Role of MITF in IgE and MRGPRX2-dependent mast cell activation

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Acknowledgments

When I started to write my doctoral dissertation, I was excited, because I am going to end my Ph.D. study. I felt like time flowed slowly and fast. A lot of memories and details come to my mind, from when I came to Barcelona and started my work of Ph.D. It is not easy. After starting my Ph.D. study, I learned and contacted many techniques and scientific ideals. I became more and more interested in my projects and focused on them. Of course, work and life do not always go well. I met many difficulties and challenges I had never met in my previous life. But I overcame them and fixed them, finally. I grow a lot and get stronger and more independent. During this period, many people gave me help and support. Otherwise, it would be super difficult for me to finish my Ph.D. study. From this part, I would like to sincerely appreciate those who helped and supported me during my Ph.D. study.

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Abstract

Anaphylaxis is a severe allergic reaction, even life-threatening. The main effector cells are mast cells, which locates in antigen portal entries such as mucose and skin. Mast cells have cytosolic granules which contain proinflammatory mediators that can be released after various stimuli. The common allergens are venoms, drugs, pollen, dust, and food. There are two distinct molecular pathways of mast cell activation: IgE-dependent and IgE-independent. Related to the last, MRGPRX2, G-protein-coupled-seven transmembrane domain receptor, has emerged as responsible for pseudoallergic reactions to several drugs: antibiotics (vancomycin, ciprofloxacin), neuromuscular blocking agents (atracurium), and opiates (morphine).

Microphthalmia-associated transcription factor (MITF) is essential for the differentiation of mast cells. It regulates the expression of several genes critical for mast cell degranulation, including proteases, tryptase and chymase. MITF is also responsible for the biosynthesis of mediators such as Prostaglandin D2 (PGD2), a mast cell-specific eicosanoid derived from arachidonic acid. PGD2 has bronchoconstriction and vasoactive properties. Recently, it has been reported that MITF controls IgE-mast cell-mediated anaphylaxis by regulating histidine decarboxylase (*HDC*), the enzyme that catalyzes histamine synthesis. Histamine can cause smooth muscle contraction, increased vascular permeability, and vasodilation-inducing anaphylaxis.

In quiescent mast cells, MITF activity is suppressed through its interaction with Histidine triad nucleotide-binding protein 1 (HINT1) at the nucleus. When allergen-IgE-FcεRI crosslinks, Lysyl-tRNA synthetase (LysRS) is translocated to the nucleus, which plays a role in MITF activation. LysRS is a moonlighting protein with a canonical function in protein synthesis and a non-canonical role in Ag dependent-FcεRI activation in mast cells. LysRS is phosphorylated on Serine 207 in IgE-activated mast cells in a MAPK-dependent manner. This phosphorylation causes LysRS to undergo a conformational shift that translocates it to the nucleus and produces Ap4A, which binds

to HINT1 and releases MITF, allowing subsequent activation of MITF and transcription of MITF-targeted genes.

In collaboration with clinicians from the allergy department in Hospital Clinic-Barcelona, part of this thesis is related to a clinical case of a patient with severe anaphylaxis to wasp allergy. This patient has a mutation in the *KARS* gene, which encodes the LysRS. In this thesis, we examined the mutation at the molecular level. Biochemical and functional approaches: cell transfection, western blot, confocal microscopy, cell degranulation, PGD2 secretion, and proteases gene transcription were analyzed. Structural analysis using Molecular Dynamics Simulations and Well-tempered Metadynamics was also performed in collaboration with Dr. Orozco's group at IRB-Barcelona. We found that LysRS mutation, P542R (proline was replaced by arginine at residue 542), changes the location of the protein in quiescent mast cells, as we showed by biochemical and structural analyses. Mutation resembles active LysRS and causes constitutive activation of MITF. The structural study also reveals how LysRS functions in mast cell activation. So, it was possible to establish a connection between the abnormal LysRS P542R function and the over-activation of mast cells with an increase in the release of pro-inflammatory mediators following an Ag-IgE-dependent response. Moreover, this study has clinical implications and highlights a signaling pathway: IgE-LysRS-MITF, that can set the severity of anaphylaxis. In addition, MITF downregulation reduces IgE-dependent mast cell degranulation.

MRGPRX2 is mainly involved in skin immunity and pain and is expressed in mast cells and neurons. It is implicated in the pathophysiology of non-IgE-mediated immediate hypersensitivity and has been related to adverse drug reactions. Our group and others have shown that various drugs induce mast cell activation through MRGPRX2-dependent mechanisms. Moreover, a role for this receptor has been proposed in asthma, atopic dermatitis, contact dermatitis, and chronic spontaneous urticaria. Although it has a significant role in disease, its signaling transduction is poorly understood. Another part of this thesis explores the role of MITF in MRGPRX2-

dependent mast cell activation. MRGPRX2 activation with substance P, a known ligand for MRGPRX2, showed increased LysRS translocation to the nucleus and enhanced MITF phosphorylation and MITF activity. Therefore, overexpression of LysRS increased MITF activity after MRGPRX2 activation, indicating that MRGPRX2 involves in LysRS and MITF pathway. Furthermore, MITF silencing reduced MRGPRX2-dependent calcium influx and mast cell degranulation. Moreover, MITF pathway inhibitor ML329 impaired MITF expression, calcium influx, and mast cell degranulation. Additionally, drugs known to trigger MRGPRX2-dependent degranulation, including atracurium, vancomycin, and morphine, increased MITF activity. Altogether, MRGPRX2 signaling augments MITF activity and its abrogation by silencing or inhibition causes MRGPRX2 degranulation impairment. We conclude that MRGPRX2 signaling involves the LysRS-MITF pathway, and MITF plays a role in calcium influx and degranulation in mast cells. In addition, this thesis shows preliminary data presenting that silencing and inhibition of MITF decrease STIM 1 expression. STIM 1 is a calcium sensor that activates calcium channels in the plasma membrane. Unpublished data of this thesis shows that MITF is also involved in late MRGPRX2-dependent cell signal events regulating proinflammatory IL-8 secretion.

In conclusion, this thesis shows the relevance of MITF in IgE and MRGPRX2-dependent mast cell activation. Thus, MITF and MITF-dependent genes may become targets to treat IgE or MRGPRX2-dependent pathologies.

Keywords: LysRS, MITF, FcεRI, MRGPRX2, mast cells degranulation, anaphylaxis, adverse drug reactions.

Abbreviations

aaRS: Aminoacyl tRNA Synahetases.

ACD: Allergic Contact Dermatitis.

AD: Atopic Dermatitis.

AD-HIES: Autosomal Dominant Hyper IgE Syndrome.

ALR: Absent-In-Melanoma (AIM)-Like Receptor.

Ap_4A: Diadenosine Tetraphosphate.

APC: Antigen-Presenting Cell.

BAT: Basophils Activation Test.

C/EBPα: CCAAT/Enhancer-Binding Protein alpha.

CCL1: Chemokine C-C Motif Ligand 1.

CCL2: Chemokine C-C Motif Ligand 2, (also known as MCP1).

CLP: Common Lymphoid Progenitor.

CLR: C-Type Lectin-Like Receptor.

CMP: Common Myeloid Progenitor.

CXCL10: Chemokine CXC Motif Ligand 10.

CXCL8: Chemokine CXC Motif Ligand 8.

CXCR1: C-X-C Motif Chemokine Receptor 1.

CysLTs: Cysteinyl Leukotrienes.

ECL: Extracellular Loop.

ECM: Extracellular Matrix.

FcεRII: Low-affinity IgE Receptor.

FcεRI: High-affinity IgE receptor.

GM-CSF: Granulocyte Macrophage - Colony Stimulating Factor.

GMP: Granulocyte-Monocyte Progenitors.

GIST: Gastrointestinal Stromal Tumor

GPCR: G-protein-coupled receptors.

HDC: Histidine Decarboxylase.

HDP: Host Defense Peptide.

HINT1: Histidine Triad Protein.

HMC-1: Human Mast Cell 1: Human Mast Cell Line with Oncogenic KIT Receptor.

HSPCs: CD34+ Progenitor Cells.

IFN I: Type I Interferon.

IL-13: Interleukin 13.

IPDef1: IP defensin 1.

IRDef2: IR defensin 2.

ITAM: Immunoreceptor Tyrosine-based Activation Motif.

KIT: Cluster of differentiation 117, CD117.

LAD2: Laboratory of allergic diseases 2.

LADR cells: Laboratory of Allergic Diseases Re-established.

LAT: Linker for Activation of T Cells.

LTB4: Leukotriene B4.

LTC4: Leukotriene C4.

LysRS: Lysyl-tRNA Synthetase.

MAPK: Mitogen-Activated Protein Kinase.

MAT: Mast Cell Activation.

MAZR: also known as PATZ1.

MC(T): Mast Cell Mucosal.

MC(TC): Mast Cell Connective Tissue.

MITF: Microphthalmia-associated transcription factor.

MPP: Multiple Potential Progenitors.

Mrgprb2: Mas-related G protein-coupled receptor B2.

Mrgprs: Mas-related G protein-coupled receptors.

MRGPRX2: Mas-Related G Protein-coupled Receptor X2.

MSC: Multi-tRNA Synthetase Complex.

NFAT: Nuclear Factor of Activated T-cells.

NFκB: Nuclear Factor kappa-light-chainenhancer of activated B cells.

NLRs: Nucleotide-Binding Oligomerization Domain (NOD)-Like Receptor.

NMBA: Neuromuscular Blocking Agent.

NSAID: Non-Steroidal Anti-Inflammatory Drugs.

PAF: Platelet-Activating Factor.

PAF-AH: PAF-acetylhydrolase.

PAMP: Proadrenomedullin N-terminal Peptide.

PGE2: Prostaglandin E2.

PI3K: Phosphoinositide 3-Kinase (γ or δ).

PLCγ: Phospholipase C gamma.

RBL: Rat Basophilic Leukemia cells.

Rh: Recombinant Human.

RLR: Retinoic acid-Inducible Gene I (RIG-I) Like Receptor.

SCF: Stem Cell Factor.

SHIP: SH3-containing Inositol Phosphatase.

SHP1 and SHP2: cytoplasmic SH2 Domain-Containing Protein Tyrosine Phosphatase 1and 2.

SPT: Skin Prick Test.

STAT: Signal Transducer and Activator of Transcription.

STIM 1：Stromal Interaction Molecule 1.

SH3BP2: SH3 Domain Binding Protein 2

TAMC: Tumor-Associated Mast Cells.

TF: Transcription Factor.

TFE3: The Transcription Factor E3.

TFEB: The Transcription Factor EB.

Tfh13: T follicular Helper Cells.

TLR: Toll-Like Receptor.

TNF: Tumor Necrosis Factor.

TNFα: Tumor Necrosis Factor alpha.

TSLP: Thymic Stromal Lymphopoietin.

VEGF: Vascular Endothelial Growth Factor.

1. Introduction

1.1 Mast cells

1.1.1 Mast cell origin

Mast cells originate from $CD34^+$ progenitor cells (HSPCs) [1][2]. Then give rise to multiple potential progenitors (MPPs). MPPs, in turn, differentiate into both common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs); CMPs can give rise to granulocyte-monocyte progenitors (GMPs) [3]. GMPs grow into mast cells and basophils in different conditions [4]. Selective expression of two transcription factors is relevant for that. MITF is upregulated and C/EBPα is further downregulated during the commitment of mast cell progenitors, but during the commitment of basophil progenitor, MITF is downregulated, and C/EBPα is upregulated [5].

Mast cell precursors migrate into tissues, differentiating to mature mast cells. Mast cells are widely distributed throughout the body, and their distribution is particularly concentrated in the skin [6], respiratory tract [7], and gastrointestinal tract [8]. β7 integrin is essential for mast cells progenitor migration to the small intestine, as shown in β7 integrin-deficient mice (on the C57BL/6 background), where mast cells are present in the lung, spleen, and bone marrow but not in the small intestine, indicating that β7 integrin is critical for homing of these cells to the small intestine [9]. Mast cells, in some tissues, developed at an early stage. One literature reported that mast cell precursors were observed in the skin of mouse embryos on day 15 of the liver, being more mast cells found in the skin on day 17 [10]. Various factors regulate the differentiation of mast cells. The most important is the stem cell factor (SCF)， which is an essential growth factor promoting mast cell generation and survival after binding to the KIT receptor [11]. IL-4 regulates proliferation and mediator release in mature human mast cells [12]. There are two types of mast cells, connective tissue (MC(TC)) and mucosal (MC(T)) mast cells, regarding protease granular content. MC(TC)

contains tryptase and chymase, and MC (T) produces tryptase [13].

Several mast cell lines have been generated for different research purposes. Laboratory of allergic diseases 2 (LAD2) cells are $CD34^+$-derived mast cells from a mastocytosis patient with no identified mutations in KIT. LAD2 cells can be used for degranulation assay, surface receptors research, and granular production. LAD2 cells require SCF for survival and proliferation. LADR is similar to LAD2 but with low proliferation [14]. HMC-1 cell line, a cell model for mastocytosis, has two KIT mutations (V560G, D816V). From this, two sublines have been generated HMC-1.1 (carrying KIT V560G)) and HMC-1.2 (carrying both mutations). HMC-1.2 exhibits a higher rate of cell division compared to HMC-1.1. It has been suggested that the increased proliferative potential observed in HMC-1.2 may be attributed to the presence of the KIT D816V mutation [15].

Primary cells, $CD34^+$ derived human mast cells are isolated from human peripheral blood and cultured in media containing recombinant human (rh) SCF, rhIL-6, and rhIL-3 (added only during the first week), SCF, and rhIL-6 for the rest culture time (2-8 weeks), until cells are available. Human mast cells are determined by two surface makers, KIT and high-affinity IgE receptors (FcεRI) [16].

RBL cells (Rat Basophilic Leukemia cells) are easy to transfect and have been extensively studied in FcεRI signal transduction studies as a murine model [17]. Another cell line is the MC/9 cell line, a murine mast cell line derived from the bone marrow cells of C57BL/6 mice. The MC/9 cells can be stimulated to release histamine and other mediators of allergic responses, making them an avaliable tool for studying the mechanisms of mast cell activation [18][19].

1.1.2 Mast cell mediators

Upon mast cells activation by an allergen, or another stimulus, they release a variety of chemical mediators that can cause inflammation, anaphylactic reactions, and

other immune responses. All of the symptoms of an inflammation and anaphylactic reaction are brought on by the actions of the proinflammatory mediators generated by sensitized cells (mast cells and basophils) after antigen exposure or other stimulants [20]. These mediators are commonly divided into two categories: firstly, preformed granule products, histamine, tryptase, carboxypeptidase, chymase, heparin, chondroitin sulfate, and some cytokines; secondarily, newly formed lipid mediators, which includes platelet-activating factor, prostaglandin D2 (PGD2), leukotriene B4 (LTB4), leukotriene C4 (LTC4), and cytokines and chemokines, as shown Fig 1. Histamine, for example, is strongly linked with the pathophysiology of human anaphylaxis. At the same time, lipid mediators, including PAF, prostaglandins, and leukotrienes, clearly contribute in mice and, to a different extent, in humans [20]. The severity of the initial reaction has also been linked to the peak serum levels of IL-2, IL-6, IL-10, and TNFRI [21].

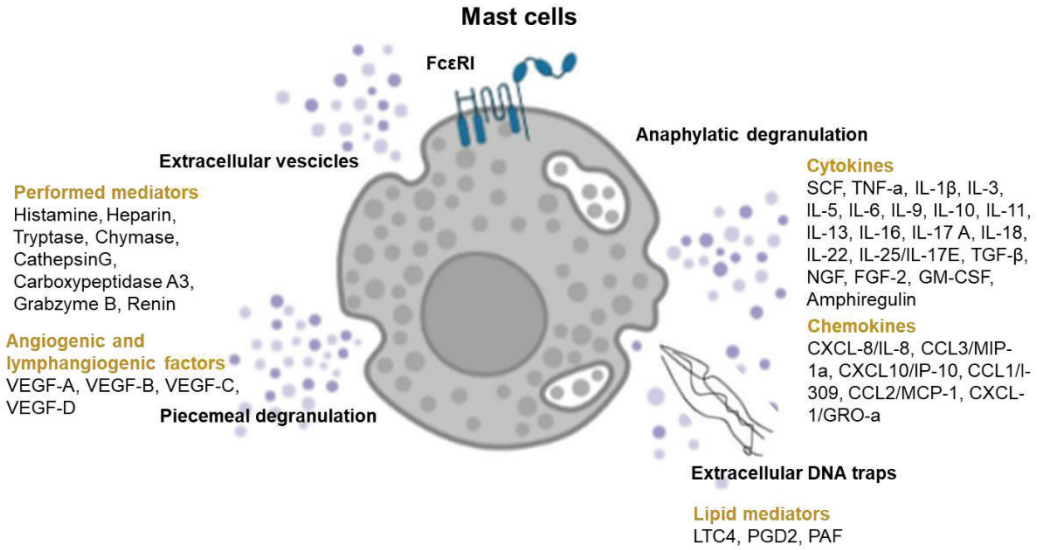

Fig 1. Proinflammatory and immunomodulatory mediators of human mast cells. Secretory granules of human mast cells selectively contain several preformed mediators (i.e., histamine, heparin, tryptase, chymase, cathepsin G, carboxypeptidase A3, granzyme B, and renin). Activated mast cells can produce a constellation of cytokines (SCF, TNF-α, IL-1β, IL-3, IL-5, IL-6, IL-9, IL-10, IL-11, IL-13, IL-16, IL-17A, IL-18, 1L-22, IL-25/IL-17E, TGF-β, NGF, FGF-2, GM-CSF, and amphiregulin), chemokines (CXCL-8/IL-8, CCL3/MIP-1α, CXCL10/IP-10, CCL1/I-309,

CCL2/MCP-1, CXCL-1/GRO-α), lipid mediators (LTC4, PGD2, and PAF), and angiogenic (VEGF-A and VEGF-B) and lymphangiogenic (VEGF-C and VEGF-D) factors. Mast cell activation can be accompanied by releasing of extracellular vesicles containing specific proteases and the formation of extracellular DNA traps, the figure was modified from [22].

Histidine decarboxylase (HDC), which catalyzes the decarboxylation of histidine, produces histamine, a vasoactive amine. Histamine, mostly secreted by mast cells and basophils, induces systemic hemodynamic alterations, systemic hypotension, and airway obstruction and is the prototypical mediator of anaphylaxis in humans and animals. Blood histamine levels are correlated with symptom severity and persistence in diseases [23]. These effects derive from its binding to the H1 and H4 receptors [24]. In addition, histamine affects the neurological conditions associated with hippocampal neuroinflammation and neurodegeneration [25].

Tryptases, known as serine proteases, are the main components of mast cell granules (a lesser extent of basophils). The gold standard for the diagnosis of anaphylaxis is the total serum level of tryptase, which correlates with the severity of the reactions as a mediator of some of the clinical symptoms, including urticaria [26], angioedema [27], and bronchospasm [28]. During an anaphylactic reaction, the serum tryptase values in the blood start to increase approximately 5 to 30 minutes after the onset of the event. The levels peak within 1 to 3 hours and gradually return to the baseline value within 16 to 24 hours following the reaction. The half-life of tryptase is estimated to be around 1.5 to 2.5 hours, indicating that it takes much time for half of the enzyme to be degraded and eliminated from the body. Measuring serum tryptase levels during the post-reaction period can help diagnose the event and assess the reaction severity level. However, other clinical evaluations are also required to confirm the diagnosis [29]. Different levels of tryptase can affect the accuracy of diagnosis in anaphylaxis [30]. The human mast cell tryptases comprise four genes: *TPSG1*, *TPSB2, TPSAB1*, and *TPSD1*. The solitary membrane-bound member of the family is encoded by the first (γ tryptase); the other tryptases are soluble. *TPSB2* encodes for βII and βIII

tryptases, *TPSAB1* encodes for α and βI, and *TPSD1* for δ-tryptase. Only α and β are likely to contribute to circulating tryptase levels, with β being the principal active tryptase in anaphylaxis [31]. The genes that encode proteases mTMT, mMCP6, and mMCP7 in mice are designated *Tpsg1*, *Tpsb2*, and *Tpsab1* (*TPSD1* is not expressed in mice). The first one is soluble, while the others are transmembrane. Additional diversity and allelic variety contribute to increased diversity in both species. An increased risk for severe anaphylaxis in humans has recently been linked to hereditary variations in the number of copies of the gene *TPSAB1* that codes for α-tryptase [32]. According to some reports, not all cases of anaphylaxis increase blood tryptase levels; this may be due to the mast cell subtype (mucosal mast cells typically have less tryptase per cell than skin mast cells) or antigen administration (tryptase levels in cases of gut anaphylaxis may end up in the gut rather than the bloodstream) [31].

Platelet-activating factor (PAF) is a phospholipid-derived proinflammatory mediator produced by mast cells, basophils, and platelets. PAF-acetylhydrolase (PAF-AH), which hydrolyzes PAF into an inert molecule, adversely regulates it. Deficiency of PAF-AH predisposed patients to severe anaphylaxis [33]. PAF injection causes anaphylaxis in mouse models, which can be prevented to varying degrees with a PAF antagonist [34].

Prostaglandins and Cysteinyl Leukotrienes (CysLTs: LTC4, LTD4, and LTE4) are produced by mast cells from arachidonic acid. Mastocytosis increases the urine metabolites PGD2 and LTC4 respectively. Although they can function as a biomarker, it is unclear how they contribute to the pathophysiology of anaphylaxis [35].

Cytokines and Chemokines are newly generated substances, and the blood concentration of these substances is delayed in human anaphylaxis. Several cytokines may influence the pathophysiology of anaphylaxis [20]. Proinflammatory cytokines such as IL-1β, IL-6, TNFα, and TNFRI enhance responses in mast cells and other immune cells, while IL-10 has a detrimental effect by dampening responses. Cytokines IL-4, IL-5, and IL-13 are produced by Th2 cells and contribute to IgE production, which

amplifies the response to vasoactive mediators. The severity of anaphylaxis has been linked to persistently elevated levels of IL6, IL-10, and TNFRI [21] [36]. Recently a study showed that circulating levels of TNF-like weak inducer of apoptosis and its receptor (Fn14) had been linked to an increase in patients with anaphylaxis, with changes in vascular permeability underlying this pathological event [37]. When it comes to chemokines, human anaphylaxis results in an increase in the chemokine CCL2, and as a result, the signaling cascade it triggers may be crucial for basophil chemotactic activity [38]. It was previously established that histone deacetylase 3 binds to the CCL2 promoter regions in the absence of an allergen to inhibit CCL2 expression. Histone deacetylase 3 binds to FcεRI and causes mast cell production of CCL2 to increase in response to allergen stimulation [39].

1.1.3 Mast cell physiologic functions

As we know that mast cells are important immune cells, playing the role of immunity because of the distribution of mast cells and mediators by interacting with other cells, as Fig 2 shows.

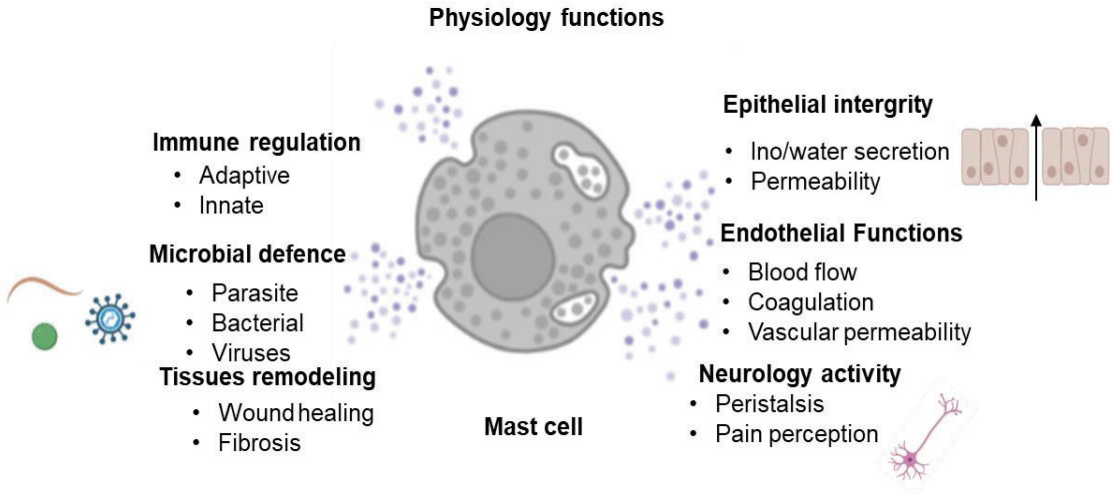

Fig 2. Physiological functions of mast cells in the immune system. Mast cells play an important role in multiple functions necessary for homeostasis, including epithelial, endothelial and neurological functions, tissue transformation, host defense, and immunity.

Interestingly, cutaneous mast cell levels rise with proximity to the epidermis in various organs distance from the center of the body, exhibiting two distinct gradients of distribution [40]. In other words, where the possibility of infection is lowest, contains significantly more mast cells than the hands, feet, and face, where the risk of bacterial infection is highest. Since their discovery, it has been assumed that mast cells perform advantageous innate immunological functions. For example, it has long been thought that mast cells digest infection function [41]. For parasites infection is involved in strong primary and secondary IgE responses, and it has long been believed that mast cells help the host defend against parasites by immunologically activating via FcεRI. Indeed, research on mice lacking in mast cells has demonstrated that mast cells play a key role in the evacuation of several parasites [42]. Another function of mast cells is limiting the toxicity of some host-produced substances that can have negative consequences at high concentrations is another protective role of mast cells during innate responses to bacterial infection. As an example, by releasing proteases contained in their granules that may break down the poisonous peptide endothelin 1 (ET-1), whose concentrations are significantly greater during acute bacterial peritonitis and sepsis, mast cells might reduce the toxicity of this peptide [43][44]. When injected human tryptase β1 into the trachea of mast cell-deficient mice, which protects against lung infection brought on by Klebsiella pneumoniae [45].

The accumulation of mast cells and release of proinflammatory and immunomodulatory mediators have been well documented in wound healing. One piece of literature claimed that the in vivo recruitment of mast cells is associated with the release of monocyte chemoattractant protein-1 (MCP-1) by keratinocytes and macrophages. The proximity of mast cells and fibroblasts created by MCP-1-mediated recruitment of mast cells to the site of injury allows for fibroblast proliferation to be induced by mast cells-released IL-4 [46]. Mast cells have been shown to stimulate fibroblast proliferation via IL-4, VEGF, and basic fibroblast growth factor (bFGF) [46] [47].

Mast cells affect the integrity of epithelial cells. Tryptase is released from mast cells and activates the protease-activated receptor (PAR2) expressed on epithelial cells [48]. Mast cell chymase is essential for inducing bronchial epithelial damage in asthma [49].

Mast cells are important cells of many vascular functions by releasing vascular endothelial growth factor (VEGF) [50]. Histamine released from mast cells increases the vascular hyperpermeability [51].

A study demonstrating the relationships between mast cells and nerves found that stimulation of spinal nerves activated mast cells, which in turn released mediators, which then activated submucosal neurons in the colon [52]. Clinically, there was a significant correlation between the intensity of pain and tryptase levels in patients who are with the complex regional pain syndrome [53].

1.1.4 Mast cell pathologic functions

Mat cells are an essential function of the immune system, which may promote a number of disease processes. In this thesis, following diseases were focused to be introduced.

Anaphylaxis

Allergy is our the body's immune system response to normally harmless substances, such as drugs, venoms, and food. Mast cells and basophils are ain effectors, here, we talk about mast cells being involved in allergies. For the activation of mast cells, there are two typical mechanisms,IgE-dependent and IgE-independent pathways. Activated mast cells release mediators inducing allergic reaction, with symptoms, a runny nose, coughing, wheezing or breathlessness, pain or tenderness around your cheeks, eyes or forehead, itchy skin or a raised rash (hives) [54].

The term "anaphylaxis," which derives from the Greek words "ana," which means

"against," and "phylax," which means "guard or protection," refers to a major allergic reaction that affects several body systems, including the respiratory and cardiovascular and may even be life-threatening if left untreated [55]. Different triggers can cause anaphylactic reactions, the most common include drugs [56], insect venom [57], and food, the latter of which is more common in children [58], as Fig 3 shows.

Drug consumption has been rising, due to the development of medicine for decades, various drugs can induce anaphylaxis: antibiotics (penicillins, cephalosporins), radiocontrast media, neuromuscular blocking agents, chemotherapy, nonsteroidal anti-inflammatory drugs (aspirin, and so on) [59]. Most anaphylaxis and anaphylactic shock induced by drugs are type I hypersensitivity reactions following gender and age, 20.15% of cases are drug-induced elicitors of anaphylaxis following the female and age [60]. Traditionally due to this small molecular size, these drugs would rather qualify as haptens, which can bind large molecular substances to induce anaphylaxis an [61]. As we know now, some drugs induce anaphylaxis through the MRGPRX2 receptor of mast cells [62].

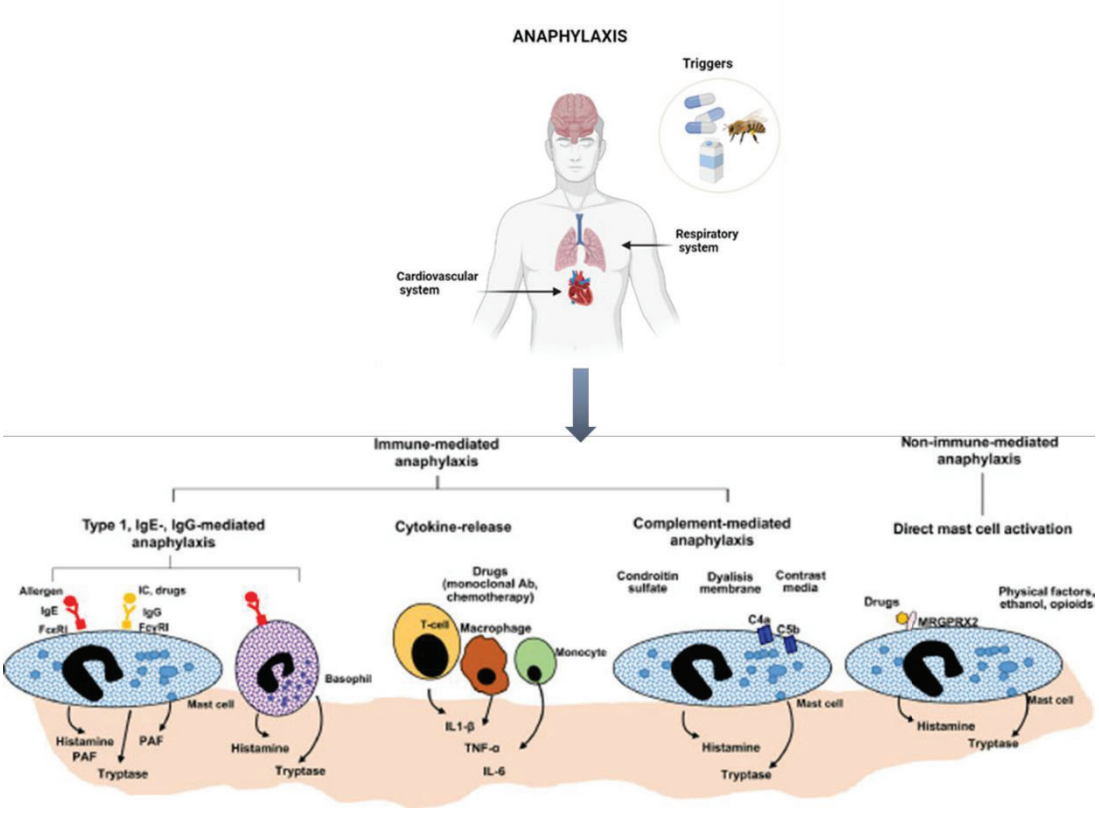

Fig 3. Anaphylaxis is when an individual's allergic reaction to a specific allergen causes substantial danger to life. In this condition, exposure to the allergen causes an instantaneous response, and without immediate and adequate medical care, the immune system cannot cope with the sudden riposte and fails. The pathophysiological classification of anaphylaxis (upper part) and its pathways are illustrated along with triggers, cell targets and receptors, when present, (middle part) and biological effect mediators (lower part). The immune system plays a role in type 1 IgE- and IgG-mediated anaphylaxis (left), cytokine-release (middle), mixed-reactions (not shown for clarity), where the previously described mechanisms are both at play, and complement-mediated anaphylaxis (middle). The last mechanism depicted (right) is the direct activation of mast cells, which can be either mediated by the interaction of drugs (e.g, vancomycin and quinolones) with Mas-Related G protein-coupled receptor X2 (MRGPRX2) or due to direct, e.g, without the engagement of receptors, membrane perturbation of mast cells by physical factors (hot and cold temperatures, osmolality variation, ethanol, etc.). Type 1 IgE-mediated anaphylaxis, the prototypical form of anaphylaxis, is triggered by the crosslinking of IgεRI by allergens on mast cells and basophils, leading to the production of the vast array of mediators (histamine, tryptase and others, see text). Immunocomplexes, chimeric IgG monoclonal antibodies, protamine-containing drugs bound by IgG crosslink FcγRs on mast cells but also neutrophils, macrophages and platelets, not shown, and determine the production of mediators, such as PAF. In cytokine-release reactions, mainly induced by chemotherapy agents and monoclonal antibodies, the release of inflammatory mediators, such as, IL-1β, IL-6, and tumor necrosis factor α (TNF-α), by T cells, macrophages, and monocytes, is responsible for the clinical picture. Oversulfated chondroitin sulfate, dialysis membrane, contrast media, dextran, some excipient (PEG, polysorbates) activate the complement system and determine mast cell degranulation. For clarity, immunoglobulin (Ig)-mediated activation of mast cells and basophils is shown after the engagement of only one Ig receptor, in complement-mediated anaphylaxis only one mast cell, without a basophil, is shown. Modified from [63].

Hymenoptera venom allergy is a common cause of anaphylaxis resulting in life-threatening. There are two main families of Hymenoptera in Europe, Apidae, and Vespide which induce anaphylaxis [64]. Allergens in the venom can trigger the immune system to produce antibodies, which can lead to an allergic reaction upon

subsequent stings. For the detection of antigens caused by wasps, there are currently clear antigens 5 [65] and PLA1 (Vesp v 1) [66]. Antigen 5 is found in the venom of yellow jackets, hornets, and wasps. And is the most common venom allergen found in bee and wasp stings. Two hyaluronidase isoforms (Vesp v 2A and Vesp v 2B) has been partially purified and characterized as potential allergens, although specific immunoglobulin IgE against these isoforms has not yet been stated.

Food anaphylaxis also is a severe, potentially life-threatening allergic reaction to certain foods. In the case of food anaphylaxis, the allergen is typically a protein found in certain foods, such as peanuts, tree nuts, shellfish, fish, milk, eggs, and soy. Food allergy is common, but some allergies can happen at a certain age. Peanut, milk, and shellfish allergies are more likely to occur in children [67]. For adults, exercise, Non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, and sleep deprivation cooperated with food allergy as the most frequent cofactors [68].

The mechanism of mast cell activation is IgE-dependent and IgE-independent in anaphylaxis, as Fig 3 shows. For IgE-dependent mast cell activation, the latter of this thesis will be extended. The mechanisms of IgE-independent mast cell activation include IgG-mediated anaphylaxis, cytokines-release, complement-mediated anaphylaxis and MRGPRX2-mast cell activation, which is going to be extended in the later of this thesis.

IgG-mediate anaphylaxis [69], FcγRs signaling can cause the activation of mast cells and basophils in human and mouse models [70], although IgG-dependent anaphylaxis has not been demonstrated in humans. One piece of the literature showed that IgG-mediated activation induces anaphylaxis in mice [71].

Cytokines release is a cytokines storm caused by such as TNF-α, IL-1B, and IL-6, these proinflammatory mediators released through triggered mast cells and other immune cells expressed FcγR. Chimeric, humanized, and human mAbs as well as chemotherapeutic drugs like oxaliplatin can cause these reactions [55].

Complement-dependent anaphylaxis, where C3a, C4a and C5a peptides (also called anaphylatoxins) can activate mast cells, basophils and macrophages, leading to the release of inflammatory mediators. Blood levels of C3a, C4a and C5a correlate with the severity of anaphylaxis in human subjects [36].

Diagnosis of allergy and anaphylaxis

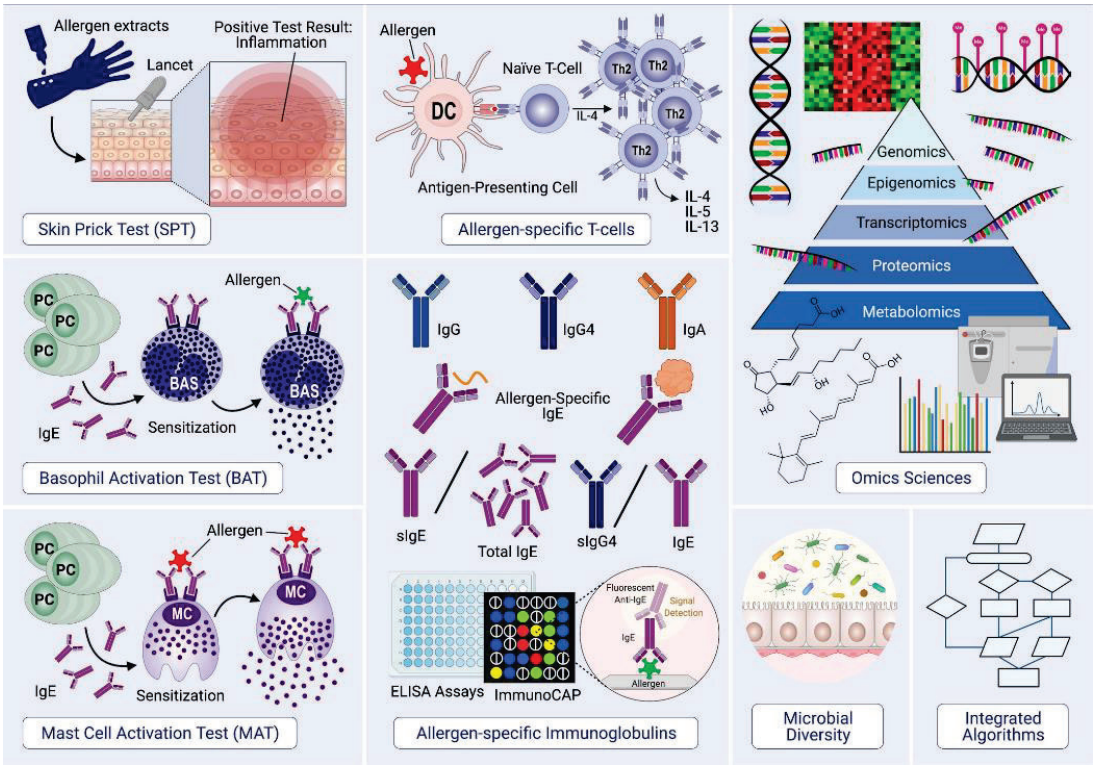

Fig 4. Biomarkers for the diagnosis, prognosis, and management of allergy, modified from [72].

