THE ROLE OF MAST CELLS IN IMMEDIATE HYPERSENSITIVITY REACTIONS

EDITED BY: Marcelo Vivolo Aun, Natalia Blanca Lopez, Mariana C. Castells and Pedro Giavina-Bianchi

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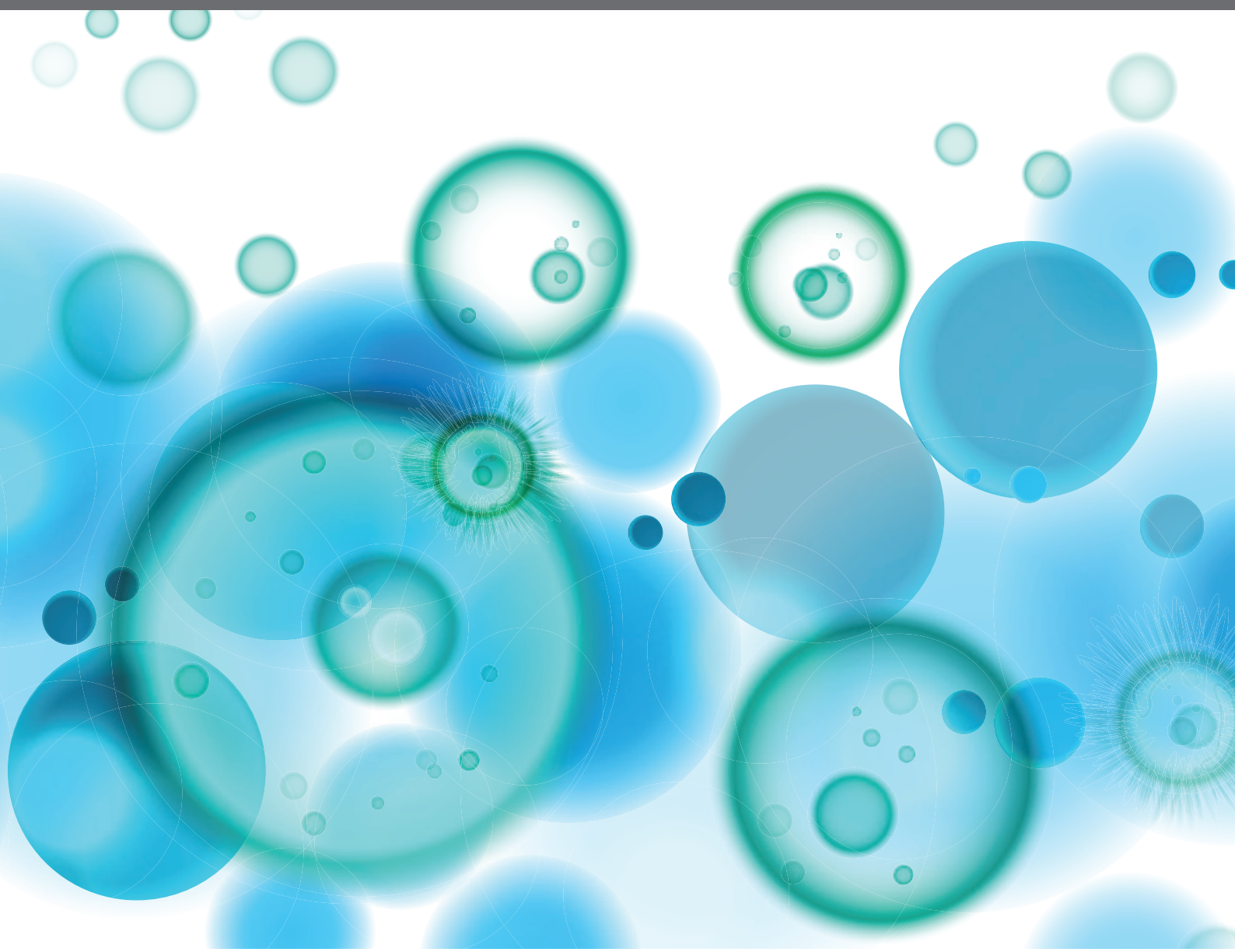

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THE ROLE OF MAST CELLS IN IMMEDIATE HYPERSENSITIVITY REACTIONS

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Table of Contents

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EDITORIAL
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Editorial: The Role of Mast Cells in Immediate Hypersensitivity Reactions

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Editorial on the Research Topic

The Role of Mast Cells in Immediate Hypersensitivity Reactions

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Mast cells are important cells at the cross roads of innate and adaptive immunity. In an interesting review paper about mast cells functions, Krystel-Whittemote described that they are present in all organs systems and thought to play essential roles in the maintenance of many physiological functions as well as in the pathophysiology of many diseases. They originate from pluripotent progenitor cells in the bone marrow, and, under normal conditions, immature mast cell progenitors travel in the blood stream, migrate into peripheral tissues and differentiate into mature mast cells under the influence of stem cell factor and various homing cytokines (1). Typically, mature mast cells do not circulate in the bloodstream.

Allergic diseases are associated with mast cell activation, degranulation, and release of pre-formed mediators, leading to clinical manifestations such as urticaria, angioedema, bronchospasm and anaphylaxis. These symptoms can be induced by many triggers which can induce IgE-and non IgE-mediated reactions, and can occur as the result of an increased mast cell burden, as in mastocytosis (2).

In the current Research Topic of Frontiers in Immunology, articles describing recent advances in mast cell activation disorders are presented, which discuss new pathophysiological mechanisms and emerging therapeutic targets in the management of those conditions.

One important receptors involved in mast cell activation is the Mas-related G-protein-coupled receptor X2 (MRGPRX2), which has been linked to several mast cell-related diseases, such as chronic spontaneous urticaria, atopic dermatitis and asthma. This receptor is expressed by different mast cell subsets and it induces degranulation upon binding by different ligands such as quinolone antibiotics, general anesthetics such as atracuronium and rocuronium and positively charged, hydrophobic molecules such as vancomycin and morphine, as cited in a recent review manuscript by McNeil. (3, 4). Working on *in vitro* cells, Hermans et al. showed that HMC1 cells express the

receptor MRGPRX2, at lower levels compared to LAD2 and HuMC cells and pre-incubating these cells with latrunculin-B leads to overexpression of MRGPRX2, which can be activated by compound 48/80, resulting in efficient HMC1 degranulation. Their findings suggest that HMC1 cells may be used to study mast cell activation through MRGPRX2. Oliveira et al. described that annexin A1, an endogenous 37 KDa glucocorticoid induced monomeric protein, which inhibits MC degranulation in murine models, is capable of interfering with the activation of HMC-1 cells. *In vivo* intraperitoneal administration of AnxA1to wild type and IL-4 knock-out mice reduced mast cell activation, suggesting its potential therapeutic use to reduce the release of MC mediators in inflammatory allergic processes.

Two further studies address new pathways of mast cells inhibition that could be investigated as potential future therapeutic targets. Li et al. describe the orosomucoid-like-3 (ORMDL3) gene, which regulates the endoplasmic reticulum stress (ERS)-induced unfolded protein response (UPR) and autophagy, and show that its protein product can suppresses Ag-mediated mast cell activation *via* an ATF6 UPR-autophagy dependent pathway, attenuating anaphylactic reactions.

In a murine model of active cutaneous anaphylaxis (ACA), Bonamichi-Santos et al. demonstrated that the programmed cell death ligand 1 (PDL-1), which is known for its inhibitory effect on T cell immune response and is expressed on the surface of mast cells, may have a relevant role in allergic diseases. Using a monoclonal antibody anti-PD-L1, the authors showed that PD-L1 blockade during allergen sensitization inhibited the synthesis of specific IgE and IgG1 and decreased mast cell activation. This effect was not observed when anti-PD-L1 was administered before antigen challenge, suggesting that the effect of blocking PD-L1 pathway affects the induction phase of the immune response not its effector phase.

Elst et al. provide further evidence of the functions of the MRGPRX2 using peripheral blood-derived cultured mast cells from healthy donors and drug allergic patients in order to assess mast cell activation and degranulation through MRGPRX2 and after silencing its effect. They show that atracurium, ciprofloxacin, and levofloxacin induced activation and degranulation in primary human mast cells, but only in MRGPRX2-positive and not in MRGPRX2-negative or -silenced mast cells. Sugammadex attenuated the atracurium-induced activation and degranulation of human mast cells through MRGPRX2 by reducing free atracurium levels.

Two different studies addressed cold urticaria and its association with the presence of cryoproteins, such as cryoglobulins and cold agglutinins. In the first article, Bizjak et al. investigated 35 cold urticaria patients and found that 46% of them had a positive cold agglutinin test, while 27% had a positive cryoglobulin test. They demonstrated that a positive cold agglutinin test, but not a positive cryoglobulin test, was associated with a higher rate of reactions triggered by cold weather and by exposure to cold water, aggravated by increased humidity. Patients with a positive cold agglutinin test had a higher frequency of angioedema triggered by ingestion of cold foods or drinks, and lower disease control. Ginter et al. looked for evidence of the association between cryoproteins and cold urticaria. They performed initially a systematic review and identified 14 studies including 1151 cold urticaria patients. The meta-analysis showed a low frequency of cryoproteins in those patients, from 0.7% for cold agglutinins to 3.0% of cryoglobulins. They then performed a retrospective analysis of 293 individuals in a single Center of Reference in a 5 years period and found low frequency of cryoproteins at 4.1%. Finally, they prospectively studied 49 cold urticaria individuals and found a very low frequency of cryoproteins: none with cryoglobulins, none with cryofibrinogens, and 2/46 (4.3%) with cryoagglutinins, who did not have underlying autoimmune or hematological disorders, indicating that the pathogenesis of cold urticaria is independent of cryoproteins.

In a large case series of more than 600 CSU patients, Sauer et al. investigated the relation between IgA and IgE levels and autoimmunity and autoreactivity. They found that lower IgA levels were associated with lower IgE levels, a higher frequency of recurrent angioedema, autoimmunity, and elevated levels of IgE-anti-thyroid peroxidase.

CSU patients have been successfully treated with second generation antihistamines, some of them with higher doses, but refractory CSU is very common in clinical practice. Omalizumab have been considered the first-line medication indicated for patients with CSU who do not respond to a four-fold dose of non-sedating antihistamines (5). Liao et al. described two pregnant women who presented with refractory CSU and were safely and successfully treated with omalizumab. They review the literature and found 11 pregnant women who were safely treated with omalizumab who had resolution of symptoms and successful deliveries.

Barni and colleagues reported a pediatric patient who developed refractory CSU associated with Chron's Disease (CD) and who was safely and successfully treated with omalizumab. She started therapy for CD with azathioprine and mesalazine at the age of 12 without any disease relapse. When she was 17, she began to present wheals and angioedema, which were not controlled with high doses of antihistamines. Omalizumab was added-on to CSU therapy, with a good clinical response and no relapse in CD.

Last but not least the role of mast cell activation in patients with clonal mast cell disorders is provided by Rama et al. who reported an 18-year-old male who presented a long-standing history of atypical urticarial skin lesions, allergic rhinitis, exercise-induced bronchospasm and food-related flushing and anaphylaxis. A diagnosed of systemic mastocytosis was made and successfully treated with antihistamines, montelukast and cromoglycate. Another case is reported by Sarcina et al. who described an 18-month-old female patient with cutaneous mastocytosis who had severe bullous skin eruption 24 hours after the second dose (booster dose) of inactivated-tetravalent influenza vaccine. The reaction was treated with steroids and antihistamines and further vaccination occurred without adverse events with premedication.

