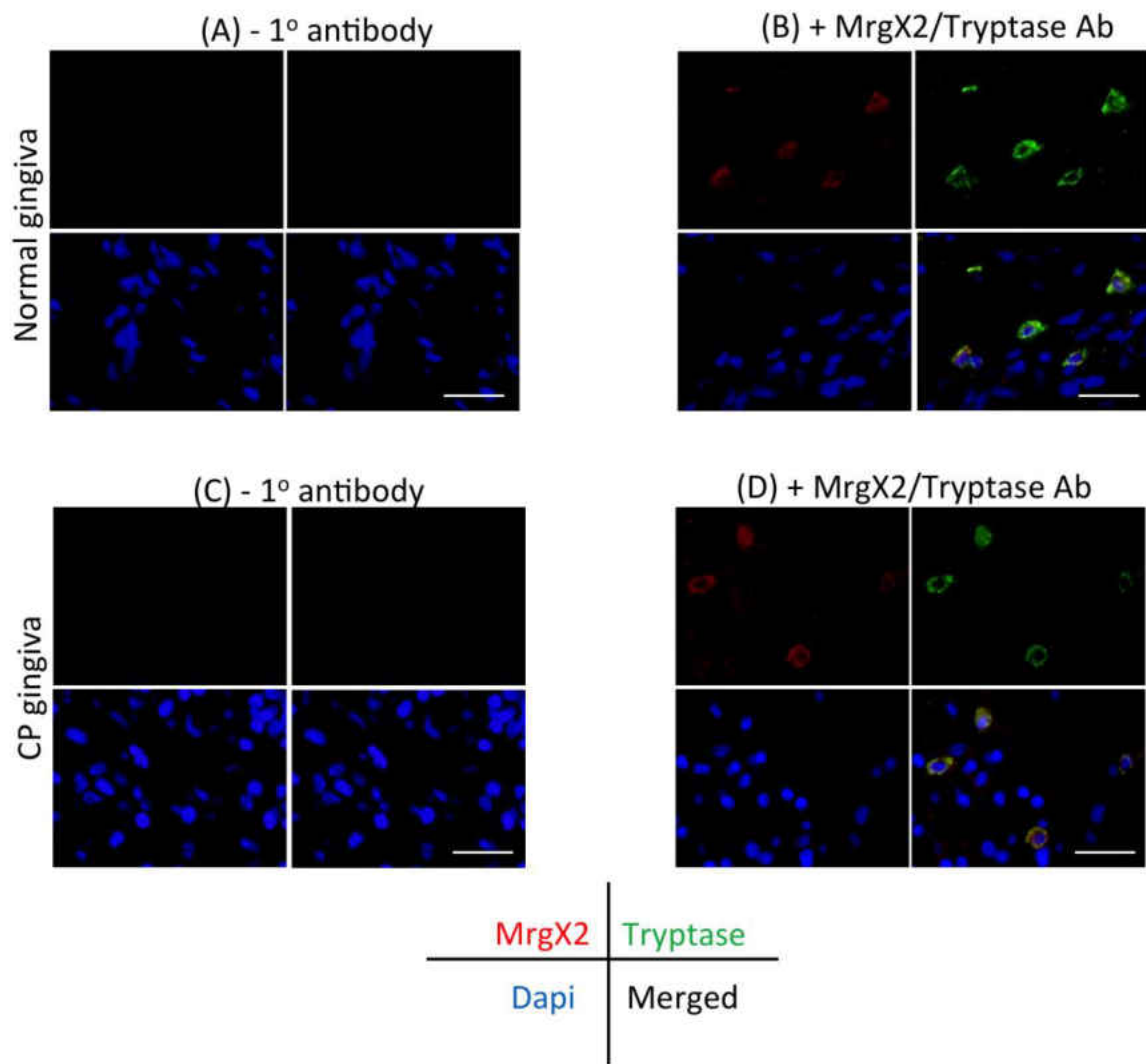


Figure 10

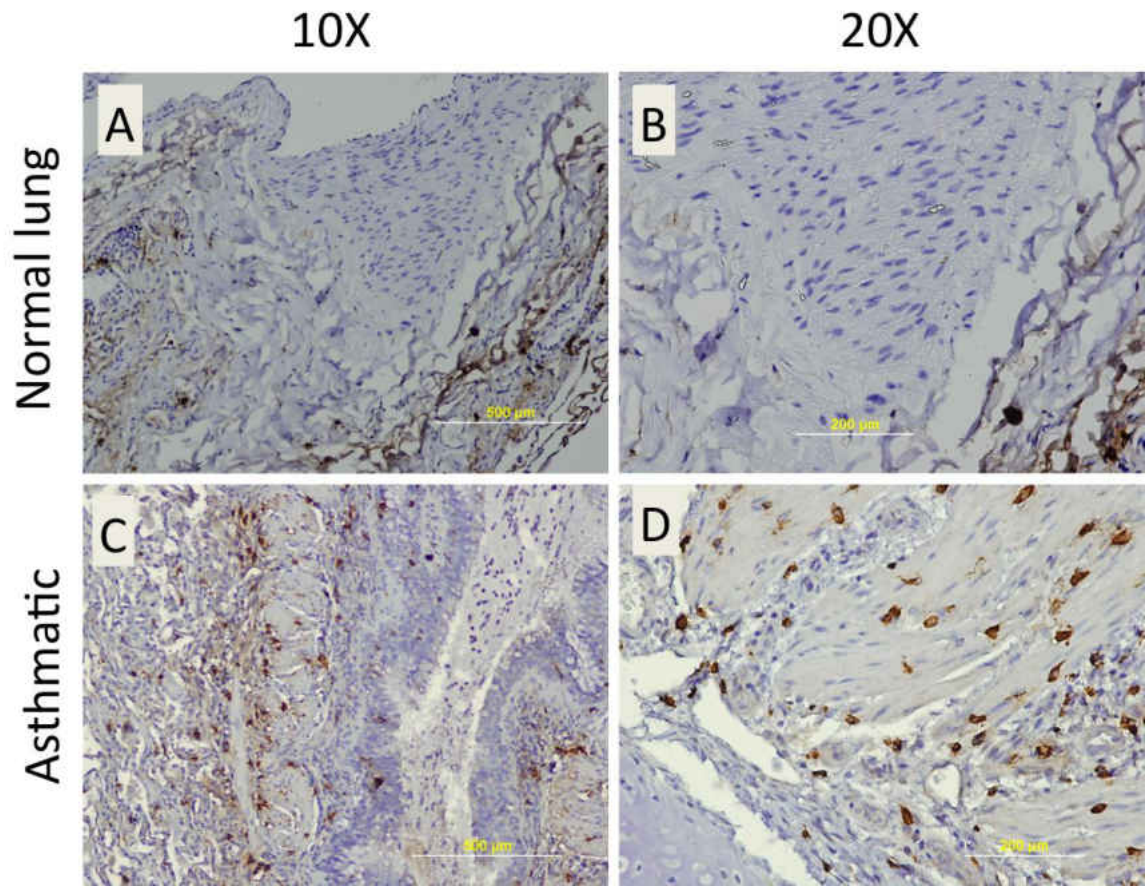


Figure 11

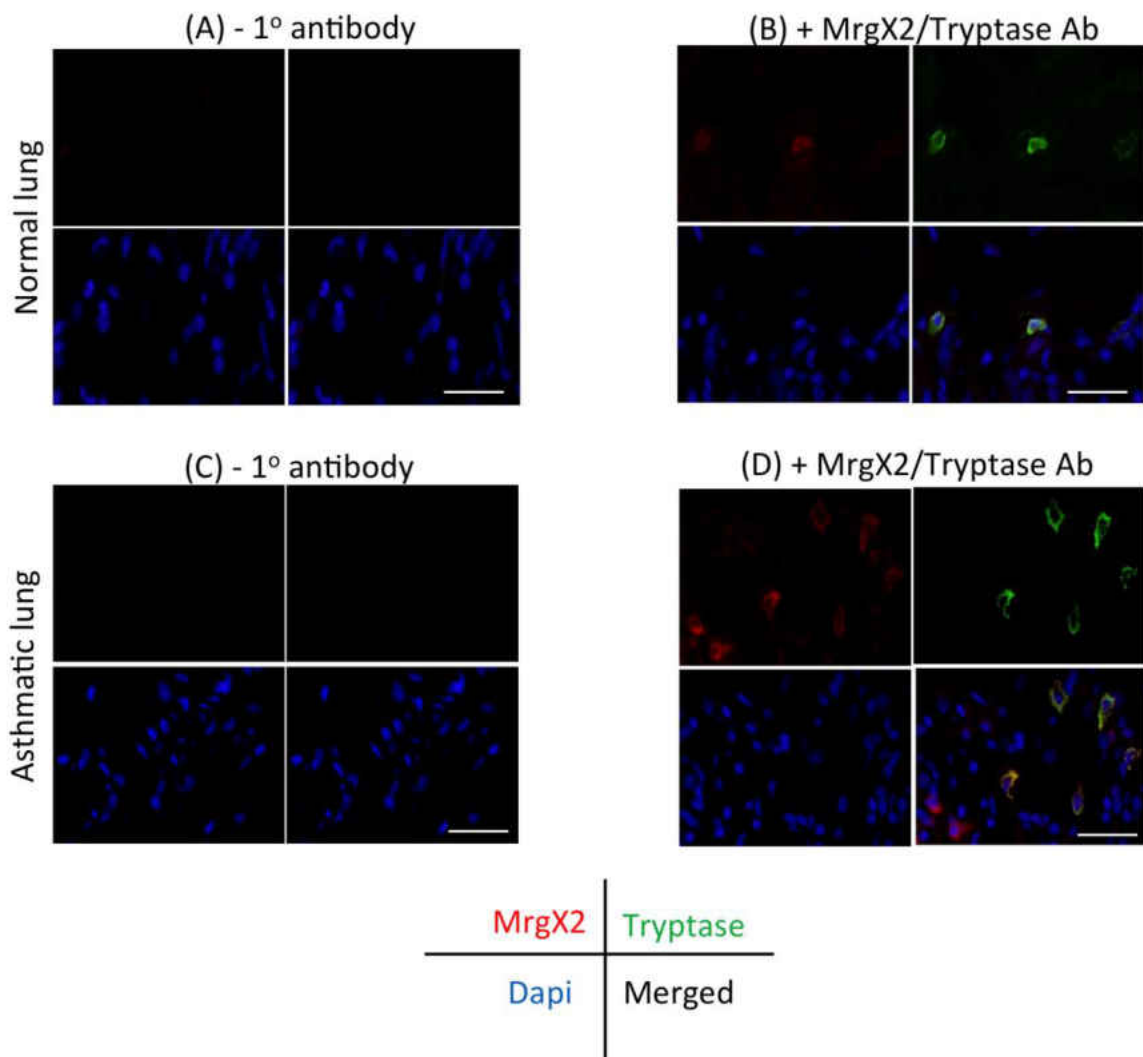
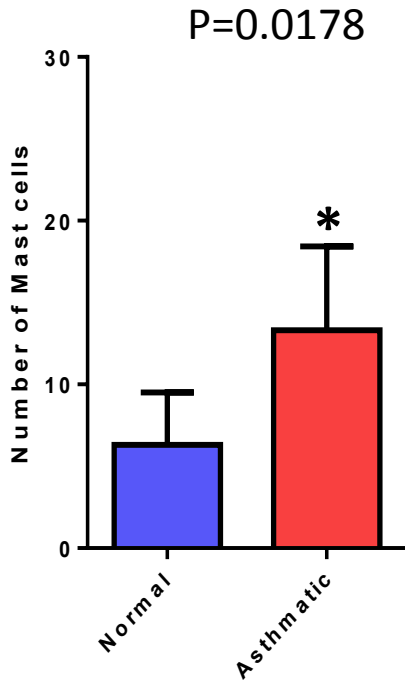