The most common way to test allergy is a skin prick test (SPT) for patients with drugs, hymenoptera venoms and food. But not for all allergens, because the extraction is limited. SPT is safe for patients, but sometimes the results are negative, due to lacking specific IgE. SPT can be done in 2-4 weeks after anaphylaxis. Basophil activation test (BAT) described BAT as those basophils from peripheral blood that is activated with specific allergen-inducing degranulation, detecting with a flow cytometer. BAT is a tool for wide diagnosis of IgE-mediated allergy reaction in the clinic and also can be a useful tool to test anaphylaxis, by detecting the CD63 or CD203c with allergens stimulation,

and accuracy is high[73], [74]. Another way to a diagnosis of allergy and anaphylaxis is a mast cells activation test (MAT), human primary mast cells are generated from peripheral blood progenitors, incubated with the patient's sera overnight, and activated cells with the allergen. CD63 is detected with a flow cytometer [75]. Measuring the specific IgE determines anaphylaxis, which is more sensitive and rapid than SPT in milk allergy [76]. Because of those allergenic extracts have been limited due to their crude nature, lack of potency assessment, and the significant variability observed among lots and manufacturers. Recently, there is a great potential to be developed for predicting and diagnosis of anaphylaxis, by integrating them with omics analysis, and genomics data. For further study, omics techniques adopt to clinical applicability needed for functional identification and validation of biomarkers [77]. As Fig 4 shows.

Mastocytosis

Mastocytosis is an abnormal proliferation and increases in the cutaneous and whole body systems. Clinic signs include flushing, pruritus, abdominal pain, diarrhea, hypotension, syncope and musculoskeletal pain. Activation of the stem cell factor receptor KIT signaling is associated with the pathophysiology of mastocytosis [78]. Mostly because of a distinctive exon 17 D816V mutation of the KIT gene, which accentuates the crucial physiological function KIT plays in mast cell proliferation and development [79]. Other KIT mutations have been found in mast cell lines, mast cell leukemia, and pediatric mastocytosis, including V560G, D816Y, D816F, D816H, and E839K [15][80]. Tryptase is a stander for the diagnosis of mastocytosis [81].

Urticaria

Urticaria is a widespread and varied inflammatory skin condition, with various subtypes, mainly, there are acute urticaria and chronic urticaria. Chronic urticaria lasts 6 weeks. Skin mast cells are the center of this disease. The activation and degranulation of cutaneous mast cells, followed by the release of histamine and other mediators that cause vasodilatation, plasma extravasation, and cellular recruitment, are the causes of

the condition [82]. Treating urticaria, there are various ways, H1 antihistamines, Omalizumab and cyclosporine [83].

Asthma

Allergic asthma is the most common asthma phenotype in children and adults, mast cells accumulate in the airways of asthma patients [84]. When compared to healthy participants, the number of MC(TC) and the MC(TC)/MC(T) ratio in the small airways were higher in biopsies from patients with severe asthma [85]. Tryptase levels in serum were higher in asthma patients who died from asthma compared to those who died from other causes [86]. Further study gave proof that the release of tryptase from the mast cells causes morphological and functional changes in bronchial epithelial cells, suggesting to crucial involvement in airway epithelial remodeling and epithelial function disturbance [87].

Infections and cancers

Mast cells play an important role in pro-inflammation dependent on infection diseases. Mast cells show a detrimental contribution of mast cells in the progress of DENV [88].

The ability of mast cells to facilitate or prevent tumorigenesis depends on the type of tumor, the stage of the cancer, the mast cell activation degree, where the mast cells are located within the tumor microenvironment, and the overall balance of pro- and anti-tumorigenic effects on the tumor cells [89]. Anatomical location, stage of mast cell development, and exposure to ambient inflammatory mediators all have a significant impact on mast cell phenotype, function, and the number of mediators they make and release.

1.2 Mast cell signaling

1.2.1 IgE-dependent mast cell activation

1.2.1.1 FcεRI signaling

In IgE-dependent mast cell activation, three factors are a must, soluble allergens, sIgEs and mast cells or basophils, representing the causative factors, which have two phases in response to an allergen, sensitization and effector phase. Sensitization phase, when an individual is first exposed to an allergen, the immune sensitization process is initiated and triggers the production of IgE, then binding to FcεRI expressed on mast cells and basophils are in a sensitization state. Effector phase, an individual is exposed to the same an allergen again, which binds two or more IgE, combined allergen-IgE complex stimulates the active mast cells or basophils and releases a large number of mediators [90]. As Fig 5 shows.

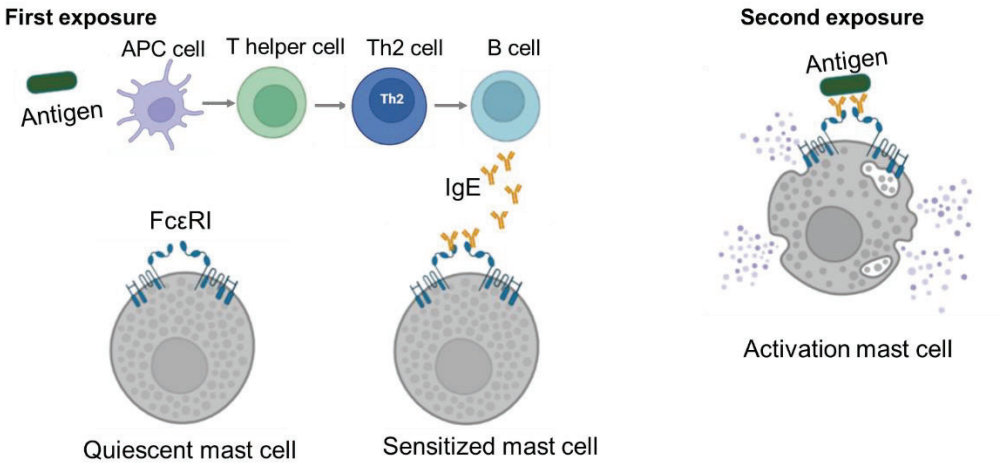

Fig 5. The classic IgE-mediated immune mechanism. The sensitization process initiates with the first exposure to an allergen. The antigen-presenting cells (APCs) capture and present the processed antigen to $CD4^+$ T cells inducing their polarization to a Th2 phenotype. These stimulate B cells which, afterward, produce and release antigen-specific IgE antibodies that bind to FcεRI. Future re-exposure(s) to the allergen induces the cross-linking of FcεRI-bound allergen-IgE

complexes, activating and inducing the degranulation of mediators by mast cells and basophils. These include histamine, platelet-activating factor (PAF), and tryptase, among others, modified from [91].

Traditionally, aggregation of, high-affinity IgE receptor, FcεRI bound IgE is the main pathway that causes mast cell and basophil activation. Numerous FcεRI comes into contact with multivalent antigens and triggers the FcεRI signaling [92]. Antigen-dependent stimulation, aggregation FcεRI of the bound α-chain of IgE complexes at the cell surface, and subsequent phosphorylation of immunoreceptor tyrosine-based activation motif (ITAMs) of β- and γ-chains interacted with Lyn and Syk kinase, respectively [93][94]. Lyn is a tyrosine kinase of the Src family with an SH2 and SH3 domain-dependent manner, which interacts with the ITAMs in the β-chain of FcεRI [95]. Syk also is another class of tyrosine kinase containing two SH2 domains, interacting with ITAMs γ-chains of FcεRI [96][97]. LAT (linker for activation of T cells) , several a substrate of activated Syk, LAT binds Grb2, Phospholipase C gamma (PLCγ), the p85 subunit of PI3K, and other critical signaling molecules indicating that LAT plays an important role in linking the IgE-dependent mast cell activation [98]. LAT also induces the downstream signaling pathways, including the MAPK, and calcium flux pathways [99]. Activated mast cells release mediators, firstly, preformed granule products, histamine, tryptase, carboxypeptides, chymase, heparin, chondroitin sulphate E. Secondarily, newly formed lipid mediators include PGD2, LTB4, LTC4 and PAF. Thirdly, cytokines contain TNF-α, basic fibroblast growth factor, interleukin-4, stem cell factor, and chemokines, such as eosinophil chemotactic factor. [100]. FcεRI signaling as Fig 6 shows.

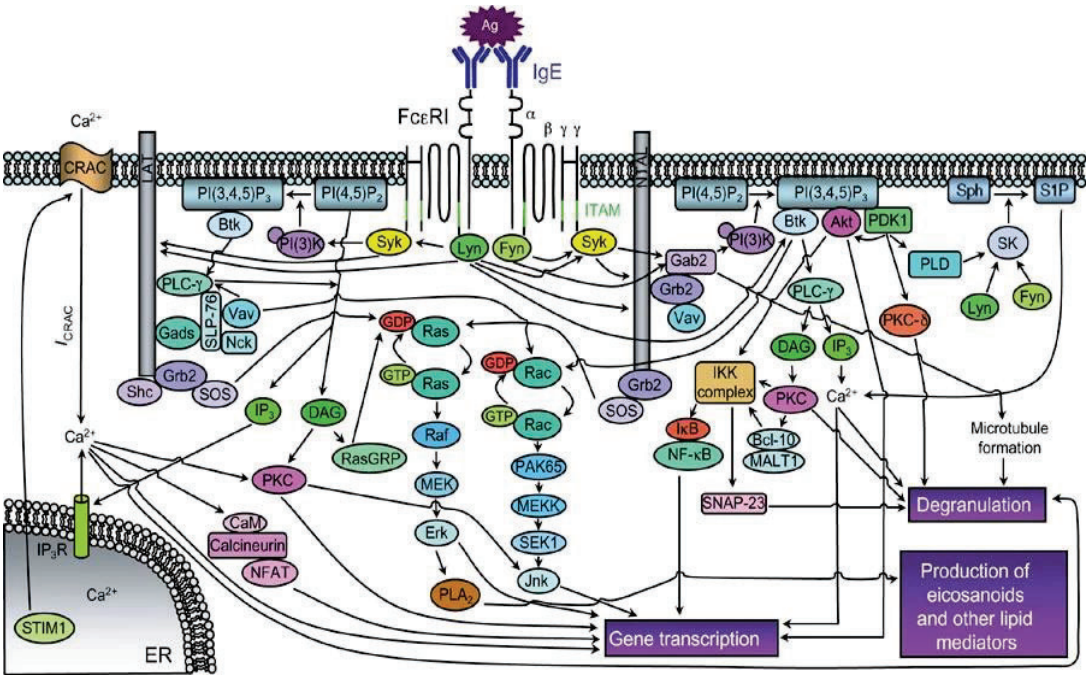

Fig 6. Antigen (Ag)-induced crosslinking of FcεRI induces activation of Lyn, which phosphorylates FcεRI ITAMs (green) and activates Syk after ITAM binding, and Fyn, which phosphorylates the adaptor Gab2 to activate the PI3K pathway. Lyn and Syk phosphorylate many adaptor molecules, such as LAT and NTAL, and enzymes to regulate activation of the GTPase Ras, phospholipase C-γ (PLC-γ) and PI3K pathways, as well as other pathways. Grb2 and SOS (son of sevenless homolog) activate the Ras-Erk (extracellular signal–regulated kinase) pathway, which regulates the activation of transcription factors and metabolism of arachidonic acid (through activation of phospholipase A2 (PLA2)). PLC-γ can be activated through the coordinated function of the adaptor molecules LAT, Gads, SLP-76 and Vav and the tyrosine kinase Btk or independently of Lat through a PI3K-Btk–dependent pathway. Activation of PLC-γ regulates the activation of classical protein kinase C (through the generation of diacylglycerol (DAG)) and calcium responses (through the generation of inositol-1,4,5-trisphosphate (IP3)). The binding of inositol-1,4,5-trisphosphate to its receptor (IP3R) triggers Ca^{2+} release from the endoplasmic reticulum (ER); STIM 1 couples depletion of endoplasmic reticulum Ca^{2+} stores with activation of CRAC channels, which leads to the influx of extracellular Ca^{2+} and a calcium release–activated current (ICRAC). The PI3K product phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) is an important lipid mediator that regulates the activity of various enzymes, such as Btk, the serine-threonine kinase Akt, the phosphoinositide-dependent kinase PDK1, phospholipase D (PLD) and sphingosine kinase (SK),

and the formation of other lipid mediators, such as diacylglycerol and sphingosine 1-phosphate (S1P). Sphingosine 1-phosphate can act intracellulary to regulate Ca^{2+} influx and degranulation (independently of phospholipase C and inositol-1,4,5-trisphosphate) and extracellularly (after secretion from the cell) by binding to the S1P1 or S1P2 surface receptor, thereby inducing cytoskeletal rearrangement or enhancing degranulation, respectively. The IKK complex consists of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit, NEMO (IKKγ); this complex phosphorylates IκB to activate NF-κB. IKKβ also phosphorylates SNAP-23 to facilitate formation of the SNARE complex. Arrows indicate the contributions of these signaling pathways to the degranulation of mast cells, the metabolism of arachidonic acid, and the production of cytokines, chemokines and growth factors (some arrows do not indicate direct interactions or targets). Bcl-10, adaptor protein; CaM, calmodulin; Gads, adaptor protein; Jnk, Janus kinase; MALT1, adaptor protein; MEK, mitogen-activated protein kinase (MAPK) kinase; MEKK, MAPK kinase kinase; PAK65, serine kinase; PKC-δ, protein kinase C-δ; Rac, small GTPase; Raf, Ras effector molecule; RasGRP, Ras guanyl nucleotide–releasing protein; SEK1, MAPK kinase; Sph, sphingosine [101].

1.2.1.2 Inhibition FcεR signaling

For IgE-dependent mast cell activation, there are serval proteins that can delay and inhibit this signaling pathway, which provides insight for mast cells related diseases. FcεR has another isoform, low-affinity immunoglobulin E (IgE) receptor, CD23 (FcεRII) binds to IgE. CD23 is a 45-kD type II membrane protein expressed in various cell types, including mast cells and B cells [102][103]. When IgE binds to CD23, further IgE synthesis is inhibited; in CD23-deficient mice, circulating IgE levels increase by several orders [104]. Moreover, CD23 binds to allergens-IgE complexes, leading to IgE clearance and resistance to allergic reactions [105].

Src homology 2-containing inositol phosphatase (SHIP), is a negative regulator, maintaining the balance of positive and negative signals in lymphocytes and myeloid cells [106], sets the threshold and limits IgE-induced degranulation as a key negative regulator or “gatekeeper” in the level of calcium [107]. SHIP1 inhibits the generation

of cytokines, mast cell hyperplasia, and allergic inflammation in vivo [108]. SHIP2 negatively regulates mast cell degranulation and cytokine expression (IL-4 and IL-13) in bone marrow-derived mast cells (BMMCs) in mice [109].

Phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1), a negative regulator [110], is the main mediator of the inhibitory action of the mast cell function-associated antigen (MAFA) on RBL-2H3 cell response to the FcεRI stimulus [111]. Conversely, SHP-2 signaling downstream of KIT is essential for mouse mast cell survival [112].

The CD300 family of myeloid immunoglobulin receptors includes, CD300a, CD300b, CD300c, CD300d, CDD300e, CD300f, CD300g and CD300h. Among them, CD300a has an inhibitory effect on mast cell activation when combined with ligands [113]. CD300a regulates the survival of mast cells. It has a long cytoplasmic region containing the consensus immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence and is required for SHP-1 recruitment by CD300a in response to its ligands in BMMCs [114]. Additionally, a specific antibody against CD300a inhibited acute murine model of cutaneous anaphylaxis [115]. CD300f negatively regulates mast cell activation through the IgE [116] and MRGPRX2-dependent pathway [117].

CD84 is a self-binding receptor from the CD150 family that is widely expressed in mast cells, which negatively regulates IgE high-affinity receptor signaling in human mast cells through SHP1 dephosphorylating Syk-LAT- PLCγ signaling axis [118].

Sialic-acid-binding immunoglobulin-like lectin (Siglec)-8 is an inhibitory receptor selectively expressed on human mast cells, down-regulating the signaling in the IgE-dependent pathway by regulating SHP-1 [119].

1.2.1.3 Aminoacyl tRNA synthetases, Lysyl-tRNA synthetase

Aminoacyl tRNA synthetases (aaRS), are essential for translation by catalyzing the ligation of amino acids to their matched tRNAs containing the corresponding

anticodon to generate aminoacyl tRNAs that are then transferred to the ribosome for translation [120][121]. Each amino acid and its cognate tRNA requires a specific aaRS to be catalyzed, so the types of amino acid tRNA synthetase are 20 [122]. Amino acid-tRNA synthetases can be divided into two categories based on the sequence and structure of the active site. Class I contains two highly conserved sequence motifs and acylation reaction occurs on the 2'-hydroxyl of adenosine on tRNA, and the active form is usually a monomer or a dimer. Class I Includes GluRS, GlnRS, ArgRS, CysRS, MetRS, ValRS, IleRS, LeuRS, TyrRS and TrpRS, among which CysRS, MetRS, TyrRS and TrpRS are homodimers, and the rest are monomers. Class II has three highly conserved sequence motifs and the amine acylation occurs on the 3'-hydroxyl of the same adenosine on the tRNA, and the active structure of the molecule is usually a dimer or a tetramer. Class II contains GlyRS, AlaRS, ProRS, SerRS, ThrRS, HisRS, AspRS, AsnRS, LysRS and PheRS, where AlaRS is a homotetramer, GlyRS and PheRS are heterotetramers, and the rest are homodimers [121][123]. AaRSs are bound to a high-molecular-weight multi-tRNA synthetase complex (MSC) in the cytosol [124][125].

AaRS catalyze the aminoacylation reaction in two steps. First step, activation of the amino acid, both the amino acid and ATP bind to the catalytic site of the aaRS, leading to condensate to aminoacyladenosine monophosphate (aa-AMP) bound to the aaRS and PPi. Second step is, the transfer of the aminoacyl group to the tRNA, the 76 nt hydroxyl group of adenine attacks the carbonyl carbon of adenosine to form aminoacyl-tRNA and AMP [126][127]. As Fig 7 shows.

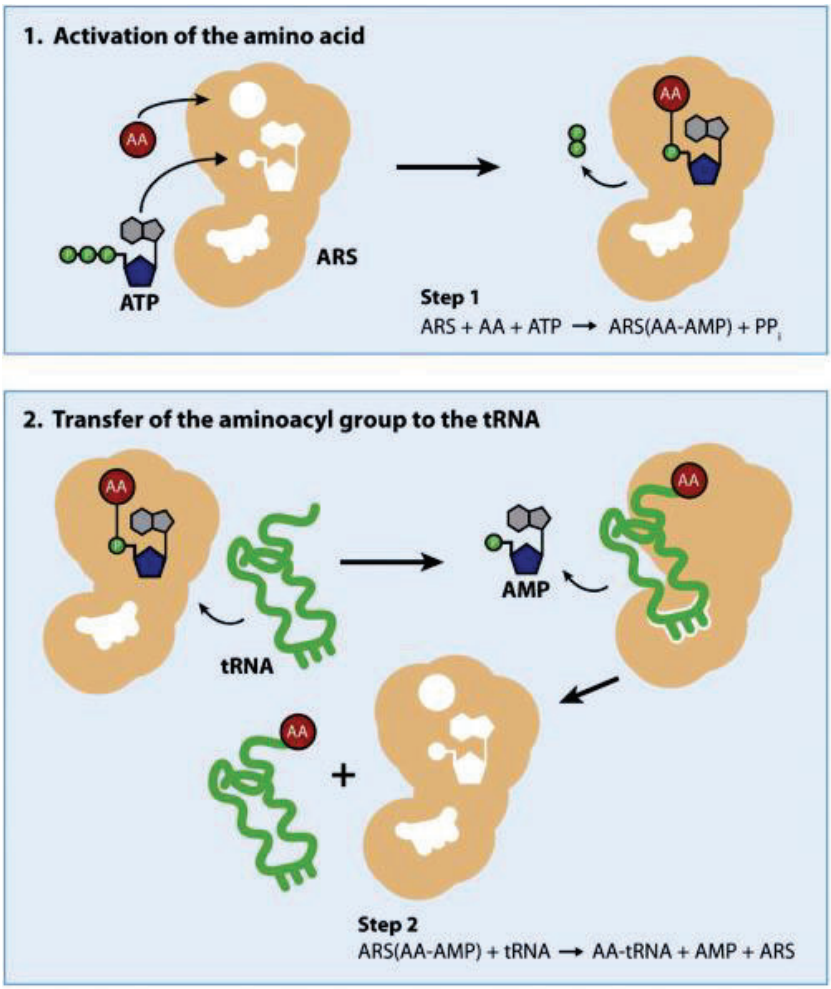

Fig 7. Mechanism of aminoacylation reaction. Step 1: The aminoacyl tRNA synthetase (ARS) first binds to amino acid (AA) and ATP, forming the ARS-AA-AMP complex by the release of inorganic pyrophosphate (PPi). Step 2: The ARS-AA-AMP complex then binds the individual tRNA molecule, thus forming ARS-AA-tRNA. Later the amino acid binds with tRNA at the CCA end by releasing AMP, detaching from the enzyme [128].

Lysyl-tRNA synthetase (LysRS) is encoded by the KARS gene in chromosome 16 in humans, which belongs to the class II of aaRS. LysRS has two isoforms: a cytosolic one of 597 amino acids and a longer mitochondrial isoform of 625 amino acids. It has two main differentiated domains, an aminoacylation domain found in the C-terminus and a tRNA anticodon-binding domain in the N-terminus. LysRS is bound to the MSC by interacting with proteins, p38 [129].

LysRS is a moonlighting protein with both canonical and non-canonical roles. The

canonical pathway is in translation like other aaRSs but specifically for Lysine. The two-step process involves Lysine and ATP binding to LysRS, forming a LysRS-Lysine-AMP complex. Then, the complex is recognized by the tRNA carrying the specific anticodon, and the second reaction releases LystRNA and AMP from LysRS [130].

In addition, LysRS plays a non-canonical role, which is involved in the IgE-dependent mast cell activation pathway. In the quiescent cells, LysRS is bound to MSC. However, in IgE-activated mast cells, LysRS is phosphorylated on Serine 207 in a MAPK-dependent manner. This phosphorylation induces a conformational change in LysRS, resulting in its translocation to the nucleus and its production of diadenosine tetraphosphate (Ap_4A), which binds histidine triad protein (HINT1) release microphthalmia-associated transcription factors (MITF) and the subsequent MITF activation and transcription of MITF-targeted genes [131]. A single conformational change triggered by IgE-mediated signaling switches the LysRS function from translation to transcription [132][133], as Fig 8 shows.

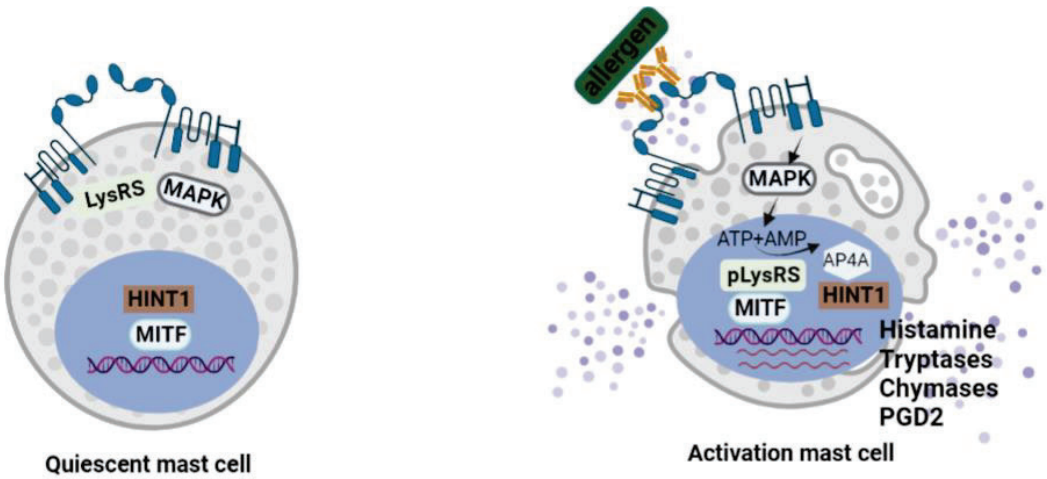

Fig 8. Proposed model for LysRS as a signaling molecule following specific stimuli. LysRS is serine phosphorylated in a MAPK-dependent fashion, dissociates from the MSC, and translocates from the cytoplasm to the nucleus. The phosphorylation on serine residue 207 elevates Ap4A levels, leads to the dissociation of HINT1 from MITF, and allows this transcription factor to activate its

responsive genes, modified from [132].

1.2.1.4 MITF/TFE family

MITF, TFEB, TFE3, and TFEC are basic helix-loop-helix leucine zipper (b-HLH-LZ) transcription factors that belong to the MiTF/TFE family. The E-box (CANNTG) motifs in the target genes' promoter region are recognized by the b-HLH-LZ [134]. MITF is mainly expressed in melanocytes, mast cells, osteoclasts, macrophages, NK cells, B cells, and the heart, while TFEC expression is only seen in cells of myeloid origin [135]. The other members, TFE3 and TFEB, show a broader pattern of expression [129][130]. The structure MiTF/TFE family is shown in Fig 9 a.

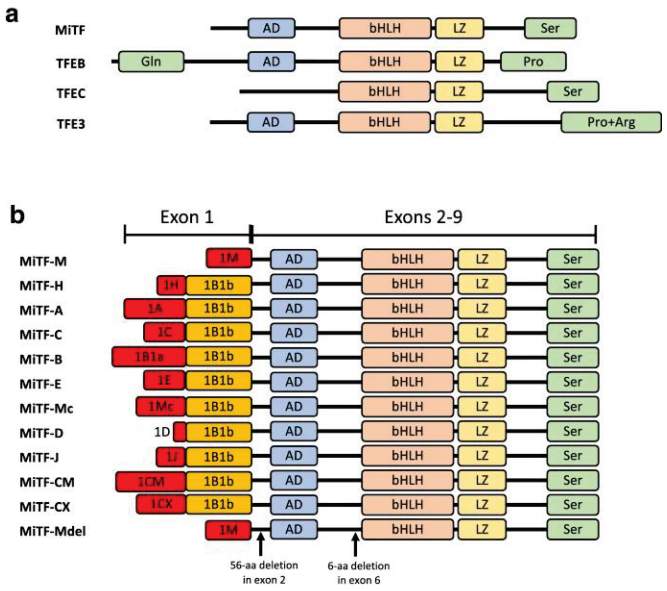

Fig 9. a Structure of the four MiT family members. AD, acidic domain; bHLH, basic helix-loop-helix; LZ, leucine zipper; Ser, serine-rich region; Gln, glutamine-rich region; Pro, proline-rich segment; Pro+Arg, proline- and arginine-rich region. b Different MITF isoforms. Each isoform is driven by its promoter and has a partially unique exon, while exons from 2 through 9 are common in all isoforms, modified from [138].

The Microphthalmia-associated transcription factor (MITF) is involved in the generation and function of mast cells [138], melanocytes [139], osteoclast [121][122],

and retinal pigmented epithelium [142]. Several isoforms described, MITF-A, -B, -C, -D, -E, -H, -M, -Mc, and -J, differ in their exon 1 and share common downstream exons from 2 to 9 [143]. All the isoforms are shown in Fig 9 b. In mice, MITF is required to transition bone marrow-derived hematopoietic stem cells into tissue-specific mast cells [144]. Moreover, IL-3 and IL-4 induce the expression of MITF [145]. Further study showed that blocking PI3K, which results in less MITF, slows the development of mast cells [146]. The development of pre-basophil and mast cell progenitors into mast cells and basophils, is dependent on STAT5 signaling, which is downstream of MITF for mast cell proliferation in mice; Instead, C/EBPα is a crucial transcription factor for the fate of basophil cells [147]. As a result, MITF and C/EBPα are regulated antagonistically, determining the destiny of basophils and mast cells, respectively. In addition, phosphorylated pyruvate dehydrogenase (PDH), crucial for mast cell degranulation, may interact with MITF in the mitochondria of mast cells [148]. Further studies demonstrate that phosphorylation of Ser73 of mitochondrial MITF, controlled by extracellular signals regulated by protein kinase (ERK1/2) activity, was related to mast cell degranulation, cytokine production, and oxidative phosphorylation (OXPHOS) activities [149].

The mucosal-type mast cells (from primary BMMCs to intestinal mast cells) have been characterized as expressing MITF-A, the most extensively expressed isoform, and the more restricted MITF-MC and MITF-E. Both human $CD34^+$ progenitor-derived mast cells (hMCs) and HMC-1 cells exhibit high levels of MITF-A, which controls tryptase-1 levels [150]. MITF-H also was found in BMMCs [151].

MITF is a pleiotropic transcription factor because it causes a variety of phenotypic abnormalities in mice. The *Mitf* mutant mouse affects the function and phenotypes of mast cells and melanocytes, including aberrant pigmentation, hearing loss, osteopetrosis, and retinal degeneration [138][117][136][137]. Any mast cell isoforms mentioned can improve the hypo granularity and SCF-dependent migration seen in BMMCs from Mitf -/- mice (homozygous genes controlling the expression of Mitf were

knockout in all the tissues in mice). These isoforms regulate both a particular collection of genes and a shared transcriptome that includes chymases (mMCP1, mMCP2, mMCP4, mMCP5), tryptase (mMCP6), Granzyme B, and adhesion molecules. MITF-MC and MITF-E may preferentially influence the expression of Cathepsin G, α4 integrin, and mMCP8, while MITF-A would control KIT expression [154]. There are two different Mitf mutant strains, $Mitf^{mi\text{-}vga9}$ and $Mitf^{Mi\text{-}wh}$, chymase enzyme activity was severely reduced, lost function of cardiac mast cells in mice [155]. Mast cell-deficient $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ sash mice without mast cells, after injection of BMMC into tail veins of $Kit^{W\text{-}sh}/Kit^{W\text{-}sh}$ mice showed limiting the migration and distribution of mast cells [156].

MITF deregulation leads to several pathologies. The most well-known is melanoma, where MITF functions as an oncogene [139]. Lately, our group found that MITF is important for gastrointestinal stromal tumors (GIST) survival and proliferation [157].

Regarding mast cell pathology, MITF is highly expressed in mastocytosis [158]. MiR-539 and miR-381 regulate MITF expression at posttranscriptional levels in response to normal KIT signaling and KIT oncogenic stimulation [159].

It has recently been demonstrated that IgE/mast cell-mediated anaphylaxis depends on the GATA2-MITF axis. According to this study, the transcription of *HDC*, which controls histamine production, is regulated primarily by GATA2 and then MITF. The *c-KIT* gene can be fully restored by overexpressing MITF without the *GATA2* gene, and the *HDC* gene can be restored in part [160]. This literature confirms that GATA2, besides promoting MITF expression, would also keep MITF accessible to the *HDC* promoter. Furthermore, MITF is important for lipid mediators on mast cells, enhancing PGD2 expression [161].

As mentioned before, HINT1 inhibits MITF function in resting mast cells. The amount of diadenosine tetraphosphate (Ap4A) in the nuclei rises in response to IgE

crosslinking; Ap4A interacts with HINT1 but not with MITF, separating the MITF/HINT1 complex and liberating MITF to bind to target genes and trigger their transcription [131]. According to a recent study, the MITF/HINT1 complex can be dissociated by Ap4A's lengthy phosphodiester linkage [162].

Another transcription factor, STAT3, inhibits the activity of MITF after IgE-dependent mast cell activation in rodents [163].

The transcription factor EB (TEFB), a member of the MiTF/TFE family, also is involved in the function of mast cells, essential for the mast cell process of secretory granule formation [164]. TFEB phosphorylated by mTORC1 is kept outside the nucleus by joining forces with 14-3-3 proteins. The transcription of genes involved in granule content and biogenesis is regulated by TFEB translocation to the nuclei due to decreased mTORC1 levels. Mast cell secretory activities and the IL-33 signaling pathway are disturbed by TFEB dysregulation [165].

The transcription factor E3 (TFE3), another member of the MiTF/TFE family, also is involved in function of mast cells, close to the function of MITF; however, it appears to affect mast cell activation rather than growth. Similar numbers of mast cells are present in Tfe3-/- animals, however the levels of KIT and FcεRI expression in peritoneal and cultured mast cells decreased, inhibiting cell degranulation and mediator release [166].

1.2.1.5 Other transcription factors involved in mast cell anaphylaxis

There are various families of transcription factors (TFs) implicated in anaphylaxis, including those responsible for maintaining the mast cell phenotype, producing proinflammatory mediators, maintaining the homeostasis of secretory granules, and regulating the Th1/Th2 balance.

STAT family

The transducer and activator of the transcription (STAT) family has seven members, including STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6. The JAK-STATS signaling pathway involves several cytokines and growth factor receptors [167]. JAKs inhibition reduces mast cell activity [168]. Inhibition JAK-STATS signaling pathway can reduce the severity of canine atopic dermatitis (AD) [169].

Different isoforms of STAT play the same or various roles in the function of mast cells and related diseases. STAT1 was reported to enhance the secretion of IL-13 and expression of IL13Rα1 in human mast cells [170].

STAT 3 and its transcriptional function also play a non-canonical role in enhancing the ATP production necessary for mast cell exocytosis events by modulating mitochondrial activities. ERK1/2 was required for the phosphorylation of mitochondrial STAT3 on serine 727 in IgE-antigen-activated RBL cells, followed by the induction of OXPHOS activity. Additionally, mice administered Stattic, a STAT3 inhibitor, showed significantly less histamine secretion [171]. Potential treatment medicines for mast-related disorders include mitochondrial STAT3 inhibitors with low concentration, which were created without impact on STAT3 transcriptional activity [172].

According to certain researchers, autosomal dominant Hyper IgE syndrome (AD-HIES) patients with dominant-negative STAT3 mutations have fewer food allergies and anaphylaxis than other hyper-IgE patients with unmutated STAT3 [173], reinforcing the idea that suppression of STAT3 activity reduces allergic symptoms and the incidence of anaphylaxis.

STAT5 lies downstream of oncogenic D816V KIT, a hallmark mastocytosis mutation [174]. Consequently, it might be a therapeutic target for systemic mastocytosis drug resistance [175]. Increased mast cell number and high levels of phosphor-STAT5 are present in patient’s skin lesions, indicating that STAT5 may also be a therapeutic

target for treating chronic inflammatory skin diseases such as atopic dermatitis [176].

Additionally, after thymic stromal lymphopoietin (TSLP) stimulation, STAT5 has been reported to act downstream of the MRGPRX2 receptor, suggesting a role in atopic skin diseases via this receptor [177], giving some insights into MRGPRX2 signaling.

GATA family

GATA transcription factors are a family of TFs (GATA1, GATA2, GATA3, GATA4, GATA5, and GATA6), characterized by their ability to bind to the DNA consensus sequence (T/A) GATA(A/G). GATA1, GATA2, and GATA3 are mostly involved in hematopoietic functions [185][186]. Non-hematopoietic organs such as the cardiovascular, gastrointestinal, endocrine, and gonadal systems express GATA4, GATA5, and GATA6 [180]. The GATA1 and GATA2 express widespread in mast cells [181].

GATA2 is critical for the development of pre-basophil and mast cell progenitors (pre-BMPs) into basophils and mast cells [182]. In addition, GATA2 is essential for maintaining the properties of mast cells. A proper cytokines inducing, mast cell that lack the GATA2 DNA domain can dedifferentiate into myeloid cells that resemble macrophages and neutrophils [183]. Furthermore, KIT and FcεRI expression on mast cells is decreased in human subjects with GATA2-deficiency, which impairs IgE-dependent degranulation [184]. The human *IL1RL1/ST2* encodes a receptor for IL-33 in mast cells and basophils, its promoter is critically transactivated by GATA2 [185].

GATA1 and GATA2 regulate protease transcription in bone marrow-derived mast cells in mice. The findings of using selective siRNAs for both factors reveal that GATA2 knockdown reduces more mast cell proteases than GATA1 does. In fact, after GATA2 silencing, *Cpa3, Mcpt4, Mcpt8,* and *Cma1* are considerably downregulated, but not in GATA1 knockdown cells [186].