Mast cells have a dual physiological and pathological role, maintaining homeostasis of connective tissues, and being involved in many clinical disorders, particularly hypersensitivity reactions. Animal models of immediate hypersensitivity, as well as *in vitro* and *ex vivo* models of human mast cell activation, in addition to investigation of case series and case reports should lead to a better understanding and management of those conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

1. Krystel-Whittemore M, Dileepan KN, Wood JG. Mast Cell: A Multi-Functional Master Cell. *Front Immunol* (2016) 6:620. doi: 10.3389/fimmu.2015.00620
2. Ebo DG, Clarke RC, Mertes PM, Platt PR, Sabato V, Sadleir PHM. Molecular Mechanisms and Pathophysiology of Perioperative Hypersensitivity and Anaphylaxis: A Narrative Review. *Br J Anaesth* (2019) 123:e38–49. doi: 10.1016/j.bja.2019.01.031
3. McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, et al. Identification of a Mast-Cell-Specific Receptor Crucial for Pseudo-Allergic Drug Reactions. *Nature* (2015) 519:237–41. doi: 10.1038/nature14022
4. McNeil BD. MRGPRX2 and Adverse Drug Reactions. *Front Immunol* (2021) 12:676354. doi: 10.3389/fimmu.2021.676354
5. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA^{2}LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73:1393–414. doi: 10.1111/all.13397

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CASE REPORT
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Case Report: Mastocytosis: The Long Road to Diagnosis

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Mastocytosis is a heterogeneous group of disorders characterized by expansion and accumulation of clonal mast cells. Patients mainly present with either cutaneous lesions, anaphylaxis, or both. Its low prevalence and unusual features often hinder its diagnosis for several years. We report the case of an 18-year-old male who was referred to our department with a long-standing history of atypical skin lesions, allergic rhinitis, exercise-induced bronchoconstriction and what was believed to be food-related flushing and anaphylaxis, that was later diagnosed with mastocytosis. This case illustrates the need to consider investigating for mastocytosis when recurrent anaphylaxis is present, especially in the presence of atypical skin lesions, even if normal serum basal tryptase levels and allergic sensitization are present.

Keywords: mastocytosis, anaphylaxis, flushing, food allergy, diagnostics

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INTRODUCTION

Mastocytosis is a heterogeneous group of disorders characterized by expansion and accumulation of clonal mast cells (MC). Patients mainly present with either skin lesions, anaphylaxis or both (1). MC mediator release and associated symptoms often occur in patients both with or without cutaneous involvement (2). Presentation with cutaneous lesions is particularly common among children and predominantly onsets during the first 6 months of life (3). Low prevalence and unusual features of mastocytosis often hinder its diagnosis for several years (4).

Here, we report the case of an 18-year-old male who was referred to our department with a long-standing history of atypical skin lesions, allergic rhinitis, exercise-induced bronchoconstriction and what was believed to be food allergy related flushing and anaphylaxis.

CASE REPORT

An 18-year-old male was referred to a Tertiary Hospital Allergy department due to recurrent anaphylaxis that was attributed to food allergy. There was a history of recurrent wheezing since early infancy, rhinitis and skin lesions on the scalp since the age of 8 months that frequently flared and relapsed. Some of these flares were associated with flushing, angioedema and presyncope. Food allergy was initially thought to be the trigger in some of these episodes. As such, at age 3 the patient was put on a strict avoidance diet and he was prescribed an adrenaline autoinjector and glucocorticoids, on demand. At this age, he also developed skin lesions suggestive of atopic dermatitis. At age 6, the patient started experiencing oral allergy symptoms with peanut and

tree nuts, and throat tightness following balloon filling. This clinical picture originated marked stress and anxiety that required Psychiatry of Childhood and Adolescence follow-up. At age 12, following ingestion of egg/egg containing food, he complained of throat constriction without any other signs and symptoms. A microarray multiple allergen component assay (ImmunoCAP ISAC, Thermo Fisher Scientific, Uppsala, Sweden) was performed, showing polysensitization to food and inhalant allergens—tree nut, peanut, olive and mugwort lipid transfer proteins (LTP), major latex allergen, kiwi thaumatin-like protein, shrimp, timothy grass pollen, groups 1 and 2 allergens of house dust mites (HDM), with a total IgE of 84 kU/L, resulting in even further dietary restrictions. He was advised not to eat apple, kiwi, and peach although no oral food challenges were performed. Also, at age 12, the patient was started on monthly anti-IgE (omalizumab) for "chronic spontaneous urticaria with angioedema" that was maintained for 2 years without any improvement. In fact, he often complained of angioedema and flushing following omalizumab administration. During endoscopic knee surgery at age 17, he reportedly presented with flushing, in spite of five previous uneventful anesthetic procedures (2 inguinal hernioplasties at 2 and 6 months of age, adenoidectomy and tonsillectomy at 9 months, appendicectomy and ankle surgery for apophysitis of the calcaneus at age 11).

At the first appointment at our department, the patient complained of spells of flushing, sometimes with angioedema and/or presyncope that occurred 3–4 times/week. At this time, he had a highly restrictive diet not eating any fresh fruits other than banana, although tolerating strawberries, mango, melon, grapes and citrus. He avoided tree nuts, peanuts, chestnuts, walnuts and despite previous tolerance, as well as apple, pear, kiwi, and olive oil, as indicated by his previous physician. He had also been advised to avoid codfish and shellfish, despite tolerating salmon, hake, sardines, sea bass, swordfish, cat-fish and shrimp. He also reported mild perennial allergic rhinitis symptoms that worsened during pollen season and exercise-induced bronchoconstriction.

Physical examination was unremarkable except for the presence of exuberant widespread keratosis pilaris involving the whole trunk (**Figure 1A**), four scalp lesions and three nodular lesions on the trunk that developed a wheal and flare reaction upon stroking (Darier's sign, **Figure 1B**). Nasal and oropharyngeal inspection, cardiopulmonary auscultation and abdominal inspection were unremarkable. He was submitted to skin prick testing (SPT), with common aeroallergens, nuts, shellfish and oral allergy syndrome specific batteries (*Laboratórios LETIPharma*, Madrid, Spain), complete blood counts, specific IgE (sIgE), and serum basal tryptase measurements, and underwent oral food challenges with apple, pear, and egg. Complete blood counts did not show anemia, leukopenia or leukocytosis, or thrombocytopenia. SPT were positive for HDM and olive tree pollen, while sIgE was positive for HDM (*Dermatophagoides pteronyssinus* 51.5 kU/L, *Lepidoglyphus destructor* 32.3 kU/L), grass mix (21.8 kU/L); olive tree (4.5 kU/L); latex (11.9 kU/L), peanut (0.70 kU/L), kiwi (0.54 kU/L), and tree nut (0.40 kU/L). sIgE for both egg yolk and white, and codfish were negative. Serum basal tryptase ranged between 7 and 9.4 ng/mL, 6 months apart. Spirometry was normal with negative bronchodilation test. All oral food challenges were negative. He was referred to the dermatology department due to the exuberance of the trunk keratosis pilaris and nodules. Skin biopsy of the latter lesions showed a CD30-positive large, round/polygonal, hypergranulated mast cell infiltrate that was compatible with well-differentiated mastocytosis (WDM) (**Figures 1C–E**). The patient was then started on sodium cromoglycate (200 mg, 3id), rupatadine (10 mg, id), and montelukast (10 mg, id) with significant improvement in both frequency and severity of the episodes of flushing and/or angioedema. A topical nasal glucocorticoid

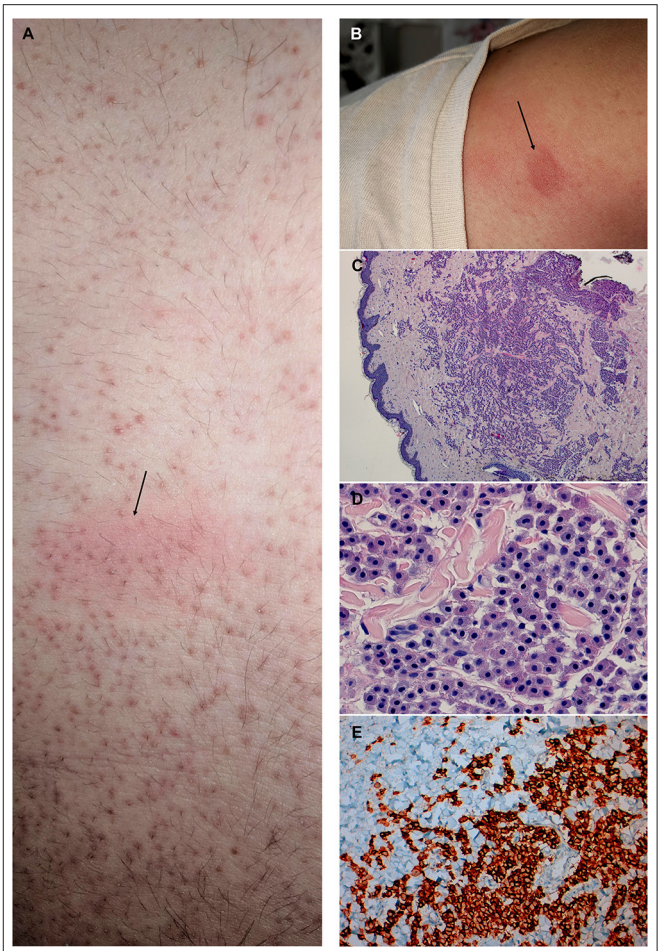

FIGURE 1 | Macroscopic and histologic findings of an 18-year-old male patient with recurrent anaphylaxis, diagnosed with well-differentiated mastocytosis. **(A)** Detail of the trunk depicting exuberant widespread keratosis pilaris and a nodule (arrow) showing a wheal and flare reaction following stroking with a tongue spatula (Darier's sign). **(B)** Supraclavicular nodule showing a wheal and flare reaction following stroking. **(C)** Skin biopsy specimen of the nodule depicted in **(B)**, showing a dense nodular aggregate of mast cell in the reticular dermis, accompanied by variable numbers of interstitial mast cell (hematoxylin and eosin stain, x40 magnification); **(D).** Mast cells with round/polygonal morphology (hematoxylin and eosin stain, x400 magnification). **(E).** Diffuse cytoplasmatic c-kit expression in mast cells (c-kit immunohistochemistry, x100 magnification).

and an on demand/pre-exertion inhaled glucocorticoid/long acting beta agonist fixed association were also prescribed. He also resumed the ingestion of egg, apple and pear with tolerance. The patient was later submitted to an abdominal ultrasound and bone densitometry which did not show any organomegalies or bone mass changes.

DISCUSSION

This report illustrates the case of a polysensitized young male with food allergy and a delayed diagnosis of mastocytosis, based on a skin biopsy histopathology supporting a well-differentiated mastocytosis. This case underlies the need to investigate for mastocytosis in patients with recurrent anaphylaxis, especially in the presence of longstanding skin lesions, and even with the coexistence of normal tryptase and allergic sensitization.

A recent classification categorizes mastocytosis in the skin into maculopapular cutaneous mastocytosis (CM) which also includes plaque and nodular forms, cutaneous mastocytoma and diffuse CM (5). Maculopapular CM has been subcategorized into monomorphic, formerly known as *urticaria pigmentosa*, and polymorphic variants (5). While monomorphic cutaneous involvement is easily recognized by experienced clinicians, that may not be the case for the polymorphic variants. The latter always onsets during childhood (6), presenting with lesions of varying sizes and asymmetric distribution throughout the cutaneous tegument and often involves the head and neck, namely the face and scalp, in contrast with monomorphic forms (5). Flushing is the most frequent MC mediator release related symptom (7). This form is mostly associated with low tryptase, KIT mutations involving exons other than 17 and displays distinct histopathological features, namely large, round hypergranulated MC that express CD30, having been called well-differentiated mastocytosis (7). These patients usually undergo partial or total remission during puberty/adolescence (8) but may recur during adulthood with aggressive systemic mastocytosis (SM) (9, 10). Our case displays a polymorphic maculopapular cutaneous mastocytosis, displaying a typical histopathology of WDM (hypergranulated, round MC) that underwent partial remission of skin lesions while maintaining significant MC mediator release related symptoms into adulthood.