Figure 12

(A) Mast cells



(B) MrgX2

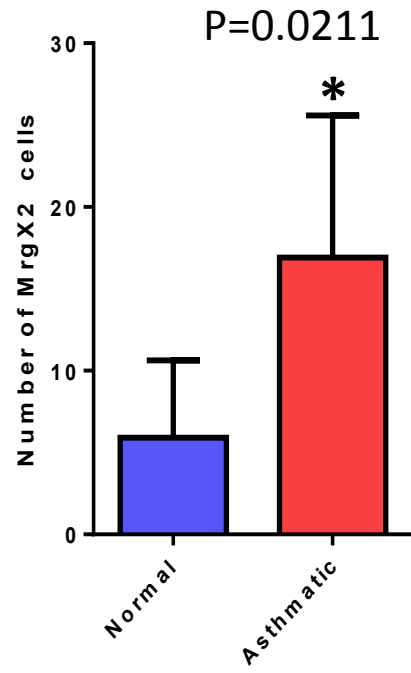


Figure 13

MrgX2⁺ Mast cells

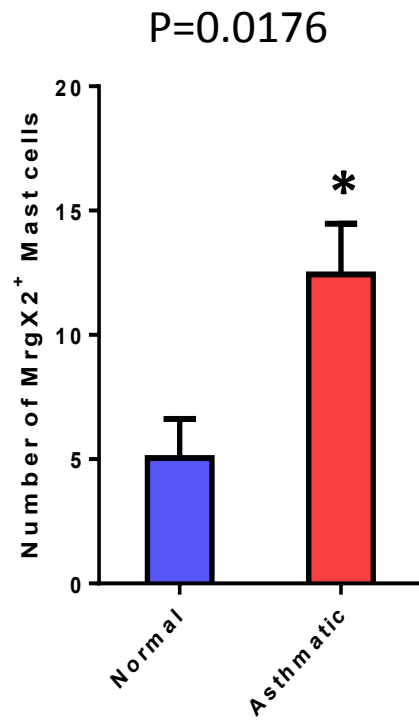


Figure 14

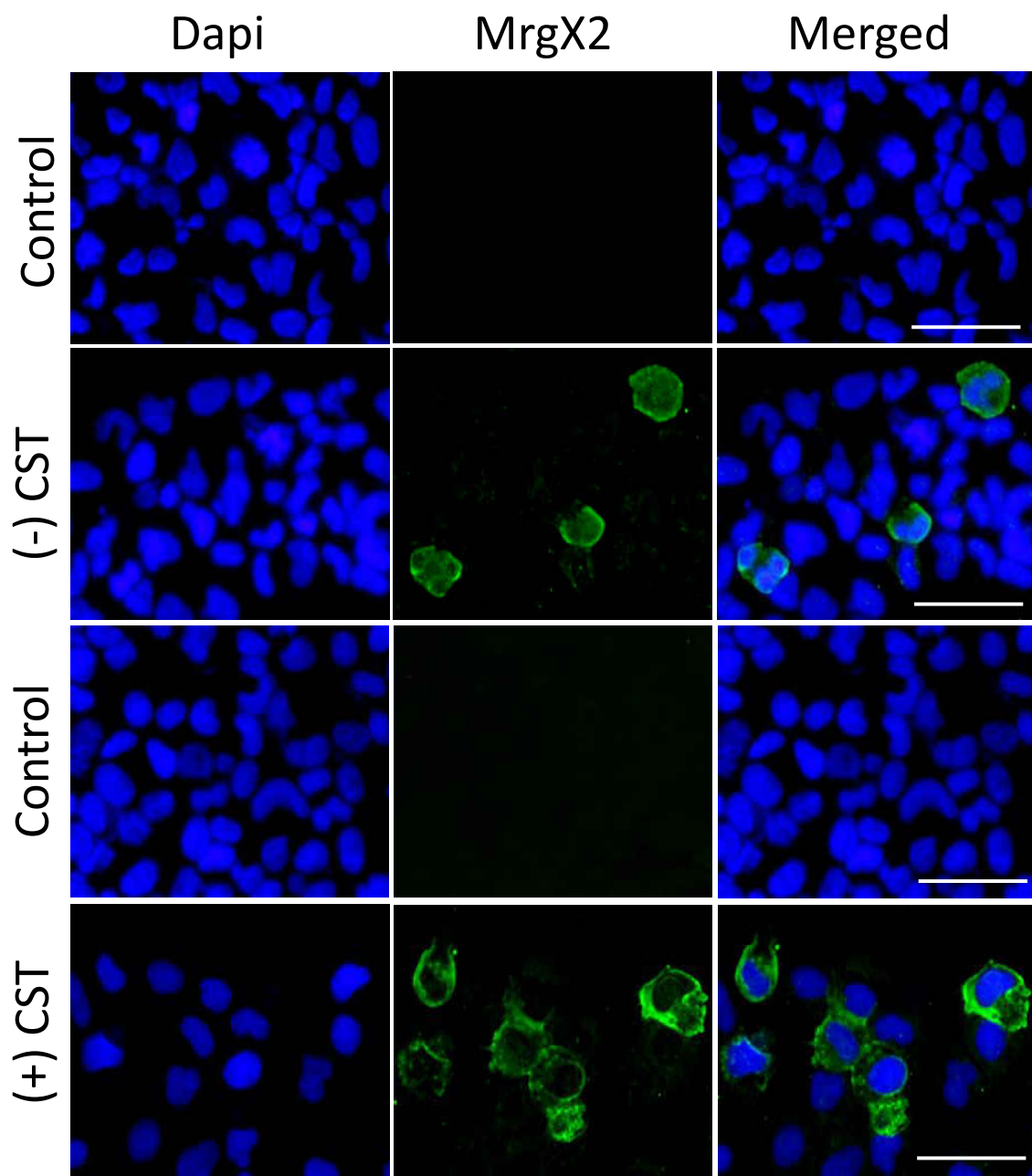


Figure 15

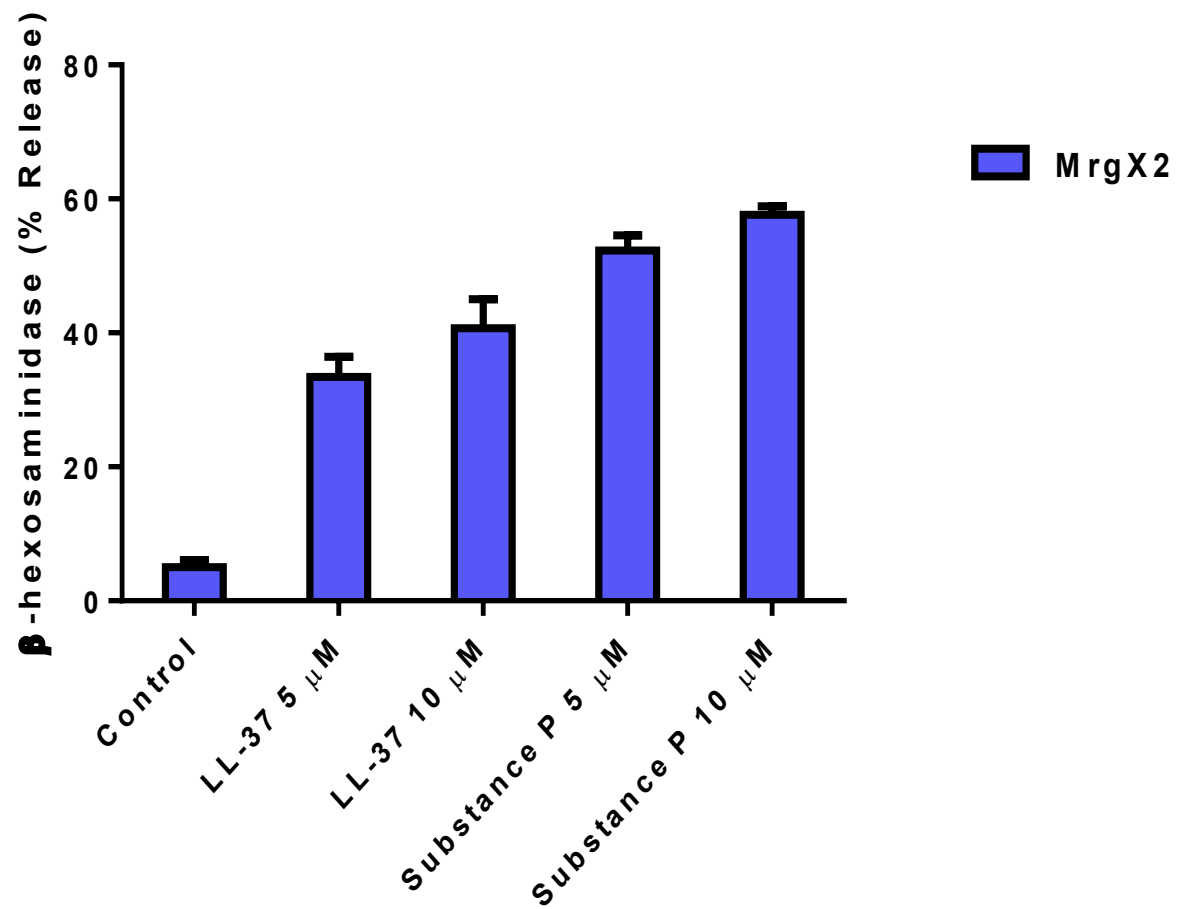


Figure 16

Busulfan-conditioned humanized mouse lung

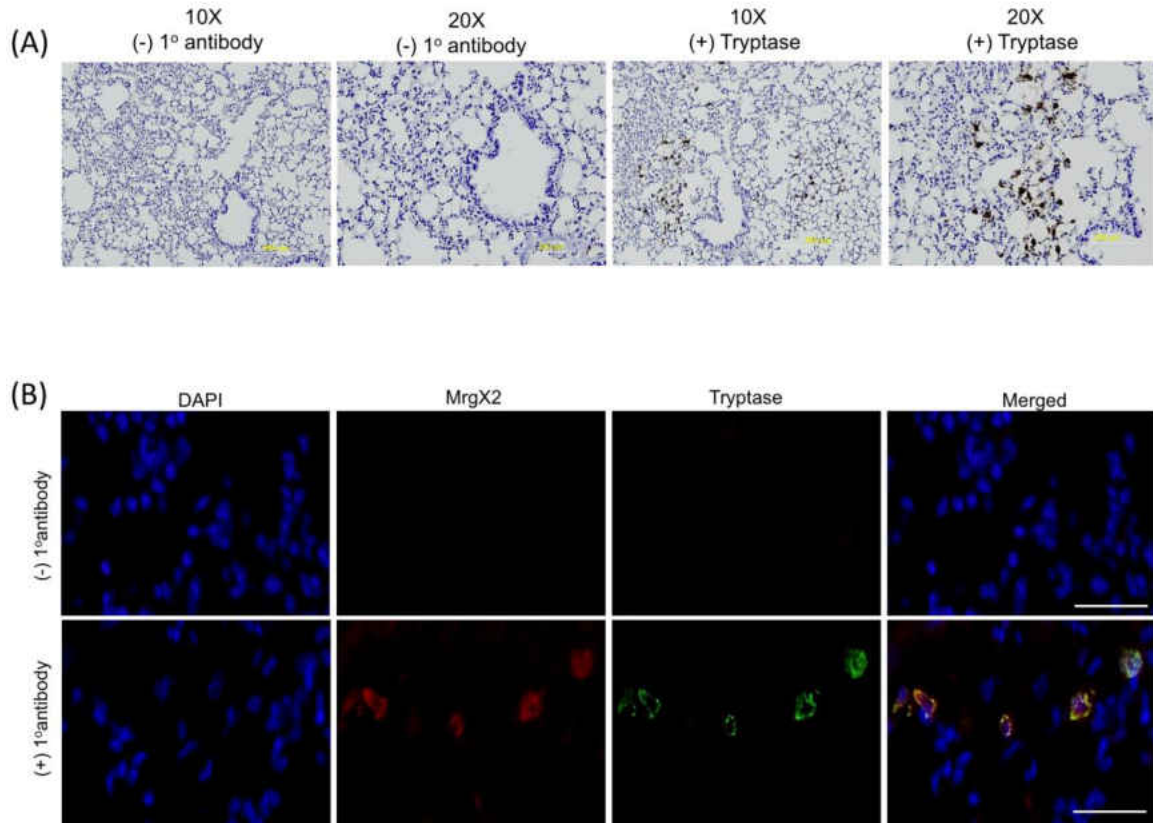


Figure 17

Gamma-irradiated humanized mouse lung

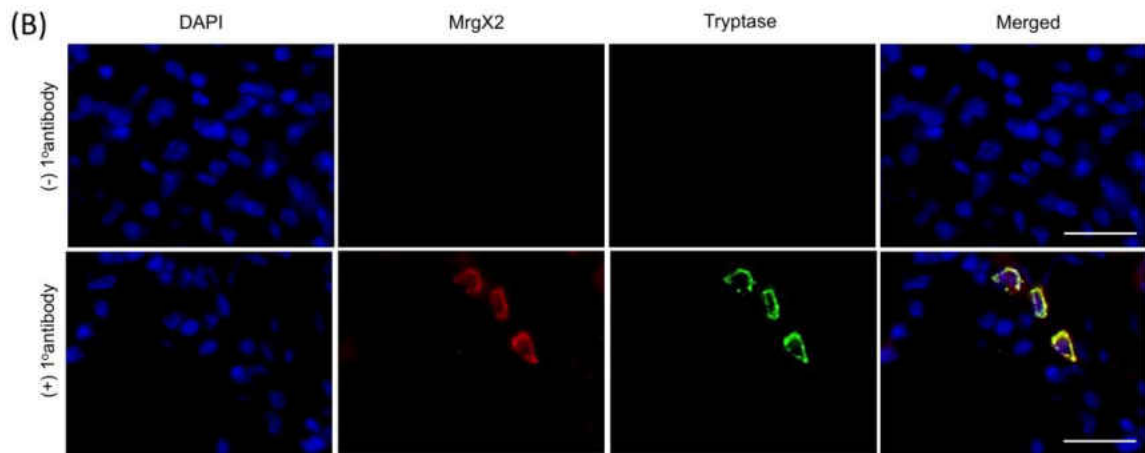
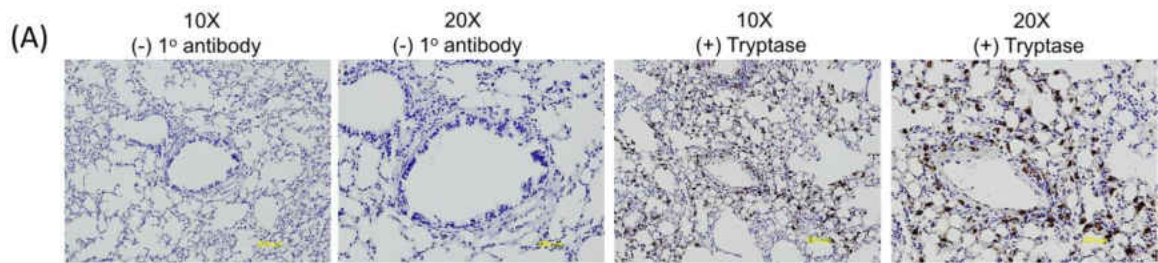


Figure 18

8. FIGURE LEGEND

Figure 1. MCs in skin of patients with AD. IHC was performed on human skin tissue from (A and B) control subjects and (C and D) patients diagnosed with AD using the MC marker tryptase. Note dark brown cytoplasmic staining of MCs. Most MCs were observed in the dermal layers in both healthy and diseased skin tissue compared to the epidermis. 4X scale bar = 2 mm while 20X scale bar = 200 μ m.

Figure 2. MrgX2 is expressed in skin MCs of normal subjects and patients with AD. IF was performed on human skin tissue from (A and B) control subjects and (C and D) AD patients. MrgX2 is shown in red, tryptase in green and the nuclei blue. Note that there is a greater degree of colocalization in AD compared with normal. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μ m.

Figure 3. MC number in normal and AD skin. Skin samples from 3 patients diagnosed with AD and 3 control patients were analyzed using IF. Pictures were taken from 5 randomly selected visual fields in each skin sample at a magnification of 40X. Image exposure, size and dimensions were exactly the same for all analyzed images. The number of skin MCs was not significantly different in skin of AD patients compared with control subjects.

Figure 4. Intensity of MrgX2 expression by MCs in AD. Using colocalized IF images, the intensity of the green tryptase staining by MCs as well as red MrgX2 staining in the same MCs was measured using Metamorph image analysis software. Image exposure was exactly the same for all analyzed images. The intensity of tryptase expression by MCs was not significantly different in skin tissues from control subjects compared with AD patients. There was also no significant difference in the intensity of MrgX2 expression by MCs between the 2 groups.

Figure 5. MCs in skin of patients with rosacea. IHC was performed on human skin tissue from (A and B) control subjects and (C and D) patients diagnosed with rosacea using the MC marker tryptase. Note dark brown cytoplasmic staining of MCs. Most MCs were observed in the dermal layers in both healthy and diseased skin tissue compared to the epidermis. 4X scale bar = 2 mm while 20X scale bar = 200 μ m.