GATA3 regulates a newly identified population of T follicular helper cells (Tfh13)

responsible for producing high-affinity anaphylactic IgE. Tfh13 was discovered in individuals with peanut or aeroallergen allergies, expressing IL-4, IL-5, and IL-13 [187]. A Tfh2 response brought on by helminth infection causes low-affinity IgE antibody production. These Tfh2 cells are unable to produce IL-13 and do not express GATA3. On the other hand, allergens cause $GATA3^{+}$ Tfh13 cells, which promote the synthesis of high-affinity IgE and anaphylaxis. It was previously revealed that IL-13 and IL-4 collaborate to generate high-affinity IgE [188] [189]. This information is supported by the genetic associations between IL-13 and asthma, food allergies, and high IgE levels [190][191][192]. Moreover, the absence of IL-13 does not affect low-affinity IgE responses to parasitic infection [193]. Future research is necessary to determine whether the ability to identify the Tfh13 population in peripheral blood from allergic patients is a valuable and promising method [194]. Mice with forced GATA3 expression are more prone to allergic airway inflammation [195]. Moreover, anaphylaxis treatment plans might focus on inhibiting IL-13 or GATA3. In this context, asthmatics have successfully used GATA3 DNAzyme to decrease GATA3 activity [196]. In addition, deacetylation of GATA3 causes the Th2 immune responses in asthma to be suppressed [197].

Other transcription factors in mast cell function

Transcription factor PU.1 is essential for mast cell homeostasis and differentiation in fetal liver cells to develop into mast cells in the presence of SCF, and IL-3 is evidence [198]. *FER1A* encodes FcεRIα, being transactivated on human mast cells, PU.1 collaborates with GATA1 and GATA2 is involved in FcεRI expression; consequently, human mast cells with PU.1 silencing exhibits reduced IgE-mediated degranulation [199].

The transcription factor MAZR (also known as PATZ1, encoded by the Patz1 gene) is a member of the Zn finger and BTB domain transcriptional regulators, which cooperates with MITF significantly increase the expression of the IL-6 in mice mast cells [200]. Further study showed that MAZR preferentially acts as a transcriptional repressor in mice mast cells and is not essential for effector functions upon FcεRI-

mediated activation [201].

1.2.2 IgE-independent mast cell activation

1.2.2.1 Mas-related G protein-coupled receptor-X2

G-protein-coupled receptors (GPCRs), GPCRs signal transduction is a significant component of vertebrates' physiology. GPCRs are among the crucial junctions of communication between the inside and outside of cells because they are receptors for hormones, neurotransmitters, ions, and other stimuli. The traditional function of GPCRs is to relate the binding of agonists to the activation of particular heterotrimeric G proteins, which modulates effector proteins downstream. The structure of GPCRs includes the human 2 adrenergic receptors (2AR), avian 1 AR, and human 2A adenosine receptor, as well as the structures of opsin and an active version of rhodopsin. According to their sequence and function, GPCRs are divided into six groups: Class A—rhodopsin-like receptors; Class B—secretin family; Class C—metabotropic glutamate receptors; Class D—fungal mating pheromone receptors; Class E—cAMP receptors; and Class F—frizzled (FZD) and smoothened (SMO) receptors [202].

The MRGPR family of receptors belongs to Class A of GPCRs. The MRGPR family of receptors, coded by the gene, *MAS1*, was discovered in 2001, including MRGPRA, MRGPRB, and MRGPRC families and families named MRGPRD-H. The MRGPR family is found in several species, from mice to monkeys [203], and shown in Fig 10. Family MRGPRX in mice and humans. The human MRGPRX family has four members: MRGPRX1-4 or SNSRs (sensory neuron-specific receptors) [204].

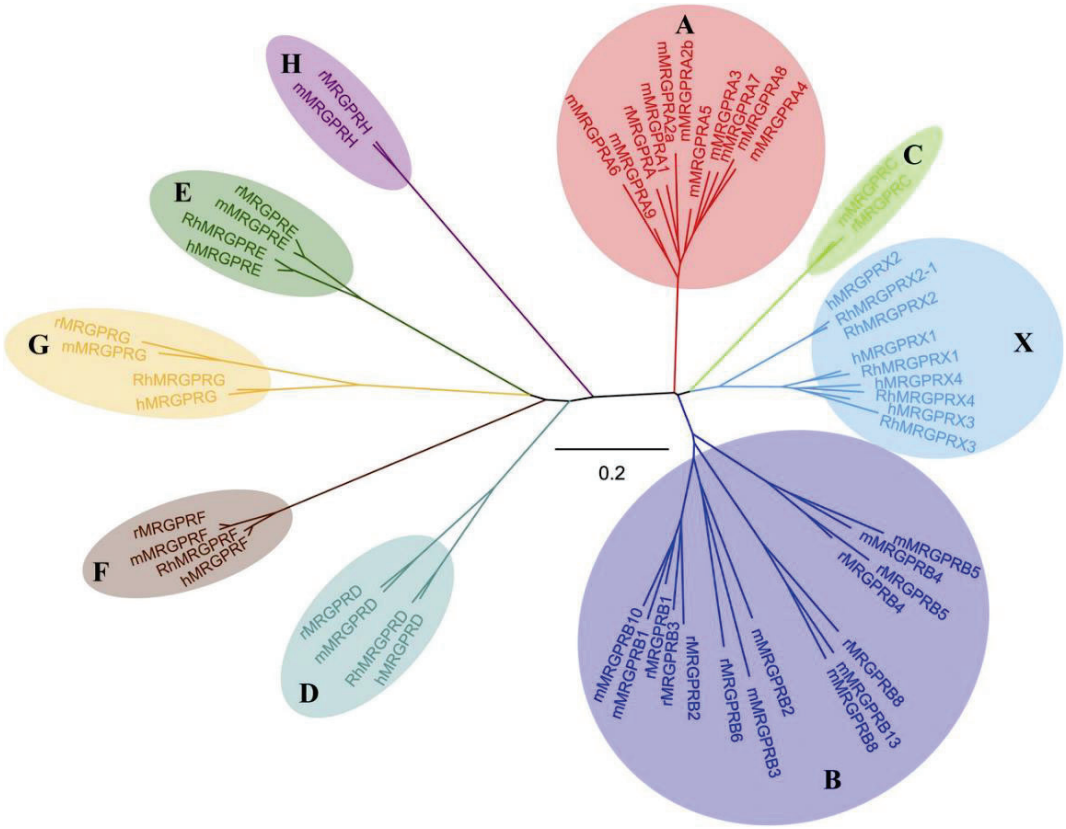

Fig 10. Phylogeny of MAS-related G-protein–coupled receptors. A phylogenetic tree of all 38 MRGPR members from the nine MRGPR subfamilies (A–H, X) of mice (m), rat (r), human (h), and rhesus monkey (Rh) was computed using Geneious 7 (Biomatters, Auckland, New Zealand; Blosum62 cost matrix, Jukes-Cantor genetic distance model, Neighbor-Joining tree build method) [203].

MRGPRs family has been described as markers for itch and pain in the peripheral nervous system [205][206][207] and was defined as the sensation of nonhistaminergic itch [208].

MRGPRX1 is just expressed in humans and primates [209], involved in the modulation of nociception and pruritus, and selectively expressed in the small-diameter primary sensory neurons. There are two endogenous ligands for MRGPRX1 in mice IP defensin 1 (IPDef1) and IR defensin 2 (IRDef2). Further research revealed that IPDef1 was more likely to cause pruritus than IRDef2 without histamine. IPDef1 induced itch through human MRGPRX1 and mouse MrgprC11 on dorsal root ganglion neurons, ion

channel TRPV1 is downstream of this signaling [210]. Previously, it was discovered that BAM8-22, a proenkephalin-derived endogenous peptide that causes itching is a particular ligand for the receptor MRGPRX1 leading to Ca^{2+} influx. In addition, it has been suggested that MRGPRX1 mediates the itch brought on by the antimalarial medication chloroquine [211] [212].

MRGPRX2, Mas-related G protein-coupled receptor-X2, belongs to the MRGPRs family. First, the research found that the receptor expresses on sensory neurons for sensation and modulation of pain [204]. It is an important receptor for non-IgE-dependent mechanisms in pseudo allergy and has been found in immune inflammation response by activation of human mast cells [213]. It has seven transmembranes connected by three extracellular (ECL) loops (ECL1, ECL2, and ECL3) and three intracellular (ICL) loops (ICL1, ICL2, and ICL3). The ECL includes the N-terminus, and the ICL part contains a C-terminal sequence. Mrgprb2 expressed on mast cells in mice is the orthologue of human MRGPRX2 [214] . In addition, Mas-related gprx2 has different N-terminal sequences in other species [215], shown in Fig 11.

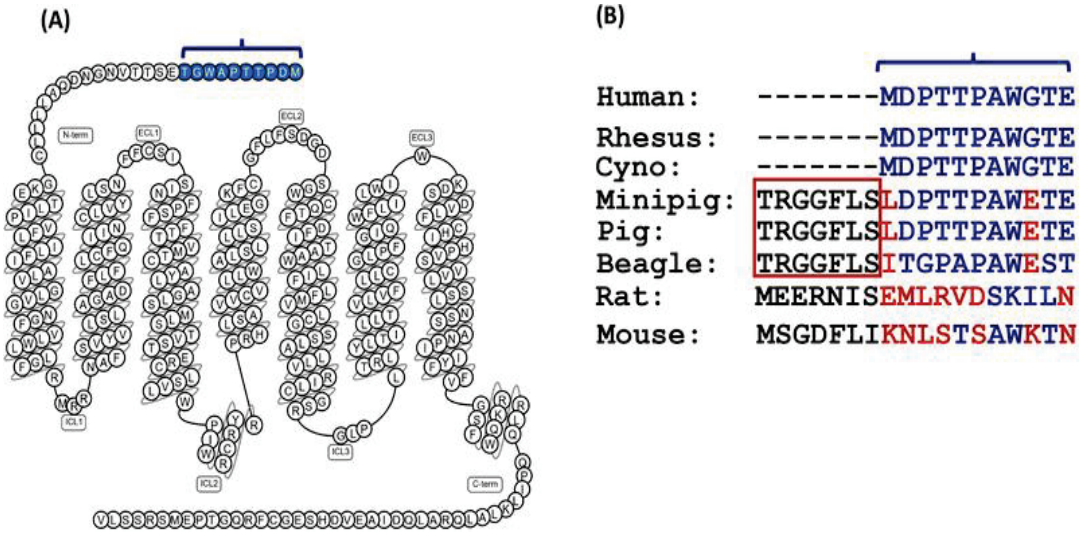

Fig 11. Sequence disparity in the N-terminus of Mas-related GPCRs in different species. (A)

Snake diagram of the secondary structure of MRGPRX2 with solid blue representing the first 10 N-terminal amino acids. (B) Comparison of N-terminal sequence of human MRGPRX2 with the corresponding rhesus, cynomolgus (Cyno), minipig, pig, beagle, rat, and mouse receptors.

Recent transcriptome analysis showed that MRGPRX2 is primarily present in skin mast cells, even though the majority of MRGPRX family members are expressed in peripheral neurons [216][217][218]. MRGPRX2 expression also was found in basophils and eosinophils. But there is a controversy about whether MRGPRX2 expresses on the surface of rest basophils [219][220]. Recently, the surface of HMC1 and $CD34^{+}$ derived mast cells expressing MRGPRX2 was found [221], and keratinocytes [222]. Adipose tissue, the esophagus, the bladder, the lungs, and the skin all have MRGPRX2 mRNA, with skin having the greatest quantities. No transcripts were found in the pancreas, ovary, liver, or kidney [223]. Mrgprb2 is present in visceral and subcutaneous mouse fat pads, adipose tissue resident cells seem to be a source of Mrgprb2 mRNA but not adipocytes. Mrgprb2 transcripts are present in the skin. Mrgprb2 mRNA in the urinary bladder was unable to detect, a result that is inconsistent with available data [224]. These inconsistencies can be related to variations in the animals immunological states, variations in RNA-seq databases, variations in primer specificity, and other factors. In spite of these alternatives, Mrgprb2 promotes the host defense against bacterial infection, but it additionally causes neurogenic inflammation, discomfort, allergic contact dermatitis (ACD), non-histaminergic itch, and pseudo allergy in mice [225][226][227]. The majority of Mrgprb2 and MRGPRX2 investigations conducted over the past five years have concentrated on cutaneous health and illness.

1.2.2.2 MRGPRX2 signaling pathway

MRGPRX2 can be activated by a series of agonists. for endogenous peptides, such as compound 48/80, substance P (SP), vasoactive intestinal peptide (VIP), cortistatin (CST), proadrenomedullin N-terminal peptide (PAMP), LL-37, PMX-53 and β-defensins [228][229][230][219]. PAMP 9-20 induced human atopic dermatitis (AD),

also can be an agonist of Mrgprb2, compared to FcεRI, Mrgprb2 activation releases more tryptase and fewer monoamines [208]. The endogenous neuropeptide, SP, promotes immune response by activating mast cells in humans and mice through MRGPRX2 and Mrgprb2 separately [225]. SP-induced mast cell response, the mast cells have small size granules with more rapidly release than the IgE-dependent pathway [231]. Human mast cell degranulation is induced by host defense peptides (HDPs), Murepavadin, via the MRGPRX2 induce the inflammation, also can activate the mast cells from mice through Mrgprb2 [232].

Additionally, it has been reported that MRGPRX2 was the mechanism by which small molecules and FDA-approved peptidermic drugs (such as morphine and neuromuscular blocking agents) caused systemic pseudoallergic and anaphylactic reactions [62][233]. The mast cell activation through MRGPRX2 is shown in Fig 12. The distinctive property of MRGPRX2 is to recognize a variety of basic amino acids as well as basic low-molecular-weight compounds. However, MRGPRX2 is not a GPCR that indiscriminately detects peptides with a basic charge [214]. On the commonly used drugs in the clinic, icatibant, cetrorelix, leuprolide, sermorelin, morphine, and neuromuscular blocking agents (NMBAs, atracurium, cisatracurium), Antibiotics (ciprofloxacin, vancomycin), intravenous (meglumine amidotrizoate, iomeprol) activate mast cells in an Mrgprx2-dependent manner [234]. NMBAs and fluoroquinolones, which are examples of small compounds with a tetrahydroisoquinoline (THIQ) motif or a related structure associated with mast cells are activated through MRGPRX2 and Mrgprb2 [214].

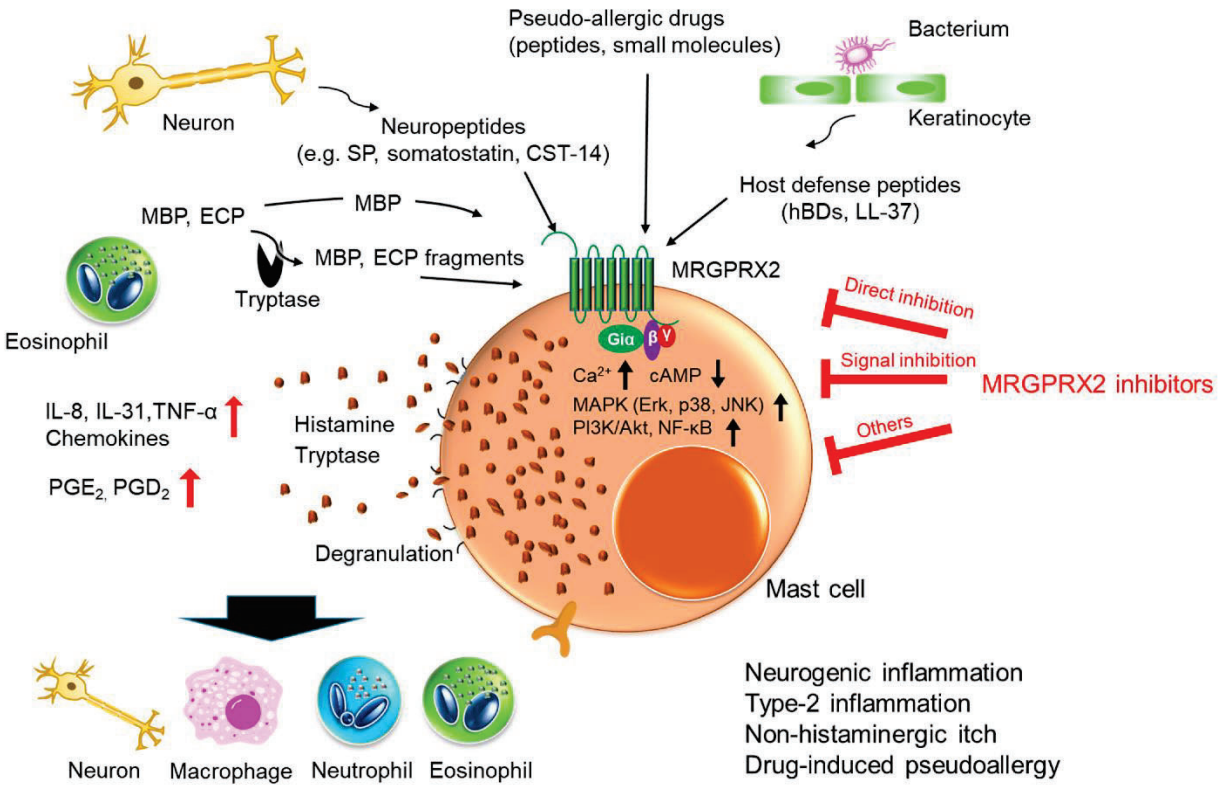

Fig 12. Schematic diagram of MRGPRX2 activation mediated by various stimuli and potential effects of MRGPRX2 inhibitors for induced diseases. MRGPRX2 is stimulated with various ligands and is thought to play important roles in neurogenic inflammation, type 2 inflammation, non-histaminergic itch, and drug-induced pseudoallergy through mast cell activation [235].

MRGPRX2 is organized by 300 amino acids, missense mutations play an important role in anaphylaxis. In the ECL and TM domains of MRGPRX2, missense variants can occur and affect the phenotypes of mast cells. Eight missense variations in the ECL/TM domains of MRGPRX2 from open-access exome-sequencing databases, However, four natural MRGPRX2 variants, G165E, D184H, W243R, and H259Y failed to respond to any of the ligands tested, these mutations (L31V, V43I, F78L, G165E) in the RBL transfectant were induced degranulation by neuropeptides (substance P and hemokinin-1), a host defense peptide (human β-defensin-3) and icatibant, a Food and Drug Administration-approved cationic drug, bradykinin B2 receptor antagonist [236]. Chalatip Chompunud Na Ayudhya et al research showed that the identification of gain function missense variants in MRGPRX2 transmembrane domains for mast cell

activation by substance P, in TM6 (I225) and TM7 (Y279) of MRGPRX2 are essential for SP-induced Ca^{2+} mobilization and degranulation in transiently transfected RBL-2H3 cell [230].

MRGPRX2 is more potently activated by cationic, research showed that Glu164 (E164) and Asp184 (D184) in MRGPRX2 fourth and fifth TM domains are negatively charged residues that interact with cationic ligands. In contrast, in changing the structure of MRGPRX2, the replacement of Glu164 with a positively charged Arg (E164R) result in loss of MRGPRX2 activation by SP, dextrorphan and morphine [237][238]. Asp184 was considered in studies, even though all opioid ligands examined, including dynorphin A, depend on this amino acid to activate the receptor. It is noteworthy that the naturally occurring D184H variation causes the side chain to change from being negatively charged to positively charged. The fact that none of the MRGPRX2 agonists examined triggered degranulation in RBL transfectants cells that were stably expressing D184H variation was a significant result of the current investigation [238].

C48/80 or SP recruits β-arrestin via MRGPRX2 in G-protein signaling to internalize and desensitize MRGPRX2 at the plasma membrane [239]. MRGPRX2 has a Ser325-containing "β-arrestin binding code" in its carboxyl terminus [240]. However, in response to LL-37, MRGPRX2 is not phosphorylated and is not desensitized [241]. When compared to the WT receptor, the naturally occurring variation (S325L, rs779903448) responds to SP for greater Ca^{2+} mobilization and degranulation [230]. In addition, a missense N62S mutation (rs10833049) in the first intracellular domain of MRGPRX2, which results in the development of a "β-arrestin binding code," has recently been discovered to act as a protective genetic variant for ulcerative colitis (UC). The authors demonstrated that receptor desensitization is linked to β-arrestin recruitment during activation of MRGPRX2 by the synthetic high affinity agonist ZINC3573 and the natural ligand PAMP12. When compared to cells expressing the WT receptor, MRGPRX2 N62S expression greatly increases the recruitment of β-arrestin

and the desensitization of the receptor produced by ZINC3573 and PAMP12. Given the PAMP12 precursor is elevated in the colon of UC patients, it has been hypothesized that people with the N62S mutation would be protected from contracting this disease because their loss-of-function phenotype for mast cells activation results from enhanced receptor function [242]. The majority of GPCR agonists also activate a signaling pathway that involves recruiting β-arrestins [243]. G protein-biased and β-arrestin-biased GPCR agonists, respectively, preferentially activate either G proteins or β-arrestins. A balanced agonist, however, is one that stimulates both routes. As a result, despite the fact that angiogenic HDP AG-30/5C, icatibant, codeine, and C48/80 caused Ca^{2+} mobilization and degranulation through MRGPRX2 via G protein-dependent route, there is a striking variation in their capacity to stimulate β-arrestin recruitment [244][245].

MRGPRX2-mediated degranulation, the level of Ca^{2+} increase is typically characteristic of activating G proteins and via PLCγ-IP3R, further study demonstrated that the stromal interaction molecule 1 (STIM 1) calcium sensor regulates GPCR-mediated Ca^{2+} responses in activation of mast cells [246]. TNF-a and IL-6 are produced when MRGPRX2 ligands SP and LL-37 activate MRGPRX2 downstream signaling, including Erk1/2, JNK, p38, and PI3K/AKT [247]. Through its downstream signals NF-B and p38/JNK MAPK, IL-33, an IL-1 family member, induces cytokine production in mast cells. It also effectively collaborates with MRGPRX2-mediated mast cell activation in the production of IL-8, TNF-α, chemokine ligand CCL1, and CCL2 [248]. MRGPRX2-induced degranulation, mast cells of skin release preformed mediators: histamine, tryptase and β-hexosaminidase, not PGD2 and LTC4 [249].

It also expresses a certain function in the modulation and activation of basophils, there are studies on factors and cellular pathways that regulate MRGPRX2, such as fMLP, IL-3 , anti-IgE, and C5a，increase the repression of MRGPRX2 [250]. In addition, some cytokines affect the activation of mast cells through MRGPRX2. One piece of literature showed that SCF and IL-4 can weaken the response in MRGPRX2-

dependent in skin mast cells [251]. IL-33 is the factor to promote MRGPRX2-triggered degranulation depending on p38 rather than JNK in mast cells of the skin [252].

1.2.2.3 Inhibition of MRGPRX2

Because MRGPRX2 is likely to contribute to inflammation, anaphylaxis, skin diseases, and other diseases. It is anticipated that MRGPRX2 inhibitors will help treat these conditions. The skin might be injected with a photosensitizer-conjugated MRGPRX2 antibody, and then exposed to near infrared light to selectively eliminate skin mast cells that express MRGPRX2 [253]. QWF is a tripeptide antagonist of NK-1, the canonical receptor for SP, a typical neuropeptide of the MRGPRX2 ligands. It has also been claimed that QWF functions as an MRGPRX2 antagonist. QWF competitively inhibits SP binding to Mrgprb2 and MRGPRX2 [254]. Compounds 1 and 2, which are small molecule MRGPRX2 antagonists discovered through the screening of a small molecule compound library, block the activation of MRGPRX2 by inhibiting MAPKs signaling, including SP and icatibant, but have no inhibitory effect on NK-1 or other GPCRs. Compounds 1 and 2 prevented the MRGPRX2 ligand from activating the mast cells, and their actions demonstrated that MAPKs were involved in MRGPRX2 downstreameam signaling. However, compounds 1 and 2 have any inhibitory effects on the mouse mast cell activation caused by SP [255]. Osthole, a coumarin derivative obtained from the Cnidium monnieri (L.) Cusson plant medicinal herbs, interact with MRGPRX2, and change structure of MRGPRX2, and it does prevent increases in intracellular Ca^{2+} concentration brought on by MRGPRX2 ligands and reduce mast cell activation. Additionally, it reduces MRGPRX2 expression on the plasma membrane [256]. A well-known anti-inflammatory medication called dexamethasone also suppresses mast cell activation by downregulating Gai and MRGPRX2 downstream signals brought on by basic secretagogues [257]. MRGPRX2 downstream signals inhibiting, such as intracellular Ca^{2+} concentration and the activation of MAPK kinase, lactic acid prevents MRGPRX2 ligand-induced mast cell activation by interacting with the basicity of MRGPRX2 ligands, it may also prevent MRGPRX2-depedndent mast

cell activation [258]. Botulinum toxin (BTX) is a neurotoxin produced by the bacterium *Clostridium botulinum* that inhibits C48/80 (agonist for MRGPRX2 and MrgprB2)-induced degranulation in human and murine mast cells [259].

In addition, one technique was developed using the single-stranded DNA (ssDNA) aptamer drugs to inhibit the MRGPRX2 as its antagonist. A model of mast cell-deficient rats transplanted with MRGPRX2-expressing mast cell lines, it has been proven that aptamer-X35, a nucleic acid aptamer, attenuates the anaphylactic reaction caused by SP. Histamine release from mast cells was 70% reduced by aptamer-X35. In the rat anaphylaxis model, a subcutaneous injection of 30 nM of aptamer-X35 prevented the anaphylactic reaction [260]. Another literature demonstrated that LL-37/MRGPRX2 signaling and mast cell degranulation was suppressed by an immunomodulatory single-stranded oligonucleotide (ssON), but IgE-mediated response was unaffected. Additionally, ssON therapy reduces inflammation brought on by LL-37 in a mouse model of rosacea [261].

A known inhibitory regulator of IgE- and LPS-stimulated mast cell activation, CD300f inhibits MRGPRX2-mediated mast cell activation [262]. Mrgprb2-mediated mast cell activation in mice and pseudo-allergic drug reactions are inhibited by the interaction between ceramide and CD300f [117]. Chronic spontaneous urticaria caused by clarithromycin with CD300f-mediated negative regulation of MRGPRX2 activation [263].

1.2.2.4 MRGPRX2 physiological functions

In addition, the activation of mast cells through MRGPRX2 plays a different role in various diseases. Pathogens like P aeruginosa that cause skin infections cause keratinocytes to produce hBD, which kills bacteria directly and activates mast cells via MRGPRX2. Histamine and PGD2 are mast cell mediators that increase the amount of hBD secreted by keratinocytes. This increases mast cell activation and the recruitment of neutrophils, which leads to the clearing of the infection [215]. An incredible find is

reported is that competence-stimulating peptide 1 (CSP-1) inhibits bacterial growth and prevents the formation of biofilms by causing degranulation, TNF-α release, reactive oxygen species (ROS), and PGD2 synthesis in mouse PMCs via Mrgprb2. It's interesting to note that MRGPRX2-silenced cells have a reduced response to LAD2 cells' reduction in biofilm viability [227]. When LAD2 cells are co-cultured with S. pneumoniae clinical isolates, which generate CSP-1, the bacterial growth is suppressed, but this suppression is decreased in MRGPRX2-silenced cells. It is well known that bacteria can thwart immune responses and create chronic illnesses by forming biofilms [264]. Local mast cell activation via MRGPRX2 aids in the eradication of skin infections caused by bacteria, promotes healing, and guards against reinfection [265]. Neurogenic inflammatory pain requires the mast cell receptor Mrgprb2 through inducing nerve injury [225].

1.2.2.5 MRGPRX2 pathological functions

MRGPRX2 activation can result in a number of pathogenic processes, further studies are needed to fully understand its role in different disease processes, here I introduce serval diseases related MRGPRX2.

Adverse drug reactions

Opiates, antibiotics, iodinated contrast media, and NMBDs (such as atracurium, mivacurium, tubocurarine, and rocuronium cisatracurium) can cause severe, life-threatening allergic reactions during taking procedures [233]. One literature reported that cisatracurium, morphine, vancomycin, meglumine amidotrizoate, and iomeprol induced LAD2 mast cells degranulation mediated by MRGPRX2 [266]. Mivacurium improves vascular permeability in mouse models of anaphylaxis; however, this reaction is noticeably decreased in mast cell-deficient Mrgprb2 animals [267]. Fluoroquinolones activated mast cells in a dose-dependent manner and reduced degranulation was observed following Mrgprb2 knock out in mice [268]. It has been demonstrated that QWF (glutaminyl-D-tryptophylphenylalanine) inhibits the activation of human

MRGPRX2 by atracurium (NMBA) and ciprofloxacin [254]. Above information suggested that MRGPRX2 is involved in drug-adverse reactions.

Atopic dermatitis

AD or atopic eczema is a long-lasting inflammatory skin condition that is clinically noted for its severe pruritus and T-helper-2 (TH2)-associated hypersensitivity to a variety of allergens, including those originating from house dust mites (HDMs) [269]. The barrier of the skin also can be associated with the occurrence of AD [270]. When compared to healthy skin, the cutaneous nerve fibers of AD patients express more SP (*TAC1*) , mast cells in the skin and its receptor MRGPRX2 may involved in the pathology [271][272][273]. The neurons express the temperature-sensitive ion channel TRPV1 and the SP precursor gene *TAC1* in skin inflammation [274]. Furthermore, Mrgprb2 mutant mice had reduced skin tissue damage brought on by TRPV1 stimulation of sensory nerves [275]. These findings suggest that MRGPRX2 in mast cells may be crucial in the development of allergic skin disorders linked to type 2 immunity.

Urticaria

Urticaria patients have a larger percentage of MRGPRX2-positive mast cells, compared to healthy persons, despite the fact that the number of mast cells in the lesions has not changed [276], SP and VIP in serum derived from urticaria patients are upregulated [277]. These reports suggest the involvement of MRGPRX2 in mast cells in the pathology of urticaria.

2. Hypothesis and objectives

2.1 Hypothesis

MITF is an essential transcription factor for mast cell differentiation; however, its function in mature mast cells remains poorly understood, although it regulates histamine synthesis. MITF activity depends on appropriate LysRS translocation to the nucleus and Ap4A production elicited by FcεRI signaling. Variations in LysRS activity may lead to differential MITF activation. Thus, MITF may play a critical role in mast cell activation, and dysregulation of the LysRS-MITF axis may be directly related to the severity of mast-cell-derived diseases. Moreover, the LysRS-MITF pathway can be a general mechanism shared in IgE-independent reactions governed by MRGPRX2.

2.2 General objectives

Based on the above hypothesis, we pursue to analyze the role of MITF in IgE-dependent mast cell activation in LysRS mutation context related to severe anaphylaxis. We extend the study of MITF in the MRGPRX2 signaling pathway.

2.3 Specific objectives

Objective 1,

1) Generate cellular models to analyze the biochemistry of LysRS P542R, a mutation found in a patient with severe anaphylaxis.

2) Analyze the structure of LysRS P542R compared to LysRS WT.

3) Study mast cell degranulation and prostaglandin synthesis in LysRS WT and LysRS P542R cellular models.

4) Determine MITF activity and MITF dependent-targets expression in LysRS WT and LysRS P542R cellular models.

5) Analyze the relevance of LysRS G189D in mast cell activity—structural, biochemical, and functional analysis.

Objective 2,

1) Determine whether MRGPRX2 signals through the LysRS-MITF pathway.

1.1 Analyze LysRS cellular location, MITF phosphorylation, and MITF activity upon MRGPRX2 activation.

2) Study whether MITF knockdown and inhibition affect MRGPRX2-dependent signals.

2.1 Determine early mast cell signals: degranulation, calcium influx upon MRGPRX2 triggering in MITF-silencing models.

2.2 Determine early mast cell signals: degranulation, calcium influx, upon MRGPRX2 engagement in ML329, an inhibitor of MITF pathway, treated cells.

2.3 Analyze cytokine synthesis in MITF knockdown cellular models.

3) Analyze whether MITF may be involved in MRGPRX2-dependent adverse drug reactions.

3.1. Study the action of drug activation of MRGPRX2 and MITF activity.

3. Publications and results

3.1 Role of MITF in IgE-dependent mast cell activation

Mutation in *KARS:* A novel mechanism for severe anaphylaxis

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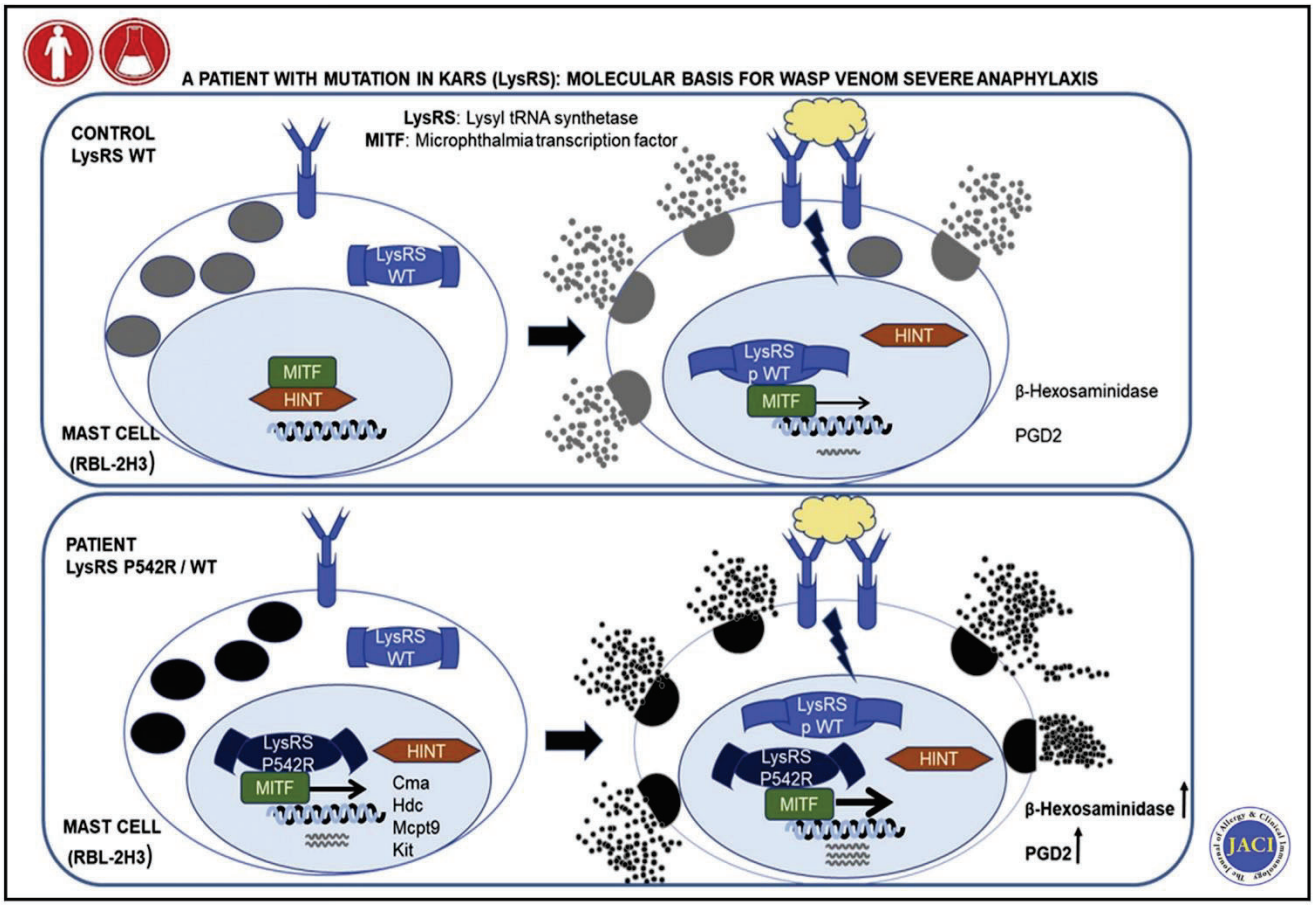
Barcelona and Madrid, Spain

GRAPHICAL ABSTRACT



Background: Anaphylaxis is a severe allergic reaction that can be lethal if not treated adequately. The underlying molecular mechanisms responsible for the severity are mostly unknown.

Objective: This study is based on a clinical case of a patient with extremely severe anaphylaxis to paper wasp venom. This patient has a mutation in the *KARS* gene, which encodes lysyl-tRNA

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synthetase (LysRS), a moonlight protein with a canonical function in protein synthesis and a noncanonical function in antigen dependent–FcεRI activation in mast cells. In this study, the objective was to characterize the mutation at the molecular level.
Methods: Analysis of the KARS mutation was carried out using biochemical and functional approaches, cell transfection, Western blot, confocal microscopy, cell degranulation, prostaglandin D_2 secretion, and proteases gene transcription. Structural analysis using molecular dynamics simulations and well-tempered metadynamics was also performed.
Results: The mutation found, P542R (proline was replaced by arginine at aminoacid 542), affects the location of the protein as we show in biochemical and structural analyses. The mutation resembles active LysRS and causes a constitutive activation of the microphthalmia transcription factor, which is involved in critical mast cell functions such as synthesis of mediators and granule biogenesis. Moreover, the structural analysis provides insights into how LysRS works in mast cell activation.
Conclusions: A link between the aberrant LysRS-P542R function and mast cell–exacerbated activation with increase in proinflammatory mediator release after antigen-IgE–dependent response could be established. (J Allergy Clin Immunol 2021;147:1855-64.)

Key words: *Anaphylaxis, lysyl-tRNA synthetase (LysRS), KARS, microphthalmia transcription factor, mast cells*

Mast cells are the effector cells in anaphylaxis, a severe allergic reaction that is rapid in onset and includes signs and symptoms that involve the skin, gastrointestinal track, respiratory system, and cardiovascular system.[1] The most severe form of anaphylaxis is anaphylactic shock, which is characterized by hypotension and can cause death. Anaphylaxis can be caused by several circumstances: allergies to foods, insect venoms, drugs, and so on. In the last decades, the incidence of anaphylaxis has risen at an alarming rate and continues to rise.[2] Therefore, understanding the molecular basis of anaphylactic shock is crucial for developing new biomarkers and effective prevention and treatment.