In contrast with the general population, the most frequent causes for anaphylaxis among mastocytosis patients are Hymenoptera venom and idiopathic causes with foods triggering only 6 to 8% of anaphlaxis (11). However, among children with mastocytosis, the prevalence of food-induced anaphylaxis may reach 20% (12) to 33% (13), only surpassed by idiopathic anaphylaxis with 60% (12) to 67% (13). Our patient displayed MC mediator release symptoms that were thought to be related to food ingestion, namely egg and tree nuts and he was sensitized to LTP, thaumatin-like protein, tree nuts, peanuts, shrimp, and codfish. Oral food challenges were negative for fresh fruits containing LTP and egg. However, tree nuts and peanuts induced reproducible oral allergy syndrome manifestations that may be mediated by LTP. This case illustrates that oral food challenges are not only safe but indicated in mastocytosis patients, with suspected food allergy.

Even when partial remission occurs, persistence of mastocytosis in the skin and MC mediator release-related symptoms into adulthood mandates a bone marrow (BM) study, in order to assess systemic involvement (14). Proposed criteria for the diagnosis of well-differentiated systemic mastocytosis (WDSM) include one major criterion that is shared with the remaining forms of SM (i.e., MC aggregates in the bone marrow) and four minor criteria—expression of CD30 and/or overexpression of carboxypeptidase A through flow cytometry; clustering of BM MCs outside BM particles forming groups of $\geq$ 2 MC; presence of mutations involving any codon of KIT or a clonal human androgen receptor assay (HUMARA) pattern; association of female sex with either pediatric disease onset or familial aggregation in adults (7). Diagnosis of WDSM is established in the presence of the major criterion and one minor, or three minor criteria (7). As such, BM flow cytometric evaluation should include assessment of BM MC expression of, at least, CD25/CD2 (generally not present in WDSM), CD30 and carboxypeptidase A (7, 15). KIT mutational analysis should be performed, starting with D816V KIT mutation, followed by KIT sequencing, if negative (16). If both D816V and sequencing are negative, clonality should be assessed through HUMARA (7). In this case, BM study is planned, although it has not yet been performed due to constraints related to the COVID19 pandemic. The term mastocytosis in the skin has been used to differentiate patients with skin biopsy compatible with mastocytosis that have not undergone BM studies (17). Our patient has morphological and immunophenotipical features that are compatible with well-differentiated cutaneous mastocytosis (7). As such, the patient may be provisionally classified as having a well-differentiated mastocytosis in the skin.

Literature on the efficacy of omalizumab in mastocytosis is still scarce (18) especially when it comes to cutaneous mastocytosis in children (19). This monoclonal antibody binds free IgE, preventing linkage and subsequent activation of its high affinity receptor (FcεRI) expressed by MC and basophils (20). Moreover, it seems to downregulate the expression of FcεRI receptors which are thought to be also involved in MC survival and non-immune activation (21). While it has been shown to be effective in the prevention of MC mediator release symptoms in both mastocytosis (19, 22–25) and food allergy (26, 27), safety concerns have been raised by the Food and Drug Administration (FDA) following reports of anaphylaxis caused by omalizumab, later sustained by several studies (28). Prevalence of such reactions was shown to reach 0.2% among patients with asthma treated with this monoclonal antibody (29). Allergy to its excipient, polysorbate (30) and activation of MC FcγRI by IgE-omalizumab immune complexes (31) have been proposed as potential causes for such reactions. While its efficacy seems to be promising in adults (18), reports of allergic reactions among mastocytosis patients, such as this case, are scarce.

Significant delays in the diagnosis of mastocytosis are infrequently reported. However, delays may be prominent in adults, averaging 6.5 to 8.1 years (4, 32) but reaching up to 50 years (4). Years lost to diagnosis are particularly important in cases without cutaneous involvement, especially in those suffering from an aggressive form of the disease (33). Nonetheless, this may also occur in patients presenting with skin lesions and it may severely impair patients' quality of life or carry more dire consequences, such as organ dysfunction and death (in advanced forms), life-threatening anaphylaxis, or pathological bone fractures from severe osteoporosis (4, 34). Delays in diagnosis may result from the use of insensitive techniques (34), a misguided belief that normal basal tryptase values preclude mastocytosis (35), or may be due to atypical symptoms (32). While skin lesions were atypical in this case, frequent spells of flushing, angioedema and presyncope should have raised the suspicion sooner, even in face of normal basal tryptase values and significant allergic disease.

CONCLUSIONS

This case underlines the relevance of considering the diagnosis of mastocytosis in the presence of recurrent anaphylaxis, while stressing the need of a thorough skin examination for mastocytosis skin lesions.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

TR conceived and designed the case report, contributed to the clinical and pathology diagnosis, collected all data and wrote the manuscript. DM and JP contributed to the pathology diagnosis, immunohistochemistry, and its photographic material. NG and AN contributed to the dermatologic evaluation and skin biopsies. LD supervised the conception, analysis, design of the work and manuscript drafting. JP and AC consulted the patient and supervised oral food challenges. All authors critically revised the manuscript for important intellectual content, provided approval of the final version and agreed to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of all parts of the work are appropriately investigated and resolved.

REFERENCES

1. Horny H-P, Akin C, Arber DA, Peterson LC, Tefferi A, Metcalfe DD, et al. Mastocytosis. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, editors. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Lyon: IARC Press (2017). 61–9.
2. Azaña JM, Torrelo A, Matito A. Update on mastocytosis (Part 1): pathophysiology, clinical features, and diagnosis. *Actas Dermo-Sifiliográficas (English Edition)*. (2016) 107:5–14. doi: 10.1016/j.adengl.2015.11.001
3. Alvarez-Twose I, Carter MC. Pediatric mastocytosis. In: Akin C, editor. *Mastocytosis*. Cham: Springer (2020) p. 93-114. doi: 10.1007/978-3-030-27820-5_6
4. Jennings S, Russell N, Jennings B, Slee V, Sterling L, Castells M, et al. The Mastocytosis Society survey on mast cell disorders: patient experiences and perceptions. *J Allergy Clin Immunol Pract*. (2014) 2:70–6. doi: 10.1016/j.jaip.2013.09.004
5. Hartmann K, Escribano L, Grattan C, Brockow K, Carter MC, Alvarez-Twose I, et al. Cutaneous manifestations in patients with mastocytosis: Consensus report of the European Competence Network on Mastocytosis; the American Academy of Allergy, Asthma & Immunology; and the European Academy of Allergology and Clinical Immunology. *J Allergy Clin Immunol.* (2016) 137:35–45. doi: 10.1016/j.jaci.2015.08.034
6. Thomas Z-RM, Hartmann K. Skin disease in mastocytosis. *Mastocytosis*. (2020)2020:69-91. doi: 10.1007/978-3-030-27820-5_5
7. Alvarez-Twose I, Jara-Acevedo M, Morgado JM, Garcia-Montero A, Sanchez-Munoz L, Teodosio C, et al. Clinical, immunophenotypic, and molecular characteristics of well-differentiated systemic mastocytosis. *J Allergy Clin Immunol.* (2016) 137:168–78 e1. doi: 10.1016/j.jaci.2015.05.008
8. Wiechers T, Rabenhorst A, Schick T, Preussner LM, Forster A, Valent P, et al. Large maculopapular cutaneous lesions are associated with favorable outcome in childhood-onset mastocytosis. *J Allergy Clin Immunol.* (2015) 136:1581–90 e3. doi: 10.1016/j.jaci.2015.05.034
9. Valent P, Sotlar K, Horny HP. Aberrant expression of CD30 in aggressive systemic mastocytosis and mast cell leukemia: a differential diagnosis to consider in aggressive hematopoietic CD30-positive neoplasms. *Leuk Lymphoma*. (2011) 52:740–4. doi: 10.3109/10428194.2010.550072
10. Morgado JM, Perbellini O, Johnson RC, Teodosio C, Matito A, Alvarez-Twose I, et al. CD30 expression by bone marrow mast cells from different diagnostic variants of systemic mastocytosis. *Histopathology*. (2013) 63:780–7. doi: 10.1111/his.12221
11. Matito A, Alvarez-Twose I, Morgado JM, Sanchez-Munoz L, Orfao A, Escribano L. Anaphylaxis as a clinical manifestation of clonal mast cell disorders. *Curr Allergy Asthma Rep*. (2014) 14:450. doi: 10.1007/s11882-014-0450-8
12. Brockow K, Jofer C, Behrendt H, Ring J. Anaphylaxis in patients with mastocytosis: a study on history, clinical features and risk factors in 120 patients. *Allergy*. (2008) 63:226–32. doi: 10.1111/j.1398-9995.2007.01569.x
13. González de Olano D, de la Hoz Caballer B, Núñez López R, Sánchez Muñoz L, Cuevas Agustín M, Diéguez MC, et al. Prevalence of allergy and anaphylactic symptoms in 210 adult and pediatric patients with mastocytosis in Spain: a study of the Spanish network on mastocytosis (REMA). *Clin Exp Allergy*. (2007) 37:1547–55. doi: 10.1111/j.1365-2222.2007.02804.x
14. Valent P, Escribano L, Broesby-Olsen S, Hartmann K, Grattan C, Brockow K, et al. Proposed diagnostic algorithm for patients with suspected mastocytosis: a proposal of the European Competence Network on Mastocytosis. *Allergy*. (2014) 69:1267–74. doi: 10.1111/all.12436
15. Morgado JM, Sanchez-Munoz L, Teodosio C, Mora LME. Identification and immunophenotypic characterization of normal and pathological mast cells. *Methods Mol Biol.* (2020) 2163:331–53. doi: 10.1007/978-1-0716-0696-4_27
16. Arock M, Sotlar K, Akin C, Broesby-Olsen S, Hoermann G, Escribano L, et al. KIT mutation analysis in mast cell neoplasms: recommendations of the European Competence Network on Mastocytosis. *Leukemia.* (2015) 29:1223–32. doi: 10.1038/leu.2015.24