Figure 6. MrgX2 is expressed in skin MCs of normal subjects and patients with rosacea. IF was performed on human skin tissue from (A and B) control subjects and (C and D) rosacea patients. MrgX2 is shown in red, tryptase in green and the nuclei blue. Note that there is a greater degree of colocalization in rosacea compared with normal. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μ m.

Figure 7. MC number in normal and rosacea skin. Skin samples from 2 patients diagnosed with rosacea and 3 control patients were analyzed using IF. Pictures were taken from 5 randomly selected visual fields in each skin sample at a magnification of 40X. Image exposure, size and dimensions were exactly the same for all analyzed images. The number of skin MCs was not significantly different in skin of rosacea patients compared with control subjects.

Figure 8. Intensity of MrgX2 expression by MCs in rosacea. Using colocalized IF images, the intensity of the green tryptase staining by MCs as well as red MrgX2 staining in the same MCs was measured using Metamorph image analysis software. Image exposure was exactly the same for all analyzed images. The intensity of tryptase expression by MCs was significantly reduced in skin tissues from rosacea patients compared with control ($P = 0.0260$). However, there was no significant difference in the intensity of MrgX2 expression by MCs between the 2 groups.

Figure 9. MCs in gingiva of patients with CP. IHC was performed on human gingiva tissue from (A and B) control subjects and (C and D) patients diagnosed with CP using the MC marker tryptase. Note dark brown cytoplasmic staining of MCs. Most of the MCs are present in the lamina propria and submucosa in both healthy and diseased gingiva tissue compared to the epithelium. Also note that gingiva samples from patients with CP have a greater number of MCs in the

epithelium compared to the healthy gingiva. 4X scale bar = 2 mm while 20X scale bar = 200 μm

Figure 10. MrgX2 is expressed in gingival MCs of normal subjects and patients with CP. IF was performed on human gingiva tissue from (A and B) control subjects and (C and D) CP patients. MrgX2 is shown in red, tryptase in green and the nuclei blue. Note that there is a greater degree of colocalization in CP gingiva compared with normal gingiva. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μm .