In this study, in a patient suffering from extremely severe anaphylaxis due to paper wasp venom allergy, manifested as immediate cardiovascular collapse (refractory hypotension and loss of consciousness), we detected a mutation in 1 of the 2 copies of the *KARS* gene that caused an amino acid change in position 542 of the cytosolic form of lysyl-tRNA synthetase (LysRS), from a proline to an arginine.

LysRS belongs to the aminoacyl-tRNA synthetases group. Aminoacyl-tRNA synthetases play key roles in charging specific tRNAs with cognate amino acids and establish the genetic code rules enabling protein translational fidelity and cellular integrity. Particularly, LysRS is the enzyme that charges lysine to its specific tRNAs. It has 2 main differentiated domains, an aminoacylation domain found in the C-terminus and a tRNA anticodon-binding domain in the N-terminus. It is encoded by the *KARS* gene located on chromosome 16. LysRS has a cytosolic isoform of 597 amino acids and a longer mitochondrial isoform of 625 amino acids generated by an unusual alternative mRNA splicing.[3] The cytosolic LysRS forms dimers that are bound to a p38 molecule of the multi-tRNA synthetase complex (MSC). Interestingly, human cytosolic LysRS has been recognized to participate in an alternative (moonlighting) function beyond translation, connecting it to immune responses. LysRS is phosphorylated at serine 207 through the mitogen-activated protein kinase pathway in stimulated mast cells.[4] Small-angle X-ray scattering data suggested that this induces a conformational change in LysRS and causes the translocation of the enzyme to the nucleus, abandoning its role in translation. Once in the nucleus, LysRS becomes the major producer of diadenosine tetraphosphate (Ap4A) during mast cell activation.[5] As a consequence, the Ap4A produced releases the microphthalmia transcription factor (MITF) from its repressor Hint-1.[6] Therefore, LysRS controls MITF transcriptional activity.[4] MITF is a basic-helix-loop-helix-leucine zipper DNA binding protein.[7] The primary cell types affected in MITF-deficient mice are mast cells, osteoclasts, and melanocytes.[8]

Abbreviations used
GFP: Green fluorescent protein
LysRS: Lysyl-tRNA synthetase
LysRS-WT: LysRS wild-type
LysRS-S207(P): LysRS where Ser207 was phosphorylated
MITF: Microphthalmia transcription factor
MSC: Multi-tRNA synthetase complex
PGD_2: Prostaglandin D_2
PMA: Phorbol 12-myristate 13-acetate
TRPM1: Transcriptional regulation of the melanoma prognostic marker melastatin
WT: Wild-type
3xNFAT: 3x Nuclear factor of activated T cells

Regarding mast cells, MITF regulates the expression of many genes critical for mast cell activation including proteases, cytokine receptors, and cell adhesion molecules.[9-12] MITF is also responsible for the biosynthesis of mediators such as prostaglandin D2 (PGD_2), a mast cell–specific eicosanoid, derived from arachidonic acid.[13] PGD_2 has bronchoconstricting and vasoactive properties.[14] Recently, it has been reported that MITF controls IgE/mast cell–mediated anaphylaxis by regulating histamine production by regulating histidine decarboxylase.[15]

In this study, we analyzed, for the first time, the structure and dynamics of LysRS and its translation to a transcription functional mechanism at a molecular level and in addition the structure and function of a mutation in 1 of the 2 copies of the *KARS* gene that caused an amino acid change in position 542 of the cytosolic form of LysRS, from a proline to an arginine. Our data show that this mutation resembles active LysRS and causes a constitutive activation of MITF, which results in an increase in degranulation and proinflammatory mediator release after IgE-dependent activation.

Our study explains for the first time the switching mechanism from translation to transcription of LysRS at a molecular level, and why specific mutations in the KARS gene deregulate its functional mechanism. Our data indicate that dysregulation of the KARS-MITF pathway can be an underlying mechanism resulting in anaphylaxis.

METHODS

Patient characteristics and allergological workup

The index patient was a 29-year-old man from a rural area north of Barcelona (Spain) referred to the Hospital Clinic, Barcelona, Spain, for recurrent anaphylactic shocks after paper wasp stings. He worked as a farmer and had a history of several paper wasp, yellow jacket, and honeybee stings with good tolerance until 2016, when he presented the first anaphylactic episode. Reactions were characterized by their severity (refractory hypotension and loss of

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consciousness), rapid onset (less than 3 minutes after a single sting), and such fast progression that the patient was unable to use his epinephrine autoinjector.

Allergological workup (see this article's Online Repository at www.jacionline.org) confirmed wasp allergy (Polistes spp). During skin prick tests, after getting an intradermal injection with Polistes spp venom, the patient suffered an anaphylaxis that required repeated doses of epinephrine. This episode was milder than the original reactions with no hypotension or cardiovascular symptoms, but with palm and sole itching, dyspnea, and impending doom. Baseline serum tryptase level (ImmunoCAP; ThermoFisher, Uppsala, Sweden) was within the normal range and did not increase during anaphylaxis. However, serum PGD_2 (ELISA PGD2-MOX; Cayman Chemicals, Mich) values increased 2.7-fold during anaphylaxis (see Table E1 in this article's Online Repository at www.jacionline.org).

Despite having a normal baseline serum tryptase level (3.62 ng/mL), REMA (Spanish Network on Mastocytosis) score[16] was 4, meaning a high probability of clonal mast cell disease. Thus, a bone marrow study was performed, including immunophenotypical analysis for detection of somatic activating exon 17 KIT mutations (D816V). Genomic DNA was extracted from peripheral blood, bone marrow, and purified cell populations using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). KIT mutational status was carried out by the allele-specific oligonucleotide real-time quantitative PCR method that allows quantification of as little as 0.003% KIT D816V mutation-positive cells.[17] KIT D816V mutation was not identified, and no morphological abnormalities or CD25/CD2 aberrant expression in mast cells was found.

Because of the loss of a daughter at a few months of age, the patient and his partner received genetic counseling. Both parents were found to have missense mutations in the KARS gene in heterozygosity that caused an amino acid substitution in the LysRS protein. The KARS mutation was identified by performing exome sequencing–next-generation sequencing (Macrogen). Moreover, the exome analysis further confirms no mutations in KIT.

Molecular dynamics simulations

See this article's Online Repository at www.jacionline.org.

Well-tempered metadynamics

See this article's Online Repository at www.jacionline.org.

Plasmid construction

Human LysRS (*KARS* wild-type [WT]) was used for the production of the LysRS mutant by site-directed mutagenesis in which proline was replaced by arginine at position 542 (LysRS P542R). Human LysRS WT and P542R variants were subcloned into the KpnI and NotI sites of the pcDNA 3.1+C-eGFP vector, and the fidelity of all constructs was verified by direct sequencing (GenScript Biotech, Leiden, The Netherlands).

Mutant RBL-2H3 cell transfectant generation

RBL-2H3 was used as a mast cell model. Cells were cultured as described elsewhere.[18] RBL-2H3 cells were transfected with *KARS* WT or *P542R or G189D* constructs with Lipofectamine (Invitrogen, Waltham, Mass) following manufacturer's instruction. G418 (Gibco, Dublin, Ireland) was used to select transfectants and further sorting with FACS ARIA was performed. Cells were finally checked with FACS CALIBUR for green fluorescent protein (GFP) and FcεRI expression.

Flow cytometry

A total amount of 10^4 cells/sample were acquired using a FACSCalibur flow cytometer (Beckton-Dickinson, Franklin Lakes, NJ), and the data were collected with CellQuest Alias and analyzed with FlowJo V10 software (Becton-Dickinson). The antibodies used were mouse primary antibody antirat FcεRI mAb (BD Pharmingen, San Diego, Calif) and secondary antibody antimouse Alexa 546 (BD Biosciences, San Jose, Calif).

Confocal microscopy staining and analysis

Cell transfectants were initially seeded at 10^5 cells/well on cover slips. The following day, cells were fixed with paraformaldehyde 4% for 15 minutes at 4°C and then nuclei were stained with Hoechst for 5 minutes. Cells were washed and cover slips were placed on microscope slides using Prolong Gold antifade reagent. Other assays included Lysotracker Red DND-99 (Molecular Probes/L7528) for 2 hours at 37°C, before fixation. Then, confocal microscopy images were taken with a Leica SP5 confocal microscope. Images were then analyzed using the Fiji/Image J software.[19]

SDS-PAGE acrylamide gel electrophoresis and Western blot

Samples were obtained as cellular fractions from 5×10^6 cells, using a centrifuge-based fractionation method.[20] The samples were quantified for protein using a Bradford technique and were then loaded accordingly into NuPAGE 4% to 12% Bis-Tris Precast Protein Gels (Thermo Fisher Scientific, Waltham, Mass). Western blot procedure was performed as described elsewhere.[18] Mouse anti-*KARS* (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) or rabbit anti–Laminin B1 (Abcam, Cambridge, UK) or mouse antitubulin (Sigma-Aldrich, St Louis, Mo) or pKIT (Tyr 719) (Cell Signalling Technology, Danvers, Mass) antibodies were used for the immunoblot. Secondary antibodies, goat antirabbit horseradish peroxidase (Sigma-Aldrich) or polyclonal rabbit antimouse horseradish peroxidase (DAKO, Carpinteria, Calif), were also used.

β-Hexosaminidase determination and PGD_2 ELISA

The degranulation assay was performed as described previously.[18] Briefly, cells were seeded at 2.5×10^4 cells/well in 96-well plates and were sensitized with anti-DNP IgE overnight with indicated concentrations. Next day, after washing, cells were incubated with the stimuli DNP-HSA, ionomycin, and phorbol 12-myristate 13-acetate (PMA) or plain medium (not stimulated). Incubation was carried out at 37°C for 1 hour. β-Hexosaminidase activity was measured in supernatants and the cell lysates as described.[18] The activity of the β-hexosaminidase enzyme was expressed as the percentage of the release: β-hexosaminidase release = [cell degranulation/(cell degranulation + total lysate)] × 100.

PGD_2 release was determined from the supernatants of cells (2.5×10^4 cells/well) activated as described above for 4 hours using a specific competitive Enzyme Immunoassay for PGD_2 (Cayman Chemical) according to manufacturer's instructions.

Luciferase assay

Firefly luciferase under the control of transcriptional regulation of the melanoma prognostic marker melastatin (TRPM1) promoter and control vector PGL3-luciferase were a gift from David Fisher (Harvard Medical School).[21] 3x Nuclear factor of activated T cells (3xNFAT)-luciferase was reported previously.[22] A total of 6×10^4 cells were transfected with the Firefly and the Renilla reporters at a ratio of 7:1, respectively. Transfections were performed using the AMAXA program T20 (Lonza, Waldshut-Tiengen, Germany).

Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega, Madison, Wis) following manufacturer's instructions. Firefly luciferase data were normalized according to Renilla luciferase data.

Gene expression analysis in cell transfectants

See Online Repository information and Table E2 in this article's Online Repository at www.jacionline.org.

Statistical analysis

All results are expressed as mean ± SEM. After determination of normal distribution of the samples and variance analysis, the unpaired student *t* test was used to determine significant differences (*P* value) between 2 experimental groups, and 2-way ANOVA with Bonferroni multiple comparison test was used to determine significant differences (*P* value) between several experimental groups. $*P < .05$, $** P < .01$, $***P < .001$, $****P < .0001$.

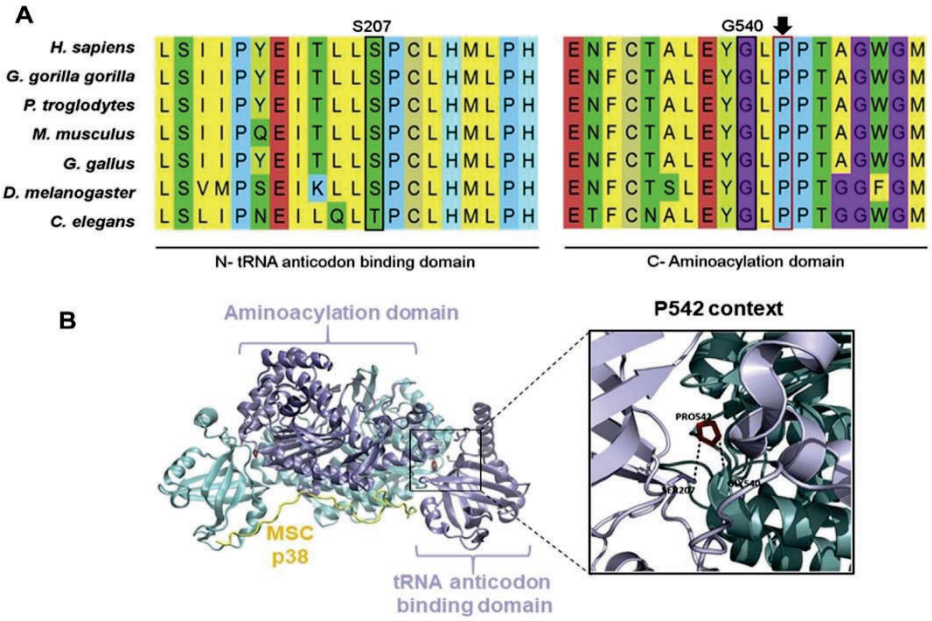

FIG 1. Proline 542 is a conserved residue in LysRS. Sequence alignment of LysRS domains Mega X: Molecular Evolutionary Genetics Analysis X software. **A,** Use of serine207, glycine540, and proline542 as the key residues is highlighted. Modified from Ofir-Birin et al.[23] **B,** Structural prediction of the proximity between proline542 and the key residues of LysRS.

RESULTS

LysRS functional mechanisms: P542R mutant characterization

As mentioned, a mutation in proline 542 in LysRS was found in a patient with anaphylaxis to hymenoptera. Our first analysis through sequence alignment showed that proline 542 is a well-conserved amino acid (Fig 1, *A*),[23] which is placed at the interface between the 2 protein domains (Fig 1, *B*), facing Ser207, which when phosphorylated triggers the opening of LysRS and, in turn, the release of LysRS from the MSC.[23] Mutations in this region, such as G540Y, lead to the opening of LysRS,[23] suggesting that mutation of P542 to a quite different residue, such as arginine, may profoundly affect the binding of LysRS to the MSC and the entire functionality of the protein. To check this, we performed molecular dynamics simulations and well-tempered metadynamics simulations of different LysRS systems: LysRS wild-type (LysRS-WT), LysRS where Ser207 was phosphorylated (LysRS-S207(P)), and LysRS carrying the P542R mutation (see this article's Methods section and Online Repository information in the Online Repository at www.jacionline.org).

Free energy profiles along the opening path (Fig 2, *A*) obtained for the LysRS-WT and LysRS-S207(P) systems show a single minimum for the LysRS-WT system in the closed state, whereas opening is spontaneous for the LysRS-S207(P) system, a result that was reproduced by unbiased molecular dynamics simulation (see Online Repository information, Fig E1, *A*, and Video E1 in this article's Online Repository at www.jacionline.org), which confirmed the spontaneous opening induced by phosphorylation. This result agrees with qualitative estimates from small-angle X-ray scattering obtained by collecting spectra of the wild-type and LysRS-S207D variants.[23] Our simulations demonstrate (see Fig 2, *B*) that the major conformational changes occur at the N-terminal tRNA anticodon-binding domain, which opens and moves away from the C-terminal aminoacylation domain, leading to a major global conformational rearrangement.

Mutation P542R is quite disruptive, because it introduces a positively charged residue at a region of the interdomain interface, which is mainly nonpolar (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). In the closed state, arginine 542 interacts with both E150 and D313 (see Fig 2, *C*, closed state inset, and Fig E2, *B*). Interestingly, both residues are placed in the binding region with the MSC-p38 protein (see Fig E2, *C*), and sequestering them should impact binding between LysRS and the MSC. However, in the opened state, the N-terminal tRNA anticodon-binding domain has moved far from the interdomain interface and residue R542 is found mostly exposed to the solvent and interacts only with residue D313 (see Fig 2, *C*, open state inset). Metadynamics simulations show that although only the "closed" state is populated for the unphosphorylated wild-type protein, both open and closed states are populated (almost identically) for the LysRS carrying the P542R mutation system (see Fig 2, *C*; see Video E2 in this article's Online Repository at www.jacionline.org). Thus, the P542R mutation first alters the MSC-p38–binding region in both the closed and opened states by trapping 1 or 2 residues involved in the interaction and recognition of the MSC-binding loop. Furthermore, the P542R mutation deregulates functional motions of LysRS by promoting the system to explore an open state that could unbind from the MSC, triggering translocation to the nucleus, a behavior that is only triggered in the LysRS-WT system by the phosphorylation of S207.

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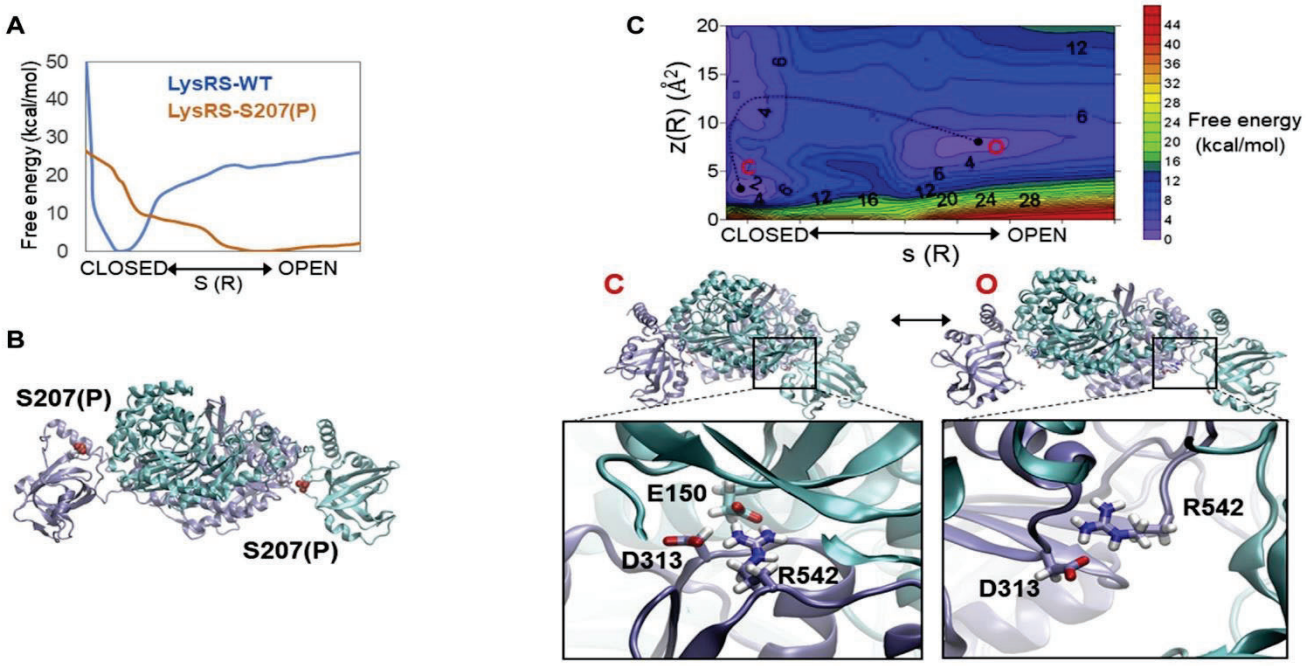

FIG 2. LysRS-WT and LysRS-P542R functional motions at an atomic level. **A,** Free energy profiles obtained for the opening process of the LysRS-WT and LysRS-S207(P) proteins. **B,** Phosphorylation of S207 (shown in van der Waals representation) provokes the spontaneous opening of LysRS (shown as ice-blue and cyan ribbons) during MD simulation. **C,** FES associated with the opening/closing motion of the LysRS-P542R–mutated protein; isoenergetic lines are shown in kcal/mol. The 2 free energy minimum structures that correspond to the opened and closed states are depicted as ribbons (in cyan and ice-blue for each of the monomers). Insets for both states show the interaction between R542 and residues involved in MSC-p38 binding. *FES,* Free energy surface; *LysRS-P542R,* LysRS carrying the P542R mutation; *MD,* molecular dynamics.

Transfectant generation and LysRS cellular location

Next, we pursued the analysis of the functional consequences of this mutant in a mast cellular model. RBL-2H3 cell transfectants expressing LysRs WT-GFP or LysRS P542R-GFP were obtained after 2 consecutive sortings, and cells were tested for FcεRI and GFP expression of the respective transfectants (Fig 3, *A*). We checked endogenous expression of LysRs versus transfected lysyl-tRNA-synthetase-GFP (Fig 3, *B*).

Next, we determined the cellular localization of the lysyl-tRNA-synthetase-GFP recombinant proteins, RBL-2H3 cells expressing the recombinant LysRs-WT gene, or LysRS-P542R by confocal microscopy. LysRs WT was expressed only in the cytoplasm as expected. As known, lysyl-tRNA synthetase is attached to the MSC in quiescent conditions. Interestingly, LysRS-P542R (Fig 3, *C*) was found mainly in the nucleus. We confirmed results by performing cell fractioning and Western blot analysis. The whole cellular fraction indicates slightly lower levels of LysRS-P542R compared with LysRS-WT (Fig 3, *D*). Nevertheless, in the cytoplasmic fraction, *P542R* levels were severely diminished and most of the LysRS-P542R was found in the nucleus (Fig 3, *D*).

LysRs mutant P542R enhances degranulation and PGD_2 synthesis

Having established the protein localization, we proceeded to check whether IgE-dependent mast cell activation was altered. Cell transfectants were sensitized with the indicated concentrations of anti-DNP IgE overnight. The following day cells were stimulated for 1 hour with 50 ng/mL of the antigen DNP-HSA or ionomycin plus PMA (1 μM + 50 ng/mL, respectively). Our data show that degranulation is enhanced in P542R compared with WT. Both transfectants have the same ability to degranulate after a general stimulus such as ionomycin and PMA (Fig 4, *A*). Interestingly, when we use lower doses of ionomycin, we observe an increase in degranulation in P542R cells, suggesting that this mutation may affect IgE-independent signals (Fig 4, *B*). Afterwards, IgE-dependent eicosanoid production was also assessed (Fig 4, *C*). PGD2, a hallmark of *de novo* mediators' synthesis in mast cell granules, was analyzed by ELISA. Our data show that PGD_2 was significantly enhanced in P542R. In both cases, the basal levels (nonstimulated conditions) of β-hexosaminidase and PGD_2 release were similar in WT and P542R, indicating that proinflammatory mediators are released on IgE crosslinking. These data agree with the increase in PGD_2 found in the patient carrying the mutation (see Online Repository information and Table E1).

Granule analysis in cell transfectants

To assess whether there was any difference in the size and amount of the granules in both transfectants, 2 different assays were performed (Fig 5). In both experiments, cells were stained with lysotracker, a red-fluorescent dye for acidic organelles. First,

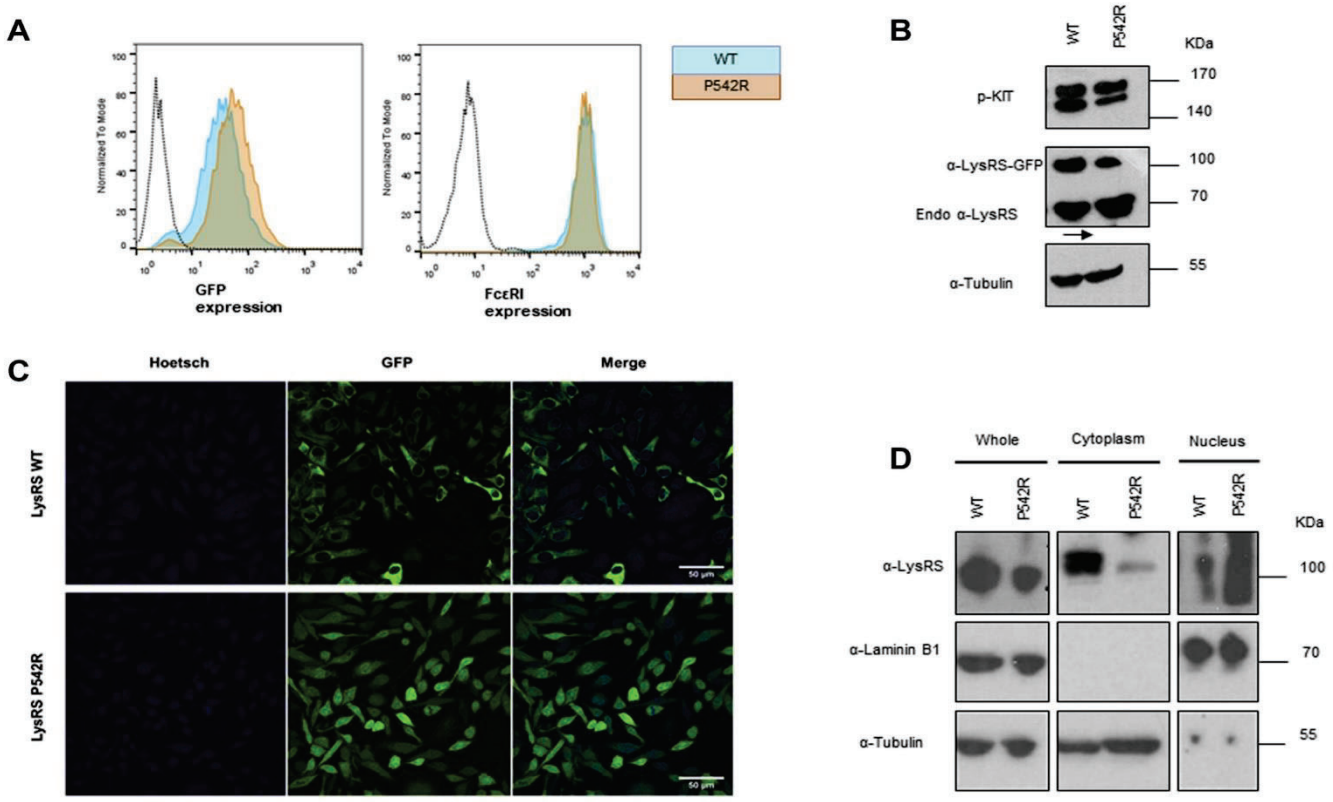

FIG 3. LysRS-WT and LysRS-P542R show distinct patterns of expression. **A,** Flow cytometry histograms for GFP and FcεRI expression in WT and *P542R* RBL-2H3 transfectants. Parental RBL-2H3 cells are used as control. **B,** pKIT and LysRS expression levels. **C,** Confocal microscopy of WT and P542R cells. GFP shows the distinct pattern of LysRS expression in WT and P542R transfectants. Nuclei are stained with Hoechst (blue). **D,** Western blot of the whole-cell lysate and cytoplasmic and nuclear fractions of WT and P542R cells. LysRS was detected by blot. Laminin B1 and α-tubulin are markers for nuclei and cytoplasm, respectively.

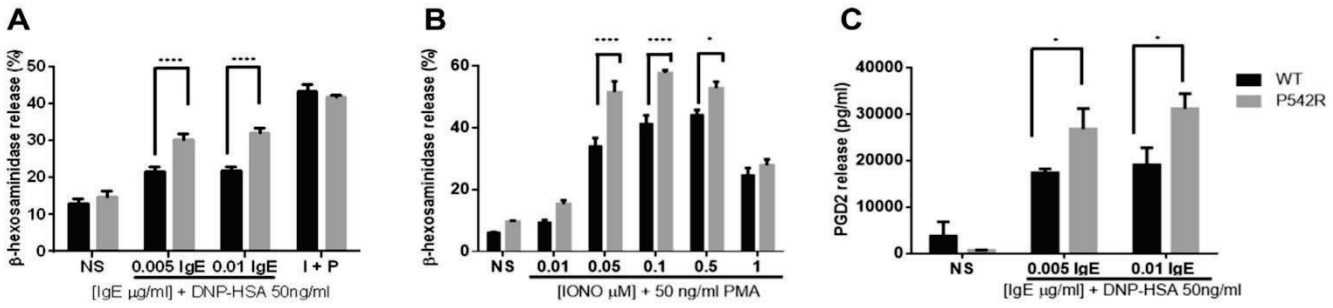

FIG 4. LysRS P542R mutation enhances β-hexosaminidase and PGD_2 release on IgE activation. **A,** β-Hexosaminidase assay in NS conditions and IgE + DNP-HSA or ionomycin-PMA (I + P) (1 μM + 50 ng/mL) stimulation in WT and P542R RBL-2H3 cells. **B,** Ionomycin titration (from 0.01 μM to 1 μM) was performed in WT and P542R cells plus 50 ng/mL of PMA. **C,** PGD_2 ELISA for P542R and WT cell transfectants with NS conditions, IgE + DNP-HSA stimulus. Data were collected in triplicate and are the mean of 3 independent experiments. Black and gray columns correspond to WT and P542R cells, respectively. *NS,* Nonstimulated.

the cells were dyed and submitted to flow cytometry analysis (Fig 5, *A*). Unexpectedly, the mutant cell transfectants did not show an increase in staining. To further corroborate, cells were prepared for analysis with confocal microscopy. This time cells were stained with lysotracker (granules) and Hoechst (nucleus), as depicted (Fig 5, *B*). To ensure an impartial analysis, we developed a macro for the Image J software. This less-cumbersome approach enabled the quantification of individual cells, up to a total of 120

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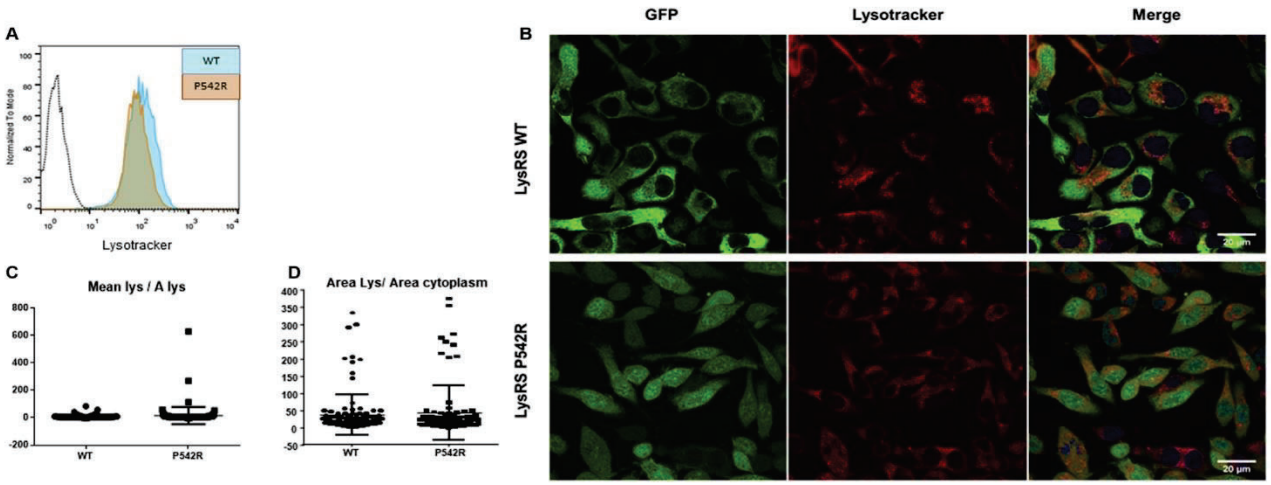

FIG 5. Granule staining with lysotracker does not show differences in LysRS-WT and LysRS-P542R transfectants. Mast cell granule analysis was performed with lysotracker red dye. **A,** Flow cytometry histogram for WT and *P542R*-stained cells. **B,** Confocal analysis of WT and P542R cells including Hoechst staining for the nucleus. **C** and **D,** ImageJ software analysis of the WT and P542R cells, showing intensity (Fig 5, *C*) and total amount of granules (Fig 5, *D*). Data are representative of 3 independent experiments.

for each cell transfectant. Only cells within the microscope field of view were counted; hence, the cells at the edge of each image were not included. The analysis was limited to the cytoplasm by subtracting the nucleus and delimiting the outer membrane of each cell unit. To evaluate any existent difference in the content of the granules, a ratio of the mean intensity of lysotracker versus the area stained with this dye was performed (Fig 5, *C*). In addition, a ratio of the area stained with lysotracker versus the area of the cytoplasm was carried out to examine possible variances in the number of granules (Fig 5, *D*). This set of analyses both presented no differences between the 2 cell transfectants, confirming the previous result obtained by flow cytometry (Fig 5, *A*).

MITF activity analysis

Next, we assessed whether LysRS-P542R constitutive nuclear translocation was concomitant with an increase in MITF activity. For this purpose, we used a reporter gene methodology, melastatin 1 (TRPM1) promoter-controlled firefly luciferase. MITF is a major transcriptional regulator of TRPM1.[24] Thus, firefly luciferase expression correlates with MITF activity.[25] This reporter gene was transfected to the cells together with a Renilla luciferase reporter gene, and results were normalized to the empty vector-transfected conditions. Results show that P542R transfectants present a significant increase in MITF activity compared with WT cells in unstimulated conditions (Fig 6, *A*) and after IgE stimulation (Fig 6, *B*). It should be noted that overexpression of LysRS-WT significantly increases TRPM1 transcription compared with control vector (PGL3-luciferase). In parallel, we transfected cells with 3xNFAT-luciferase and measured basal 3XNFAT activation in WT and P542R transfectants. As shown in Fig 6, *C*, no activation was observed in P542R in unstimulated conditions. As expected, after PMA + ionomycin stimulation, 3xNFAT-luciferase was induced, indicating that the reporter gene was working properly and it was susceptible to activation after the right stimulus (Fig 6, *D*). These data indicate that MITF is active in P542R unstimulated cells, whereas other transcription factors such as NFAT are not.

MITF-dependent gene expression

Next, we analyzed some described MITF-dependent genes involved in mast cell biology, such as mast cell protease 2, 4, and 9 and chymase 1, among others (see Table E2). The levels of some of them increased significantly in P542R transfectants compared with WT. Interestingly, chymase 1 was found particularly augmented in contrast to other proteases (Table I).

DISCUSSION

Anaphylaxis is a critical illness in emergency medicine, which is described as a severe allergic reaction with an immediate onset, led by mast cells and basophils.[26] The mediators released by mast cells and basophils on contact with FcεRI-bound IgE to a specific allergen characterize the pathology of this disease, which can lead to death if not controlled. Unlike other allergic diseases, patients with anaphylaxis barely have prophylactic treatments. Frequently, the only option for these patients is to avoid the stimuli that trigger these reactions. However, it becomes a huge challenge when the trigger is a ubiquitous and/or hidden allergen (foods such as milk, egg, or wheat, among others) or when the trigger avoidance escapes the patient's control (hidden allergens, hymenoptera, or presence of cofactors, for example). In addition, with the exception of tryptase and the D816V mutation of the KIT receptor (CD117), we barely have reliable biomarkers that allow us to unequivocally identify those patients who have a higher risk of reaction, or serious reaction.[27] Therefore, there is a clear interest in the field to better understand the molecular mechanism underlying severe allergic reactions. This study is based on a particular clinical case of an individual with extremely severe anaphylactic reactions in response to *Hymenoptera* stings, reacting even at concentrations used for routine allergological workup (skin tests) and pretreated with omalizumab (anti-IgE) to tolerate wasp venom

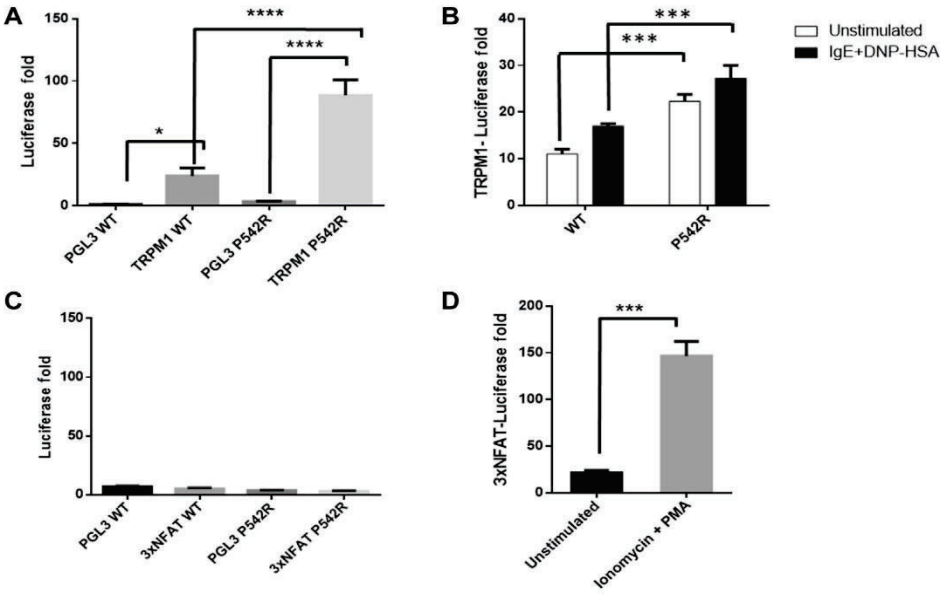

FIG 6. LysRS P542R increases MITF activity in the absence of stimulation. **A,** LysRS-WT and LysRS-P542R were transfected with TRPM1-luciferase reporter gene or PGL3-luciferase plus the thymidine kinase promoter-*Renilla* luciferase reporter plasmid (pRL-*TK*) . Next day, cells were lysed and luciferase activity was measured. **B,** Cells were stimulated with IgE + DNP-HSA (0.25 μg/mL + 50 ng/mL). **C,** The same procedure was carried out with 3XNFAT instead of TRPM1. **D,** WT cells were activated with ionomycin + PMA (1 μM + 50 ng/mL, respectively) as a positive control of 3XNFAT. Measurements were performed in triplicate, and the data are representative of 3 independent experiments.