17. Valent P, Akin C, Escribano L, Födinger M, Hartmann K, Brockow K, et al. Standards and standardization in mastocytosis: consensus statements on diagnostics, treatment recommendations and response criteria. *Eur J Clin Invest.* (2007) 37:435–53. doi: 10.1111/j.1365-2362.2007.01807.x
18. Jendoubi F, Gaudenzio N, Gallini A, Negretto M, Paul C, Bulai Livideanu C. Omalizumab in the treatment of adult patients with mastocytosis: a systematic review. *Clin Exp Allergy.* (2020) 50:654–61. doi: 10.1111/cea.13592
19. Hughes JDM, Olynyc T, Chapdelaine H, Segal L, Miedzybrodzki B, Ben-Shoshan M. Effective management of severe cutaneous mastocytosis in young children with omalizumab (Xolair(R)). *Clin Exp Dermatol.* (2018) 43:573–6. doi: 10.1111/ced.13408
20. Brownell J, Casale TB. Anti-IgE therapy. *Immunol Allergy Clin North Am.* (2004) 24:551–68, v. doi: 10.1016/j.iac.2004.06.002
21. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol.* (2002) 2:773–86. doi: 10.1038/nri914
22. Broesby-Olsen S, Vestergaard H, Mortz CG, Jensen B, Havelund T, Hermann AP, et al. Omalizumab prevents anaphylaxis and improves symptoms in systemic mastocytosis: Efficacy and safety observations. *Allergy.* (2018) 73:230–8. doi: 10.1111/all.13237
23. Matito A, Blazquez-Goni C, Morgado JM, Alvarez-Twose I, Mollejo M, Sanchez-Munoz L, et al. Short-term omalizumab treatment in an adolescent with cutaneous mastocytosis. *Ann Allergy Asthma Immunol.* (2013) 111:425–6. doi: 10.1016/j.anai.2013.08.014
24. Douglass JA, Carroll K, Voskamp A, Bourke P, Wei A, O'Hehir RE. Omalizumab is effective in treating systemic mastocytosis in a nonatopic patient. *Allergy.* (2009) 65:926–7. doi: 10.1111/j.1398-9995.2009.02259.x
25. Carter MC, Robyn JA, Bressler PB, Walker JC, Shapiro GG, Metcalfe DD. Omalizumab for the treatment of unprovoked anaphylaxis in patients with systemic mastocytosis. *J Allergy Clin Immunol.* (2007) 119:1550–1. doi: 10.1016/j.jaci.2007.03.032
26. Nadeau KC, Schneider LC, Hoyte L, Borras I, Umetsu DT. Rapid oral desensitization in combination with omalizumab therapy in patients with cow's milk allergy. *J Allergy Clin Immunol.* (2011) 127:1622–4. doi: 10.1016/j.jaci.2011.04.009
27. Andorf S, Purington N, Block WM, Long AJ, Tupa D, Brittain E, et al. Anti-IgE treatment with oral immunotherapy in multifood allergic participants: a double-blind, randomised, controlled trial. *Lancet Gastroenterol Hepatol.* (2018) 3:85–94. doi: 10.1016/S2468-1253(17)30392-8
28. Lieberman PL, Jones I, Rajwanshi R, Rosen K, Umetsu DT. Anaphylaxis associated with omalizumab administration: Risk factors and patient characteristics. *J Allergy Clin Immunol.* (2017) 140:1734–6 e4. doi: 10.1016/j.jaci.2017.07.013
29. Limb SL, Starke PR, Lee CE, Chowdhury BA. Delayed onset and protracted progression of anaphylaxis after omalizumab administration in patients with asthma. *J Allergy Clin Immunol.* (2007) 120:1378–81. doi: 10.1016/j.jaci.2007.09.022
30. Perino E, Freymond N, Devouassoux G, Nicolas JF, Berard F. Xolair-induced recurrent anaphylaxis through sensitization to the excipient polysorbate. *Ann Allergy Asthma Immunol.* (2018) 120:664–6. doi: 10.1016/j.anai.2018.02.018
31. Balbino B, Herviou P, Godon O, Stackowicz J, Goff OR, Iannascoli B, et al. The anti-IgE mAb omalizumab induces adverse reactions by engaging Fcgamma receptors. *J Clin Invest.* (2020) 130:1330–5. doi: 10.1172/JCI129697
32. Hermans MAW, Rietveld MJA, van Laar JAM, Dalm V, Verburg M, Pasmans S, et al. Systemic mastocytosis: A cohort study on clinical characteristics of 136 patients in a large tertiary centre. *Eur J Intern Med.* (2016) 30:25–30. doi: 10.1016/j.ejim.2016.01.005
33. Butterfield JH. Systemic mastocytosis: clinical manifestations and differential diagnosis. *Immunol Allergy Clin North Am.* (2006) 26:487–513. doi: 10.1016/j.iac.2006.05.006
34. Sanchez-Munoz L, Morgado JM, Alvarez-Twose I, Matito A, Garcia-Montero AC, Teodosio C, et al. Diagnosis and classification of mastocytosis in non-specialized versus reference centres: a Spanish Network on Mastocytosis (REMA) study on 122 patients. *Br J Haematol.* (2016) 172:56–63. doi: 10.1111/bjh.13789
35. Hermans MAW, Schreurs MWJ, van Daele PLA. Systemic mastocytosis with normal serum tryptase: rule or exception? *J Eur Acad Dermatol Venereol.* (2019) 33:e472–e4. doi: 10.1111/jdv.15800

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ORMDL3 Functions as a Negative Regulator of Antigen-Mediated Mast Cell Activation *via* an ATF6-UPR-Autophagy–Dependent Pathway

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Antigen (Ag)-mediated mast cell activation plays a critical role in the immunopathology of IgE-dependent allergic diseases. Restraining the signaling cascade that regulates the release of mast cell-derived inflammatory mediators is an attractive therapeutic strategy to treat allergic diseases. Orosomucoid-like-3 (ORMDL3) regulates the endoplasmic reticulum stress (ERS)-induced unfolded protein response (UPR) and autophagy. Although ERS/UPR/autophagy pathway is crucial in Ag-induced mast cell activation, it is unknown whether ORMDL3 regulates the ERS/UPR/autophagy pathway during mast cell activation. In this study, we found that ORMDL3 expression was downregulated in Ag-activated MC/9 cells. Overexpression of ORMDL3 significantly inhibited degranulation, and cytokine/chemokine production, while the opposite effect was observed with ORMDL3 knockdown in MC/9 cells. Importantly, ORMDL3 overexpression upregulated mediators of ERS-UPR (SERCA2b, ATF6) and autophagy (Beclin 1 and LC3BII). Knockdown of ATF6 and/or inhibition of autophagy reversed the decreased degranulation and cytokine/chemokine expression caused by ORMDL3 overexpression. Moreover, *in vivo* knockdown of ORMDL3 and/or ATF6 enhanced passive cutaneous anaphylaxis (PCA) reactions in mouse ears. These data indicate that ORMDL3 suppresses Ag-mediated mast cell activation *via* an ATF6 UPR-autophagy dependent pathway and thus, attenuates anaphylactic reaction. This highlights a potential mechanism to intervene in mast cell mediated diseases.

Keywords: orosomucoid-like 3, mast cell activation, degranulation, activating transcription factor 6, autophagy, passive cutaneous anaphylaxis

INTRODUCTION

Mast cells are the key effector cells inducing immunoglobulin E (IgE)-mediated inflammatory responses to allergens in sensitized individuals (1). Mast cells express high-affinity FcεRI which binds to antigen (Ag)-specific IgE resulting in mast cell sensitization. Upon subsequent exposure to the specific antigen, these sensitized mast cells undergo degranulation and release histamine and lipid mediators (prostaglandins, leukotrienes) followed by a diverse range of cytokines and chemokines (2). These inflammatory mediators trigger acute allergic reactions as observed in allergic disorders, such as allergic asthma, atopic dermatitis, allergic rhinitis, and life-threatening anaphylaxis (3). Hence, a better understanding of the regulatory mechanisms of mast cell activation and subsequent release of inflammatory mediators and how this can be restrained to restore homeostasis is critical for the identification of novel therapeutic targets to treat mast cell mediated diseases.

Orosomucoid-like 3 (*ORMDL3*) gene was first identified in 2007 as an asthma risk gene (4). To date, a number of studies have been performed to investigate the molecular mechanisms by which ORMDL3 contributes to the pathogenesis of asthma (5–7). Airway epithelial cells overexpressing ORMDL3 showed increased transcriptions of genes encoding matrix metalloproteinase, chemokine and CXC chemokine (IL-8, CXCL-10 and CXCL-11), oligoadenylate synthetases (OAS), and ATF6 (8). Overexpression of ORMDL3 in bone marrow-derived eosinophils causes increased rolling, distinct cytoskeletal rearrangement and nuclear translocation of nuclear factor kappa B. Knockdown of ORMDL3 significantly inhibits activation-induced eosinophils shape changes, adhesion and recruitment to sites of inflammation *in vivo* (9). In yeast ORMDL proteins control sphingolipid biosynthesis by regulating the bioactivity of serine palmitoyl transferase (SPT), the rate-limiting enzyme of *de novo* pathway (10). However, the regulatory role of mammals ORMDL proteins in lipid metabolism appears to be much more complicated. Kiefer and his colleagues demonstrated that mammalian SPT activity seems to be affected only when simultaneously enhancing the expression of ORMDL1, 2, and 3 while solo manipulation of any member had no effect (11–13). As an endoplasmic reticulum (ER)-resident transmembrane protein, ORMDL3 also regulates ER stress (ERS) and unfolded protein response (UPR) (10, 14). UPR is comprised of three major signaling pathways, which are initiated by the activation of three protein sensors—activating transcription factor 6 (ATF6), inositol —requiring enzyme 1α (IRE1α) and PKR—like ER kinase (PERK). All three arms of UPR regulate autophagy (15–19). Multiple studies have attempted to uncover the physiological role of ORMDL3 in the cells involved in allergic asthma including airway epithelial cells, eosinophils, macrophages and B cells (8, 9, 20, 21). ORMDL3 specifically binds to and inhibits the sarcoendoplasmic reticulum calcium ATPase (SERCA) 2b resulting in reduction of ER Ca^{2+} concentration and activation of ERS-induced UPR signaling in HEK293 cells (12, 22). Conversely, ORMDL3 has been shown to increase ATF6α level and subsequent induction of SERCA2b expression in human bronchial epithelial cells (BEC) (8), suggesting ORMDL3 mediated ERS-UPR response is cell-specific.

ORMDL3 negatively regulates mast cell activation (23), with ORMDL3 expression found to be lower in Ag-activated mast cells, without affecting the degranulation process. However, the molecular mechanism by which ORMDL3 regulates mast cell function remains largely unknown. The high secretory demand of mast cells is largely dependent on a well-developed ER and, consequently, UPR signal (24, 25). Activation of mast cells initiates the onset of dramatic Ca^{2+} mobilization and triggers degranulation (26, 27). Autophagy, a regulatory process of removing and degrading malfunctioning proteins and organelles, and pathogens (28), is also critical for the degranulation of mast cells. Bone marrow-derived mast cells (BMMCs) deficient in the autophagy related gene (Atg)-7 exhibit normal granule formation, but defective IgE-mediated degranulation demonstrating the importance of autophagic machinery in granule movement and release (29). Given the role of ORMDL3 in ERS-UPR and autophagy in different immune/non-immune cells and the requirement of ERS-UPR and autophagy in mast cell degranulation, we hypothesized that ORMDL3 induces ERS-UPR as well as autophagy in mast cells and thus, ORMDL3 regulates mast cell degranulation and cytokine/chemokine responses.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies against ORMDL3, ATF6, XBP1, p-eIF2α, SERCA2 ATPase, LC3B, and Beclin 1 were purchased from Abcam (Cambridge, MA, USA). FITC-Concanavalin A was obtained from MKbio (Shanghai, China). 3-MA was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture and Treatment

The MC/9 mouse mast cells (ATCC CRL-8306) were cultured in DMEM supplemented with 10% FBS, 0.05 mM 2-mercaptoethanol, 0.1 mM MEM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml recombinant murine IL-3 and 10 ng/ml recombinant murine SCF at 37°C with 5% CO_2. To inhibit autophagy, cells were serum starved overnight, then treated with 3-MA (Sigma-Aldrich, USA) at indicated concentrations for 24 h. LC3B expression was measured in cell lysates by western blot to confirm the inhibitory effect of 3-MA.

Vector Construction

To construct the overexpression vector of ORMDL3, mouse *ORMDL3* gene coding sequence was synthesized according to the gene sequence (NM_025661) in the GenBank and inserted into the vector pLenti-GFP-IRES (provided by Novobio Shanghai, China) *via* NheI and AscI restriction endonuclease sites. To generate the knockdown vector of ORMDL3, shRNA was prepared by synthesizing and annealing two oligonucleotides (Forward primer 5'-CACCGCCAAGTATGACCA AGTCCATTCGA AAATGGACTTGGTCATACTTGG-3' and Reverse complementary primer 5'-AAAACCAAGTATG ACCAAGTCCATTTTCGAATGGACTTGGTCATACTTGGC-3') and then cloned into the vector pLenti-U6-shRNA-GFP (provided by Novobio Shanghai, China) *via* two BsmBI sites.