Figure 11. MCs in lung of patients with asthma. IHC was performed on human lung tissue from (A and B) control subjects who died from non-asthma related causes and (C and D) patients who died from asthma complications using the MC marker tryptase. Note dark brown cytoplasmic staining of MCs. An increased number of MCs are present in the lung smooth muscles of asthma patients compared to healthy lung. 10X scale bar = 500 μm while 20X scale bar = 200 μm

Figure 12. MrgX2 is expressed in lung MCs of normal subjects and patients with asthma. IF was performed on human lung tissue from (A and B) control subjects who died from non-asthma related causes and (C and D) asthma patients who died from asthma complications. MrgX2 is shown in red, tryptase in green and the nuclei blue. Note that there is a greater degree of colocalization in

asthma lung compared with normal lung. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μ m.

Figure 13. MC number in normal and asthma lung. Lung tissue from 6 patients who died from asthma-related complications and 6 control patients who died from non-asthma related causes were analyzed using IF. Pictures were taken from 3 randomly selected visual fields in each sample at a magnification of 20X. Image exposure, size and dimensions were exactly the same for all analyzed images. The number of lung MCs was significantly greater in lung tissue from patients who died from asthma complications compared with control subjects ($P= 0.0178$). The number of MrgX2-positive cells was also significantly greater in lung tissue from patients who died from asthma complications compared with control subjects ($P= 0.0211$). In both healthy and asthmatic lungs, a proportion of MrgX2-positive cells were not MCs.

Figure 14. Number of MrgX2-positive MCs in normal and asthma lung. Image analysis was performed as in Fig. 13. The number of MrgX2-positive MCs was significantly greater in lung tissue from patients who died from asthma complications compared with control subjects ($P=0.0176$).

Figure 15. MrgX2 localization in RBL-2H3 cells transiently expressing HA-tagged MrgX2. IF analysis was performed on RBL-2H3 cells transiently expressing HA- tagged MrgX2 before and after stimulation with CST using anti-

HA antibody for the detection of MrgX2. Prior to stimulation with CST, MrgX2 is diffusely expressed on the plasma membrane as well as the cytoplasm. On stimulation with CST, the receptor remained on the plasma membrane as well as in the cytoplasm but with a more peri-nuclear and punctate appearance. Images taken at 20X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 100 μ m.