TABLE I. MITF-dependent gene array

Gene	P542R vs WT
Cdk2	−1.11
Cma	**5.31***
Gzmb	−2.72
Kit	**1.59†**
Mcpt2	1.46
Mcpt4	1.69
Mcpt9	**2.0‡**
Serpine 1	−1.80
Tph1	−1.49
Hpgd	−1.07
Tpsb2	1.52
Sort1	−1.47
Lyst	1.11
Cdk5r1	1.25
Hdc	**1.77‡**

Gene expression fold changes between WT and P542R are shown. Boldface indicates statistical significance.
*Statistical significance of upregulated gene expression in P542R vs WT: $P < .001$.
†Statistical significance of upregulated gene expression in P542R vs WT: $P < .01$.
‡Statistical significance of upregulated gene expression in P542R vs WT: $P < .05$.

immunotherapy. In this study, we found that dysregulation of the FcεRI-LysRS-MITF pathway due to mutations in the *KARS* gene may explain a hypereactive phenotype that can lead to anaphylaxis.

LysRS (encoded by the *KARS* gene) as a moonlight protein has a dual and crucial role in protein translation or gene transcription in mast cells.[6] Some mutations of KARS have been reported, and the associated clinical phenotype of them is heterogeneous ranging from early-onset encephalopathy to isolated peripheral neuropathy, nonsyndromic hearing impairment, or progressive leukoencephalopathy.[28-30] Some other mutations have been associated with suspected mitochondrial disorder,[31] or with combined respiratory chain complex deficiencies (I and IV).[32]

Nevertheless, no naturally occurring mutations have been identified nor have they been connected to anaphylactic reactions. Here, we attempted to link the noncanonical role of LysRS involving the activation of transcription factor MITF with the release of key mediators in anaphylaxis.

Our findings indicate that the P542R amino acid substitution in LysRS promotes the translocation of mutant LysRS to the nucleus in the absence of stimuli. This could be explained by the substitution of a proline, an amino acid that introduces rigidity to the polypeptide backbone, for a positively charged amino acid with a considerable side chain, such as arginine. Our findings also suggest that mutations placed in the region of the interdomain interface of LysRS are candidates to deregulate its functionalities. The constitutive nuclear location of LysRS-P542R results in a higher release of both preformed and "*de novo*" mediators in mast cells. Interestingly, the LysRS-P542R cell transfectant showed that MITF activity was enhanced, as well as specific MITF-dependent gene transcription, in the absence of stimulation. One of the most intriguing aspects of human anaphylaxis is that some patients with severe symptoms do not show an elevated tryptase level. This raises the possibility of the involvement of other, unidentified pathways and opens up the possibility of finding unidentified biomarkers. Remarkably, chymase (Cma) expression was significantly increased in P542R cells, whereas tryptase (Tph1) was not. This result agrees with the clinical data of this patient where there was no increase in tryptase level

(hallmark of anaphylaxis) in the acute phase in this clinical case (see Online Repository information, patient characterization, in this article's Online Repository at www.jacionline.org). Chymase is a serine protease basically found in the secretory granules in mast cells. The evidence of chymase as a potential biomarker for anaphylaxis comes from the significant results found in 2 different studies of autopsy cases with anaphylaxis.[33,34] One consideration is that chymase stability in serum appears to be higher than that of tryptase. Chymase is detectable 4 hours hours after the anaphylactic reaction, whereas tryptase levels return to baseline by this time point.[35] Consequently, it would be interesting to extend the analysis of chymase in those cases of anaphylaxis where tryptase is negative.

This study is based on a particular clinical case of an individual with anaphylactic reactions in response to *Hymenoptera* stings due to such severe allergic reactions, even at concentrations used for routine allergological workup (skin tests). Indeed, this patient requires omalizumab (anti-IgE) treatment to tolerate wasp venom immunotherapy administration. Because of the loss of a daughter at a few months of age, the patient and his partner received genetic counseling. Both parents were found to have missense mutations in the *KARS* gene, which caused an amino acid substitution in the LysRS protein. The allergic patient has mutation P542R, whereas the partner, with no allergic background, has mutation G189D. In the Online Repository data, we compared both mutations at the structural level and also at the functional level. Our metadynamics simulations showed that the G189D mutation does not promote the opening mechanism as is the case of the P542R mutation. A single free energy minimum is found in the closed state of the LysRS-G189D system as in the LysRS-WT system (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, G189D is found far from both the interdomain interface and the S207 residue (see Fig E3, *C*, bottom structure), and it is not found to interact with residues in charge of binding with MSC-p38, as opposed to the P542R mutation. Therefore, G189D would neither perturb the possibility of S207 being phosphorylated nor the binding of LysRS to MSC-p38.

Interestingly, the G189D LysRS protein is not observed intrinsically in the nucleus (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org) as we observed for P542R and consequently does not increase MITF activity using the TRPM1-luciferase gene reporter assay (Fig E4, *C*). Accordingly, we found that an MITF-dependent event such as PGD_2 generation (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org) or MITF-dependent gene expression was similar in LysRS G189D and LysRS-WT (Fig E5, *B*), indicating that this mutation does not enhance the noncanonical function in mast cells. To conciliate our results with the clinical data, where inheriting both mutations produced a deleterious phenotype, we demonstrated that mutation P542R translocates the protein to the nucleus, increasing MITF activity, thereby committing this protein to its noncanonical function. The increase in MITF activity allows crucial genes for anaphylaxis such as histidine decarboxylase to be enhanced.[15] On IgE activation, the proinflammatory mediator release will be more pronounced, resulting in a more harmful inflammatory phenotype. It is of note that spontaneous degranulation is not enhanced in P542R transfectants, indicating that the presence of IgE plus allergen would be needed to trigger the anaphylaxis. The preventive treatment of the patient with omalizumab reduces the IgE bound to mast cells[36] and the eventual anaphylactic shock in the presence of the allergen. Regarding mutation G189D, this substitution, based on our results, does not lead to a more susceptible anaphylactic profile on allergen encounter. In fact, this individual has not reported any allergic episode so far. We believe that this mutation impairs the LysRS canonical function in protein synthesis. As depicted in Fig E3, *C*, the G189D mutation is placed on the surface of the tRNA anticodon-binding domain. Thus, this mutation might distort the mode of tRNA binding and alter the LysRS translational mechanism.

Altogether, this study shows how a mutation in LysRS triggers its translocation to the nucleus independently of stimuli and that this translocation enhances MITF activity and proinflammatory mediator release on IgE crosslinking. Besides the mitogen-activated protein kinase-LysRS-Ap4A-HINT1 signaling pathway, multiple pathways have been reported to regulate MITF activity and/or stability in mast cells, including the c-KIT and PI3K pathways.[37-39] The analysis of the MITF signaling cascade in the context of IgE and beyond IgE could provide further insight into the molecular pathways involved in anaphylaxis to *Hymenoptera* and it may be extended to food or idiopathic anaphylaxis.

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Clinical implications: Our study highlights a signaling pathway, IgE-LysRS-MITF, that can set the degree of severity of anaphylaxis.

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ALLERGOLOGICAL STUDY

Skin tests (prick and intradermal tests) with yellow jacket, paper wasp (*Vespula spp and Polistes spp*, ALK-Abelló, Madrid, Spain), and honeybee venom, as the most prevalent hymenoptera insects in our area, were performed following the standard procedure.[E1] Intradermal tests were performed at 0.001, 0.01, and 0.1 μg/mL and results were positive only at 0.1 μg/mL for both *Vespula spp* and *Polistes spp*. Specific IgE (ImmunoCAP; ThermoFisher, Uppsala, Sweden) were positive for *Polistes spp* (2.33 kU/L) and *Vespula spp* (0.89 kU/L), and negative for bee venom (<0.1 kU/L). Basophil activation test (Flow2CAST; Bühlmann Laboratories AG, Schönenbuch, Switzerland), measured as expression of CD63, result was positive only for *Polistes spp* at 0.25 μg/mL and negative for Vespula *spp* at all tested concentrations (1, 0.5, 0.25, and 0.1 μg/mL).

Considering the severity of the reaction, venom immunotherapy (VIT) for *Polistes spp* was prescribed on the basis of results of the allergological study and the higher prevalence of *Polistes spp* in our area. With the first dose of VIT (0.01 μg), the patient presented again an anaphylaxis, similar to that observed with the skin test, without cardiovascular involvement, and required 0.5 mg of epinephrine. For this reason, we decided to use omalizumab 300 mg as a pretreatment. Thus, the patient received 2 doses of omalizumab without VIT, and thereafter a monthly dose of omalizumab was administered. Using that strategy, we were able to reach 100 μg of venom using a conventional VIT schedule, with no relevant symptoms. Basophil activation test result with *Polistes spp* venom was negative after 12 months of VIT and omalizumab.

GENE EXPRESSION ANALYSIS IN LysRS-WT/ LysRS-P542R AND LysRS-G189D TRANSFECTANTS

Samples were performed in triplicate. RNA extraction was performed by following the Trizol Reagent protocol (Thermo Fisher Scientific). Reverse transcription was performed using StaRT Reverse Transcription Kit (AnyGenes, Paris, France). Before quantitative PCR (qPCR) array analysis, a quality control for all cDNA was performed by qPCR on a Light Cycler 480 instrument (Roche Diagnostic, Rotkreuz, Switzerland) with 2X Perfect Master Mix SYBR Green (PMS) (Anygenes) and Actb primer set (Actin beta, House-Keeping Gene, known to be constitutively expressed in all tissues). Briefly, each qPCR reaction contains 10 μL of PMS, 1 μL of Actb Validated qPCR Primer Sets (Reverse + Forward, 10 μM each: Anygens), 1 μL of diluted cDNA, and 8 μL nuclease-free water, for a final volume of 20 μL. All qPCR reactions were performed on the Light Cycler 480 instrument (Roche Diagnostic) with 2x Perfect Master Mix SYBR Green (PMS) (AnyGenes). All the Validated qPCR primer sets used are described in Table E2. All cDNA samples were analyzed in qPCR triplicates for each studied or House-Keeping gene. The mRNA expression for each gene was normalized using the mean of Cp values for the 8 housekeeping genes, and defined by $2^{\wedge(-\mathrm{dCp})} \times 10{,}000$ values. Fold changes, *P* value via Student test, and SD have been calculated, to generate tables and graphs. The entire analysis was performed by the AnyGenes SignArrays Service.

MOLECULAR DYNAMICS SIMULATIONS

Methods

We performed long classical molecular dynamics (MD) simulations for the LysRS-WT system where Ser207 was phosphorylated (LysRS-S207(P)), the LysRS carrying the P542R mutation (LysRS-P542R) system, and the LysRS-G189D mutant system. The starting conformations of the LysRS systems were modeled from the crystal structure of LysRS with PDB ID 6ILD,[E2] which is a homodimeric structure. To insert the ligands in the active site of our systems, we aligned the 6ILD crystal structure with the PDB ID 1E22[E3] crystal structure. 1E22 is crystallized as a monomer with a lysine residue, 3 Mg^{2+} ions, and a cofactor analogue of ATP. The alignment showed a root-mean-square deviation of less than 0.5 Å between the structures. The LysRS subfamily of enzymes is known to belong to class II of Aminoacyl-RSs, which work as homodimers and, as a first step of their canonical function, catalyze the formation of an aminoacyl adenylate in the presence of 3 Mg^{2+} cations.[E4] Thus, we inserted the missing ligands (a modeled ATP molecule and 3 MG^{2+} ions) inside the 2 active sites of our homodimer. Finally, we reconstructed the missing amino acids in the 6ILD chains, making use of SWISS PROT utility.[E5] The initial structures were subjected to several cycles of minimization and equilibration as explained below.

The AMBER 18 program[E6] was used to carry out the MD simulations using the AMBER ff14SB force field[E7] for the protein. Force field parameters of ATP were developed by Meagher et al,[E8] Mg^{2+} parameters by Allnér et al,[E9] and parameters of phosphorylated serine by Homeyer et al.[E10] The protonation state of LysRS residues was assigned making use of propKa[E11] at pH 7. Then, the system was solvated using the LEAP module of AMBER into a truncated octahedron box of TIP3P[E12] water molecules. A buffer of water molecules extending for 12 Å in every direction around the system was applied, and the system was neutralized with potassium ions by adding an excess of 150 mM of KCl. Long-range electrostatic interactions were computed by using the Particle Mesh Ewald method[E13] using standard defaults and a cutoff in the real-space of 10 Å. A cutoff of 10 Å was used to compute the nonbonded interactions. The SHAKE algorithm[E14] was applied to restrain bond lengths involving hydrogen atoms, and the time step of the simulation was 2 fs. The systems were energy minimized, thermalized, and preequilibrated for 50 ns in the canonical and isobaric-isothermal ensembles before the production run. Production MD simulations were carried out in the isothermal-isobaric ensemble with a pressure of 1 atmosphere and a temperature of 300 K, making use of the Berendsen thermostat and barostat. Unrestrained MD simulations were performed for 1 μs.

Results

Initially, we performed molecular dynamics simulations of 1 μs for LysRS-WT, LysRS-WT-S207(P), LysRS-P542R systems, and the system containing the G189D mutation (LysRS-G189D) (see the Methods section for details). Our unbiased MD simulation for the LysRS-S207(P) system was able to capture the opening motion of LysRS, leading to an open state in agreement with the published Small-angle X-ray data.[E15] The opening of the protein occurs in a relatively short simulation time (see root-mean-square deviation in Fig E1, *B*), suggesting that the introduction of a negative charge in a mainly apolar interdomain interface should provoke steric clashes and the opening of the protein dimer in a short time scale. As expected, the LysRS-WT system did not open during the simulation run and remained in its closed conformation (Fig E1, *A*). In addition, we did not observe much change in the overall structure of the

LysRS-P542R system (Fig E1, *C*, root-mean-square deviation), which remained closed. The mutated residue P542R is found interacting with residues E150 and D313 (see Fig E2, *B*) along part of the MD simulation as in the closed state free energy minimum found in the metadynamic simulation (see Fig 2, *C*). Finally, the G189D-mutated LysRS system remained in the closed state throughout the simulation. As can be seen in Fig E1, *D*, where D189 is depicted as van der Waals spheres, this mutation is placed far from the interdomain interface and is exposed to the solvent and the overall structure did not suffer any conformational changes.

WELL-TEMPERED METADYNAMICS

Methods

The Amber18 program patched with PLUMED 2.6[E16] was used to carry out metadynamics simulations with path collective variables.[E17] This enhanced sampling methodology allows us to explore processes, such as the opening of a protein that may occur at higher time scales not accessible by state-of-the-art unbiased MD simulations. We selected as our collective variable, or reaction coordinate in which a bias potential is added during the simulation, the progress along the path $s(R)$ and the distance from the path $z(R)$. The path used was the opening path obtained from the unbiased MD simulation of the LysRS-S207 (P) system where we captured the opening motion spontaneously. Thus, this path was defined on 90 equally spaced structures that were aligned on its Cα atoms extracted from the MD trajectory. Finally, the free energy surface of the process was computed by using the added bias along the collective variable. A cumulative time of 1 μs of well-tempered metadynamics simulation for each of the LysRS systems was carried out. A wall potential with force constant of 300 kcal/mol/Å^4 was used to restrain $z(R)$ to a maximum value of 20 Å^2, thus allowing the system to explore regions different from the reference path while preserving an efficient sampling and computational cost. The addition frequency of the Gaussians was set to 1 ps. For both s and z, the height and the width of the Gaussians were 0.2 kcal/mol and 0.1, respectively.

Results

To further analyze the opening motion and its thermodynamics, we performed well-tempered metadynamics simulations on the aforementioned systems. This enhanced sampling methodology (see Methods) allows us to explore processes, such as the opening of a protein that may occur at higher time scales not accessible by state-of-the-art unbiased MD simulations. In Fig 2 and Fig E3, *B* and *C*, free energy surfaces monitoring the opening of the different LysRS systems are shown. As can be observed, a single minimum is found for the LysRS-WT system in the closed state (see Fig 2 and Fig E3, *A*). As already seen for the LysRS-S207 (P) phosphorylated system in our MD simulation, the opening occurs spontaneously from the closed state to the open state, and a free energy minimum is found in the opened state (see Fig 2 and Fig E3, *B*). We found 2 accessible states for the LysRS-P542R system separated by a free energy barrier of 6.3 kcal/mol and thermodynamically equally stable (see Fig 2, *C*). Thus, mutation P542R critically impacts the dynamical behavior of the LysRS by promoting the system to explore an open state that could unbind from the MSC and might provoke translocation to the nucleus.

Finally, the G189D mutation does not alter the functional behavior of the LysRS protein. As can be observed in the calculated free energy surface, a free energy minimum is found in the closed state as for the LysRS-WT system (see Fig E3, *C*). The G189D mutation is found far from the interdomain interface, which is not affected by the mutation. If we observe the closed state in both our MD simulations (see Fig E1, *D*) and the one obtained by MD simulations, we can see that D189 is found exposed to the solvent and therefore not altering the LysRS structure. Moreover, the G189D mutation is also far from the MSC-p38 binding region, and no interactions were found between D189 and any of the residues that interact with the p38 protein during our simulations. These results are in agreement with our experiments, which show that mutation G189D is not observed intrinsically in the nucleus and this mutation does not enhance the noncanonical function in mast cells.

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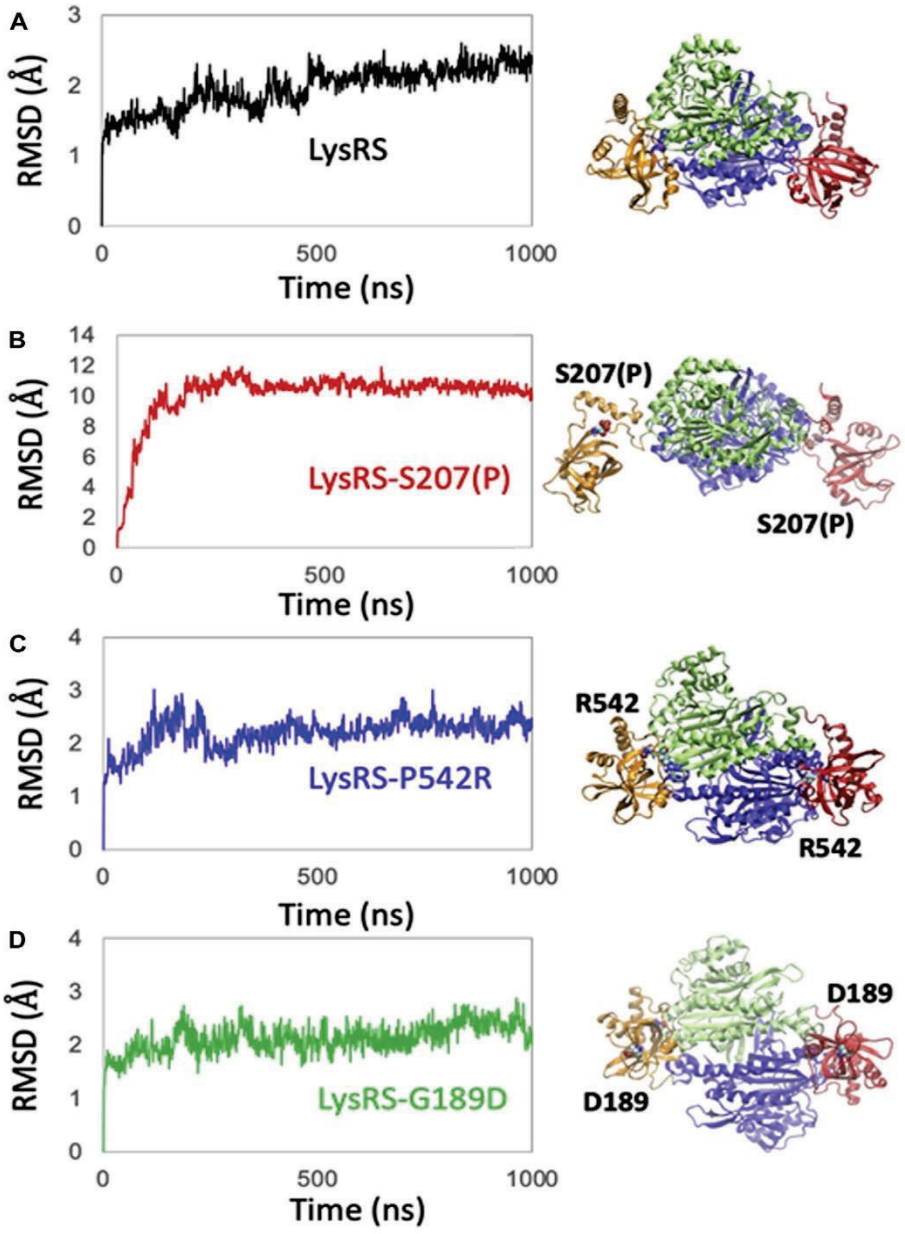

FIG E1. Root-mean-square deviations calculated for the backbone atoms of the (**A**) Lys-RS WT, (**B**) LysRS-S207 (P), (**C**) LysRS-P542R, and (**D**) LysRS-G189D systems. tRNA anticodon-binding domains are shown in ribbons in red and orange, whereas the aminoacylation domains are shown in blue and green. S207 (P), R542, and D189 are shown as van der Waals spheres in Fig E1, *B*, *C*, and *D*. *RMSD*, Root-mean-square deviation.

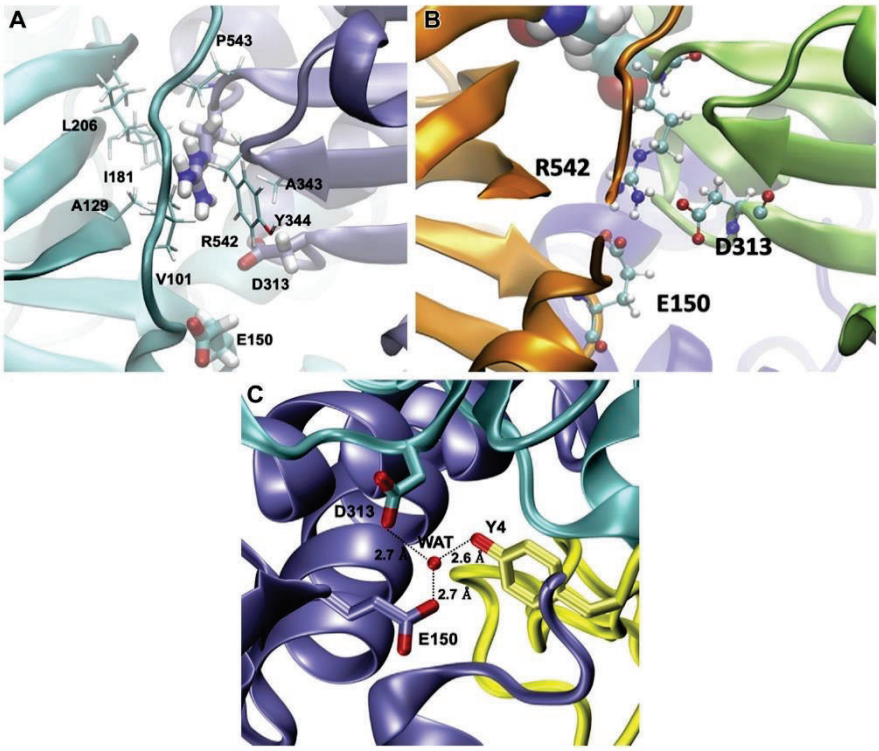

FIG E2. **A,** Interdomain interface inset of the starting structure selected for the MD simulations corresponding to the mutated P542R system. The P542R mutation introduces a positively charged residue in a nonpolar environment, which creates steric clashes between R542 and the surrounding amino acids. The closest amino acids that can form a salt bridge are D313 and E150. **B,** Interactions between E150 and D313 residues of the LysRS-P542R system (shown in orange and green) and the mutation R542 found during our MD simulation. **C,** Interactions between E150 and D313 residues of LysRS (shown in ice-blue and cyan) and Y4 residue of the MSC-p38 (shown in yellow) proteins found in the X-ray structure with PDB ID:6ILD.[E2]

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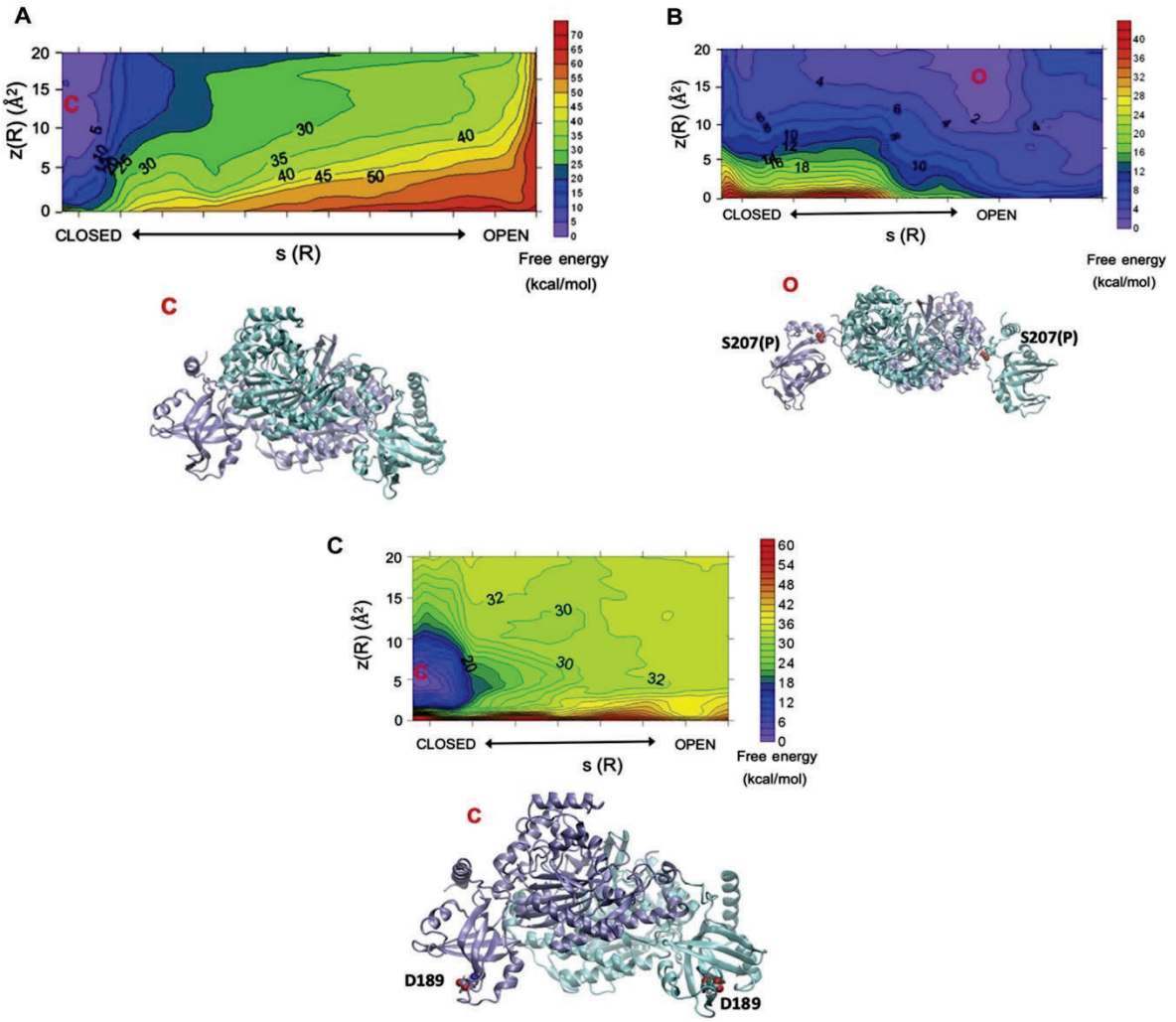

FIG E3. A, FES reconstructed along the opening path, *s*(R), and the distance from the path, *z*(R), computed for the LysRS-WT system. Structure of the closed state (C) free energy minimum found is depicted as ribbons (in cyan and ice-blue for each of the monomers) at the bottom. **B,** FES reconstructed along the opening path, *s*(R), and the distance from the path, *z*(R), computed for the LysRS-S207 (P) system. Structure of the open state (O) free energy minimum found is depicted as ribbons (in cyan and ice-blue for each of the monomers) at the bottom. Phosphorylated serine is shown as van der Waals representation. **C,** FES reconstructed along the opening path, *s*(R), and the distance from the path, *z*(R), computed for the LysRS-G189D system. Structure of the closed state (C) free energy minimum found is depicted as ribbons (in cyan and ice-blue for each of the monomers) at the bottom. The G189D mutation is shown as van der Waals representation. *FES,* Free energy surface.

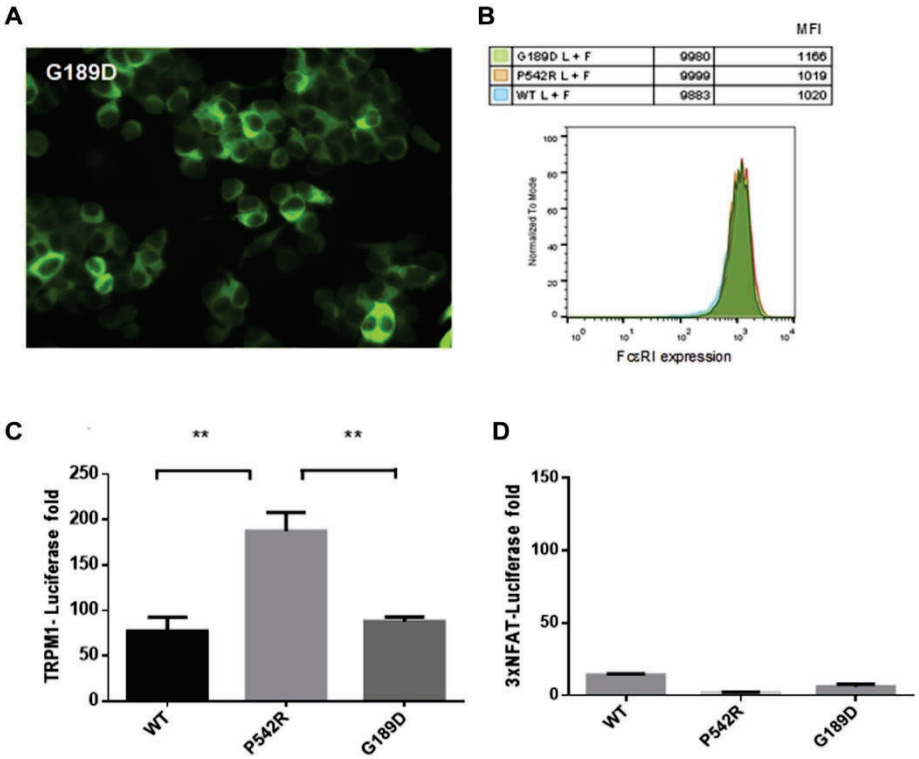

FIG E4. LysRS G189D expression is found mainly in the cytosol and does not increase MITF activity. **A,** LysRS G189D was mainly in the cytosol. **B,** The FcεRI expression was similar in all the transfectants. **C,** Cells carrying the different LysRS mutations were transfected with TRPM-1-luciferase reporter gene and TK Renilla. **D,** Similarly, cells were transfected with 3XNFAT. Data were collected in triplicate and are representative of 3 independent experiments. *MFI,* Mean fluorescence intensity.

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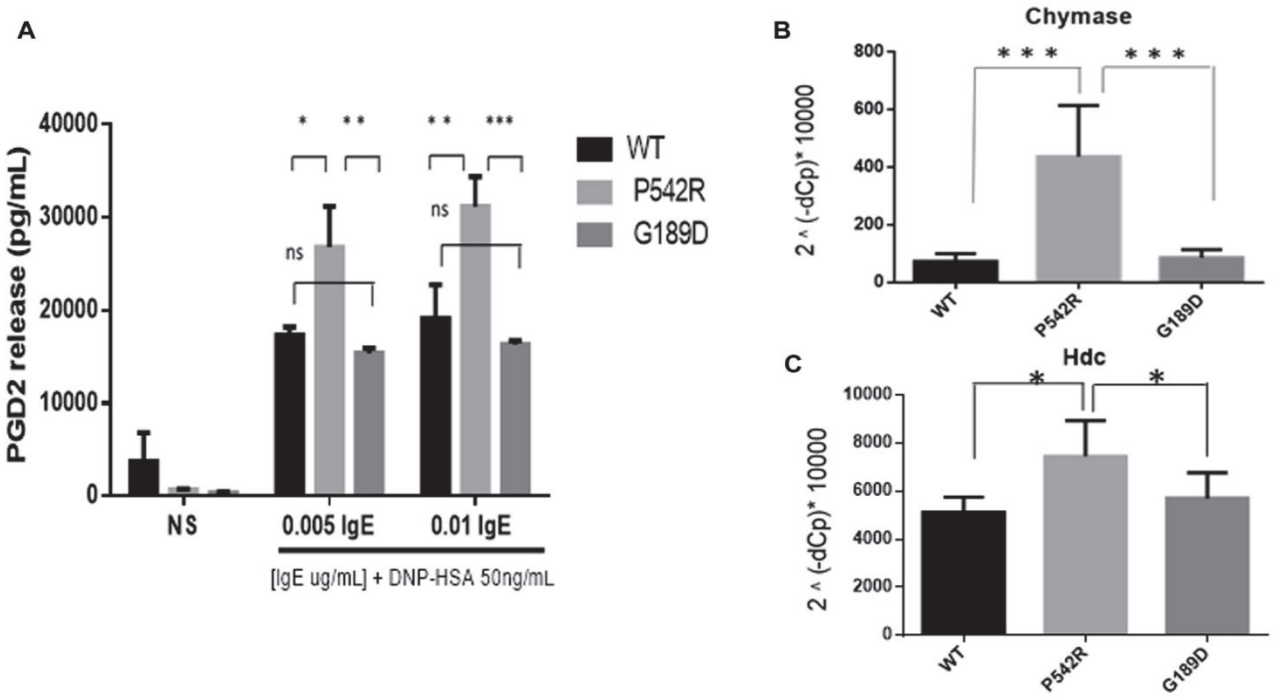

FIG E5. LysG189D does not increase MITF-dependent targets like LysP542R does. **A,** PGD2 ELISA for WT, P542R, and G189D cell transfectants with nonstimulated (NS) conditions and IgE-DNP stimulus was carried out. (**B**) Chymase and (**C**) HdC transcripts were also analyzed. Data were collected in triplicate and are the mean of 3 independent experiments. *HdC*, Histidine decarboxylase; *NS*, not stimulated.

TABLE E1. Serum tryptase and PGD_2 values at baseline and during anaphylaxis while performing skin tests

Time from anaphylaxis onset	Tryptase (ng/mL)	PGD_2 (pg/mL)
Baseline	3.62	40.81
30 min	3.50	111.65
60 min	3.52	ND
240 min	3.22	ND

ND, Not done.

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TABLE E2. Gene array information

Gene name (symbol)	Ref. Seq
Cyclin-dependent kinase 2 (Cdk2)	NM_199501.1
Chymase 1 (Cma1)	NM_013092.1
Granzyme B	NM_138517.3
KIT proto-oncogene receptor tyrosine kinase (KIT)	NM_022264.1
Mast cell protease 2 (Mcpt2)	NM_172044.1
Mast cell protease 4 (Mcpt4)	NM_019321.2
Mast cell protease 9 (Mcpt9)	NM_019323.1
Serpin family E member 1 (Serpine 1)	NM_012620.1
Tryptophan hydroxylase 1 (Tph1)	NM_001100634.2
15-Hydroxyprostaglandin dehydrogenase (Hpgd)	NM_024390.2
Tryptase beta 2 (Tpsb2)	NM_019180.2
Sortilin 1 (Sort1)	NM_031767.1
Lysosomal trafficking regulator (Lyst)	NM_053518.1
Cyclin-dependent kinase 5 regulatory subunit 1 (Cdk5r1)	NM_053891.1
Histidine decarboxylase (Hdc)	NM_ 017016.2
*Peptidylprolyl isomerase A (cyclophilina A)	NM_017101.1
*Actin, beta (Actb)	NM_031144.3
*TATA box binding protein (Tbp)	NM_ 001004198.1
*Beta-2 microglobulin (B2m)	NM_012512.2
*Ribosomal protein lateral stalk subunit P0 (Rplp0)	NM_022402.2
*Hypoxanthine phosphoribosyltransferase 1 (Hprt1)	NM_012583.2
*Transferrin receptor (Tfrc)	NM_022712.1
*Glucuronidase, beta (Gusb)	NM_017015.2

*Housekeeping genes.

3.2 Role of MITF in MRGPRX2-dependent mast cell activation

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MRGPRX2 signaling involves the Lysyl-tRNA synthetase and MITF pathway

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MRGPRX2, a G-protein-coupled-seven transmembrane domain receptor, is mainly expressed in mast cells and neurons and is involved in skin immunity and pain. It is implicated in the pathophysiology of non-IgE-mediated immediate hypersensitivity and has been related to adverse drug reactions. Moreover, a role has been proposed in asthma, atopic dermatitis, contact dermatitis, and chronic spontaneous urticaria. Although it has a prominent role in disease, its signaling transduction is poorly understood. This study shows that MRGPRX2 activation with substance P increased Lysyl t-RNA synthetase (LysRS) translocation to the nucleus. LysRS is a moonlighting protein with a dual role in protein translation and IgE signaling in mast cells. Upon allergen- IgE-FcεRI crosslinking, LysRS is translocated to the nucleus and activates microphthalmia-associated transcription factor (MITF) activity. In this study, we found that MRGPRX2 triggering led to MITF phosphorylation and increased MITF activity. Therefore, overexpression of LysRS increased MITF activity after MRGPRX2 activation. MITF silencing reduced MRGPRX2-dependent calcium influx and mast cell degranulation. Furthermore, a MITF pathway inhibitor, ML329, impaired MITF expression, calcium influx, and mast cell degranulation. Moreover, drugs such as atracurium, vancomycin, and morphine, reported to induce MRGPRX2-dependent degranulation, increased MITF activity. Altogether, our data show that MRGPRX2 signaling enhances MITF activity, and its abrogation by silencing or inhibition resulted in defective MRGPRX2 degranulation. We conclude that MRGPRX2 signaling involves the LysRS and MITF pathway. Thus, MITF and MITF-dependent targets may be considered therapeutic approaches to treat pathologies where MRGPRX2 is implicated.