The knockdown vector of ATF6 was constructed by using designed shRNA oligonucleotides (Forward primer 5'-CCG GGCACTTTGATGCAGCACATGACGAATCATGTGCTGCA TCAAAGTGCTTTTT-3' and Reverse complementary primer 5'-AATTAAAAAGCACTTTGATGCAGCACATGATTCGT CATGT GCTGCATCAAAGTGC-3') which were then inserted into an inducible knockdown system pLKO-Tet-On (Addgene 21915) *via* AgeI and EcoRI sites. All the constructs were verified by Sanger sequencing.

Virus Like Particles Production

HEK293T cells were seeded in a 10-cm dish (5×10^6 cells) 1 day before transfection. The vectors of ORMDL3 overexpression, ORMDL3-shRNA and ATF6-shRNA were respectively transfected into HEK293T cells together with lentiviral packaging plasmids pSPAX2 (Addgene 12260) and pMD2G (Addgene 12259) at a ratio of 10 μg: 10 μg: 5 μg by using linear polyethylenimine (PEI). Six hours after transfection, media was changed to fresh DMEM with 5% FBS plus Pen/Strep. Forty-eight hours after transfection, VLPs in the supernatant were harvested by filtration with a sterile 0.45 μm filter and stored in -80°C. VLPs were quantified by using CAp24 ELISA.

Cell Transduction

MC/9 cells were plated at a density of $2 \times 10_4$ cells per 35 mm plate and transduced with VLPs (equivalent to 50 ng of CAp24) conveying ORMDL3, ORMDL3-shRNA or ATF6-shRNA with Polybrene at a concentration of 8 μg/ml. Transduced cells were selected with 2.5 μg/ml blasticidin S (Sigma-Aldrich, MO, USA). To induce varying levels of ATF6 downregulation, 10 ng/ml or 100 ng/ml of doxycycline was added in the cell culture. The transduction results were obtained using fluorescence microscope 48 h post-transduction. The transduction efficiency was assessed by measuring mRNA levels using qRT-PCR and protein expression using Western blot.

Assessment of Mast Cell Degranulation

MC/9 cells were sensitized overnight with 1 μg/ml of anti-DNP mouse IgE (SPE-7 monoclonal, Sigma, St. Louis, USA) and then washed three times with PBS. Cells were stimulated with the indicated concentrations of DNP–BSA (Santa Cruz, CA, USA) for 30 min. The supernatants were collected and cell pellets were lysed with 0.5% Triton-X 100 at 37°C for 30 min. Commercial ELISA kits were used to detect the concentrations of histamine (Elabscience, Wuhan, China), β-glucuronidase (Elabscience, Wuhan, China) and tryptase (Cusabio, Wuhan, China) in both supernatant and cell lysates. The percentage released was calculated using the following formula: release (%) = $[S/(S + L)] \times 100$, where S and L refer to the concentrations in supernatant and cell lysate, respectively.

Quantitative Real-Time PCR

Total RNA was isolated from cells using TRIZOL reagent following manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. qRT-PCR reaction was performed using the ChamQ SYBR qPCR Master Mix (Vazyme, China) on a CFX96™ Real-Time System. The data was normalized to the level of β-actin gene expression in the cell samples and calculated as a fold change of the corresponding control. All qRT-PCR primer sequences are listed in **Supplementary Table 1**.

Western Blot Analysis

Total protein (30 μg) was separated from cell lysates by SDS-PAGE at 100 V for 90 min and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). After blocking with 5% non-fat milk in TBS containing 0.01% Tween-20 (TBST) at room temperature for 1 h, the membrane was incubated with primary antibodies diluted at 1:1000 (Antibodies against ORMDL3, ATF6, XBP1, p-eIF2α, SERCA2 ATPase, LC3B, and Beclin 1, Abcam, USA) overnight at 4°C. After washing with TBST, the membrane was incubated with HRP-conjugated secondary antibodies diluted at 1:2000 in 5% non-fat milk at room temperature for 1 h. After washing with TBST three times, immunoreactive bands were detected using an ECL Kit (Thermo Scientific, MA, USA) and visualized using a ChemiDoc MP imager (Bio-Rad, California, USA) according to the manufacturer's instructions. Protein levels were normalized to the amount of GAPDH used as a loading control and to the corresponding controls.

Confocal Microscopy

For ORMDL3, ATF6 and LC3B staining, treated MC/9 cells were first fixed with 4% paraformaldehyde for 30 min and permeabilized using 0.5% Triton X-100 in PBS for 10 min. Non-specific binding was blocked with 10% normal goat serum in PBS and then incubated with anti-ORMDL3 (1:100 dilution; Abcam, USA), anti-ATF6 (1:100 dilution; Abcam, USA) or anti-LC3B (1:100 dilution; Abcam, USA) overnight. Following labeling with corresponding secondary antibodies (1:1000 dilution; iFluor 555 goat anti-rabbit IgG (H+L), AAT Bioquest, CA, USA) for 1 h at room temperature, the cells were counterstained using DAPI (1:10,000 dilution; Sigma, USA) and imaged on a Super-resolution Multiphoton Confocal Microscope (Leica, Germany). For ER staining, cells were incubated with FITC labelled Concanavalin A (5 μg/ml; MKbio, China) at room temperature for 30 min after DAPI.

Passive Cutaneous Anaphylaxis

All experiments and animal care procedures conform to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Shanghai General Hospital (No. 2016KY246). Eight-week-old BALB/c mice were injected into both ears (intradermally) with 20 μl of PBS containing different combinations of ORMDL3 overexpressing VLPs (20 ng of CAp24), ORMDL3 knockdown VLPs (20 ng of CAp24) and ATF6 knockdown VLPs (20 ng of CAp24) as follows: ORMDL3-OE group was given ORMDL3 overexpression VLPs; ORMDL3-KD group was given ORMDL3 knockdown VLPs; ATF6-KD group was given ATF6 knockdown VLPs; ORMDL3-OE + ATF6-KD group was given ORMDL3 overexpression VLPs and ATF6 knockdown VLPs; ORMDL3-KD + ATF6-KD group was given ORMDL3 knockdown VLPs and ATF6 knockdown VLPs. Negative and positive control groups were not given any VLPs. Forty-eight

hours later, 20 µl PBS containing 100 ng anti-DNP IgE (SPE-7 monoclonal, Sigma, St. Louis, USA) was intradermally injected into both ears. The next day, 200 µl PBS containing 10 µg DNP-BSA (Santa Cruz, CA, USA) and 1% Evans blue was injected intravenously, negative control group was given PBS containing Evans blue only. Two hours later, the mice were euthanized and skin areas were photographed. Skin samples were harvested and Evans blue dye was extracted by incubating the samples in 0.5 ml DMSO for 24 h at 37 °C, and optical density (O.D.) was measured at 650 nm. ORMDL3 and ATF6 gene expression were measured by qRT-PCR to confirm the efficiency of overexpression and knockdown strategies.

Statistical Analysis

Data were presented as mean ± standard deviation (SD). One-way ANOVA or two-way ANOVA with a Turkey's multiple comparisons test was applied as appropriate. GraphPad Prism 8.2.1 software (La Jolla, CA, USA) was used for statistical analyses. (NS denotes not significant; * denotes $P < 0.05$; ** denotes $P < 0.01$; *** denotes $P < 0.001$; **** denotes $P < 0.0001$).

RESULTS

ORMDL3 Is Downregulated in Ag-Activated Mast Cells

Firstly, mouse MC/9 mast cells were sensitized with DNP-specific IgE and activated with DNP-BSA at different concentrations. The expression of ORMDL3 in mast cells was measured by qRT-PCR and western blot. Ag-activation dose-dependently suppressed ORMDL3 expression at both mRNA and protein levels in MC/9 cells with the maximum response observed with the challenge of DNP-BSA at 100 ng/ml (**Figures 1A, B**). This was corroborated by an increase in the release of β-glucuronidase, a marker of mast cell degranulation (**Figure 1C**). Reduction in ORMDL3 expression was not associated with increased death of activated mast cells (data not shown). Next, we stained ER using FITC-Concanavalin A and found that ORMDL3 is localized to the ER in MC/9 cells (**Figure 1D**). Importantly, the expression of ORMDL3 was lower in Ag-activated mast cells (**Figure 1D**). Taken together, this data confirms that Ag-activation downregulates ORMDL3 expression.

ORMDL3 Negatively Regulates Degranulation and the Production of Cytokines and Chemokines in Ag-Activated Mast Cells

To further investigate the role of ORMDL3 in the activation of mast cells, we generated MC/9 cells with knockdown or overexpression of ORMDL3. ORMDL3 mRNA was knocked down approximately 70% by ORMDL3 shRNA (ORMDL3-KD) in MC/9 cells compared to the negative control shRNA (ORMDL3-KD-NC) and cell only groups (**Figure 2A**). The knockdown level of ORMDL3 protein was confirmed by western blot analysis. The level of ORMDL3 protein in the knockdown cells reduced by approximately 60% when compared to that of the negative control and cell only groups

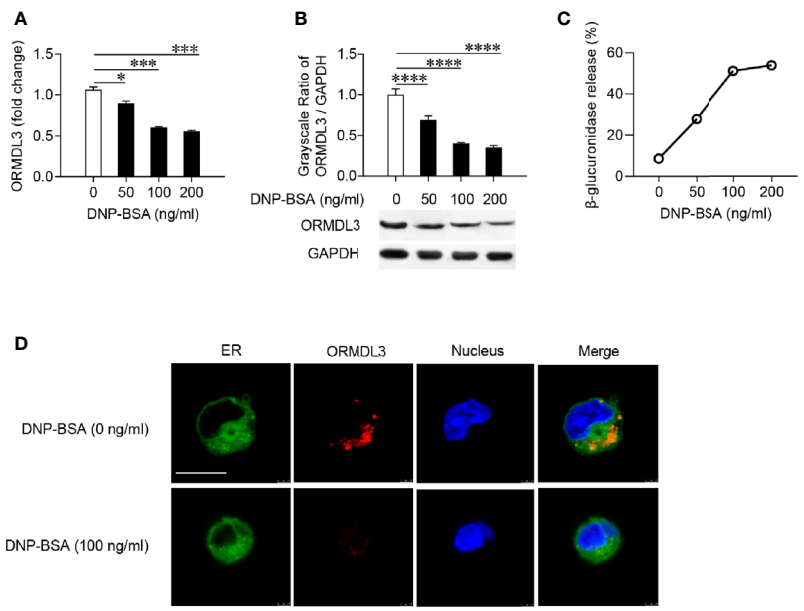

FIGURE 1 | ORMDL3 is downregulated in Ag-activated mast cells. MC/9 cells were sensitized overnight with 1 µg/ml of anti-DNP mouse IgE followed by stimulation with PBS (0 ng/ml DNP-BSA) or indicated concentrations of DNP-BSA for 30 min. **(A)** Expression of ORMDL3 mRNA was measured by qRT-PCR. Data were normalized to the amount of β-actin and calculated as a fold change of the non-stimulated group. **(B)** Expression of ORMDL3 protein was measured by western blotting. GAPDH served as a loading control. **(C)** The release of β-glucuronidase was determined by ELISA. **(D)** Representative images depict ORMDL3 expression [red, stained with iFluor 555 goat anti-rabbit IgG (H+L)] in MC/9 cells. Endoplasmic reticulum (ER, green) was stained with FITC-Concanavalin A and nuclei (blue) were stained with DAPI. Scale bar 10 µm. All the results are shown as mean ± SDs of three independent experiments. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

as shown in **Figure 2B**. Overexpression of ORMDL3 (ORMDL3-OE) was conducted by stably expressing ORMDL3 with a lentiviral vector containing ORMDL3 cDNA in MC/9 cells. The increase of ORMDL3 expression was checked at both the mRNA (**Figure 2A**) and protein levels (**Figure 2B**). The mRNA level of ORMDL3 was increased ~10-fold when compared to that of cells expressing the empty vector (ORMDL3-OE-NC) and cell only. The protein level of ORMDL3 increased approximately 6-fold when compared to controls.