Figure 16. Effects of LL-37 and SP-induced degranulation on RBL-2H3 cells stably expressing MrgX2. RBL-2H3 cells stably expressing MrgX2 were exposed to buffer (control), LL-37 at 5 μ M and 10 μ M and SP at 5 μ M and 10 μ M. Percentage degranulation (β -hexosaminidase release) was determined. Data are Mean \pm SEM of 3 experiments.

Figure 17. Human MCs in the lung of NSG-SGM3 mice preconditioned with busulfan express MrgX2. IHC performed with tryptase antibody demonstrates that human MCs develop in the lung of humanized mice preconditioned with busulfan (Fig. 17A). IF was also performed on the humanized mice lung tissue (Fig. 17B). MrgX2 is shown in red, tryptase in green and the nuclei blue. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μ m.

Figure 18. Human MCs in the lung of NSG-SGM3 mice preconditioned by irradiation express MrgX2. IHC performed with tryptase antibody demonstrates

that human MCs develop in the lung of humanized mice preconditioned by irradiation (Fig. 18A). IF was also performed on the humanized mice lung tissue (Fig. 18B). MrgX2 is shown in red, tryptase in green and the nuclei blue. Images taken at 40X magnification using a Nikon Eclipse fluorescent microscope. Scale bar = 50 μ m.

9. BIBLIOGRAPHY

1. Galli SJ, Grimaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol*. 2008 Jun;8(6):478-86.
2. Sismanopoulos N, Delivanis DA, Alysandratos KD, Angelidou A, Therianou A, Kalogeromitros D, et al. Mast cells in allergic and inflammatory diseases. *Curr Pharm Des*. 2012;18(16):2261-77.
3. Cruse G, Bradding P. Mast cells in airway diseases and interstitial lung disease. *Eur J Pharmacol*. 2015 May 8.
4. Galli SJ, Tsai M. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. *Eur J Immunol*. 2010 Jul;40(7):1843-51.
5. Weller CL, Collington SJ, Williams T, Lamb JR. Mast cells in health and disease. *Clin Sci (Lond)*. 2011 Jun;120(11):473-84.
6. Bachelet I, Levi-Schaffer F, Mekori YA. Mast cells: not only in allergy. *Immunol Allergy Clin North Am*. 2006 Aug;26(3):407-25.
7. Olivera A, Rivera J. Paradigm shifts in mast cell and basophil biology and function: an emerging view of immune regulation in health and disease. *Methods Mol Biol*. 2014;1192:3-31.
8. Theoharides TC, Alysandratos KD, Angelidou A, Delivanis DA, Sismanopoulos N, Zhang B, et al. Mast cells and inflammation. *Biochim Biophys Acta*. 2012 Jan;1822(1):21-33.
9. Reber LL, Sibilano R, Mukai K, Galli SJ. Potential effector and immunoregulatory functions of mast cells in mucosal immunity. *Mucosal Immunol*. 2015 May;8(3):444-63.
10. Amin K. The role of mast cells in allergic inflammation. *Respir Med*. 2012 Jan;106(1):9-14.
11. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A*. 1986 Jun;83(12):4464-8.
12. Schwartz LB. Mast cells: function and contents. *Curr Opin Immunol*. 1994 Feb;6(1):91-7.
13. Galli SJ, Kalesnikoff J, Grimaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. *Annu Rev Immunol*. 2005;23:749-86.
14. Broide DH, Finkelman F, Bochner BS, Rothenberg ME. Advances in mechanisms of asthma, allergy, and immunology in 2010. *J Allergy Clin Immunol*. 2011 Mar;127(3):689-95.
15. Kalesnikoff J, Galli SJ. Anaphylaxis: mechanisms of mast cell activation. *Chem Immunol Allergy*. 2010;95:45-66.
16. Moon TC, St Laurent CD, Morris KE, Marcet C, Yoshimura T, Sekar Y, et al. Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol*. 2010 Mar;3(2):111-28.

17. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. *Nat Rev Immunol*. 2010 Jun;10(6):440-52.
18. Mekori YA, Metcalfe DD. Mast cells in innate immunity. *Immunol Rev*. 2000 Feb;173:131-40.
19. Dawicki W, Marshall JS. New and emerging roles for mast cells in host defence. *Curr Opin Immunol*. 2007 Feb;19(1):31-8.
20. Mekori YA, Metcalfe DD. Mast cell-T cell interactions. *J Allergy Clin Immunol*. 1999 Sep;104(3 Pt 1):517-23.
21. Tkaczyk C, Frandji P, Botros HG, Poncet P, Lapeyre J, Peronet R, et al. Mouse bone marrow-derived mast cells and mast cell lines constitutively produce B cell growth and differentiation activities. *J Immunol*. 1996 Aug 15;157(4):1720-8.
22. Suto H, Nakae S, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cell-associated TNF promotes dendritic cell migration. *J Immunol*. 2006 Apr 1;176(7):4102-12.
23. Noli C, Miolo A. The mast cell in wound healing. *Vet Dermatol*. 2001 Dec;12(6):303-13.
24. Frandji P, Oskeritzian C, Cacaraci F, Lapeyre J, Peronet R, David B, et al. Antigen-dependent stimulation by bone marrow-derived mast cells of MHC class II-restricted T cell hybridoma. *J Immunol*. 1993 Dec 1;151(11):6318-28.
25. Frandji P, Tkaczyk C, Oskeritzian C, David B, Desaymard C, Mecheri S. Exogenous and endogenous antigens are differentially presented by mast cells to CD4+ T lymphocytes. *Eur J Immunol*. 1996 Oct;26(10):2517-28.
26. Malaviya R, Twosten NJ, Ross EA, Abraham SN, Pfeifer JD. Mast cells process bacterial Ags through a phagocytic route for class I MHC presentation to T cells. *J Immunol*. 1996 Feb 15;156(4):1490-6.
27. Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol Rev*. 2003 Mar;55(1):27-55.
28. Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest*. 2003 Jun;111(11):1665-72.
29. Bowdish DM, Davidson DJ, Hancock RE. Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol*. 2006;306:27-66.
30. Weinberg A, Jin G, Sieg S, McCormick TS. The yin and yang of human Beta-defensins in health and disease. *Front Immunol*. 2012 Oct 8;3:294.
31. Lehrer RI, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol*. 2002 Feb;14(1):96-102.
32. Subramanian H, Gupta K, Lee D, Bayir AK, Ahn H, Ali H. beta-Defensins activate human mast cells via Mas-related gene X2. *J Immunol*. 2013 Jul 1;191(1):345-52.

33. Schaubert J, Dorschner RA, Yamasaki K, Brouha B, Gallo RL. Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. *Immunology*. 2006 Aug;118(4):509-19.
34. Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I. Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol*. 2001 Apr;31(4):1066-75.
35. Schiemann F, Brandt E, Gross R, Lindner B, Mittelstadt J, Sommerhoff CP, et al. The cathelicidin LL-37 activates human mast cells and is degraded by mast cell tryptase: counter-regulation by CXCL4. *J Immunol*. 2009 Aug 15;183(4):2223-31.
36. Niyonsaba F, Ushio H, Hara M, Yokoi H, Tominaga M, Takamori K, et al. Antimicrobial peptides human beta-defensins and cathelicidin LL-37 induce the secretion of a pruritogenic cytokine IL-31 by human mast cells. *J Immunol*. 2010 Apr 1;184(7):3526-34.
37. Soruri A, Grigat J, Forssmann U, Riggert J, Zwirner J. beta-Defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved. *Eur J Immunol*. 2007 Sep;37(9):2474-86.
38. Niyonsaba F, Iwabuchi K, Matsuda H, Ogawa H, Nagaoka I. Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway. *Int Immunol*. 2002 Apr;14(4):421-6.
39. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, et al. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology*. 2002 May;106(1):20-6.
40. Chen X, Niyonsaba F, Ushio H, Hara M, Yokoi H, Matsumoto K, et al. Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *Eur J Immunol*. 2007 Feb;37(2):434-44.
41. Bienenstock J, MacQueen G, Sestini P, Marshall JS, Stead RH, Perdue MH. Mast cell/nerve interactions in vitro and in vivo. *Am Rev Respir Dis*. 1991 Mar;143(3 Pt 2):S55-8.
42. Metcalfe DD, Baram D, Mekori YA. Mast cells. *Physiol Rev*. 1997 Oct;77(4):1033-79.
43. Pundir P, Kulka M. The role of G protein-coupled receptors in mast cell activation by antimicrobial peptides: is there a connection? *Immunol Cell Biol*. 2010 Aug;88(6):632-40.
44. Gilfillan AM, Beaven MA. Regulation of mast cell responses in health and disease. *Crit Rev Immunol*. 2011;31(6):475-529.
45. Okayama Y, Ono Y, Nakazawa T, Church MK, Mori M. Human skin mast cells produce TNF-alpha by substance P. *Int Arch Allergy Immunol*. 1998 Sep;117 Suppl 1:48-51.
46. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides activate human mast cell degranulation and chemokine production. *Immunology*. 2008 Mar;123(3):398-410.
47. Erjavec F, Lembeck F, Florjanc-Irman T, Skofitsch G, Donnerer J, Saria A, et al. Release of histamine by substance P. *Naunyn Schmiedebergs Arch Pharmacol*. 1981 Aug;317(1):67-70.