KEYWORDS

MRGPRX2, LysRS, MITF, mast cell degranulation, adverse drug reactions

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Introduction

Mast-related G-protein-coupled receptor member X2 (MRGPRX2) is mainly expressed on mast cells (MC) (abundantly in skin MC) and neurons. MRGPRX2 is physiologically involved in host defense, tissue homeostasis and repair, nociception, inflammatory pain, and itch (1, 2). Endogenous and exogenous ligands have been described to bind MRGPRX2. Endogenous ligands such as substance P, human β-defensins, or vasointestinal peptide, directly involved in pain and itch, are directly related to its physiological functions (3). These effects can become chronic inflammation in a pathological state (4–6). Regarding exogenous ligands, several have been identified, such as mastoparan (bee wasp venom component), cationic peptidergic drugs, neuromuscular blocking agents (NMBAs) (e.g., atracurium, cisatracurium, and rocuronium), opiates, and antibiotics such as fluoroquinolones and vancomycin. Most of these exogenous ligands are the molecular basis of MRGPRX2-dependent adverse drug reactions (7, 8). Several molecules, such as natural compounds, cytokines, peptides, and DNA aptamers, have been described to prevent MRGPRX2-mediated pseudo-allergic responses and inflammation (9–11).

Yet, MRGPRX2 signaling is still poorly understood. To better understand how to modulate MRGPRX2 actions to dodge pathological states, some progress in unrevealing MRGPRX2 signaling has been made; however, more effort is needed. Proteins G, Gαi, and/or Gαq are involved in the early signals (12, 13), and calcium mobilization is rapid and transient (14). Further downstream events include MAP kinases (ERK1/2), p38, and PI3K (15). Furthermore, GTPase activation (Cdc42) upstream of the unconventional class I myosin 1f (MYO1F) regulates actin cytoskeleton dynamics and granule release (16). Lately, β arrestins 1 and 2 (involved in GPCR desensitization) have been proposed to interfere with MRGPRX2-dependent degranulation (17).

Microphthalmia associated-transcription factor (MITF) is a basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family member. MITF involves many biological processes, including cell differentiation, survival, senescence, metabolism, and DNA damage repair (18). The principal cell types affected in MITF-deficient mice are MCs, osteoclasts, and melanocytes (19). Moreover, MC and basophil cell fates are determined by the antagonistic regulation of C/EBPα and MITF (20). Additionally, MITF is downstream of KIT and FcεRI pathways (21–23). Nevertheless, most information about MITF stems from its essential role in melanocyte biology and melanoma as an oncogene (24–26). Post-translational modifications are primarily subject to the transcriptional activity of MITF. MITF is phosphorylated by ERK1/2 at Ser73 and by p90 ribosomal S6 kinase (p90RSK) at Ser409 (27–29). MITF phosphorylation at Ser73 has been associated with increased activity and double phosphorylation at Ser73 and Ser409 has been related to proteasome degradation (29). MITF activity can be repressed by HINT1 (histidine triad nucleotide-binding protein 1) (30).

Lysyl-tRNA synthetase (LysRS), a paradigm of a multifunctional protein in MCs, is involved in protein translation (canonical function) and upon FcεRI triggering in MITF activation (non-canonical role) (23). After IgE crosslinking, MAP kinase activation phosphorylates cytosolic LysRS (Ser 207). Phosphorylation in LysRS alters its binding to the multi-tRNA synthetase complex (MSC)-p38 in the cytosol and allows translocation to the nucleus, synthesizing diadenosine tetraphosphate (Ap4A). The Ap4A accumulated in the nuclei of IgE-activated MCs binds to HINT1, a MITF repressor, and liberates MITF, activating MITF-dependent gene expression. MITF regulates numerous genes encoding essential proteins involved in MC proinflammatory events, such as histidine decarboxylase (Hdc) (31), which catalyzes histamine synthesis; granzyme B (GrB), which participates in the cytotoxic action of MCs (32) or PGD2 synthase increasing PGD2 levels (33).

Our group recently characterized the mutation LysRS P542R in a patient with severe anaphylaxis to Hymenoptera. A substitution of proline by arginine disrupts the protein's functional motion, promoting an open state similar to the phosphorylated wild-type form. Altogether, this results in a constitutive increase in MITF activity (34).

MRGPRX2 has been related to several pathologies, from immediate-type hypersensitivity reactions (adverse drug reactions, Hymenoptera venoms reactions) to prolonged type- 2 inflammation (such as chronic asthma or chronic spontaneous urticaria) (1, 2, 4). Therefore, it is essential to understand the molecular groundwork of MRGPRX2 and the associated intracellular signaling components to manage the treatment of MRGPRX2-associated diseases. The current study explores the MRGPRX2-LysR-MITF pathway in mast cell exocytosis.

Materials and methods

Antibodies and reagents

Anti-MITF (clone D5G7V) and phospho-p44/42 MAPK antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-MITF (Ser73/180) and mouse anti-αTubulin antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-Lamin β1 antibody was purchased from Abcam (Cambridge, UK). PE anti-human FcεRI antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Anti-LysRS (D-4) and PE anti-human CD117 were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). PE anti-human MRGPRX2 was from Bio Legend (San Diego, CA, USA). APC anti-human CD63 and FITC Annexin V were from ImmunoTools GmbH (Friesoythe, Germany). Biotinylated human IgE was from Abbiotec (San Diego, CA, USA), and streptavidin was from Sigma (St. Louis, MO, USA). Substance P was from AnaSpec (Fremont, CA). ML329 was obtained from Axon Med Chem (Groningen, The Netherlands). Morphine was obtained from B. Braun Medical S.A (Spain), the muscle relaxant atracurium was from Pfizer Inc (NY, USA), meglumine amidotrizoate was from Juste Laboratories (Spain), and the vancomycin antibiotic was from Normon (Spain).

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Cell culture

The LAD2 human mast cell line was a kind gift from Drs. A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD), culturing in StemPro-34 media, supplemented with StemPro-34 nutrient (Thermo Fisher Scientific; Waltham, MA, USA) and 2mM L-glutamine (Lonza), 100 U/mL penicillin (Lonza) and 100 µg/mL streptomycin (Lonza), and 100 ng/mL SCF (ImmunoTools GmbH, Friesoythe, Germany) (35). Primary human mast cells (huMCs) derived from $CD34^+$ -positive peripheral blood cells were obtained from healthy donors, CD117 MicroBeads Kit (Mitenyi Biotec, Germany) for $CD34^+$ progenitor cell isolation. Cells were cultured for 0-2 weeks with 100 ng/ml SCF, IL-6, and IL-3 (ImmunoTools GmbH, Friesoythe, Germany) and 2-6 weeks with 100 ng/ml IL-6 and SCF. After six weeks, $CD34^+$ -derived human MCs were assessed by surface expression of FcεRI and CD117. The expression of anti-human PE-MRGPRX2 was also checked for experiments. The HEK 293LTV cell line (Cell Biolabs Inc, San Diego, CA, USA) was used for lentivirus production.

Western blotting

$7{\cdot}10^6$ cells were activated with substance P (MedChemExpress, NJ, USA) at different times. Cellular fractioning was performed as described elsewhere (36). The protein concentration was determined using the Protein Assay Dye Bio-Rad Kit (Bio-Rad Laboratories, Inc. USA) according to the manufacturer's recommendations. Electrophoresis was performed using NuPage TM 4-12% Bis-Tris Gel, 1.5 mm 15 w (Invitrogen, USA), and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). In all blots, proteins, after specific antibody incubation, were visualized by enhanced chemiluminescence (Western Bright TM ECL, Advansta, USA).

Lentiviral transduction

Lentiviral particles were generated in HEK293LTV. The non-target (NT) sequence was as follows: 5'CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT 3', MITF shRNA 2 sequence: 5'CCGGCGGGAAACTTGATTGATCTTTCTCGAGAAAGATCAATCAAGTTTCCCGTTTTTG 3'. MITF shRNA 3 sequence: 5' CCGGGGGAGCTCACAGCGTGTATTTCTCGAGAAATACACGCTGTGAGCTCCCTTTTTG 3'.

According to the manufacturer's instructions, lentiviral particles to silence MITF gene expression were generated using Mission ® shRNA technology (Sigma, St. Louis, MO, USA). LAD2 cells were transduced with polybrene 8 µg/ml (Santa Cruz) and selected with puromycin 1µg/ml. Cells were maintained with puromycin until the day of the experiment, when it was removed prior to cell activation.

FACS staining

FcεRI and MRGPRX2 expression were detected by direct staining with the indicated Abs for 30 minutes at 4°C. KIT expression was detected using PE- anti-human CD117. Cells were analyzed using a FACSCalibur flow cytometer (FACScan; BD Biosciences). Dead cells were excluded based on their Forward (FSC) and side scattering (SSC) profiles.

Degranulation assays

Degranulation was analyzed based on CD63 expression on the cell membrane assessed by flow cytometry or via levels of β-hexosaminidase activity in the supernatant, as described in previous studies (8). Briefly, for CD63 determination: $1{\cdot}10^5$ cells/point were incubated with Tyrode's buffer or SP at 37°C for 30 min. Cells were incubated with blocking buffer (0.1% NaN_3, 2% FBS, 20% rabbit serum, PBS) for 30 min on ice, and APC anti-human CD63 staining was performed for 30 min on ice. After washing, samples were incubated with propidium iodide (PI), acquired with a FACSCalibur flow cytometer, and analyzed with FlowJo software. PI-positive cells were excluded from the analysis. For β-hexosaminidase assays (37), cells were seeded at $3{\cdot}10^4$ cells/well in 96 well plates in Tyrode's buffer and then activated with SP or left untreated for 30 min. An equal number of viable cells were used in each case. Supernatants were collected and incubated with P-nitrophenyl-N-acetyl-β-D-glucopyranoside (Sigma Aldrich) for 1h at 37°C. Triton (1%) was used to obtain total cell lysates of β-hexosaminidase. Absorbance was read at 405nm. β-hexosaminidase enzyme activity was expressed as the release percentage: β-hexosaminidase release = [cell degranulation/(cell degranulation + total lysate)] x100.

PGD2 ELISA

PGD2 release was determined from the supernatants of cells ($2.5{\cdot}10^4$ cells/well) activated with substance P (2µM) overnight using a specific competitive Enzyme Immunoassay for PGD2 (Cusabio Technology LLC, Houston, TX, USA) according to the manufacturer's instructions.

Apoptosis assay

Apoptosis was measured using FITC Annexin V (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's suggested protocol and analyzed by flow cytometry. Caspase activity was assayed using the Caspases-Glo® 3/7 Assay (Promega, San Luis Obispo, CA, USA) according to the manufacturer's instructions.

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Calcium release assay

Cells were incubated with 2 µM Fluo-4, AM cell permeant (Thermo Fisher Scientific; Waltham, MA, USA), at 37°C for 30 minutes, then washed, and resuspended in Tyrode's buffer. An equal number of viable cells were used in each case. After defining basal conditions, SP (or ionomycin) was immediately added to the cells (Sigma-Aldrich, St. Louis, MO, USA), and fluorescence was determined every 10 sec using a TECAN SPARK microplate reader.

Luciferase assay

Firefly luciferase under the control of the TRPM1 promoter and control vector PGL3-Luciferase was a gift from David Fisher (Harvard Medical School) (38). $2.5{\cdot}10^4$ cells were transfected with the Firefly and Renilla reporters at a ratio of 7:1, respectively. This reporter gene was transfected to the cells with a Renilla luciferase reporter gene, and the results were normalized to the empty vector-transfected conditions. Transfections were performed with lipofectamine. We used SP at a higher concentration for this assay to see significant gene reporter activation. Luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer's instructions. Firefly Luciferase data were normalized according to Renilla luciferase data.

GFP and LysRS WT-GFP transfection in LAD2 cells

LAD2 cells were transfected with human LysRS (KARS WT) pcDNA 3.1+C-eGFP vector and GFP empty vector (obtained from GenScript Biotech, The Netherlands)) as previously reported (34).

Statistical analysis

Statistical analyses were performed using PRISM 9 (GraphPad Software, La Jolla, CA, USA). All results are expressed as mean ± standard error of the mean (SEM). After determining the normal distribution of the samples and variance analysis, a t-test was used to determine significant differences (p-value) between two experimental groups, and two-way ANOVA was used to determine significant differences (p-value) between several experimental groups. "*"P<0.05, "**" P<0.01, "***"P<0.001, "****"P<0.0001.

Results

MRGPRX2 activation increases the translocation of LysRS to the nucleus

MRGPRX2 signaling transduction increases ERK1/2 activity (15). On the other hand, Lysyl-tRNA synthetase (LysRS) is phosphorylated at Ser 207 and translocated into the nucleus downstream of IgE/FcεRI signaling activation (23, 39). We wanted to explore whether MRGPRX2 signaling involves LysRS and its translocation to the nucleus. Ligands, such as SP, have shown MRGPRX2-dependent MC activity (40, 41). Thus, MCs (LAD2) were activated with substance P, cellular fractionation was carried out, and the LysRS location was explored. Our results show a rapid and transient phosphorylation of ERK1/2 and an increase in LysRS translocation to the nucleus of activated cells (Figure 1).

MRGPRX2 activation increases MITF phosphorylation

Downstream of IgE signaling, the translocation of LysRS into the nucleus switches its catalytic activity in the production of diadenosine oligophosphate Ap4A, which binds to HINT, dissociating the MITF-HINT repressor complex and liberating MITF (23, 42). We next analyzed MITF activity in the context of MRGPRX2 activation. Since phosphorylation of MITF at Ser 73 has been associated with transcriptional activity (29), we examined whether MRGPRX2 increased MITF phosphorylation. As shown in Figure 2, MRGPRX2-dependent activation enhanced MITF phosphorylation in the cytoplasm and nucleus (note the pMITF/MITF ratio). Interestingly, MITF expression was mainly detectable in the nucleus before stimulation. However, after MRGPRX2 triggering, MITF levels increased in the cytosol. After 15 minutes, MITF returned close to the steady state. This is consistent with data regarding MITF posttranscriptional regulation in melanocytes where phosphorylation of MITF regulates its nuclear export, and via this export-import cycle, MITF activity can be tuned (43).

MRGPRX2 signaling increases MITF activity

Next, we assessed whether LysRS translocation to the nucleus and MITF phosphorylation after MRGPRX2 signaling resulted in increased MITF activity. We used a reporter gene assay, Melastatin 1 (TRPM1) promoter-controlled firefly luciferase, for that purpose. MITF is the main transcriptional regulator of TRPM1 (44). Thus, luciferase expression correlates with MITF activity (45). Results show that MITF activity increased after MRGPRX2 stimulation (Figure 3A). Moreover, when cells overexpressed LysRS WT-GFP (Figure 3B), a notorious increase in MITF activity was detected after substance P stimulation (Figure 3C). In that context, we also analyzed whether this increase in activity was concomitant to an increase in a MITF-dependent target such as PGD2 (33). Results show that PGD2 significantly increased in LysRS WT-GFP transfected cells after MRGPRX2 activation (Figure 3D).

MITF silencing decreases MRGPRX2-dependent mast cell degranulation

We next explored the role of MITF in MRGPRX2-dependent degranulation. Cells were silenced using lentivirus technology with two specific sequences to that end. The two sequences satisfactorily

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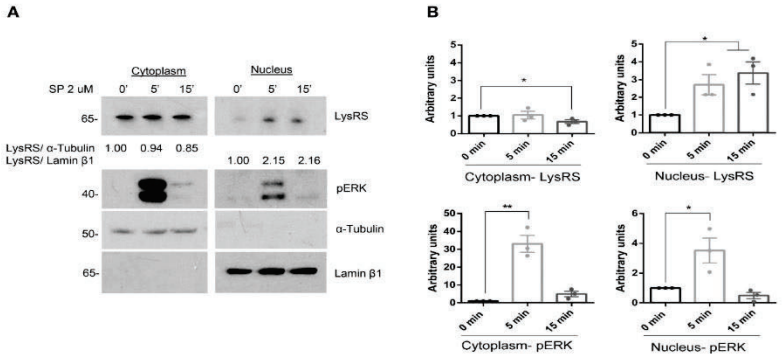

FIGURE 1
LysRS translocates to the nucleus after MRGPRX2 activation in mast cells (LAD2). **(A)** Cells were activated with 2 μM substance P (SP) at various times. Cytoplasmic and nuclear fractions were isolated, and western blots for LysRS and pERK were performed. α-tubulin and Lamin β1 markers for cytoplasm and nuclei, respectively, were used to assess cellular fraction purification and as loading controls. **(B)** The unpaired t-test was used for statistical analysis (*p < 0.05*, **p < 0.01). Experiments are the mean ± SEM (n=3).

knocked down the levels of MITF (Figure 4A). Degranulation measured by β-hexosaminidase release (Figure 4B) and CD63 expression by FACS (Figure 4C and Supplementary Figure 1A) showed a significant reduction after SP stimulation. In addition, calcium influx significantly diminished after MITF silencing in MRGPRX2 activation (Figure 4D). None or little difference was observed when ionomycin was used as a primary stimulus. MRGPRX2 expression levels after MITF silencing were consistent, and all cells were positive (Supplementary Figure 2A). The mean of fluorescence intensity was lower, however, total MRGPRX2 levels analyzed by blotting showed no reduction in MITF silencing samples compared to Non-target (NT) control (Supplementary Figure 3). MITF is involved in cell survival in different cellular models (46, 47), thus downregulation of this transcription factor may induce apoptosis. Next, we assessed apoptosis in MITF-knockdown cells. MITF silencing significantly increased cellular apoptosis (Supplementary Figure 4A). The increase in apoptosis was higher in cells with lower MITF levels. An equal number of live cells were used in all assays, exclusion of dead cells was performed when possible and MRGPRX2-dependent degranulation and calcium influx were still significantly decreased, in MITF-silenced cells.

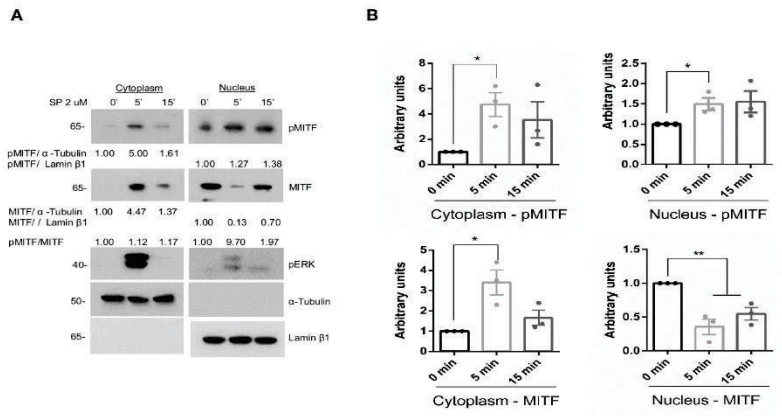

FIGURE 2
MITF is phosphorylated after MRGPRX2 activation in mast cells (LAD2). **(A)** Cells were activated with 2 μM substance P (SP) at various times. Cytoplasmic and nuclear fractions were isolated, western blots for pMITF (Ser 73/180), and MITF were performed, α-tubulin and Lamin β1 markers for cytoplasm and nuclei, respectively, were used to assess cellular fraction purification and as loading controls. **(B)** The unpaired t-test was used for statistical analysis (*p < 0.05, **p < 0.01). Experiments are the mean ± SEM (n=3).

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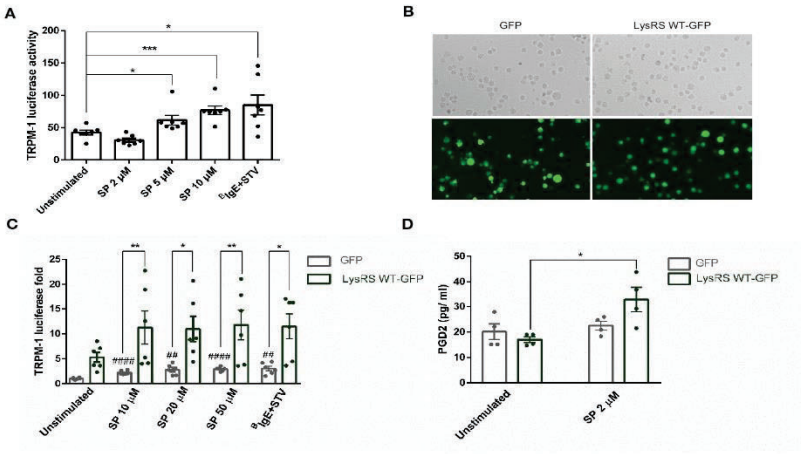

FIGURE 3
MRGPRX2 activation increases MITF activity via LysRS in mast cells (LAD2). **(A)** Cells were activated with 2 µM substance P (SP) at various concentrations for 6 hours in LAD2 cells transfected with TRPM1-luciferase gene reporter, as described in the materials and methods section. Biotinylated-IgE (0.1 µg/ml) was incubated overnight, and then, streptavidin (16 µg/mL) was added for 6 hours and used as a positive control. The unpaired t-test was used for statistical analysis (*p < 0.05, ***p < 0.001). **(B)** LAD2 cells were transfected with GFP and LysRS WT-GFP plasmids **(C)** and then activated at various SP concentrations, and biotinylated-IgE+ streptavidin was used as indicated above. The unpaired t-test used for statistical analysis (##p < 0.01, #### p<0.0001, SP *vs.* unstimulated GFP transfected LAD2 cells). The two-way ANOVA test was used for statistical analysis (*p < 0.05, **p < 0.01, GFP LAD2 *vs.* LysRS WT-GFP LAD2). **(D)** PGD2 after SP activation was measured in GFP and LysRS WT-GFP LAD2 cells. A two-way ANOVA test was used for statistical analysis (*p<0.05, GFP *vs.* LysRS WT-GFP). Experiments are the mean ± SEM (n=3).

ML329 inhibits MITF expression and impairs MRGPRX2-dependent mast cell degranulation

ML329 has been described to inhibit the MITF pathway (38, 48) therefore we used this inhibitor to reinforce data about MITF involvement in MRGPRX2 signaling. Cells treated with different concentrations of ML329 showed a decrease in MITF expression (Figure 5A). MITF reduction was always more consistent after day 5 thus the following experiments were performed on that day. Cells treated with ML329 showed decreased degranulation measured by CD63 expression (Figure 5B and Supplementary Figure 1B). Calcium influx was also impaired after MRGPRX2 activation, although the ionomycin response was not significantly affected (Figure 5C). MRGPRX2 expression after ML329 was similar in all cases (Supplementary Figure 2B). Like MITF silencing, ML329 also induced significant cellular apoptosis at a higher concentration (Supplementary Figure 4B). Altogether, decreased MITF levels correlated with reduced MRGPRX2-dependent degranulation and calcium influx.

$CD34^+$ -derived human MCs were also used to analyze the effect of MITF reduction on degranulation. Human MCs fully differentiated from CD34 cells were treated with ML329 for five days. Cells were activated with SP, and CD63 staining was used to measure degranulation (Figure 6). The analysis was performed in the live cell population (propidium iodide negative). Our results show that ML329 treatment led to a significant reduction in MRGPRX2-dependent mast cell degranulation.

Drug activation of MRGPRX2 induces MITF activity

One of the fascinating biological aspects that makes this receptor a current hot research topic is its ability to interact with several drugs and be involved in adverse drug reactions.

Our group showed that vancomycin, atracurium, and morphine induce MRGPRX2-dependent degranulation (8). Meglumine amidotrizoate was also described to interact with MRGPRX2 (49). Next, we analyzed whether these drugs were able to increase MITF activity by using Melastatin 1 (TRPM1) promoter-controlled firefly luciferase. Our results show that all were able to increase MITF activity (Figure 7). These data indicate that MITF activity is induced by several drugs reported to mediate their actions through MRGPRX2 signaling thus MITF is a MRGPRX2-dependent downstream signal for endogenous (SP) and exogenous ligands (atracurium, vancomycin…).

Discussion

Despite the increasing number of studies on MRGPRX2 in recent years, the dissection of its signaling pathway in MCs is poorly understood. Several studies focus on identifying new receptor ligands and how to block ligand recognition or involvement in pathology. To advance in the knowledge of MRGPRX2, one significant limitation is the lack of the crystal structure which prevents a complete characterization of ligand binding sites and a

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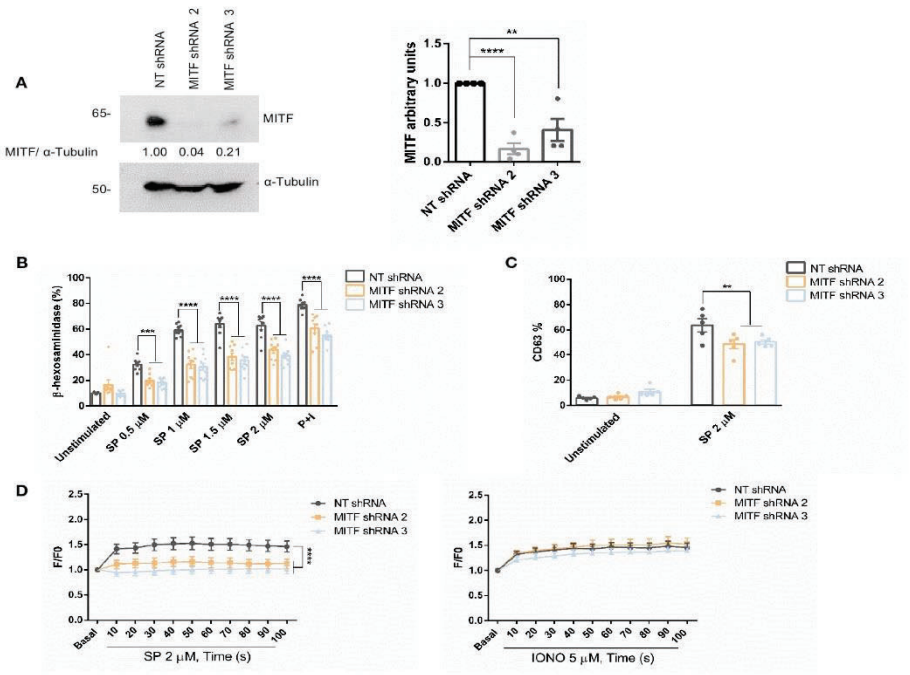

FIGURE 4
MITF silencing reduces degranulation and $Ca2^{+}$ influx. **(A)** LAD2 cells were transduced with control NT (non-target) shRNA or MITF shRNA 2 or MITF shRNA 3, and the efficiency of MITF silencing was assessed by western blot. α-Tubulin was used as a loading control. The unpaired t-test was used for statistical analysis (**p < 0.01*, ****p < 0.0001). Experiments are the mean ± SEM (n=4). **(B)** β-hexosaminidase was performed with different concentrations of SP and P+I (PMA+ionomycin, 10 ng/mL+0.5 µM) as a positive control in NT and MITF-silenced cells, n=4. **(C)** Degranulation was determined by flow cytometry using CD63 staining in unstimulated and SP (2uM) in the alive population (propidium iodide negative staining) in NT and MITF-silenced cells. **(D)** NT and MITF-silenced LAD2 cells were preincubated with Fluo4 and activated with SP (2µM) or ionomycin. A two-way ANOVA test was used for statistical analysis (**p<0.01, ***p<0.001, ****p<0.0001). Experiments are the mean ± SEM (n=3).

proper design of agonists/antagonists. Nevertheless, molecular modeling experiments have identified crucial amino acids for endogenous binding ligands, such as substance P, and exogenous ligands such as cationic agonists or opioids (12, 50, 51). MRGPRX2-triggered degranulation depends on Gαi, Gαq, Ca^{++} channels, ERK, and PI3K (14, 15). Cytoskeleton dynamics and granule exocytosis need Cdc42 GTPase activation and the participation of unconventional class I myosins, MYO1F (16), and β1 and β2 arrestins regulate the kinetics and the extent of MRGPRX2 cellular activity (17). The present study adds more insights into MRGPRX2 signals and demonstrates the involvement of the LysRS-MITF pathway.

LysRS is a moonlighting protein that belongs to the aminoacyl-tRNA synthetases (aaRS), which catalyze the aminoacylation reaction linking amino acids to their cognate tRNAs. They are highly conserved cytoplasmic and mitochondrial enzymes, one for each amino acid, and are responsible for the fidelity of gene code reading. During evolution, some aaRS acquired newly evolved domains that are not crucial for tRNA aminoacylation but are responsible for non-canonical functions (52). In this respect, LysRS, the aaRS that attaches lysine to its corresponding tRNA, is also involved in IgE-dependent signals upstream of the microphthalmia-associated transcription factor (MITF) in MCs (23). Upon IgE crosslinking, LysRS is phosphorylated (Ser 207) and translocated to the nucleus, where it catalyzes the synthesis of Ap4A as non-canonical activity. Ap4A binds to HINT, releasing MITF from the MITF-HINT1 inhibitory complex and causing an increase in the transcription of its target genes (23). MITF is a crucial hub in MC biology and function, according to the *Mitf*-mutant mouse phenotype, characterized by a reduced number and abnormal MCs (53–55).

In this study, we found that MRGPRX2 increases ERK1/2 phosphorylation rapidly and transiently. Our results agree with data showing that ERK1/2 is efficiently phosphorylated after MRGPRX2 in skin MCs and is more transiently induced than FcεRI signals (15). Nevertheless, this rapid signal allowed increased LysRS recruitment in the nucleus of MRGPRX2-activated cells. LysRS in the nucleus performs its non-canonical function, releasing MITF from the inactive HINT1-MITF complex (23). Moreover, MRGPRX2 signaling increased MITF phosphorylation (Ser 73).

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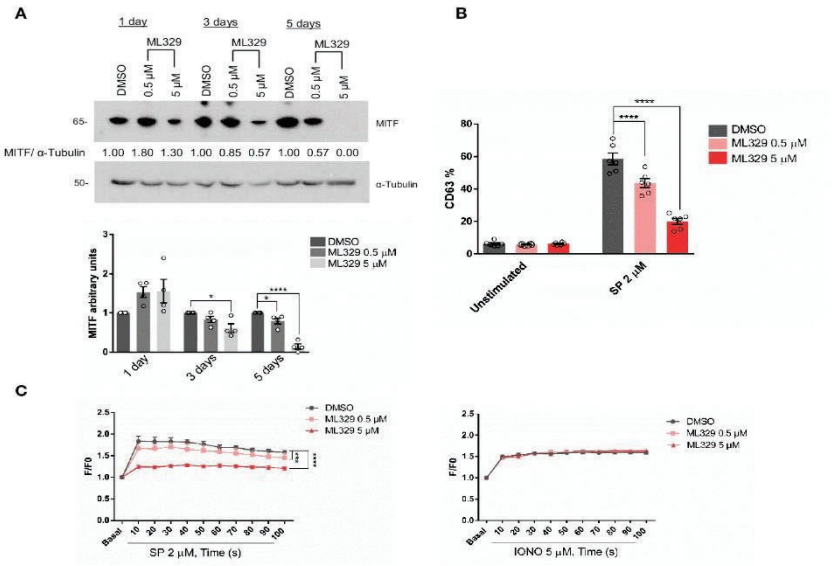

FIGURE 5
The MITF inhibitor ML329, reduces degranulation and $Ca2^{+}$ influx. **(A)** LAD2 cells were treated with either DMSO or ML329 at different concentrations on various days, and the efficiency of MITF silencing was assessed by western blot. α-Tubulin was used as a loading control. The unpaired t-test was used for statistical analysis after 5 days (*p < 0.05, ****p<0.0001). Experiments are the mean ± SEM (n=4). **(B)** Degranulation was determined by flow cytometry using CD63 staining in unstimulated and ML329 -treated cells (after 5 days) in the live population (propidium iodide negative staining). **(C)** DMSO and ML329 -treated cells (after 5 days) were preincubated with Fluo4 and activated with SP (2µM) or ionomycin. A two-way ANOVA test was used for statistical analysis (***p<0.001, ****p<0.0001). Experiments are the mean ± SEM (n=3).

The transcriptional activity of MITF is primarily regulated post-translationally. Most studies relating to MITF regulation have been performed in melanocytes showing that mainly ERK1/2 phosphorylates MITF at Ser73, p90 ribosomal S6 kinase (p90RSK) at Ser409, glycogen synthase kinase-3β (GSK3β) at Ser298, and p38 MAPK at Ser307 (27–29). MITF phosphorylation usually augments its activity. MAPK-mediated phosphorylation of MITF at Serine 73 combines the two coactivators, E1A binding protein p300 and cAMP-response element binding protein (p300/CBP), enhancing MITF transcriptional activity (56). However, dual phosphorylation at Ser73 and Ser409 endorses its ubiquitination and proteasome degradation (29). MITF A is the longest and most abundant isoform in mast cells (57). Ser 73 in MITF-M, the specific isoform in melanocytes, corresponds to Ser 180 in MITF-A.

Donor 1		
CD34⁺ derived mast cells		
Receptors	%	MFI
Iso/ KIT	4.89/ 96.10	3.63 / 40.90
Iso/ FcεRI	4.35/ 52.30	3.98 / 10.60
Iso/ MRGPRX2	5.85/ 73.00	3.69 / 12.70

Donor 2		
CD34⁺ derived mast cells		
Receptors	%	MFI
Iso/ KIT	5.03 / 94.70	3.02 / 43.00
Iso/ FcεRI	4.28 / 92.00	2.24 / 22.00
Iso/ MRGPRX2	4.43 / 99.80	3.71 / 95.70

Donor 3		
CD34⁺ derived mast cells		
Receptors	%	MFI
Iso/ KIT	5.88 / 63.10	3.65 / 25.10
Iso/ FcεRI	5.72 / 47.50	3.63 / 6.69
Iso/ MRGPRX2	5.61 / 25.70	3.75 / 5.30

Iso, Isotype ; MFI, mean fluorescence intensity

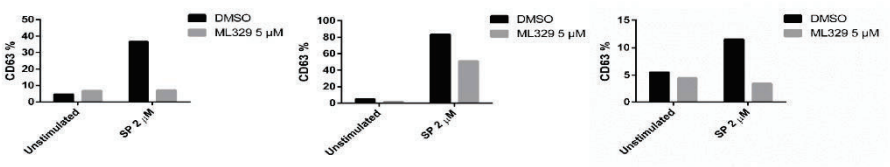

FIGURE 6
MITF inhibitor ML329 reduces $CD34^{+}$ derived hMCS degranulation. $CD34^{+}$ derived hMCs from three different healthy donors were assessed. KIT, FcεRI, and MRGPRX2 were checked by flow cytometry. Degranulation was determined by flow cytometry using CD63 staining in unstimulated and ML329-treated cells (after 5 days) in the live population (propidium iodide negative staining).

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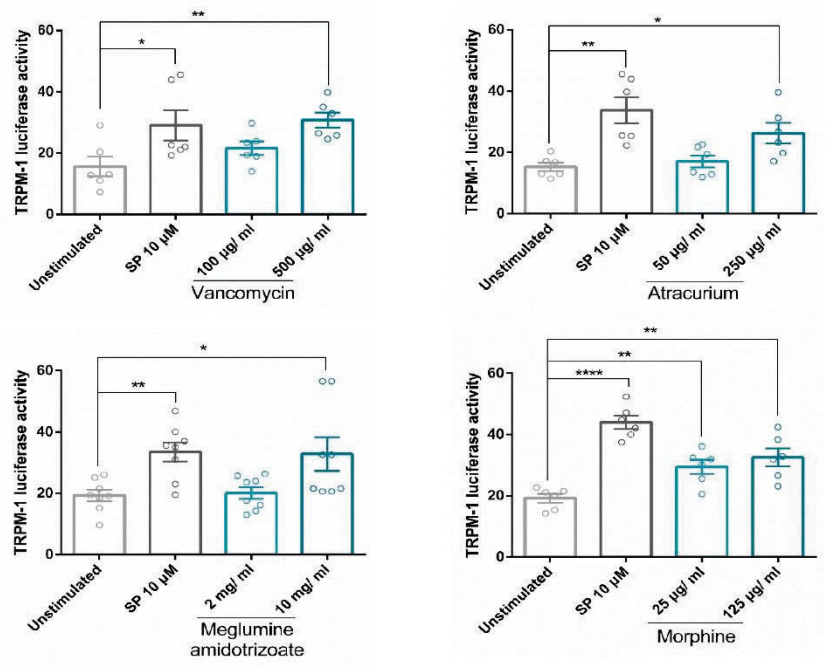

FIGURE 7
Drug activation of MRGPRX2 induces MITF activity. Cells were activated with SP or drugs (vancomycin, atracurium, morphine, and meglumine amidotrizoate) reported to induce MRGPRX2-dependent MC degranulation at various concentrations for 6 hours in LAD2 cells transfected with TRPM1-luciferase gene reporter. Experiments are the mean ± SEM (n=3 for vancomycin and atracurium, n=4 for meglumine amidotrixoate and morphine). The unpaired t-test was used for statistical analysis (*p < 0.05, **p < 0.01, ****p < 0.0001).