Using the cell models established above, we assessed the effects of ORMDL3 on Ag-induced degranulation of MC/9 cells, which was evaluated by the release levels of histamine, β-glucuronidase and tryptase. Antigen induction significantly increased degranulation in the cell only and ORMDL3 controls (ORMDL3-OE-NC and ORMDL3-KD-NC) as evidenced by increased release of histamine, β-glucuronidase and tryptase (**Figure 2C**). Importantly, Ag-induced degranulation was significantly down-regulated by the overexpression of ORMDL3 (ORMDL3-OE) and up-regulated by the knockdown of ORMDL3 (ORMDL3-KD; **Figure 2C**). Consistent with the release results, the concentrations of histamine, β-glucuronidase and tryptase were decreased in the supernatant of Ag-activated cells in the ORMDL3-OE group and increased in the ORMDL3-KD group (**Supplementary Figure 1**). This was coupled with an increase of the intracellular concentrations of these markers in ORMDL3-OE cells and a decrease in ORMDL3-KD cells (**Supplementary Figure 1**).

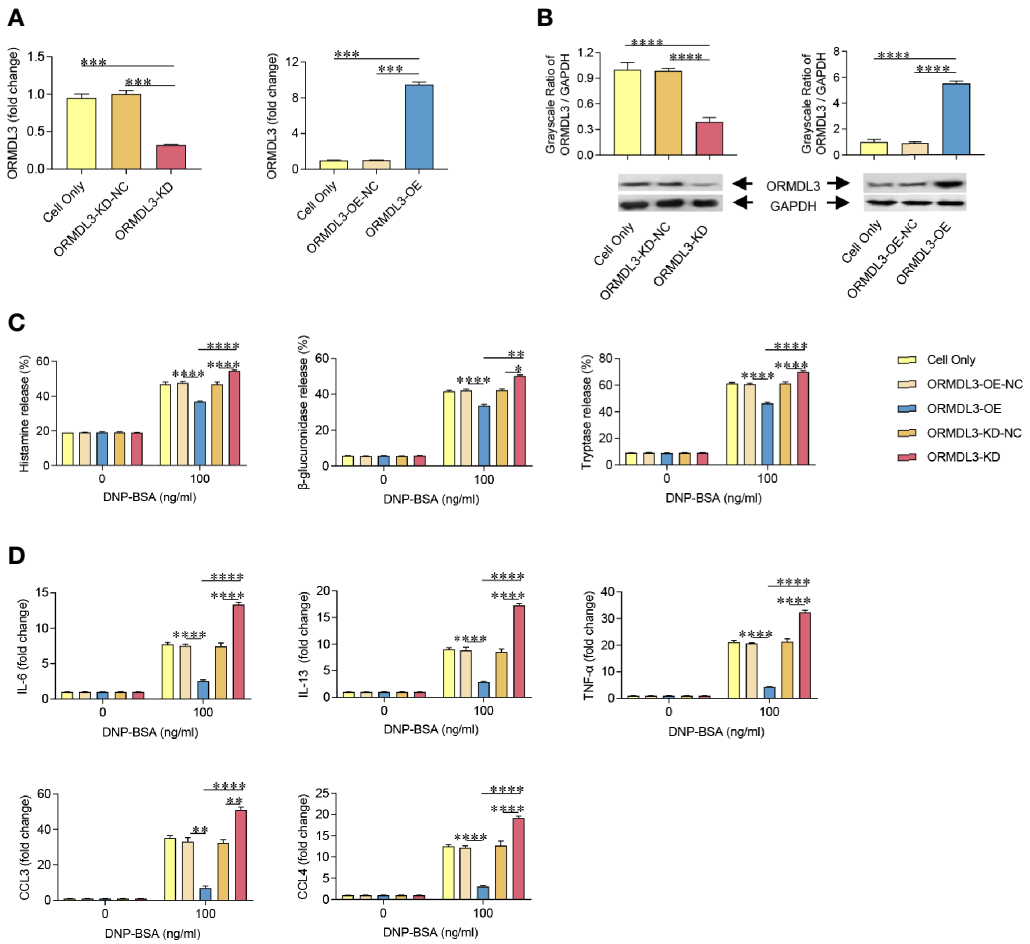

FIGURE 2 | ORMDL3 negatively regulates degranulation and the production of cytokines and chemokines in Ag-activated mast cell. MC/9 cells were transduced with VLPs conveying ORMDL3-shRNA and ORMDL3 and referred to as ORMDL3-KD and ORMDL3-OE respectively. Controls transduced with empty VLPs are referred to as ORMDL3-KD-NC and ORMDL3-OE-NC. **(A, B)** The knockdown and overexpression efficiency were determined by qRT-PCR and western blotting. ORMDL3 mRNA level was normalized to the amount of β-actin and calculated as a fold change of cell only group. For western blotting, GAPDH was used as a loading control. **(C, D)** Cells were sensitized overnight with 1 μg/ml of anti-DNP mouse IgE followed by stimulation with PBS (0 ng/ml DNP-BSA) or 100 ng/ml of DNP-BSA for 30 min. Release of histamine, β-glucuronidase and tryptase was measured by ELISA. Quantification of cytokines (IL-6, IL-13, and TNF-α) and chemokines (CCL3 and CCL4) was performed by qRT-PCR. Data were normalized to the amount of β-actin and calculated as a fold change of the non-stimulated cell only group. Results are shown as mean ± SDs of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. VLPs, virus like particles; KD, knockdown; OE, overexpression; NC, negative control.

Mast cells also produce cytokines and chemokines critical for inflammatory responses. Hence, we next measured the expression of cytokines and chemokines in Ag-activated mast cells. Ag-induction promoted the release of key effector cytokines IL-6, IL-13, and TNF-α and chemokines CCL3 and CCL4. Importantly, the release of these cytokines/chemokines were significantly impaired in ORMDL3-OE cells and increased in ORMDL3-KD cells (**Figure 2D**). In the non-activated MC/9 cells, there were no differences in the productions of studied cytokines and chemokines. Collectively, these findings suggest that ORMDL3 is a negative regulator of Ag-activated mast cell degranulation as well as cytokine/chemokine production.

ORMDL3 Regulates ATF6-UPR and Autophagy in Ag-Activated Mast Cells

To investigate whether the ERS-induced UPR-autophagy pathway participates in the ORMDL3-mediated negative regulation of mast cell activation, we compared the ERS/UPR signaling and autophagy biomarkers between ORMDL3-OE and ORMDL3-KD cells. The mRNA levels of ERS markers SERCA2b/ATF6 and autophagy markers Beclin 1 and light chain 3B (LC3B) showed a significant increase in the activated ORMDL3-OE cells and a decrease in ORMDL3-KD cells (**Figure 3A**). The protein levels of SERCA2b, ATF6, Beclin 1 and LC3B II/I also increased in ORMDL3-OE cells and decreased in ORMDL3-KD cells (**Figure 3B**). Other UPR signaling molecules including XBP1u, XBP1s, Perk and BiP were independent of ORMDL3 expression level (**Supplementary Figure 2A**), but the protein level of phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α) was up-regulated in activated ORMDL3-OE cells and down-regulated in ORMDL3-KD cells (**Supplementary Figure 2B**). Confocal microscopy analysis further verified the effects of ORMDL3 on the ATF6-autophagy pathway. As shown in **Figures 3C, D**, overexpression of ORMDL3 led to an increased nuclear localization of ATF6 and a significant

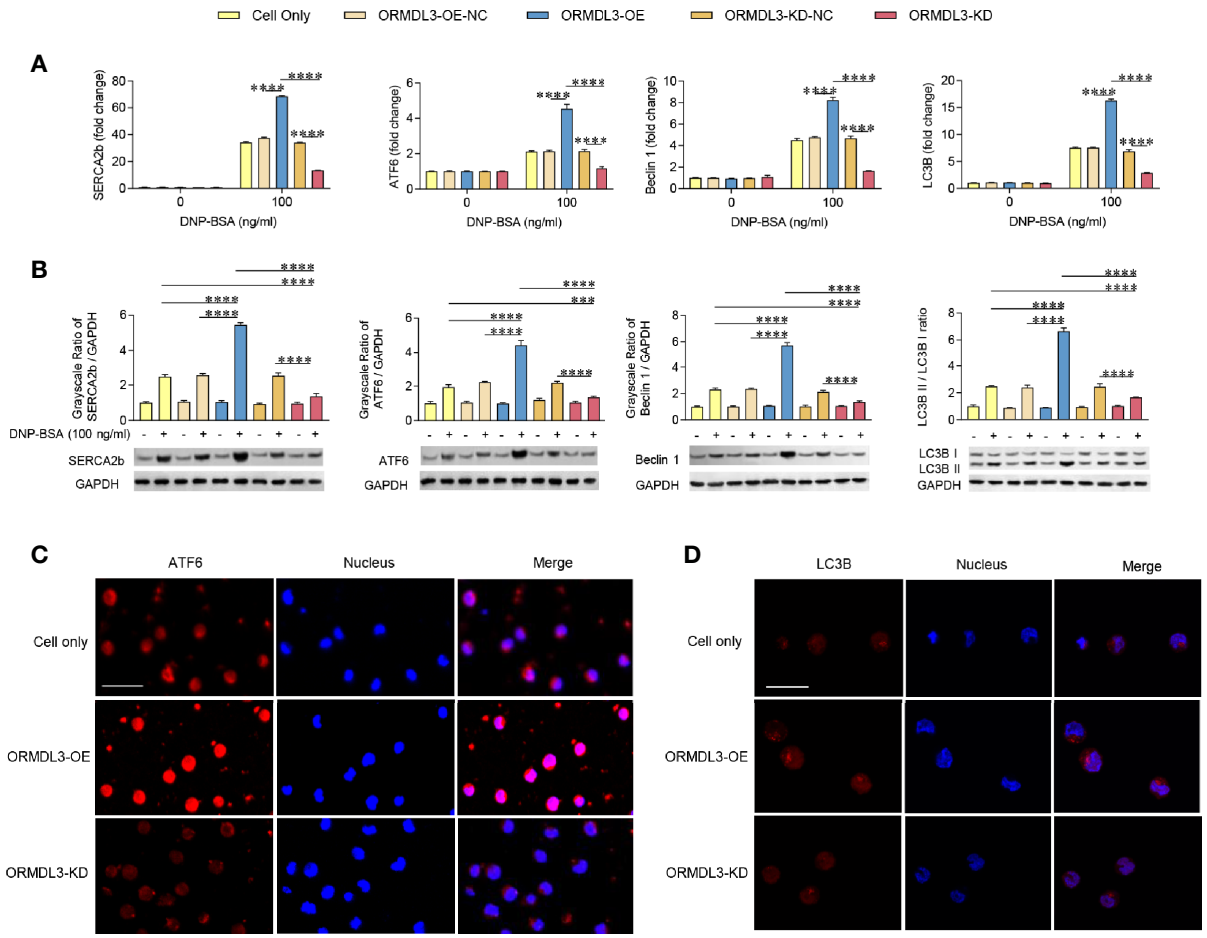

FIGURE 3 | ORMDL3 regulates ATF6-UPR and autophagy in Ag-activated mast cell. MC/9 cells were treated as described in **Figure 2**. mRNA **(A)** and protein **(B)** expression of ERS (SERCA2b, ATF6) and autophagy (Beclin 1, LC3B and LC3B II/LC3B I) markers in non-activated and Ag-activated mast cells was measured by qRT-PCR and western blotting. mRNA level was normalized to the amount of β-actin and calculated as a fold change of the non-stimulated cell only group. For western blotting, GAPDH served as a loading control. **(C, D)** Representative images depict expressions of ATF6 (red) and LC3B (red) in Ag-activated mast cells. Scale bar 25 μm. Nuclei (blue) were stained with DAPI. Results are shown as mean ± SDs of 3 independent experiments. ***$P < 0.001$; ****$P < 0.0001$. KD, knockdown; OE, overexpression; NC, negative control.

increase of LC3B puncta in Ag-activated MC/9 cells, whereas these markers were down-regulated in ORMDL3-KD cells. No significant difference was observed between non-activated groups (**Supplementary Figures 3A, B**).