48. Lowman MA, Benyon RC, Church MK. Characterization of neuropeptide-induced histamine release from human dispersed skin mast cells. *Br J Pharmacol.* 1988 Sep;95(1):121-30.
49. Grundemar L, Hakanson R. Neuropeptide Y, peptide YY and C-terminal fragments release histamine from rat peritoneal mast cells. *Br J Pharmacol.* 1991 Dec;104(4):776-8.
50. Maurer M, Theoharides T, Granstein RD, Bischoff SC, Bienenstock J, Henz B, et al. What is the physiological function of mast cells? *Exp Dermatol.* 2003 Dec;12(6):886-910.
51. Artuc M, Hermes B, Steckelings UM, Grutzkau A, Henz BM. Mast cells and their mediators in cutaneous wound healing--active participants or innocent bystanders? *Exp Dermatol.* 1999 Feb;8(1):1-16.
52. Meininger CJ, Zetter BR. Mast cells and angiogenesis. *Semin Cancer Biol.* 1992 Apr;3(2):73-9.
53. Clark RA. Biology of dermal wound repair. *Dermatol Clin.* 1993 Oct;11(4):647-66.
54. Woodley DT, Chen JD, Kim JP, Sarret Y, Iwasaki T, Kim YH, et al. Re-epithelialization. Human keratinocyte locomotion. *Dermatol Clin.* 1993 Oct;11(4):641-6.
55. Moulin V. Growth factors in skin wound healing. *Eur J Cell Biol.* 1995 Sep;68(1):1-7.
56. Rao KN, Brown MA. Mast cells: multifaceted immune cells with diverse roles in health and disease. *Ann N Y Acad Sci.* 2008 Nov;1143:83-104.
57. Maurer M, Paus R, Czarnetzki BM. Mast cells as modulators of hair follicle cycling. *Exp Dermatol.* 1995 Aug;4(4 Pt 2):266-71.
58. Silberstein R, Melnick M, Greenberg G, Minkin C. Bone remodeling in W/W^v mast cell deficient mice. *Bone.* 1991;12(4):227-36.
59. Nagasaka A, Matsue H, Matsushima H, Aoki R, Nakamura Y, Kambe N, et al. Osteopontin is produced by mast cells and affects IgE-mediated degranulation and migration of mast cells. *Eur J Immunol.* 2008 Feb;38(2):489-99.
60. Dong X, Han S, Zylka MJ, Simon MI, Anderson DJ. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell.* 2001 Sep 7;106(5):619-32.
61. Lembo PM, Grazzini E, Groblewski T, O'Donnell D, Roy MO, Zhang J, et al. Proenkephalin A gene products activate a new family of sensory neuron--specific GPCRs. *Nat Neurosci.* 2002 Mar;5(3):201-9.
62. Subramanian H, Kashem SW, Collington SJ, Qu H, Lambris JD, Ali H. PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in human mast cells. *Mol Pharmacol.* 2011 Jun;79(6):1005-13.
63. Subramanian H, Gupta K, Guo Q, Price R, Ali H. Mas-related gene X2 (MrgX2) is a novel G protein-coupled receptor for the antimicrobial peptide LL-37 in human mast cells: resistance to receptor phosphorylation, desensitization, and internalization. *J Biol Chem.* 2011 Dec 30;286(52):44739-49.

64. Tatemoto K, Nozaki Y, Tsuda R, Konno S, Tomura K, Furuno M, et al. Immunoglobulin E-independent activation of mast cell is mediated by Mrg receptors. *Biochem Biophys Res Commun*. 2006 Nov 3;349(4):1322-8.
65. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *J Allergy Clin Immunol*. 2014 Sep;134(3):622,633.e9.
66. Ferry X, Brehin S, Kamel R, Landry Y. G protein-dependent activation of mast cell by peptides and basic secretagogues. *Peptides*. 2002 Aug;23(8):1507-15.
67. Hernanz A, Muelas G, Borbujo J. Plasma neuropeptide pattern in acute idiopathic urticaria. *Int Arch Allergy Appl Immunol*. 1989;90(2):198-200.
68. Smith CH, Atkinson B, Morris RW, Hayes N, Foreman JC, Lee TH. Cutaneous responses to vasoactive intestinal polypeptide in chronic idiopathic urticaria. *Lancet*. 1992 Jan 11;339(8785):91-3.
69. Robas N, Mead E, Fidock M. MrgX2 is a high potency cortistatin receptor expressed in dorsal root ganglion. *J Biol Chem*. 2003 Nov 7;278(45):44400-4.
70. Kashem SW, Subramanian H, Collington SJ, Magotti P, Lambris JD, Ali H. G protein coupled receptor specificity for C3a and compound 48/80-induced degranulation in human mast cells: roles of Mas-related genes MrgX1 and MrgX2. *Eur J Pharmacol*. 2011 Oct 1;668(1-2):299-304.
71. Okayama Y, Saito H, Ra C. Targeting human mast cells expressing g-protein-coupled receptors in allergic diseases. *Allergol Int*. 2008 Sep;57(3):197-203.
72. McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature*. 2015 Mar 12;519(7542):237-41.
73. Pitcher JA, Freedman NJ, Lefkowitz RJ. G protein-coupled receptor kinases. *Annu Rev Biochem*. 1998;67:653-92.
74. Krupnick JG, Benovic JL. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol*. 1998;38:289-319.
75. Langkabel P, Zwirner J, Oppermann M. Ligand-induced phosphorylation of anaphylatoxin receptors C3aR and C5aR is mediated by "G protein-coupled receptor kinases. *Eur J Immunol*. 1999 Sep;29(9):3035-46.
76. Kaplan AP, Greaves M. Pathogenesis of chronic urticaria. *Clin Exp Allergy*. 2009 Jun;39(6):777-87.
77. Ferrer M, Nunez-Cordoba JM, Luquin E, Grattan CE, De la Borbolla JM, Sanz ML, et al. Serum total tryptase levels are increased in patients with active chronic urticaria. *Clin Exp Allergy*. 2010 Dec;40(12):1760-6.
78. Tidman MJ. Managing urticaria in primary care. *Practitioner*. 2015 Feb;259(1779):25,8, 3.

79. Metz M, Krull C, Hawro T, Saluja R, Groffik A, Stanger C, et al. Substance P is upregulated in the serum of patients with chronic spontaneous urticaria. *J Invest Dermatol*. 2014 Nov;134(11):2833-6.
80. Borici-Mazi R, Kouridakis S, Kontou-Fili K. Cutaneous responses to substance P and calcitonin gene-related peptide in chronic urticaria: the effect of cetirizine and dimethindene. *Allergy*. 1999 Jan;54(1):46-56.
81. Peters MS, Schroeter AL, Kephart GM, Gleich GJ. Localization of eosinophil granule major basic protein in chronic urticaria. *J Invest Dermatol*. 1983 Jul;81(1):39-43.
82. Piliponsky AM, Gleich GJ, Nagler A, Bar I, Levi-Schaffer F. Non-IgE-dependent activation of human lung- and cord blood-derived mast cells is induced by eosinophil major basic protein and modulated by the membrane form of stem cell factor. *Blood*. 2003 Mar 1;101(5):1898-904.
83. Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev*. 2011 Jul;242(1):233-46.
84. Akdis CA, Akdis M, Bieber T, Bindslev-Jensen C, Boguniewicz M, Eigenmann P, et al. Diagnosis and treatment of atopic dermatitis in children and adults: European Academy of Allergology and Clinical Immunology/American Academy of Allergy, Asthma and Immunology/PRACTALL Consensus Report. *J Allergy Clin Immunol*. 2006 Jul;118(1):152-69.
85. Eichenfield LF, Tom WL, Chamlin SL, Feldman SR, Hanifin JM, Simpson EL, et al. Guidelines of care for the management of atopic dermatitis: section 1. Diagnosis and assessment of atopic dermatitis. *J Am Acad Dermatol*. 2014 Feb;70(2):338-51.
86. Nutten S. Atopic dermatitis: global epidemiology and risk factors. *Ann Nutr Metab*. 2015;66 Suppl 1:8-16.
87. Bieber T. Atopic dermatitis. *N Engl J Med*. 2008 Apr 3;358(14):1483-94.
88. Wen HJ, Chen PC, Chiang TL, Lin SJ, Chuang YL, Guo YL. Predicting risk for early infantile atopic dermatitis by hereditary and environmental factors. *Br J Dermatol*. 2009 Nov;161(5):1166-72.
89. Schultz Larsen FV, Holm NV. Atopic dermatitis in a population based twin series. Concordance rates and heritability estimation. *Acta Derm Venereol Suppl (Stockh)*. 1985;114:159.
90. Mu Z, Zhao Y, Liu X, Chang C, Zhang J. Molecular biology of atopic dermatitis. *Clin Rev Allergy Immunol*. 2014 Oct;47(2):193-218.
91. Irvine AD, McLean WH, Leung DY. Filaggrin mutations associated with skin and allergic diseases. *N Engl J Med*. 2011 Oct 6;365(14):1315-27.
92. Palmer CN, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. *Nat Genet*. 2006 Apr;38(4):441-6.
93. Wollenberg A, Seba A, Antal AS. Immunological and molecular targets of atopic dermatitis treatment. *Br J Dermatol*. 2014 Jul;170 Suppl 1:7-11.