In our study, MRGPRX2 activation with SP increased MITF activity measured by the TRPM1-reporter gene. Overexpression of LysRS greatly enhanced MITF activity after SP stimulation. PGD2 production depends on MITF activity (33), thus, we observed that the augmented MITF activity was associated with an increase in PGD2. Altogether, MRGPRX2 ligation with substance P led to LysRS translocation to the nucleus, raising MITF activity. In parallel, MRGPRX2 signaling induced MITF phosphorylation, contributing to the increase in MITF activity.

MITF phosphorylation has been described as a mechanism of nuclear export-import of MITF to regulate the activity of this transcription factor in melanocytes (43). Interestingly, we found increased MITF in the cytosol after MRGPRX2 activation. Thus, it would be plausible that similar regulation events could occur in MCs. Nevertheless, this subject deserves further study.

MRGPRX2 ligation to exogenous ligands that can result in adverse drug reactions such as to atracurium, vancomycin, morphine, and meglumine amidotrizoate increases MITF activity, suggesting that endogenous and exogenous ligands use the LysRS-MITF pathway.

Selective silencing with MITF was effective after day 5, and previous experiments showed that the half-life of MITF was longer than expected for a transcription factor. Cycloheximide experiments from our group confirmed a complete reduction of protein levels after 96h drug treatment (data not shown). Similarly, using ML329, we see a significant decrease in protein levels at day 5. Our study indicates that MITF downregulation by shRNA silencing or ML329 treatment results in decreased MRGPRX2-dependent degranulation measured by β-hexosaminidase and CD63 plasma membrane expression. MITF function has been related to the biogenesis of lysosomes (58), and bone marrow-derived MCs from *Mitf* -/- mice display hypogranularity that can be restored with MITF addition (59). We examined whether our MITF-silenced cells had reduced granular content compared to control cells. Unfortunately, we could not find any apparent differences in our staining with Lysotracker in MITF-silenced cells compared to non-target controls (data not shown). Interestingly, downregulated MITF levels significantly decreased Ca2+ influx after substance P activation. MRGPRX2-dependent $Ca2^{+}$ is regulated by store-operated $Ca2^{+}$ entry (SOCE) *via* the calcium sensor, stromal interaction molecule 1 (STIM1) (60). STIM1 is an endoplasmic reticulum (ER) $Ca2^{+}$ sensor that, upon activation and decreased ER $Ca2^{+}$ levels, oligomerizes and activates Orai channels (Orai1/2/3), resulting in $Ca2^{+}$ influx (61). Indeed, STIM1 is crucial in promoting

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the $Ca2^+$ influx needed for IgE-dependent mast cell activation and anaphylaxis (62). Recently, MITF has been reported to regulate STIM1 and SOCE expression in melanocytes (63). Chromatin immunoprecipitation (ChIP) and luciferase assays with truncated STIM1 promoters validated the MITF-STIM1 interaction. Functional assays confirmed that MITF regulates STIM1 expression and activity in primary human melanocytes (63). Further experiments are needed to see MITF-dependent STIM1 regulation in MCs.

A pool of MITF resides in mitochondria and regulates proteins independently of its function as a transcription factor of nuclear genes. MITF interacts with one of the three subunits of the pyruvate dehydrogenase (PDH) complex, an enzyme that catalyzes the conversion of pyruvate to acetyl CoA (64). Moreover, mitochondrial MITF regulates PDH activity, which is crucial to maintain glucose homeostasis and essential as a source of mitochondrial ATP to maintain energetic expenses for MC degranulation and cytokine secretion. MITF activation related to the phosphorylation of Serine 73 mediated by ERK1/2 activity accounts for increased mast cell activity (65).

MITF downstream of the KIT receptor governs mast cell differentiation and proliferation (66). KIT receptor signaling regulates MITF through miR-539 and miR381 downregulation (22). At the same time, MITF fosters KIT expression (67), showing a reciprocal regulation. The reduced and abnormal MC in *Mitf*-mutated mice is partly due to the low levels of KIT receptor and, hence, the low response to its ligand, SCF (68). In that context, our group reported that the adaptor protein 3BP2 participates in the KIT-MITF axis, delivering survival signals in MCs. Thus, silencing of 3BP2 reduced KIT and MITF protein levels and induced MC apoptosis. MITF overexpression rescued KIT protein levels in 3BP2 knockdown cells (69).

Since a reduction in MITF may affect cell viability, apoptosis was monitored. Indeed, MITF downregulation is also involved in the termination of MC-mediated response, apoptosis induction, and removal of exhausted cells (70, 71). Conversely, increased MITF levels and activity have been linked to increased mast cell activity and proliferation. MITF is required for the transformed phenotype of mastocytosis (72). Indeed, MITF downregulation leads to apoptosis of HMC-1 carrying the gain of function mutation KIT D816V (73). Moreover, MITF is highly expressed in bone marrow biopsies from patients with systemic mastocytosis and activating KIT mutations (22). Recently, we identified that a mutation in LysRS (P542R) in a patient with severe anaphylaxis to wasp venom favors the recruitment of this molecule to the nucleus resulting in a constitutive increase in MITF activity (34). This increased MITF activity accounts for an increase in histamine and PGD2 secretion. It is known that transcription factors GATA2 and MITF regulate Hdc gene expression, which is necessary for IgE/mast cell-mediated anaphylaxis (31).

Altogether, the LysRS-MITF pathway should be considered in MRGPRX2 signaling, and it is shared with FcεRI. Patients with alterations in this pathway may increase their range of susceptibility to a broad spectrum of substances that can trigger both receptors.

Increased knowledge of MRGPRX2 signaling may provide new approaches for upregulating responses, which may help treat antibiotic-resistant cutaneous infections, or downregulate MRGPRX2 to ameliorate allergic and inflammatory diseases *via* this receptor.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by University of Barcelona. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YG performed and designed the experiments and wrote and reviewed the manuscript. LO, EP-P, and CA performed the experiments and reviewed the manuscript. MG provided technical support and reviewed the manuscript. RM-C conceived the experiments and reviewed the manuscript. MM conceived the experiments, provided secure funding, and wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1154108/full#supplementary-material

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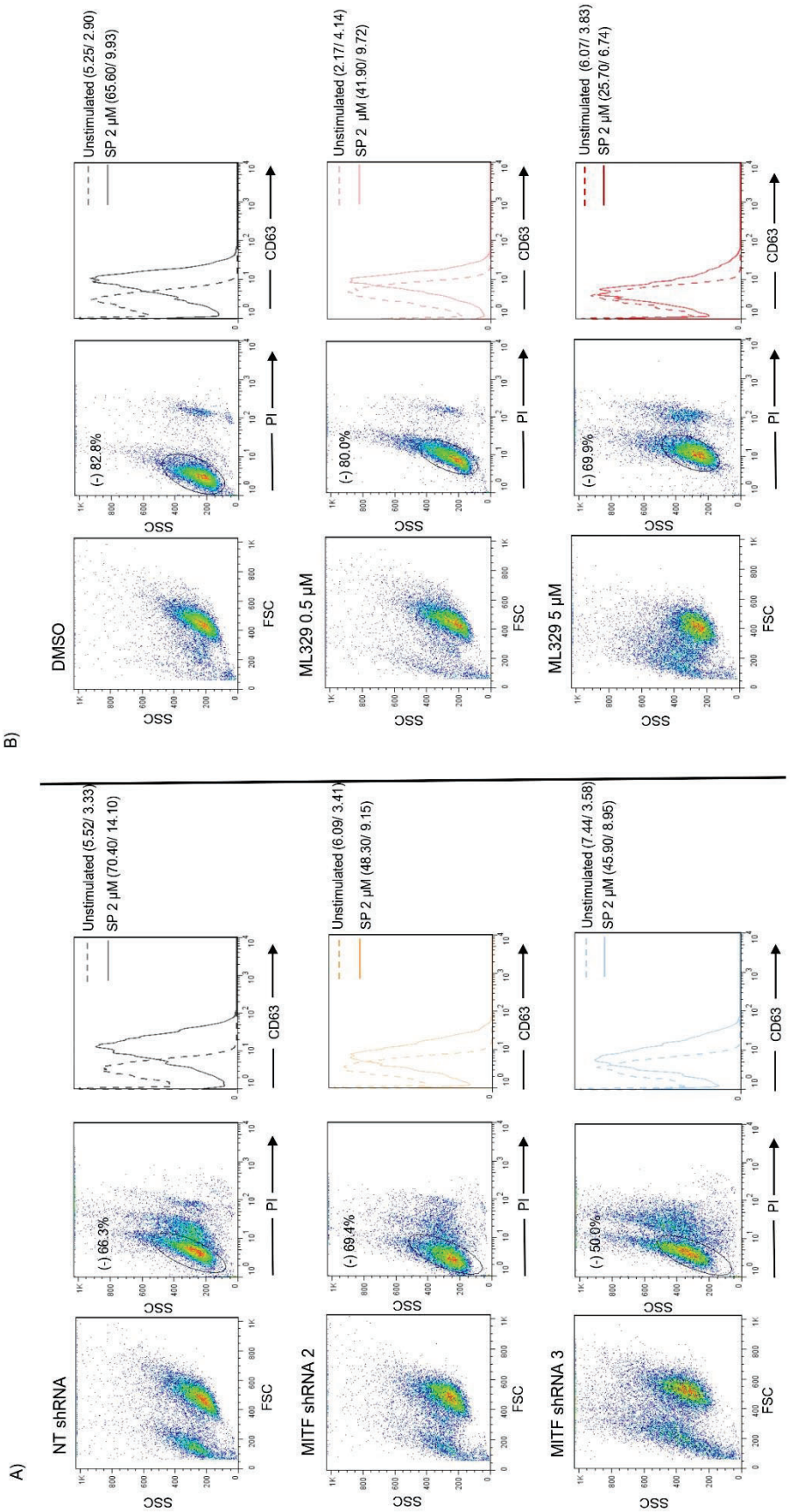
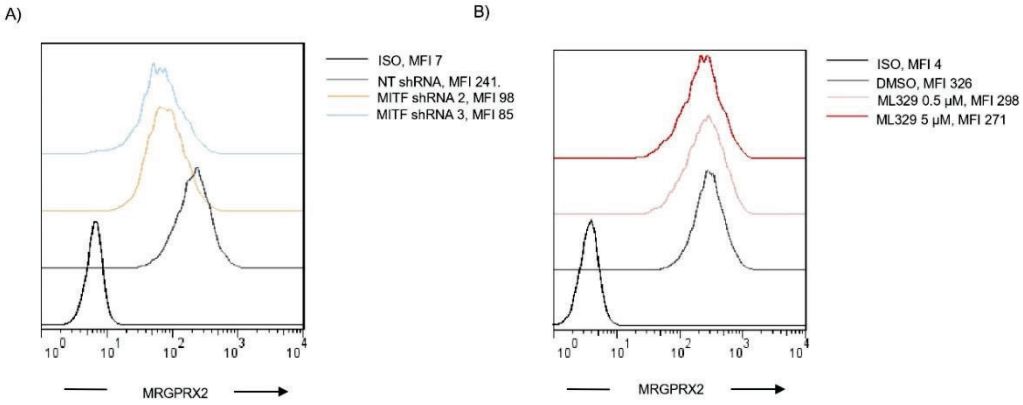
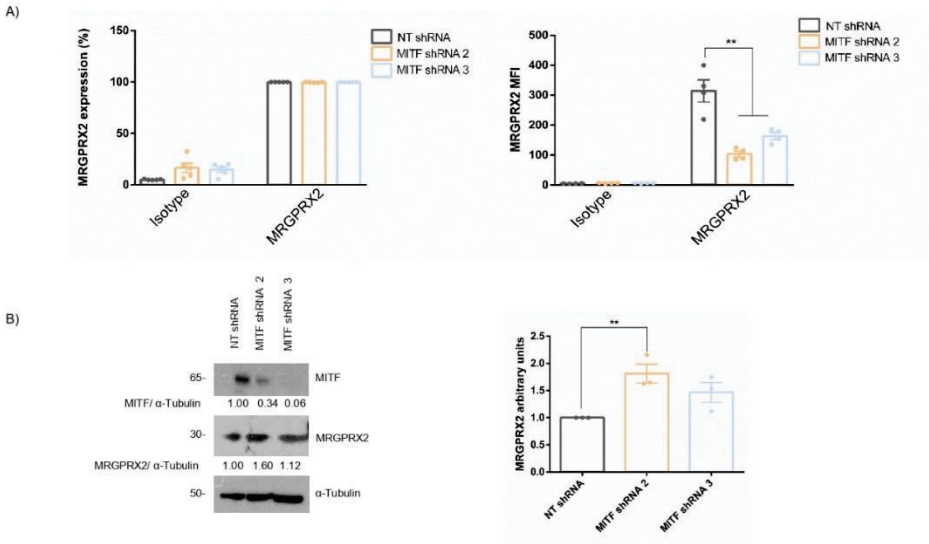


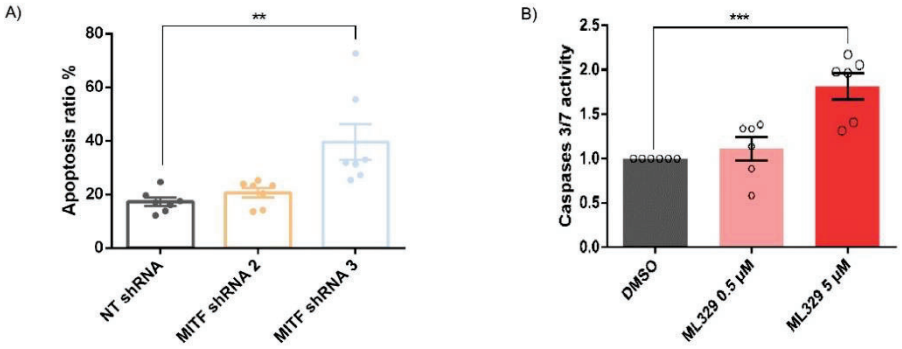
Supplementary Figure 1. CD63 expression after MITF silencing or ML329 treatment in propidium iodide (PI) negative gated LAD2 cells. (A) CD63 expression was measured in NT shRNA, MITF shRNA 2, and MITF shRNA 3 transduced cells, after five days of infection, by flow cytometry; and (B) after DMSO or 0.5 µM or 5µM ML329 treatment for five days. The (%/ MFI) of CD63 was shown for unstimulated and 2 µM SP stimulated cells in the same plot. MFI: Mean of Fluorescence Intensity. Notice that ML329 has some intrinsic fluorescence.



Supplementary Figure 2. MRGPRX2 levels after MITF silencing or ML329 treatment in LAD2 cells. (A) MRGPRX2 expression was measured in NT shRNA, MITF shRNA 2, and MITF shRNA 3 transduced cells (day five after infection) by flow cytometry. (B) MRGPRX2 expression was measured in cells incubated with DMSO, 0.5 µM ML329, or 5 µM ML329 for five days by flow cytometry. MFI: Mean of Fluorescence Intensity.



Supplementary Figure 3. MRGPRX2 expression after MITF silencing. (A) MRGPRX2 expression (% and Mean of Fluorescence Intensity (MFI) was measured by flow cytometry (n=4). (B) Total MRGPRX2 levels were measured by western blot (n=3). The unpaired t-test was used to determine significant differences, ** p< 0.01.



Supplementary Figure 4. MITF knockdown increases apoptosis in mast cells. (A) Apoptosis assays (Annexin %) in NT shRNA, MITF shRNA 2 and MITF shRNA 3 transduced LAD2 cells were assessed. (B) Caspase 3/7 activity was measured after incubating LAD 2 cells with DMSO, or ML329 (day 5). The unpaired t-test was used for statistical analysis (**p<0.01, ***p<0.001). Experiments are the mean ±SEM (n=3).

Annex 1 MITF downregulation reduces IgE-dependent mast cell degranulation

Introduction

MITF is required for IgE-dependent anaphylaxis by regulating histamine synthesis in mast cells [160]. MITF is also involved in the synthesis of other allergic mediators such as tryptase, chymase, granzyme B [278], and Prostaglandin D_2 [161]. SH3BP2 is a cytoplasmic adaptor initially identified as a protein interacting with the SH3 domain of the protein kinase ABL[279]. Our group previously showed that SH3BP2 is essential for mast cell signaling through the binding of Lyn, Syk, and PLCγ [280]. SH3BP2 regulates human mast cell survival and participates in KIT-mediated signal transduction by directly controlling KIT expression [281]. Our group found that SH3BP2 silencing decreases MITF by increasing miR-1246 and miR-5100 in GIST [282]. We explored whether the downregulation of MITF levels with miR-1246 and miR-5100, or ML329, MITF inhibitor, is consistent with a decrease in IgE-dependent mast cell degranulation.

Methods

Cell culture

The LAD2, human mast cell line, was a gift from Drs. A. Kirshenbaum and D.D. Metcalfe (National Institutes of Health, Bethesda, MD), culturing in StemPro-34 media, supplemented with StemPro-34 nutrient (Thermo Fisher Scientific; Waltham, MA, USA) and 2mM L-glutamine (Lonza), 100 U/ ml penicillin (Lonza) and 100 μg/mL streptomycin (Lonza), and 100 ng/mL SCF (ImmunoTools GmbH, Friesoythe, Germany) (35). Primary human mast cells (huMCs) derived from $CD34^+$-positive peripheral blood cells were obtained from healthy donors, CD117 MicroBeads Kit (Mitenyi Biotec, Germany) for $CD34^+$ progenitor cell isolation. Cells were cultured for 0-2 weeks with 100 ng/ml SCF, IL-6, and IL-3 (ImmunoTools GmbH, Friesoythe, Germany) and 2-6 weeks with 100 ng/ml IL-6 and SCF. After six weeks, $CD34^+$-

derived human mast cells were assessed by surface expression of FcεRI and CD117. The expression of MRGPRX2 was also checked for experiments.

GFP-miRNA overexpression

MiR-CTL, miR-1246, or miR-5100 were overexpressed by lentiviral transduction. LAD2 cells were transduced in the presence of 8 μg/ ml of polybrene (Santa Cruz, CA, USA), and puromycin selection (1 μg/ ml) was carried out after one day from transduction. miRNA-GFP expression was corroborated in both cell lines on the fourth day by microscopy fluorescence (Leica AF600, Wetzlar, Germany).

ML329 inhibition with $CD34^+$ derived human mast cells

$CD34^+$ derived human mast cells were treated with DMSO or ML329 5 μM in 5 days for degranulation.

CD63 FACS analysis

Briefly, for CD63 determination: $1 \cdot 10^5$ cells/ point were incubated with Tyrode's buffer or SP at 37°C for 30 min. Cells were incubated with blocking buffer (0.1% NaN3, 2% FBS, 20% rabbit serum, PBS) for 30 min on ice, and APC anti-human CD63 staining was performed for 30 min on ice. After washing, samples were incubated with propidium iodide (PI), acquired with a FACSCalibur flow cytometer, and analyzed with FlowJo software.

Results and discussion

In this study, we aimed to analyze the relationship between MITF and IgE-dependent mast cell degranulation. Therefore, we investigated MITF downregulation using miRNAs already described that target MITF in mast cells and the MITF inhibitor ML329. Before miRNAs were used for mast cells, to validate these miRNAs in HMC-1, a quantitative real-time PCR was performed in SH3BP2-silenced HMC-1 cells,

results showed that knockdown SH3BP2 increases miR-5100-GFP and miR-1246-GFP [283] . Firstly, LAD2 cells were transfected with miR-CTL-GFP, miR-5100-GFP, and miR-1246-GFP. Levels of transfection were assessed with GFP expression under microscopy (Fig 1). MITF levels were reduced in cells transfected with miRNA-5100 and miRNA-1246 (Fig 2 A). Then CD63 expression was checked in IgE-dependent mast cell activation, in the living cell population (propidium iodide negative) by flow cytometry (Fig 2 B). $CD34^{+}$-derived mast cells were performed the further study. Since the number of cells obtained after differentiation was low, we pursued the analysis of MITF using ML329. Inhibition of MITF reduced CD63 expression in the living cell population (Fig 2 C).

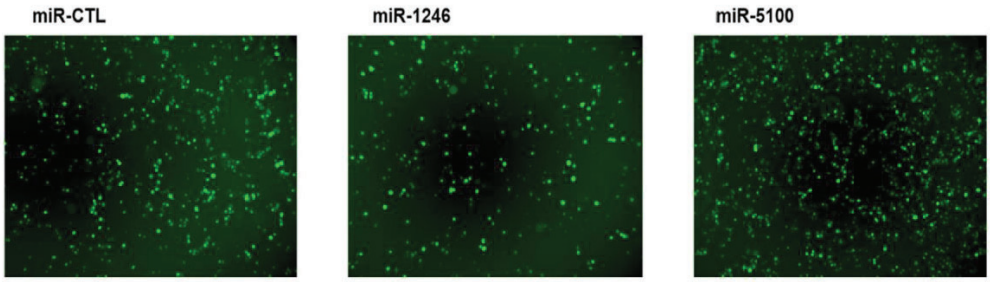


Fig 1. GFP-miRNAs expression in LAD2. GFP expression was corroborated for control lentiviral transduction in both cell lines at 4^{th} day miR-CTL, miR-5100, and miR-1246 were measured by fluorescence microscopy in LAD2 cells.

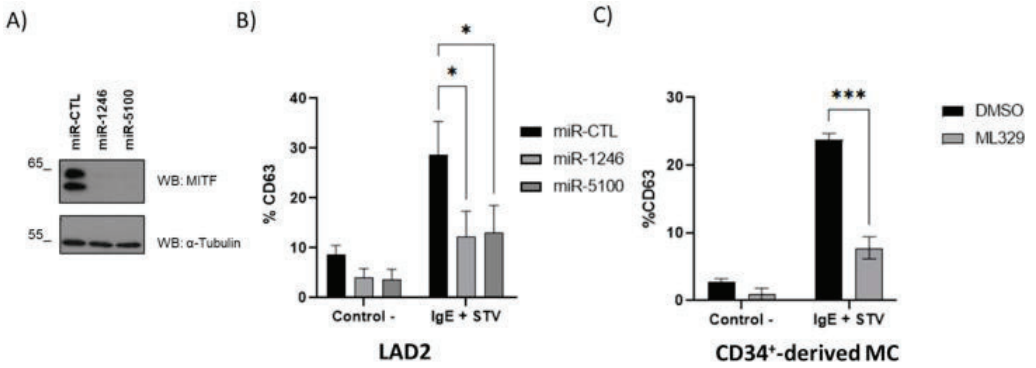


Fig 2. MITF downregulation impairs IgE-dependent degranulation in LAD2 cells and $CD34^{+}$-derived mast cells. (A) Western blot shows MITF levels in miR-1246 and miR-5100 overexpressed LAD2. (B) Percentage of CD63 expression in miR-CTL, miR-1246, and miR-5100 overexpressed

LAD2 cells. (C) Percentage of CD63 expression in $CD34^+$-derived mast cells treated with ML329 5 μM. Data show the mean ± SEM. Statistical significance (* $p < 0.05$, *** $p < 0.001$; two-way ANOVA with Tukey's multiple comparison analysis). The figure comes from paper authors Elizabeth Proaño-Pérez, Laia Ollé, Yanru Guo, et al. [283].

Our results showed that downregulated MITF decreases the CD63 expression in LAD2 cells and $CD34^+$-derived human mast cells with IgE-dependent activation, indicating that MITF is involved in early mediators' release of mast cell activation. Previously, in article 2 of this thesis, we showed that MITF was involved in MRGPRX2 degranulation. This data suggested a general involvement of MITF in mast cell degranulation that must be explored in the near future.

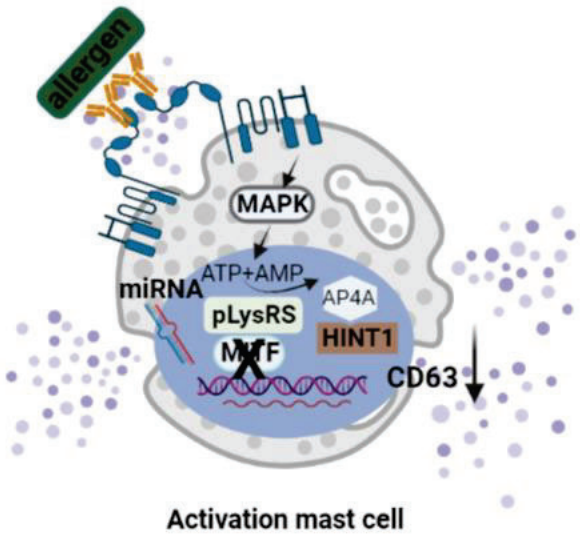


Fig 3. Down regulation of MITF which affects the degranulation of mast cells.

Annex 2 MITF regulates Ca^{2+} sensor STIM 1

Introduction

Our previous data showed that MITF silencing and inhibition decrease calcium influx. STIM 1 is involved in the calcium influx in mast cell activation through MRGPRX2 [284]. Recently, it has been reported that MITF regulates the function of STIM 1 in melanocytes [285]. So, we explored if MITF targets STIM 1 expression controlling the calcium influx in mast cells.

Materials and Methods

MITF-silencing using lentivirus construction

Lentiviral particles to silence MITF gene expression were generated using Mission ® shRNA technology (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Lentiviral particles were generated in HEK293LTV. We used the following sequences reported elsewhere. These sequences were used from our group [157] [283] and the second publication of this thesis.

The non-target (NT) sequence:
5'CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTG TTG TTTTT 3',

MITF shRNA 2 sequence:
5'CCGGCGGGAAACTTGATTGATCTTTCTCGAGAAAGATCAATCAAGTTTCC CGTTTTTG 3'.

MITF shRNA 3 sequence: 5'
CCGGGGGAGCTCACAGCGTGTATTTCTCGAGAAATACACGCTGTGAGCTCC CTTTTTG 3'.

LAD2 cells were transduced with polybrene at 8 µg/ ml (Santa Cruz) and selected with 1µg/ ml of puromycin. Cells were maintained with puromycin until the day of the experiment when it was removed before cell activation. Experiments were performed five days after silencing.

ML329 cell treatment

LAD2 cells were incubated with DMSO or the MITF inhibitor ML329 at 0.5 µM or 5 µM for 5 days for experiments.

Luciferase assay

Firefly luciferase under the control of STIM 1 promoter was a gift of Jonathan Soboloff (Institute for Cancer Research and Molecular Biology, Pennsylvania, USA). After MITF silencing and inhibition for 5 days, $2.5 \cdot 10^4$ cells were transfected with the Firefly and the Renilla reporters at 7:1, respectively. Transfections were performed with lipofectamine. After transfection for 24 hours, stimulate transfected LAD2 cells with SP for 6 hours. For IgE-dependent mast cell activation, transfected LAD2 cells were sensitized with biotinylated IgE overnight, and stimulated by STV for 6 hours. Luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) following the manufacturer's instructions. Firefly Luciferase data were normalized according to Renilla Luciferase data.

Results and discussion

LAD2 cells were silenced using two different MITF shRNAs, and MITF and STIM 1 level was assessed by western blot. STIM 1 expression was decreased following the silencing of MITF (Fig 1A). One of the MITF sequences did not silence properly, so STIM1 was not reduced. Recently it has been described that MITF binds to STIM 1 promoter controlling STIM 1 expression in melanocytes [285]. So, next, we analyzed whether increased MITF activity after MRGPRX2-dependent activity participates in STIM 1 expression in LAD2. For that purpose, NT and MITF shRNAs

cells were transfected with the STIM 1-promoter luciferase reporter gene, and substance P (SP) was used to activate MRGPRX2. Our results showed that STIM 1 expression increased with the activation of SP, compared with unstimulated in NT shRNA. This result agrees with the role of MRGPRX2 in increasing STIM 1 expression previously reported [284]. Interestingly, a significant decrease of STIM 1 expression was observed in silencing MITF, compared with NT shRNA (Fig 1, B), suggesting that silencing MITF decreased the STIM 1 transcription.

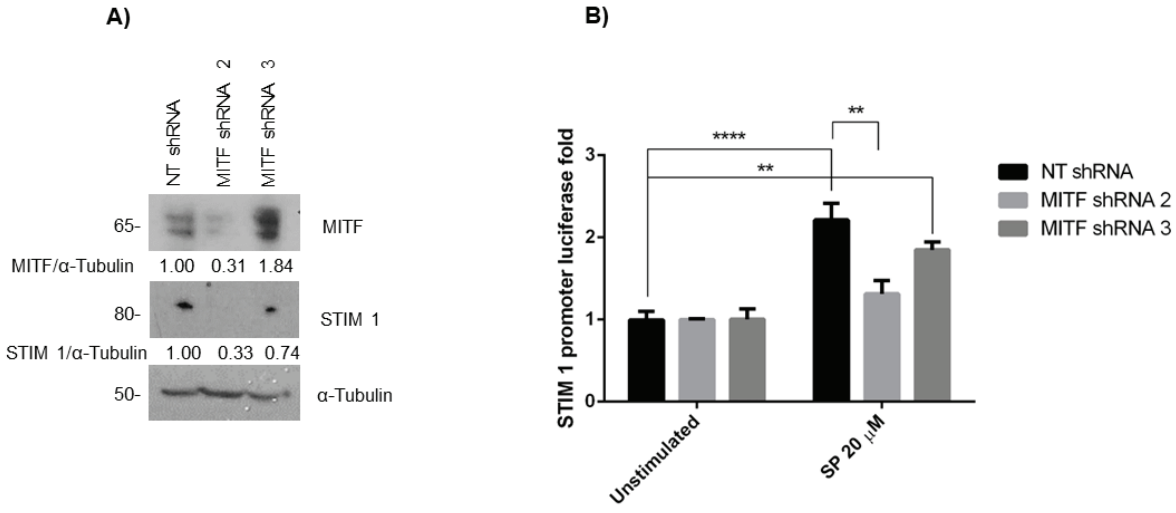


Fig 1. MITF silencing downregulated the calcium sensor STIM 1 in mast cells (LAD2). **A)** LAD2 cells were transduced with either control NT (non-target) shRNA or MITFshRNA 2 or MITFshRNA 3, and the efficiency of MITF silencing was assessed by western blot. α-Tubulin was used as a loading control. **B)**, Cells were activated with SP 20 μM for 6 hours in LAD2 cells transfected with the STIM 1-luciferase reporter gene. The two-way ANOVA test was used for the statistical analysis (**p < 0.01, ****p < 0.0001). The data are from one representative experiment.

In parallel, we used the MITF inhibitor, ML329, at 0.5 μM and 5 μM for 5 days. In this case, we assessed the IgE-dependent pathway, sensitizing cells with biotinylated IgE overnight and challenging them with streptavidin. Afterward, the expression of MITF and STIM1 was examined by western blot (Fig 2 A). As we expected, STIM 1 expression was decreased following MITF inhibition (Fig 2 B). In parallel, STIM 1

promoter-controlled firefly and Renilla luciferase were transfected into the DMSO-treated and ML329-treated cells. Cells were stimulated with SP or ^{B}IgE+STV (0.1 µg/ ml + 16 µg/ ml) for 6 hours. As shown after activation with SP or ^{B}IgE+STV in DMSO control cells, STIM 1 promoter activity increased, compared with unstimulated. Interestingly, the activity of the STIM 1 promoter decreased after MITF inhibition, compared with DMSO (Fig 2 B).

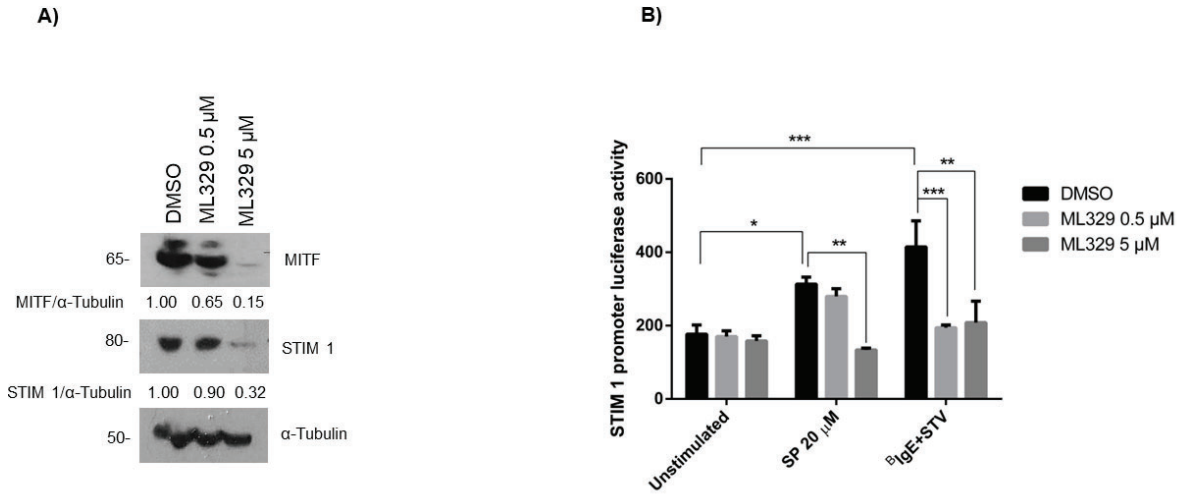


Fig 2. MITF inhibition decreases the calcium sensor STIM 1 expression in mast cells (LAD2). **A)** LAD2 cells were incubated with DMSO or ML329 0.5 µM or 5 µM for5 days, and the efficiency of MITF silencing and STIM was assessed by western blot. α-Tubulin was used as a loading control. **B)** Cells were activated with SP 20 µM for 6 hours in LAD2 cells transfected with STIM1-luciferase gene reporter. Biotynilated-IgE (0.1 µg/ ml) sensitized overnight plus streptavidin (16 µg/ ml) for 6 hours was used as a control. The two-way ANOVA test was used for the statistical analysis (*p < 0.5, **p < 0.01, ***p < 0.001). The data are from one representative experiment.

Although it is known that MRGPRX2 activation raises intracellular Ca^{2+} levels, STIM 1 is involved in this mechanism, as has been reported [246] [284]. STIM 1 is essential for IgE-dependent mast cell activation [286]. Our results showed that MITF regulates STIM 1. These data suggested that MITF controls STIM 1, which is needed for proper calcium influx in IgE and MRGPRX2-dependent mast cell activation.

Annex 3 MITF regulates IL-8 in MRGPRX2-dependent mast cell activation

Introduction

MITF is involved in the synthesis of other allergic mediators such as tryptase, chymase, granzyme B [278], and prostaglandin D_2 [161]. But cytokines induction is still unknown.

Materials and Methods

shRNAMITF lentivirus construction

Lentiviral particles to silence MITF gene expression were generated using Mission ® shRNA technology (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Lentiviral particles were generated in HEK293LTV. We used the following sequences reported elsewhere. These sequences were used from our group [157] [283] and the second publication of this thesis.

The non-target (NT) sequence:
5'CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTG TTG TTTTT 3',

MITF shRNA 2 sequence:
5'CCGGCGGGAAACTTGATTGATCTTTCTCGAGAAAGATCAATCAAGTTTCC CGTTTTTG 3'.

MITF shRNA 3 sequence: 5'
CCGGGGGAGCTCACAGCGTGTATTTCTCGAGAAATACACGCTGTGAGCTCC CTTTTTG 3'.

LAD2 cells were transduced with polybrene at 8 µg/ ml (Santa Cruz) and selected with 1µg/ ml of puromycin. Cells were maintained with puromycin until the day of the experiment when it was removed before cell activation. Experiments were performed five days after silencing.

ML329 inhibition with LAD2 cells

LAD2 cells were treated with DMSO or ML329 at 0.1 µM or 0.5 µM for 5 days before experiments.

ELISA assays

For IL-8 and GM-CSF release, cells ($1 \cdot 10^5$ cells/ well) were preincubated with stem-pro media or SP for 24 hours; the supernatant was collected and measured using IL-8 ELISA assay kit or GM-CSF ELISA assay kit according to the manufacturer's instructions (R&D Systems).

Results and discussion

MRGPRX2 has been described to induce IL-8 and GM-CSF secretion after SP activation [225]. We explored the release of these cytokines in MITF-silenced LAD2 cells. MITF was satisfactorily silenced with both MITF shRNA sequences (Fig 1A). Our results show that MRGPRX2 activation with SP increases the release of IL-8 and GM-CSF in NT cells (Fig 1 B and C). Interestingly, IL-8 significantly decreased after SP stimulation in MITF silencing (Fig 1 B). In contrast, GM-CSF secretion was unaffected (Fig 1 C).

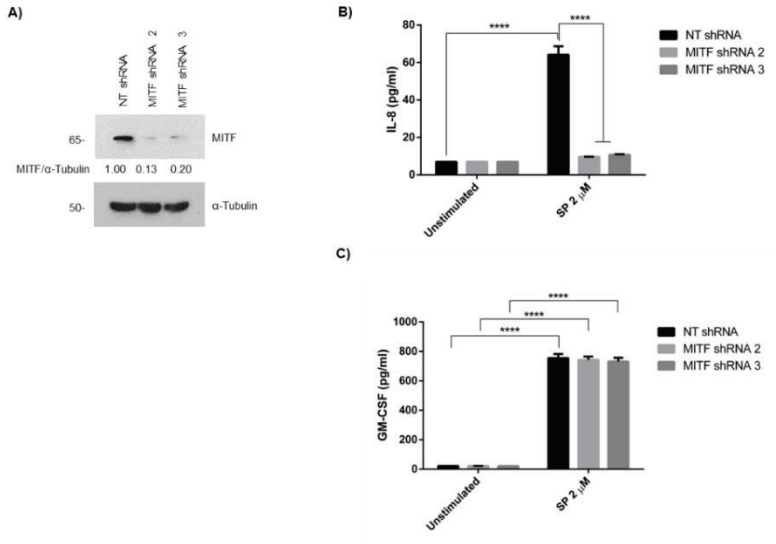


Fig 1. MITF-silencing affected the release of IL-8. **A)**, LAD2 cells were transduced with either control NT (non-target) shRNA or MITFshRNA 2 or MITFshRNA 3, and the efficiency of MITF silencing was assessed by western blot. α-Tubulin was used as a loading control. **B)**, IL-8, and **C)**, GM-CSF release with activation of SP. A two-way ANOVA test was used for the statistical analysis (****p<0.0001). The data are from one representative experiment.