Inhibition of Either ATF6 or Autophagy Reverses ORMDL3 Overexpression-Mediated Suppression of Mast Cell Activation

ATF6 is an important protein sensor and is activated during ORMDL3-mediated ERS/UPR signaling. To validate whether ATF6-UPR facilitates ORMDL3-mediated negative regulation of MC/9 function, we adapted a Tet-on system to knockdown the expression of ATF6 to different levels in ORMDL3-OE cells. The result showed that the expression of ATF6 was decreased by doxycycline (Dox) in a dose-dependent manner, which was confirmed by qRT-PCR and western blot analysis (**Figure 4A**). Interestingly, Ag-induced degranulation, and the production of cytokines and chemokines was up-regulated in ATF6-KD (Dox 100 ng/ml) ORMDL3-OE cells, suggesting that impairments of degranulation and cytokine and chemokine production caused by overexpression of ORMDL3 were overcome by ATF6 downregulation, albeit not to the level of un-manipulated cells (**Figures 4B, C**). In line with this, the activated ATF6-KD (Dox 100 ng/ml) ORMDL3-OE cells had higher concentrations in supernatant and lower intracellular concentrations of histamine, β-glucuronidase and tryptase compared with ORMDL3-OE cells (**Supplementary Figure 4**). However, downregulation of ATF6 by Dox at 10 ng/ml did not rescue the impairment of degranulation caused in ORMDL3-OE cells, but it did partially reinitiate the production of IL-6, IL-13, TNF-α and CCL4 (**Figure 4C**). Moreover, as shown in **Figures 4D, E**, the increased Beclin1 and LC3B mRNA expression as well as the increased protein level of Beclin1 and LC3B II/I in ORMDL3-OE

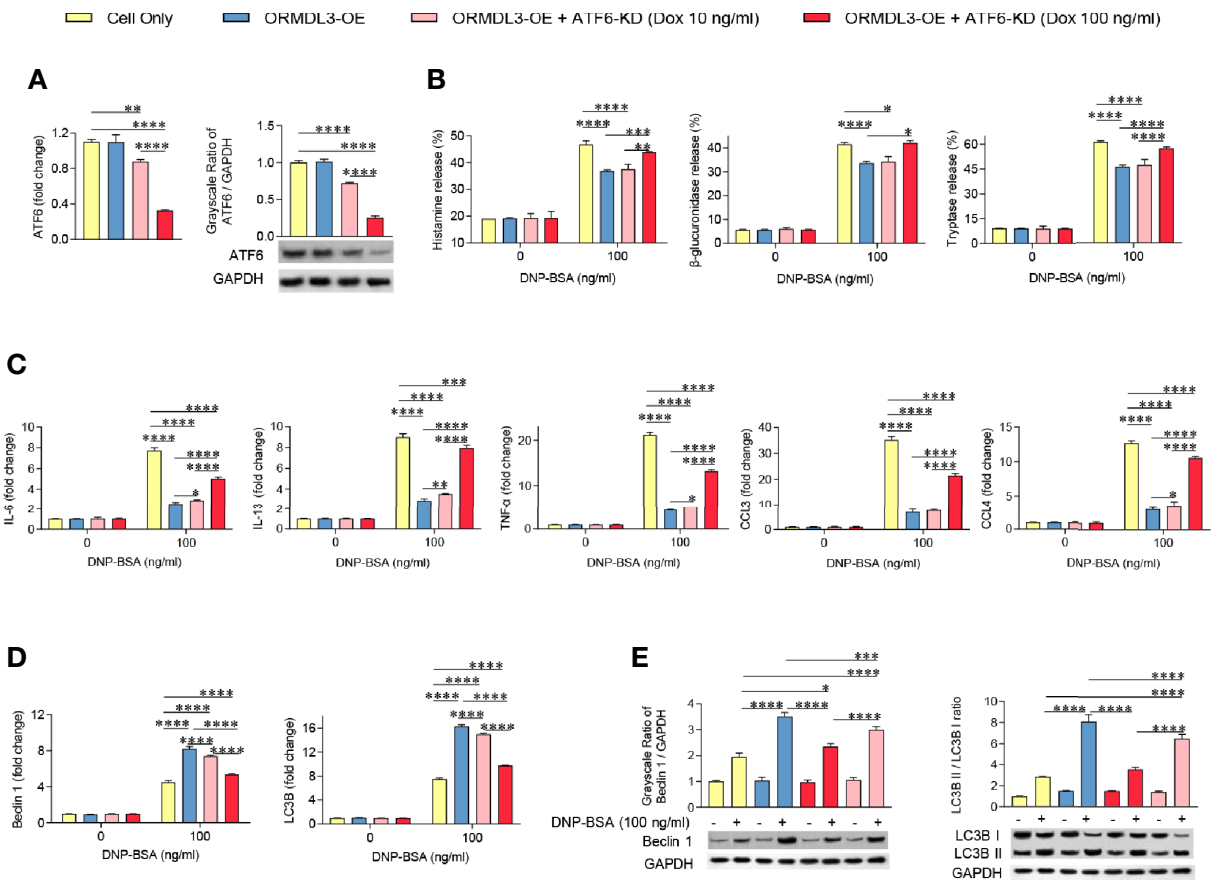

FIGURE 4 | Knockdown of ATF6 reverses ORMDL3 overexpression-mediated suppression of mast cell activation. Knockdown of ATF6 was conducted by transducing ORMDL3-OE cells with VLPs conveying ATF6-shRNA and the addition of doxycycline (Dox, 10 ng/ml or 100 ng/ml). Cells were sensitized overnight with 1 μg/ml of anti-DNP mouse IgE followed by stimulation with PBS (0 ng/ml DNP-BSA) or 100 ng/ml of DNP-BSA for 30 min. **(A)** The knockdown efficiencies were determined by qRT-PCR and western blotting. mRNA level was normalized to the amount of β-actin and calculated as a fold change of the cell only group. For western blotting, GAPDH served as a loading control. **(B)** Release of histamine, β-glucuronidase and tryptase was measured by ELISA. **(C)** mRNA expression of cytokines (IL-6, IL-13, and TNF-α) and chemokines (CCL3 and CCL4). Data were normalized to the amount of β-actin and calculated as a fold change of the non-stimulated cell only group. **(D, E)** qRT-PCR and western blot analysis of autophagy markers (Beclin 1, LC3B, and LC3B II/LC3B I). mRNA level was normalized to the amount of β-actin and calculated as a fold change of the non-stimulated cell only group. For western blotting, GAPDH served as a loading control. Results are shown as mean ± SDs of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. KD, knockdown; OE, overexpression.

cells were markedly reversed by ATF6 knockdown (100 ng/ml and 10 ng/ml Dox), suggesting that autophagy appears to be downstream of the ORMDL3/ATF6 pathway.

To further determine the contribution of autophagy in the inhibition role of ORMDL3 on mast cell activation, ORMDL3-OE cells were treated with 3-MA, which is widely used as an inhibitor of autophagy. The inhibition of autophagy was demonstrated by the low expression of LC3B II/LC3B I, which was confirmed by western blot analysis (**Figure 5A**). As shown in **Figures 5B, C**, induction of degranulation and cytokine/chemokine production in the 3-MA treated Ag-activated MC/9 cells was significantly increased when compared to the cell only group. When ORMDL3-OE cells were treated with 3-MA and activated, the decreased degranulation and the production of cytokines and chemokines caused by ORMDL3 overexpression was reversed. A similar result was observed in the 3-MA treated ATF6-KD ORMDL3-OE cells. Taken together, these results suggest that ORMDL3 negatively regulates degranulation as well as cytokine and chemokine production of Ag-activated MC/9 cells *via* the ATF6-autophagy pathway.

Knockdown of ORMDL3 and/or ATF6 Enhances PCA Reactions

To evaluate the *in vivo* relevance of the ORMDL3-mediaed ATF6 UPR pathway in mast cell functions, mice were intradermally injected into both ears with ORMDL3 overexpressing virus like particles (VLPs), ORMDL3 knockdown VLPs and/or ATF6 knockdown VLPs to change ORMDL3 and/or ATF6 expression locally (**Figure 6A**). After 48 h of injection, the efficiency of VLPs was confirmed by qRT-PCR (**Figure 6B**). To assess local IgE-dependent mast cell degranulation by PCA reactions, the mice were intradermally injected with anti-DNP IgE into both ears and challenged intravenously with DNP-BSA and Evans blue 24 h later (**Figure 6A**). As presented in **Figures 6C, D**, Evans blue extravasation at PCA reaction sites was inhibited in mice injected with ORMDL3 overexpression VLPs, whereas, ORMDL3 knockdown VLPs and/or ATF6 knockdown VLPs injection enhanced Evans blue extravasation. As expected, the ORMDL3-OE + ATF6-KD group had higher Evans blue extravasation than the ORMDL3-OE group, which suggesting that ATF6 knockdown reversed the inhibited PCA reaction caused by ORMDL3 overexpression. Thus, we concluded that the ORMDL3-mediated ATF6 UPR pathway plays a crucial role in the degranulation of Ag-activated mast cells *in vivo*.