94. Man MQ, Hatano Y, Lee SH, Man M, Chang S, Feingold KR, et al. Characterization of a hapten-induced, murine model with multiple features of atopic dermatitis: structural, immunologic, and biochemical changes following single versus multiple oxazolone challenges. *J Invest Dermatol*. 2008 Jan;128(1):79-86.
95. Mihm MC, Jr, Soter NA, Dvorak HF, Austen KF. The structure of normal skin and the morphology of atopic eczema. *J Invest Dermatol*. 1976 Sep;67(3):305-12.
96. Irani AM, Sampson HA, Schwartz LB. Mast cells in atopic dermatitis. *Allergy*. 1989;44 Suppl 9:31-4.
97. Soter NA. Morphology of atopic eczema. *Allergy*. 1989;44 Suppl 9:16-9.
98. Sugiura H, Hirota Y, Uehara M. Heterogeneous distribution of mast cells in lichenified lesions of atopic dermatitis. *Acta Derm Venereol Suppl (Stockh)*. 1989;144:115-8.
99. Liu FT, Goodarzi H, Chen HY. IgE, mast cells, and eosinophils in atopic dermatitis. *Clin Rev Allergy Immunol*. 2011 Dec;41(3):298-310.
100. Terada M, Tsutsui H, Imai Y, Yasuda K, Mizutani H, Yamanishi K, et al. Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice. *Proc Natl Acad Sci U S A*. 2006 Jun 6;103(23):8816-21.
101. Ong PY, Leung DY. Immune dysregulation in atopic dermatitis. *Curr Allergy Asthma Rep*. 2006 Sep;6(5):384-9.
102. Horsmanheimo L, Harvima IT, Jarvikallio A, Harvima RJ, Naukkarinen A, Horsmanheimo M. Mast cells are one major source of interleukin-4 in atopic dermatitis. *Br J Dermatol*. 1994 Sep;131(3):348-53.
103. Obara W, Kawa Y, Ra C, Nishioka K, Soma Y, Mizoguchi M. T cells and mast cells as a major source of interleukin-13 in atopic dermatitis. *Dermatology*. 2002;205(1):11-7.
104. Spergel JM, Mizoguchi E, Oettgen H, Bhan AK, Geha RS. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest*. 1999 Apr;103(8):1103-11.
105. Black PH. Stress and the inflammatory response: a review of neurogenic inflammation. *Brain Behav Immun*. 2002 Dec;16(6):622-53.
106. Jarvikallio A, Harvima IT, Naukkarinen A. Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. *Arch Dermatol Res*. 2003 Apr;295(1):2-7.
107. Salomon J, Baran E. The role of selected neuropeptides in pathogenesis of atopic dermatitis. *J Eur Acad Dermatol Venereol*. 2008 Feb;22(2):223-8.
108. Kapp A. The role of eosinophils in the pathogenesis of atopic dermatitis--eosinophil granule proteins as markers of disease activity. *Allergy*. 1993 Jan;48(1):1-5.
109. Berg M, Liden S. An epidemiological study of rosacea. *Acta Derm Venereol*. 1989;69(5):419-23.

110. Feldman SR, Huang WW, Huynh TT. Current drug therapies for rosacea: a chronic vascular and inflammatory skin disease. *J Manag Care Spec Pharm*. 2014 Jun;20(6):623-9.
111. Doe PT, Asiedu A, Acheampong JW, Rowland Payne CM. Skin diseases in Ghana and the UK. *Int J Dermatol*. 2001 May;40(5):323-6.
112. Khaled A, Hammami H, Zeglaoui F, Tounsi J, Zermani R, Kamoun MR, et al. Rosacea: 244 Tunisian cases. *Tunis Med*. 2010 Aug;88(8):597-601.
113. Del Rosso JQ. Management of cutaneous rosacea: emphasis on new medical therapies. *Expert Opin Pharmacother*. 2014 Oct;15(14):2029-38.
114. Wollina U. Recent advances in the understanding and management of rosacea. *F1000Prime Rep*. 2014 Jul 8;6:50,50. eCollection 2014.
115. Two AM, Wu W, Gallo RL, Hata TR. Rosacea: part I. Introduction, categorization, histology, pathogenesis, and risk factors. *J Am Acad Dermatol*. 2015 May;72(5):749,58; quiz 759-60.
116. Heiberger K, Brenman S. Common triggers of facial erythema in adults. *JAAPA*. 2001 Sep;14(9):49,50, 53-4.
117. Landow K. Unraveling the mystery of rosacea. Keys to getting the red out. *Postgrad Med*. 2002 Dec;112(6):51,8, 82; quiz 9.
118. Yamasaki K, Gallo RL. Rosacea as a disease of cathelicidins and skin innate immunity. *J Investig Dermatol Symp Proc*. 2011 Dec;15(1):12-5.
119. Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, et al. Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. *Nat Med*. 2007 Aug;13(8):975-80.
120. Schaubert J, Gallo RL. The vitamin D pathway: a new target for control of the skin's immune response? *Exp Dermatol*. 2008 Aug;17(8):633-9.
121. Yamasaki K, Schaubert J, Coda A, Lin H, Dorschner RA, Schechter NM, et al. Kallikrein-mediated proteolysis regulates the antimicrobial effects of cathelicidins in skin. *FASEB J*. 2006 Oct;20(12):2068-80.
122. Meyer-Hoffert U, Schroder JM. Epidermal proteases in the pathogenesis of rosacea. *J Investig Dermatol Symp Proc*. 2011 Dec;15(1):16-23.
123. Larrick JW, Hirata M, Zhong J, Wright SC. Anti-microbial activity of human CAP18 peptides. *Immunotechnology*. 1995 May;1(1):65-72.
124. Yamasaki K, Kanada K, Macleod DT, Borkowski AW, Morizane S, Nakatsuji T, et al. TLR2 expression is increased in rosacea and stimulates enhanced serine protease production by keratinocytes. *J Invest Dermatol*. 2011 Mar;131(3):688-97.
125. Aroni K, Tsagrani E, Kavantzias N, Patsouris E, Ioannidis E. A study of the pathogenesis of rosacea: how angiogenesis and mast cells may participate in a complex multifactorial process. *Arch Dermatol Res*. 2008 Mar;300(3):125-31.