In parallel, ML329, MITF inhibitor, was incubated with LAD2 cells for 5 days, and IL-8 and GM-CSF release after SP stimulation was assessed. Consistently with silencing data, IL-8 secretion was impaired, whereas GM-CSF was unaltered after MITF inhibition (Fig 2 A and B).

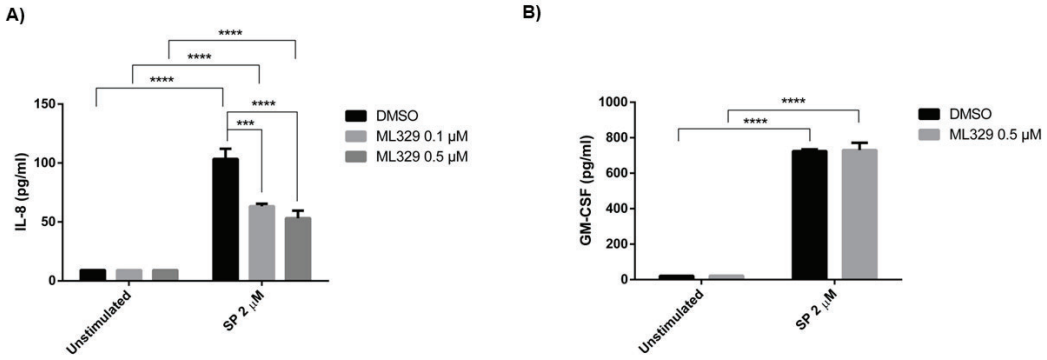


Fig 2. MITF inhibition decreased the release of IL-8. **A)**, IL-8 release in LAD2 cells with SP activation in DMSO or ML329 0.1 µM or ML329 0.5 µM for 5 days. **B)**, GM-CSF release in LAD2 cells with SP activation in DMSO or ML329 0.5 µM for 5 days. A two-way ANOVA test was used

for the statistical analysis (***p<0.001, ****p<0.0001). The data are from one representative experiment.

Our results showed that MITF could specifically regulate IL-8 in mast cells through MRGPRX2 activation. Previous studies showed that IL-8 is essential for recruiting neutrophils and contact with T cells in inflammation response [287]. Some literature showed that IL-8 is associated with psoriatic skin [288] and AD [289], and MRGPRX2 is mainly expressed in mast cells in the skin, which may suggest that IL-8 release could be induced with MRGPRX2 mast cell activation. In addition, the release of IL-8 from mast cells causes the epithelial-to-mesenchymal transition and migration and accelerates non-small cell lung cancer growth [290]. And it's a biomarker of melanomas [291]. It indicated that MITF can be a central role for regulating more cytokines which are the keys to the pathology of the disease. Because some cytokines are more important for differentiation, growth, distribution, recruiting immune cells and involved in immune response. In the future, more deep and wide research studies can be done for understanding the role of MITF.

4. Results summary

Anaphylaxis is a severe allergic reaction triggered by allergens to certain foods, medicines, insect venom, and other substances. Anaphylactic shock can be fatal if not treated. Mast cells are the effector cells in anaphylaxis, and mast cell transcriptional factors are crucial in controlling the production of mediators. Microphthalmia associated-transcription factor (MITF) is required for IgE-dependent anaphylaxis by regulating the histidine decarboxylase enzyme responsible for histamine production. In quiescent mast cells activity of MITF is suppressed through its interaction with the HINT1 repressor at the nucleus. The release of HINT1 is driven explicitly by antigen-IgE-induced production of a signaling molecule, Ap4A, and the consequent binding of HINT1 to Ap4A. In IgE-activated mast cells, LysRS, a component of the translation apparatus, is phosphorylated on Serine 207 in a MAPK-dependent manner. This phosphorylation induces a conformational change in LysRS, resulting in its translocation to the nucleus and its production of Ap4A, which causes HINT1 release from MITF and the subsequent MITF activation and transcription of MITF-targeted genes.

In this thesis, we characterize the mutation in LysRS P542R found in a patient with severe anaphylaxis to insect venom. We identified the conformational change using cell transfectants and protein structure analysis. LysRS Proline 542 faces Serine 207 and is close to the G540, interacting with the multi-aminoacyl-tRNA synthetase complex (MSC) and controlling the LysRS transfer into the nucleus. There are two states for the LysRS WT. The closed state is the unphosphorylated LysRS that remains in the MSC's cytosol. The open state corresponds to the phosphorylated LysRS (Ser 207) translocated to the nucleus. In a closed state, LysRS P542R interacts with the Glutamate at position 150 (E150) and Aspartate at position 313 (D313), residues involved in the interaction and recognition of the MSC-binding loop. In the open state, the LysRS P542R just interacts with the D313. The P542R mutation changes the trapping residues jointing

with the MSC-p38. On the other hand, metadynamics stimulation postulates both open and close states for LysRS P542R. These analyses suggest that LysRS P542R may be located in the nucleus and acts as the Ser207 phosphorylated LysRS form. To verify that cell transfectants (LysRS WT and LysRS P542R) in RBL-2H3 cells were generated. Confocal microscopy and cell fractioning studies revealed that LysRS P542R is constitutively localized in the nuclei. Furthermore, we used TRPM-1 luciferase reporter gene assay to measure MITF activity; TRPM-1 encodes melastatin, and its promotor has multiple binding sites for MITF. Our results showed MITF activity was increased in P542R compared with WT. In addition, we analyzed NFAT activity in that context using a gene reporter assay. Our results showed that LysRS P542R activated NFAT activity similarly to LysRS WT. Thus NFAT activity was not altered in this mutation. Moreover, LysRS P542R enhanced MITF activity and MITF-dependent targets such as Histamine. Functional analysis revealed that LysRS P542R transfectants increase degranulation and PGD2 synthesis compared to LysRS WT. MITF has been related to lysosome biogenesis, so we analyzed granular content in the transfectants. Our results showed that the granular staining with lysotracker has no differences between LysRS P542R and LysR WT. Since modulation of the LysRS-MITF pathway is crucial in IgE-dependent mast cell responses, we wanted to further extend the study to IgE-independent mast cell activation focusing on MRGPRX2.

MRGPRX2 has been recognized as a key receptor in adverse drug reactions leading to anaphylaxis. Research from our group and others has demonstrated that several drugs can cause the degranulation of mast cells by acting on this receptor. According to recent research, MRGPRX2 signals through MAPK in mast cells. Studies on the regulation of MITF in melanocytes and mast cells have revealed that ERK1/2 is primarily responsible for phosphorylating MITF at Ser73. We set out to investigate the involvement of MITF in MRGPRX2 signaling.

Firstly, our results showed that MRGPRX2 activation increased the translocation of LysRS to the nuclei and increased MITF phosphorylation and activity.

Overexpression of LysRS increased MITF activity after MRGPRX2 activation and PGD2 secretion. Moreover, MITF silencing with two different sequences reduced MRGPRX2-dependent degranulation and calcium influx. At the same time, another approach was carried out using MITF inhibitor ML329. After inhibition, our results showed that CD63 and calcium decreased compared with DMSO. Since MITF knockdown and inhibition affect cell viability, as we previously published, the experiments were carried out in life cells excluding non-viable cells when possible. Several drugs reported by our group and others that activates MRGPRX2 receptor activates MITF activity. Our results indicate that MRGPRX2 signaling involves Lysyl-tRNA synthetase and MITF pathway. Interestingly, our unpublished data show that the calcium sensor STIM 1, needed for calcium influx and degranulation, is also downregulated after MITF knockdown with shRNAS-specific sequences or ML329 in mast cells. Preliminary analysis of late events also shows that MITF can regulate IL-8.

Conclusions, MITF has involved in IgE and MRGPRX2-dependent mast cell activation; it regulates the synthesis and release of proinflammatory mediators, calcium, and cell viability. Alterations in the LysRS-MITF signaling axis may be related to the severity of mast cell-derived pathologies.

5. Discussion

Anaphylaxis is a serious and complex allergic reaction that includes respiratory and circulatory system symptoms and may be life-threatening, particularly if untreated immediately [55]. The most frequent triggers of anaphylactic reactions include drugs [56], insect venom [57], and food allergy, the last with a higher prevalence in children [58]. The main effector cells for anaphylaxis are mast cells and basophils. The distribution of mast cells is the reason for various symptoms of anaphylaxis. Mast cell mechanisms involved in anaphylaxis can be distinguished depending on the presence or absence of IgE, defining an IgE-dependent or IgE-independent anaphylaxis [35] [60].

In IgE-dependent mast cell activation, LysRS, a component of the translation apparatus, is phosphorylated on Serine 207 in a MAPK-dependent manner. This phosphorylation induces a conformational change in LysRS, resulting in its translocation to the nucleus and production of Ap_4A, which bind to HINT1, HINT1 release from MITF, and the subsequent MITF activation and transcription of MITF-targeted genes[131]. A single conformational change triggered by IgE-mediated signaling switches the LysRS function from translation to transcription [132][133]. MITF is also involved in the synthesis of other allergic mediators such as tryptase, chymase, granzyme B [278], and Prostaglandin D_2 [161].

5.1 LysRS-MITF pathway deregulation can lead to anaphylaxis

In this study, a patient suffering from severe anaphylaxis to wasp venom holds a mutation in one of the two copies of the *KARS* gene that caused an amino acid substitution (arginine instead of proline) in position 542 of the cytosolic form of Lysyl-tRNA synthetase.

LysRS, a moonlighting protein coded by the *KARS* gene, has two isoforms, a cytosolic one of 597 amino acids, and a longer mitochondrial isoform of 625 amino acids. LysRS isoforms have each role in both the cytosolic and the mitochondrial

translation. The cytosolic isoform has a non-canonical role and is essential for protein translation and MITF activity transcription in mast cells upon IgE activation [131]. A variety of KARS mutations have been identified, and their associated clinical phenotypes range from early-onset encephalopathy to isolated peripheral neuropathy, non-syndromic hearing loss, or progressive leukoencephalopathy [292][293][294][295]. There have been several additional mutations linked to a possible mitochondrial disease [296] or combined respiratory chain complex deficiencies (I and IV) [297].

Apart from tryptase and the D816V mutation of the KIT receptor (CD117), we rarely have reliable biomarkers to identify patients at a higher risk of severe anaphylactic reactions [55]. There is a general interest in understanding the molecular process underlying severe allergic reactions. Here, we tried to connect LysRS's non-canonical function, which involves the activation of the transcription factor MITF, with the release of proinflammatory mast cell mediators. In this study, we discovered that the overactive phenotype that might cause anaphylaxis might be explained by the deregulation of the FcεRI-LysRS-MITF pathway caused by mutations in the *KARS* gene.

5.1.1 Protein and structural analysis of LysRS P542R compared to LysRS WT

LysRS is located in the cytoplasm [298], in the Multi-tRNA Synthetase Complex (MSC) [299], which has three non-enzymatic proteins (p43, p18, and p38) interacting with nine distinct aaRSs [300][301]. LysRS is bound to MSC-p38; however, phosphorylation of Ser207 of LysRS triggers an open conformation that dissociates from MSC and translocates into the nucleus, producing AP4A, which binds to the MITF repressor HINT and activates MITF [132].

Protein structure analysis of LysRS-Proline 542 Arginine shows that arginine interacts with residues (E150 and D313) critical for the LysRS location to the cytoplasm at the MSC. A Proline, an amino acid that introduces rigidity to the polypeptide backbone, is substituted for a positively-charged amino acid with a big side chain, like

Arginine. This predicts an unstable MSC binding. The free energy profile diagram analysis shows each closed and open state molecular frequency. The closed state is mainly found in the cytosol in quiescent mast cells. In contrast, LysRS Ser207 phosphorylation drives the open state, making the detachment from MSC more favorable and the translocation to the nucleus. The open state is found in activated cells upon allergen-IgE-FcεRI interaction. The analysis for LysRS WT and LysRS P542R shows an increase in free energy for the closed state in LysRS WT, while LysRS P542R shows similar frequencies for both states.

The analysis of LysRS WT and P542R-RBL-2H3 cell transfectants by confocal microscopy and protein fraction confirmed that the P542R amino acid substitution in LysRS promotes the translocation of LysRS P542R to the nucleus in the absence of stimuli. Our findings also suggest that mutations placed in the region of the interdomain interface of LysRS are candidates to deregulate its functions.

5.1.2 Mast cell degranulation and MITF activity are enhanced in the presence of P542R upon IgE stimulation

Based on the FcεRI-LysRS-MITF pathway, we took advantage of our cellular models, activated them via FcεRI, and analyzed cellular degranulation, β-hexosaminidase release, and PGD2 assays. No differences were observed without stimuli between LysRS WT and LysRS P542R; however, LysRS P542R showed a significant increase in β-hexosaminidase and PGD2 compared to LysRS WT. The release of PGD2 in LysRS P542R is consistent with MITF-specific regulation of the PGD_2 [161]. Interestingly, in P542R transfectants, spontaneous degranulation is not amplified, indicating that IgE-dependent allergen activation would be required to cause anaphylaxis.

Next, we analyze whether LysRS P542R constitutive nuclear translocation was concomitant with increased MITF activity. The results from TRPM-1 showed that activities of MITF were increased in quiescent mast cells and after IgE-dependent mast

cell activation. Interestingly, NFAT activity was not enhanced in LysRS P542R transfectant compared to LysRS WT, showing that the mutation selectively triggers MITF.

LysRS P542R cell transfectants show MITF activity increased, so next, we analyzed an array of genes that encode proinflammatory mediators and reported MITF-dependent targets. Hdc was found significantly increased in P542R as expectable. The increased MITF activity enhances crucial genes for anaphylaxis such as *HDC* [160]. Surprisingly, tryptase (*TPSB2*) expression was not significantly elevated in P542R transfected cells, but chymase (*CAM*) expression was. This finding is consistent with the patient's clinical data, which shows that tryptase levels did not rise. Some anaphylaxis patients have severe symptoms without increased tryptase, which is a confusing feature. Tryptase increases the probability of other unknown pathways being involved and allows the discovery of unidentified biomarkers. The serine protease called chymase is mostly located in the secretory granules of mast cells. The increase in chymase found in two separate studies of autopsy cases with anaphylaxis supports the hypothesis that chymase may be a biomarker for anaphylaxis [302][303]. Chymase stability in serum is higher than tryptase, which is one factor to consider. Tryptase levels return to baseline 4 hours after the anaphylactic reaction; however, chymase is still detectable at that time [304]. Consequently, it would be interesting to analyze chymase in those cases of anaphylaxis where tryptase is negative.

5.1.3 G189D LysRS analysis in the context of MITF and mast cell activation

This research is based on a specific clinical patient who experienced anaphylactic reactions to Hymenoptera stings because of extremely severe allergic reactions, even used for standard allergological work-up (skin tests). This patient needs omalizumab (anti-IgE biological) therapy to tolerate the administration of wasp venom immunotherapy. This patient and her partner underwent genetic counseling due to the death of their baby daughter. Missense mutations in the KARS gene were discovered in both parents, which led to an amino acid change in the LysRS protein. While the

partner, who has no history of allergies, has mutation LysRS G189D, the allergic patient has mutation LysRS P542R. We compared both mutations on a structure and functions. Metadynamics simulations revealed that, unlike the P542R mutation, the G189D mutation does not promote the open state. The closed state of the LysRS-G189D system shows a single free energy minimum, just like the LysRS-WT system (Suppl. Fig E3, from the first article in the thesis, pages 70). The interdomain interface and the S207 residue are also far from G189D (Suppl. Fig E3 C bottom structure, from the first article in the thesis pages 70). Unlike the P542R mutation, it is not observed to interact with the residues responsible for interaction with MSC-p38. Therefore, G189D does not affect LysRS binding to MSC-p38.

Consequently, the LysRS G189D protein is not located intrinsically in the nucleus of the LysRS G189D cellular transfectant (Suppl. Fig E4 A, the first article in the thesis, pages 71). Subsequently, MITF activity is not enhanced in this transfectant (Suppl. Fig E4 C from the first article in the thesis, pages 71). Accordingly, PGD2 secretion (Suppl. Fig E5 A from the first article in the thesis, pages 72) and MITF-dependent gene expression were similar in LysRS G189D and LysRSWT (Suppl. Fig E5 B, from the first article in the thesis, pages 72), indicating that this mutation does not increase the non-canonical function of LysRS in mast cells. Agreeing with our findings, the mutation G189D does not increase the risk of anaphylaxis when exposed to allergens. Actually, the person who carries that mutation has not yet reported allergic reactions.

Probably, LysRS G189D would affect the canonical function in protein synthesis since the aminoacid substitution may also be quite disruptive (an aspartate, a long and negatively charged amino acid, replaces glycine-small amino acid). Besides, the LysRS G189D mutation is placed on the surface of the tRNA anticodon binding domain (from the first article in the thesis, pages 70). Thus, this mutation might affect the mode of tRNA binding and alter the LysRS translational mechanism. Both mutations reported profound changes consequently, inheriting both led to a fatal outcome.

This study shows the mechanism of a mutation in LysRS triggers its translocation to the nucleus independently. This translocation induces MITF activity and proinflammatory mediator release upon IgE crosslinking. Besides the MAPK-LysRS-Ap4A-HINT1 signaling pathway, some pathways have been reported to regulate MITF activity and/or stability in mast cells, including the c-KIT and PI3K pathways [159][146][280]. The analysis of the MITF signaling cascade could provide further insight into the molecular pathways associated with anaphylaxis to Hymenoptera, and it may be extended to food or idiopathic anaphylaxis. Fig A shows the function of MITF in LysRS P542R.

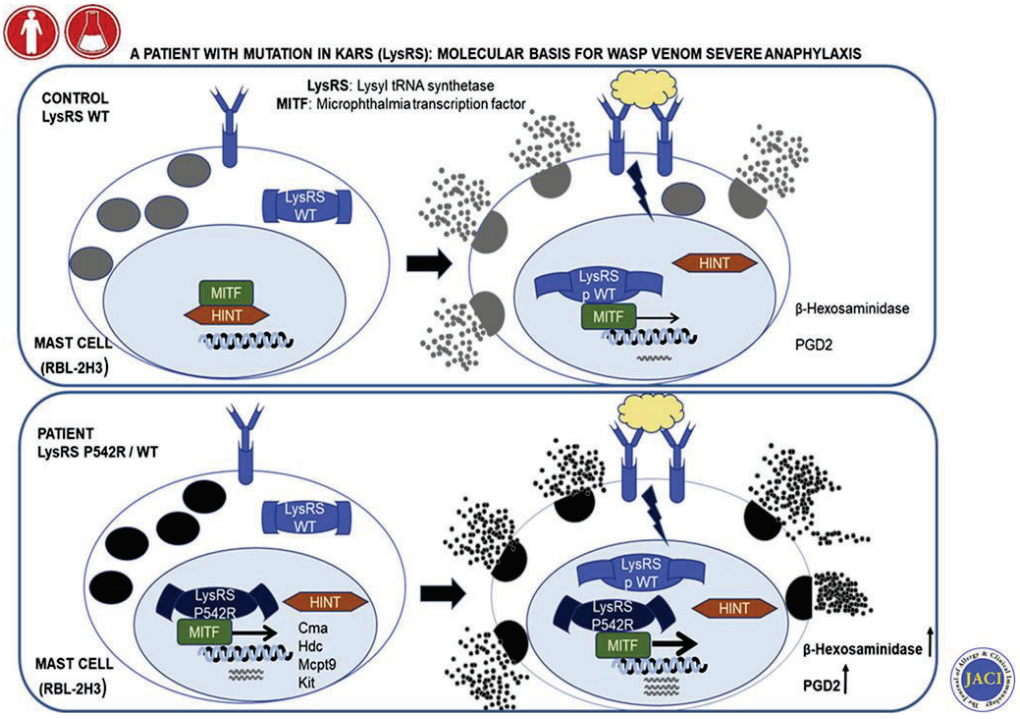


Fig A, MITF in LysRS P542R context after IgE-dependent mast cell activation. LysRS WT is serine 207 phosphorylated in a MAPK-dependent manner in response to IgE-dependent stimuli, which causes the dissociation from the MSC and moves from the cytoplasm to the nucleus, increasing the activity of MITF and its dependent genes. LysRS P542R translocates to the nucleus due to conformational change and induces the activity of MITF and target genes. After IgE-dependent activation, more degranulation and proinflammatory releases may lead to anaphylaxis.

5.1.4 MITF knockdown impairs IgE-dependent degranulation

In addition, our results in annex 1 showed that down-regulated MITF, through an increase in miR-1246 and miR-5100, decreased cell degranulation measured by CD63 expression in LAD2 cells. Consistently miR-1246 and miR-5100 downregulates MITF in GIST [283].

ML329, MITF inhibitor, inhibiting MITF expression in LAD2 cells and reduction CD63 expression triggered with SP in LAD2 cells and $CD34^{+}$-derived human mast cells, data from the second publication of this thesis. CD63 is impaired in IgE-dependent activation in ML329 inhibiting compared with DMSO, indicating MITF is involved in IgE-dependent mast cell degranulation.

5.2 Role of MITF in MRGPRX2-dependent mast cell activation.

Even though several studies concerning the MRGPRX2 receptor have been reported in recent years, its signaling transduction in mast cells is still poorly understood. Most of the research identifies novel receptor ligands, compounds for inhibiting ligand recognition, or involvement in pathology. However, the lack of crystal structure, endogenous binding ligands like substance P, and exogenous ligands such as cationic agonists or opioids have been discovered [279][280][332]. MRGPRX2-triggered degranulation depends on Gαi, Gαq, Ca^{2+} channels, ERK, and PI3K [272][319]. Cdc42 GTPase controls cytoskeleton dynamics, and Myo1f, an atypical type I myosin, participates in granule exocytosis and mitochondria fission [307]. Finally, β1 and β2 arrestins regulate the kinetics and the extent of MRGPRX2 cellular activity [308].

5.2.1 MRGPRX2 activation increases the translocation of LysRS to the nuclei

The present study adds more insights into MRGPRX2 signals and shows the involvement of the LysRS-MITF pathway. This research showed that MRGPRX2 rapidly and briefly enhances ERK phosphorylation. Our findings are consistent with evidence demonstrating that ERK1/2 is quickly phosphorylated in skin mast cells

following MRGPRX2 and signals faster than FcεRI [306]. The LysRS recruitment in the nucleus depends on MAP kinase signaling, as reported by FcεRI so it is conceivable that the LysRS translocation is due to MAPK kinase activation after MRGPRX2 triggering [132].

5.2.2 MRGPRX2 activation increases MITF phosphorylation and activity

Additionally, the MRGPRX2 signaling boosts the phosphorylation of MITF (Ser 73). MITF transcriptional activity is mostly post-translationally controlled by phosphorylation. Some studies in melanocytes have demonstrated that ERK1/2 phosphorylates MITF at Ser73, p90 ribosomal S6 kinase (p90RSK) at Ser409, GSK3 at Ser298 and p38 MAPK at Ser307 [126][357][358]. The MAPK-mediated phosphorylation of MITF at Ser73 combines the two coactivators E1A binding protein p300 and cAMP-response element binding protein (p300/CBP), which jointly promote MITF transcriptional activity [311]. However, phosphorylation at Ser73 and Ser409 both support the ubiquitination and proteasome-dependent degradation of the protein [310].

We used the TRPM1-reporter gene assay to detect MITF activity following MRGPRX2 signaling since there are no available phospho-MITF antibodies. Since overexpression of LysRS dramatically enhances even more MITF activity, MITF activity increases in response to SP stimulation due to LysRS translocation to the nucleus. Activation of the MITF is necessary for PGD2 production [161]. Thus, we observed that the increase in MITF activity was associated with an increase in PGD2. Collectively, MITF activity is increased by LysRS translocation to the nucleus as a result of MRGPRX2 interaction with substance P. MITF is phosphorylated as a result of MRGPRX2 signaling, which enhances MITF activity. The boost in MITF activity brought about by LysRS was equivalent to that generated by IgE stimuli.

According to one principle, the nuclear export-import mechanism of MITF involves phosphorylation to regulate the activity of this transcription factor in

melanocytes [312]. Interestingly, after MRGPRX2 activation, we detected an increase in MITF levels in the cytosol. Therefore, it would be plausible for mast cells to experience similar regulatory occurrences. However, more research has to be done on this topic.

Both endogenous and exogenous ligands may apply the LysRS-MITF manner because MRGPRX2 activation with exogenous ligands such as atracurium, vancomycin, morphine, and meglumine amidotrizoate promotes MITF activity.

5.2.3 MITF silencing and inhibition decreases MRGPRX2-dependent mast cell degranulation and calcium influx.

The half-life of MITF is more prolonged than predicted for a transcription factor, and prior studies have demonstrated that selective silencing sequences of MITF are effective after day 5. The cycloheximide tests results of our group showed that MITF protein levels were reduced entirely in a 96-hours. Comparably, using ML329, we see a significant decrease in MITF levels at day 5. According to our research, MRGPRX2-dependent degranulation, the expression of CD63 on the plasma membrane and β-hexosaminidase were reduced when MITF was downregulated by shRNA silencing or ML329 treated. Biogenesis of lysosomes is associated with MITF [313], and bone marrow-derived mast cells from *Mitf* -/- mice display hypogranularity that can be restored with MITF addition [154]. We investigate if the granular content of our MITF-silenced cells is lower than that of control cells. Unfortunately, when comparing MITF-silenced cells to non-target controls, we could not detect any apparent alterations in our Lysotracker stainings. Further research is needed since the kinetics of silencing may take longer than expected to show differences in the preformed granules. Interestingly, MITF downregulation drastically reduced Ca^{2+} influx upon substance P activation. Store-operated Ca^{2+} entry (SOCE), relying on the calcium sensor stromal interaction molecule 1 (STIM 1), controls MRGPRX2-dependent Ca^{2+} [284]. STIM 1 is an endoplasmic reticulum (ER) Ca^{2+} sensor that oligomerizes and activates Orai channels (Orai1/2/3), causing Ca^{2+} influx in response to activation and decreasing ER Ca^{2+} levels

[314]. Indeed, STIM 1 is necessary for promoting the Ca^{2+} influx in FcεRI-mediated mast cell activation and anaphylaxis [286]. Recently reported, MITF regulates STIM 1 and SOCE expression in melanocytes [285]. The MITF-STIM 1 relationship was confirmed by luciferase assays using STIM 1 promoters and chromatin immunoprecipitation (ChIP). Functional assays confirmed MITF regulates STIM 1 expression and activity in primary human melanocytes [285]. Preliminary results in this thesis (annex 2) showed that MITF regulates the calcium sensor, STIM 1, in IgE and MRGPRX2-dependent mast cell activation.

5.2.4 MITF silencing and inhibition decreases the viability of mast cells.

In our research, we found that MITF knockdown reduced cell viability. In fact, MITF downregulation has also been linked to the termination of the mast cell-mediated response, the induction of apoptosis, and the elimination of cells that are aged [315], [316]. Since we found that the SH3BP2 pathway regulates MITF through miR-1246 and miR-5100 in GIST [282]. The human mast cell leukemia cell line (HMC-1) with SH3BP2 silenced was used to validate miR-1246 and miR-5100 using quantitative PCR. Overexpression of miR-1246 and miR-5100 downregulates MITF and affect the viability and cell cycle progression [283]. On the other hand, elevated levels and activity of MITF have been connected to elevated mast cell activity and proliferation.

Our results showed that MITF could specifically regulate IL-8 in mast cells through MRGPRX2 activation. Previous studies showed that IL-8 is essential for recruiting neutrophils and contact with T cells in inflammation response [287]. Some literature showed that IL-8 is associated with skin diseases [288][289], MRGPRX2 is mainly expressed in mast cells in the skin, which may suggest that IL-8 release could be induced with MRGPRX2 mast cell activation. In addition, the release of IL-8 from mast cells causes the epithelial-to-mesenchymal transition and migration and accelerates non-small cell lung cancer growth [290]. And it's a biomarker of melanomas [291]. It indicated that MITF can be a central role in regulating more cytokines which are the keys to the pathology of the disease. Because some cytokines

are more important for differentiation, growth, distribution, recruiting immune cells and involved in immune response. In the future, more deep and wide research studies can be done for understanding the role of MITF.

MITF has a pool in mitochondria that controls proteins independently from its job as a nuclear gene transcription factor. One of the three subunits of the complex enzyme pyruvate dehydrogenase, which catalyzes the conversion of pyruvate to acetyl CoA, connects with MITF [148]. Additionally, mitochondrial MITF controls PDH activity, which is necessary for mast cell degranulation and cytokine production as well as for maintaining glucose homeostasis and serving as a source of mitochondrial ATP. Increased mast cell activity will be explained by MITF activation linked to the phosphorylation of Ser73 by extracellular signals regulated by protein kinase (ERK1/2) activity [149].

Altogether, the LysRS-MITF pathway should be considered in the MRGPRX2 signal, and it is shared with FcεRI. Patients with alterations in this pathway may increase the range of susceptibility to a broad spectrum of substances that can trigger both receptors. Fig B shows the signaling pathway and function of MITF in mast cell activation through MRGPRX2.

Increased knowledge in MRGPRX2 signaling may provide new approaches for upregulating response, which may help treat antibiotic-resistant cutaneous infections or downregulate MRGPRX2 to ameliorate allergic and inflammatory diseases via this receptor.

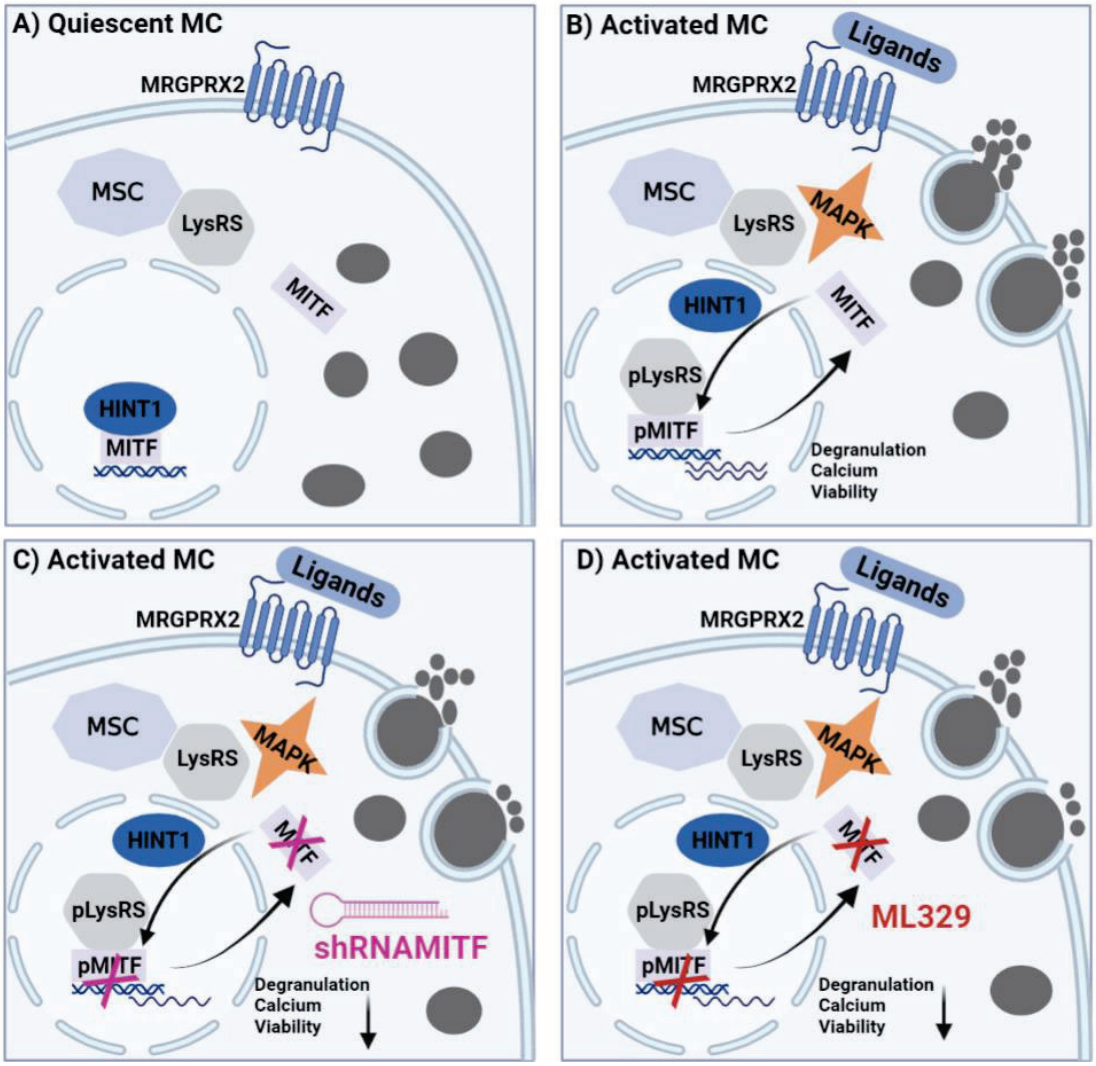


Fig B, MITF after MRGPRX2 dependent mast cell activation. A), In quiescent mast cells, LysRS binds to MSC, and HINT1 inhibits MITF. B), LysRS is phosphorylated in a MAPK-dependent manner after MRGPRX2 activation, which causes it to separate from the MSC and move from the cytoplasm to the nucleus, increasing the activity of MITF and MITF-dependent genes. MITF silencing and inhibition C) and D) decrease degranulation, calcium, and cell viability.

6. Conclusions

1) Our study highlights the important role of the IgE-LysRS-MITF signaling pathway in anaphylaxis. Alterations in this pathway can set the degree of severity of the reaction.

2) The mutation P542R in LysRS, found in a patient with severe anaphylaxis to wasp venom, leads to an exacerbating IgE-dependent mast cell response measured by β-hexosaminidase release and PGD2 synthesis and secretion.

3) Structural analysis revealed that substituting proline with arginine in aminoacid 542 in LysRS alters the binding sites interacting with P38-MSC that allow protein retention in the cytoplasm. Thus, LysRS P542R is found preferentially in the nucleus.

4) Molecular dynamics show that P542R mutation can be found in close and open states (open state resembles the active form LysRS pSer 207), indicating that it may be constitutively active in the nucleus developing its non-canonical function.

5) LysRS P542R increases MITF activity compared to LysRS WT. MITF-dependent targets: *HDC*, *CAM*, *KIT*, and *MCPT9* were enhanced in LysRS P542R compared to LysRS WT. On the other hand, NFAT activity was similar in LysRS P542R and LysRS WT.

6) LysRS G189D mutation is not associated with anaphylaxis. This missense mutation promotes a closed state and does not enhance MITF activity or exacerbated mast cell responses.

7) Down-regulating MITF using selective shRNAS or inhibitors decrease IgE-dependent mast cell degranulation.

8) MRGPRX2 activation through substance P increases MAPK activity and LysRS translocation into the nucleus. Consequently, MITF phosphorylation and activity are

enhanced.

9) Drugs such as vancomycin, atracurium, meglumine amidotrizoate, and morphine which signal through MRGPRX2, increase MITF activity, suggesting that MITF may contribute to adverse drug reactions.

10) MITF knockdown or inhibition expression decreases MRGPRX2-dependent mast cell degranulation, calcium, cell viability, and IL-8 synthesis.

11) Preliminary data shows that MITF may regulate calcium sensor STIM 1, affecting calcium influx in MRGPRX2-dependent mast cell activation

In summary, MITF is essential in IgE and MRGPRX2-dependent mast cell responses (Fig A). Dysregulation of its activity may be on the molecular basis of pathological mast cell-derived diseases.

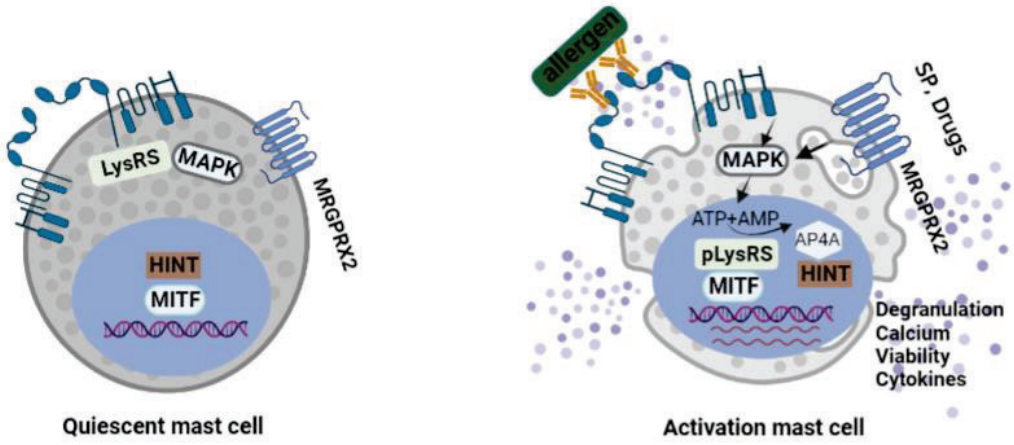


Fig A, MITF has been involved in IgE and MRGPRX2-dependent mast cell activation. In quiescent mast cells, MITF is suppressed by HINT1. After IgE and MRGPRX2-dependent mast cell activation, LysRS goes into the nucleus and induces the activity of MITF, regulating the function of mast cells, degranulation, calcium, and viability.

7. References

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