126. Schwab VD, Sulk M, Seeliger S, Nowak P, Aubert J, Mess C, et al. Neurovascular and neuroimmune aspects in the pathophysiology of rosacea. *J Invest Dermatol Symp Proc*. 2011 Dec;15(1):53-62.
127. Di Nardo A, Vitiello A, Gallo RL. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. *J Immunol*. 2003 Mar 1;170(5):2274-8.
128. Muto Y, Wang Z, Vanderberghe M, Two A, Gallo RL, Di Nardo A. Mast cells are key mediators of cathelicidin-initiated skin inflammation in rosacea. *J Invest Dermatol*. 2014 Nov;134(11):2728-36.
129. Jang YH, Sim JH, Kang HY, Kim YC, Lee ES. Immunohistochemical expression of matrix metalloproteinases in the granulomatous rosacea compared with the non-granulomatous rosacea. *J Eur Acad Dermatol Venereol*. 2011 May;25(5):544-8.
130. Wollina U. Rhinophyma--unusual expression of simple-type keratins and S100A in sebocytes and abundance of VIP receptor-positive dermal cells. *Histol Histopathol*. 1996 Jan;11(1):111-5.
131. Kurkcuoglu N, Alaybeyi F. Substance P immunoreactivity in rosacea. *J Am Acad Dermatol*. 1991 Oct;25(4):725-6.
132. Huang S, Lu F, Chen Y, Huang B, Liu M. Mast cell degranulation in human periodontitis. *J Periodontol*. 2013 Feb;84(2):248-55.
133. Batista AC, Rodini CO, Lara VS. Quantification of mast cells in different stages of human periodontal disease. *Oral Dis*. 2005 Jul;11(4):249-54.
134. Lagdive SS, Lagdive SB, Mani A, Anarthe R, Pendyala G, Pawar B, et al. Correlation of mast cells in periodontal diseases. *J Indian Soc Periodontol*. 2013 Jan;17(1):63-7.
135. Liu J, Chen J, Du X, Hu L, Chen L. The expression of hBDs in the gingival tissue and keratinocytes from healthy subjects and periodontitis patients. *Arch Oral Biol*. 2014 Feb;59(2):193-8.
136. Puklo M, Guentsch A, Hiemstra PS, Eick S, Potempa J. Analysis of neutrophil-derived antimicrobial peptides in gingival crevicular fluid suggests importance of cathelicidin LL-37 in the innate immune response against periodontogenic bacteria. *Oral Microbiol Immunol*. 2008 Aug;23(4):328-35.
137. Turkoglu O, Emingil G, Kutukculer N, Atilla G. Gingival crevicular fluid levels of cathelicidin LL-37 and interleukin-18 in patients with chronic periodontitis. *J Periodontol*. 2009 Jun;80(6):969-76.
138. Lei Y, Gregory JA, Nilsson GP, Adner M. Insights into mast cell functions in asthma using mouse models. *Pulm Pharmacol Ther*. 2013 Oct;26(5):532-9.
139. Ali H, Panettieri RA, Jr. Anaphylatoxin C3a receptors in asthma. *Respir Res*. 2005 Feb 21;6:19.
140. Maddox L, Schwartz DA. The pathophysiology of asthma. *Annu Rev Med*. 2002;53:477-98.

141. Strunk RC, Bloomberg GR. Omalizumab for asthma. *N Engl J Med*. 2006 Jun 22;354(25):2689-95.
142. Oskeritzian CA, Zhao W, Min HK, Xia HZ, Pozez A, Kiev J, et al. Surface CD88 functionally distinguishes the MCTC from the MCT type of human lung mast cell. *J Allergy Clin Immunol*. 2005 Jun;115(6):1162-8.
143. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med*. 2002 May 30;346(22):1699-705.
144. Balzar S, Fajt ML, Comhair SA, Erzurum SC, Bleecker E, Busse WW, et al. Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program. *Am J Respir Crit Care Med*. 2011 Feb 1;183(3):299-309.
145. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *Am J Respir Crit Care Med*. 2005 Mar 1;171(5):431-9.
146. Andersson CK, Bergqvist A, Mori M, Mauad T, Bjermer L, Erjefalt JS. Mast cell-associated alveolar inflammation in patients with atopic uncontrolled asthma. *J Allergy Clin Immunol*. 2011 Apr;127(4):905,12.e1-7.
147. Kajiwara N, Sasaki T, Bradding P, Cruse G, Sagara H, Ohmori K, et al. Activation of human mast cells through the platelet-activating factor receptor. *J Allergy Clin Immunol*. 2010 May;125(5):1137,1145.e6.
148. Busse WW, Lemanske RF, Jr, Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet*. 2010 Sep 4;376(9743):826-34.
149. Duits LA, Nibbering PH, van Strijen E, Vos JB, Mannesse-Lazeroms SP, van Sterkenburg MA, et al. Rhinovirus increases human beta-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. *FEMS Immunol Med Microbiol*. 2003 Aug 18;38(1):59-64.
150. Proud D. The role of defensins in virus-induced asthma. *Curr Allergy Asthma Rep*. 2006 Feb;6(1):81-5.
151. Kota S, Sabbah A, Chang TH, Harnack R, Xiang Y, Meng X, et al. Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-kappaB-mediated innate antiviral response against human respiratory syncytial virus. *J Biol Chem*. 2008 Aug 15;283(33):22417-29.
152. Proud D, Sanders SP, Wiehler S. Human rhinovirus infection induces airway epithelial cell production of human beta-defensin 2 both in vitro and in vivo. *J Immunol*. 2004 Apr 1;172(7):4637-45.
153. Monteseirin J. Neutrophils and asthma. *J Investig Allergol Clin Immunol*. 2009;19(5):340-54.
154. Brehm MA, Shultz LD, Greiner DL. Humanized mouse models to study human diseases. *Curr Opin Endocrinol Diabetes Obes*. 2010 Apr;17(2):120-5.
155. Shultz LD, Brehm MA, Bavari S, Greiner DL. Humanized mice as a preclinical tool for infectious disease and biomedical research. *Ann N Y Acad Sci*. 2011 Dec;1245:50-4.
156. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol*. 2007 Feb;7(2):118-30.

157. Choi B, Chun E, Kim M, Kim ST, Yoon K, Lee KY, et al. Human B cell development and antibody production in humanized NOD/SCID/IL-2Rgamma(null) (NSG) mice conditioned by busulfan. *J Clin Immunol*. 2011 Apr;31(2):253-64.
158. Singh M, Singh P, Gaudray G, Musumeci L, Thielen C, Vaira D, et al. An improved protocol for efficient engraftment in NOD/LTSZ-SCIDIL-2Rgammanull mice allows HIV replication and development of anti-HIV immune responses. *PLoS One*. 2012;7(6):e38491.
159. Kambe N, Hiramatsu H, Shimonaka M, Fujino H, Nishikomori R, Heike T, et al. Development of both human connective tissue-type and mucosal-type mast cells in mice from hematopoietic stem cells with identical distribution pattern to human body. *Blood*. 2004 Feb 1;103(3):860-7.
160. Nicolini FE, Cashman JD, Hogge DE, Humphries RK, Eaves CJ. NOD/SCID mice engineered to express human IL-3, GM-CSF and Steel factor constitutively mobilize engrafted human progenitors and compromise human stem cell regeneration. *Leukemia*. 2004 Feb;18(2):341-7.
161. Wunderlich M, Chou FS, Link KA, Mizukawa B, Perry RL, Carroll M, et al. AML xenograft efficiency is significantly improved in NOD/SCID-IL2RG mice constitutively expressing human SCF, GM-CSF and IL-3. *Leukemia*. 2010 Oct;24(10):1785-8.
162. Gupta K, Kotian A, Subramanian H, Daniell H, Ali H. Activation of human mast cells by retrocyclin and protegrin highlight their immunomodulatory and antimicrobial properties. *Oncotarget*. 2015 Oct 6;6(30):28573-87.
163. Kubanov AA, Katunina OR, Chikin VV. Expression of Neuropeptides, Neurotrophins, and Neurotransmitters in the Skin of Patients with Atopic Dermatitis and Psoriasis. *Bull Exp Biol Med*. 2015 Jul;159(3):318-22.
164. Hazlett L, Wu M. Defensins in innate immunity. *Cell Tissue Res*. 2011 Jan;343(1):175-88.
165. Marshall JS, Jawdat DM. Mast cells in innate immunity. *J Allergy Clin Immunol*. 2004 Jul;114(1):21-7.
166. Ramos R, Silva JP, Rodrigues AC, Costa R, Guardao L, Schmitt F, et al. Wound healing activity of the human antimicrobial peptide LL37. *Peptides*. 2011 Jul;32(7):1469-76.
167. Duplantier AJ, van Hoek ML. The Human Cathelicidin Antimicrobial Peptide LL-37 as a Potential Treatment for Polymicrobial Infected Wounds. *Front Immunol*. 2013 Jul 3;4:143.
168. Parachin NS, Franco OL. New edge of antibiotic development: antimicrobial peptides and corresponding resistance. *Front Microbiol*. 2014 Apr 8;5:147.