DISCUSSION

Our data revealed a novel mechanism of ORMDL3 mediated regulation of mast cell activation. We found that ORMDL3 expression was downregulated in Ag-activated MC/9 cells. Overexpression of ORMDL3 suppressed mast cell degranulation and attenuated the release of inflammatory cytokines and chemokines. The opposite responses were observed with the knockdown of ORMDL3 in mast cells. ORMDL3 overexpression also upregulated the ATF6-

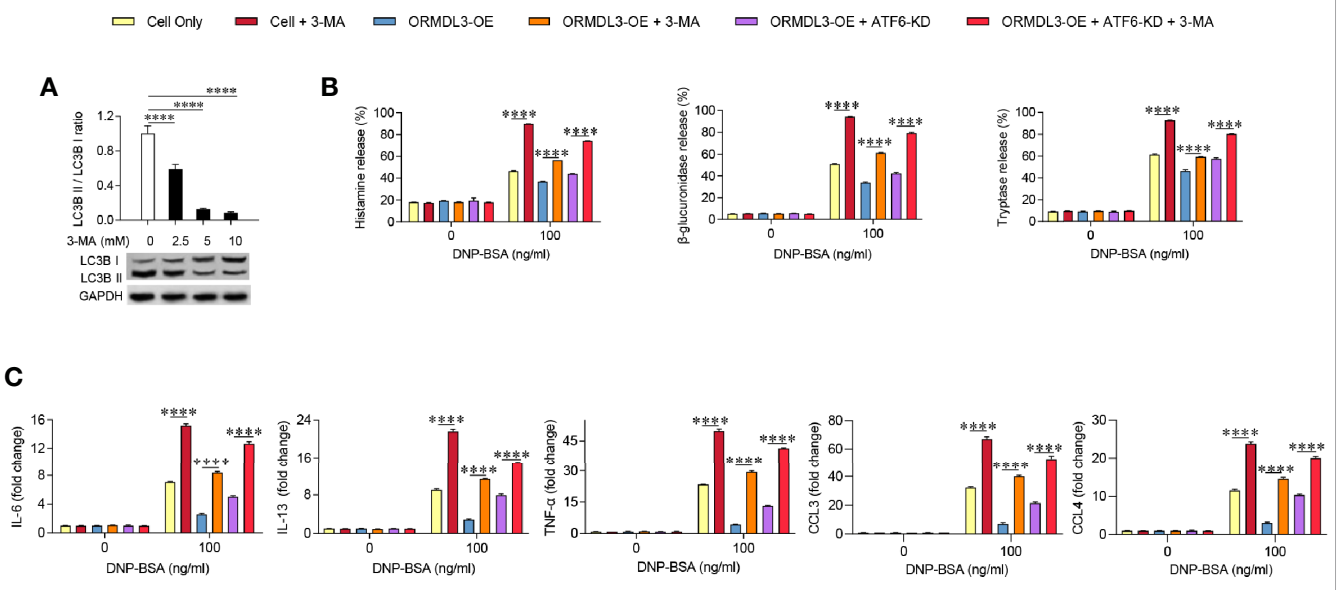

FIGURE 5 | Inhibition of autophagy reverses the effect of ORMDL3 overexpression-induced suppression of mast cell activation. **(A)** MC/9 cells were serum starved overnight, then treated with 3-MA at indicated concentrations for 24 h. Western blot analysis shows the inhibition efficiency of indicated concentrations of 3-MA on LC3B expressions. GAPDH served as a loading control. **(B, C)** ORMDL3 overexpression and ATF6 knockdown were conducted as described in **Figures 2** and **4**. Cells were serum starved overnight followed by treatment with 1 μg/ml of anti-DNP mouse IgE together with or without 5 mM 3-MA for 24 h, then stimulated with 100 ng/ml of DNP-BSA for 30 min. Release of histamine, β-glucuronidase and tryptase was measured by ELISA. Quantification of cytokines (IL-6, IL-13, and TNF-α) and chemokines (CCL3 and CCL4) mRNAs was performed by qRT-PCR. Data were normalized to the amount of β-actin and calculated as a fold change of the non-stimulated cell only group. Results are presented as mean ± SDs of three independent experiments. ****$P < 0.0001$. KD, knockdown; OE, overexpression.

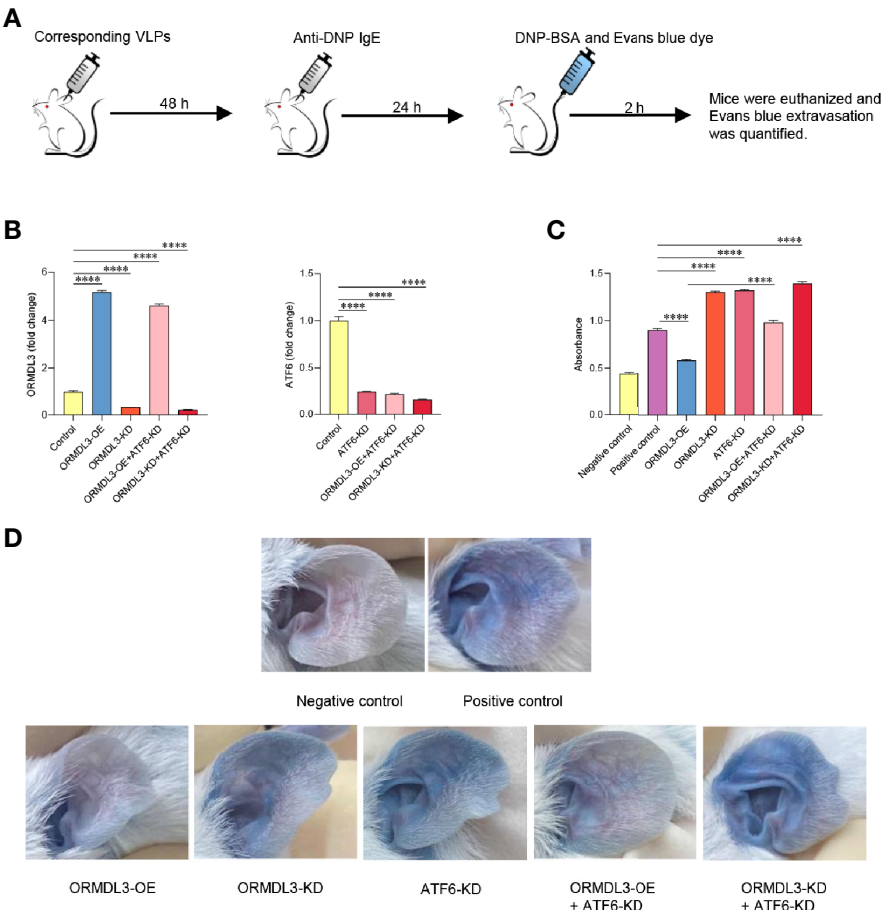

FIGURE 6 | Knockdown of ORMDL3 and/or ATF6 enhances passive cutaneous anaphylaxis (PCA) reactions. **(A)** The ears of mice were injected intradermally with corresponding VLPs (ORMDL3 overexpressing, knockdown and/or ATF6 knockdown); both PCA negative and positive control were VLPs-free. Anti-DNP IgE was injected intradermally into the ears 48 h later. DNP-BSA and Evans blue was injected intravenously another 24 h later, the negative control received Evan blue in PBS only. Two hours later, mice were euthanized. **(B)** ORMDL3 and ATF6 quantification of extracts from ears was performed by qRT-PCR. Data were normalized to the amount of β-actin and calculated as a fold change of the control group. Results are presented as mean ± SDs of 3 independent experiments. **(C)** Evans blue dye was extracted by incubating the ear samples in 0.5 ml DMSO for 24 h at 37 °C, and optical density (O.D.) was measured at 650 nm. Quantitative data are presented as mean ± SDs (n=6; pooled data from two independent experiments). **(D)** Representative photographs of ears from indicated mice 2 h after antigen challenge. ****$P < 0.0001$. VLPs, virus like particles; KD, knockdown; OE, overexpression.

dependent UPR response as well as promoted autophagy in mast cells. Intriguingly, knockdown of ATF6 and/or autophagy inhibition repressed ORMDL3-mediated inhibition of mast cell activation. This was nicely corroborated with the findings that ORMDL3-ATF6-UPR suppressed PCA reaction. Taken together these data suggest that ORMDL3 negatively regulates mast cell activation and subsequent immune responses *via* ATF6-autophagy dependent pathway (**Figure 7**).

Mast cells are the crucial effector cells in IgE-dependent allergic diseases. Mast cells release various pro-inflammatory cytokines and chemokines upon antigen activation. We have shown that knockdown of ORMDL3 in Ag-activated mast cells increased mRNA expression of pro-inflammatory cytokines IL-6, IL-13 and TNF-α, and chemokines CCL3 and CCL4. This observation is consistent with a previous study using BMMCs in which the expression of cytokines and chemokines was found to be significantly enhanced in Ag-activated ORMDL3-KD BMMCs (23). Knockdown of ORMDL3 in Ag-activated mast cells causes enhanced phosphorylation of IκBα, a master regulator of transcription factor NF-κB (23, 30). ORMDL3 knockdown is also known to enhance nuclear localization of NF-κBp65 subunit which increases the transcription of NF-κB dependent genes of cytokines/chemokines including IL-6, IL-13, TNF-α, CCL3, and CCL4. However, Bugajev and colleagues reported that the mRNA expression of these cytokines/chemokines was higher in unstimulated ORMDL3-KD BMMCs whereas we did not find any difference in unstimulated cells (23). They also reported that ORMDL3 knockdown did not affect mast cell degranulation. This can be ascribed to the inherent differences in using cell lines versus primary cells and the different antigens being used in these

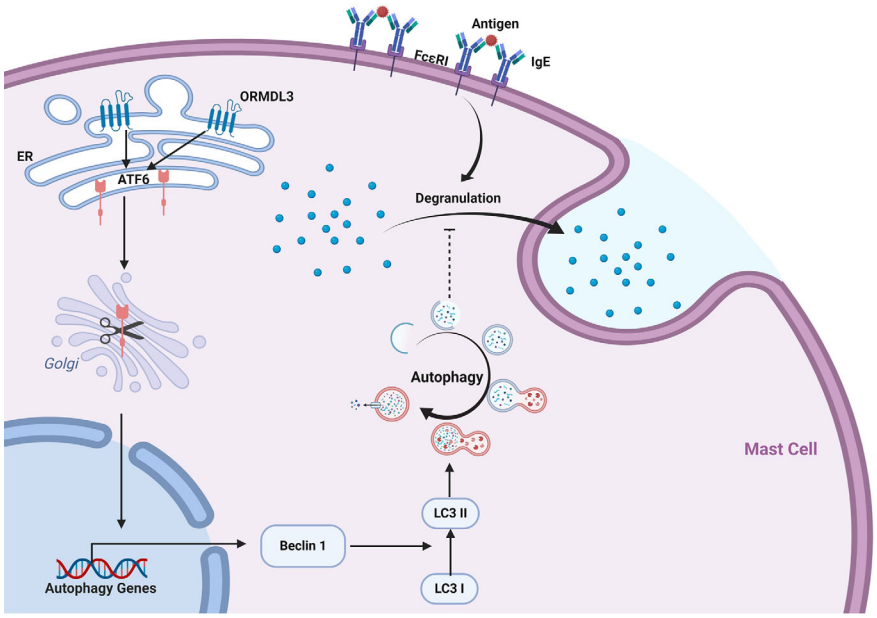

FIGURE 7 | ORMDL3 negatively regulates mast cell activation *via* an ATF6-autophagy dependent pathway. ORMDL3 activates ATF6 pathway which upregulates the transcription of autophagy gene Beclin 1. This results in increased autophagic activity in mast cells. The FcεRI-mediated mast cell activation can be inhibited by autophagy.

experiments. It would be beneficial to perform a side-by-side comparison to investigate the underlying mechanisms of such differences.

ORMDL3 exhibits a negative regulatory role in Ag-activated mast cells, one that differs from activated eosinophils (9), airway smooth muscle (31), and bronchial airway epithelial cells (8). In fact, very few negative regulatory mediators have been reported in allergic diseases compared to positive mediators, therefore it is essential to explore the mechanisms involved. As an ER localized protein, the role of ORMDL3 in the regulation of ERS-induced UPR is an important mechanism to link ORMDL3 to asthma pathogenesis (5, 8, 32, 33). UPR has three major distinct arms: IRE1, PERK, and ATF6 (15, 16). Prior studies have demonstrated that the role of ORMDL3 in the regulation of UPR signaling is cell-specific. It is reported that the overexpression of ORMDL3 inhibited SERCA2b and activated the PERK/eIF2α arm of UPR in HEK 293 cells (human embryonic kidney cells) (22). In airway epithelium, ORMDL3 was found to activate the ATF6 pathway and subsequently regulate the expression of SERCA2b, which has been implicated in airway remodeling (8). In our study, we found that both SERCA2b and ATF6 expression was significantly increased in Ag-activated ORMDL3-OE cells, whereas it was decreased in Ag-activated ORMDL3-KD cells, which suggests that the ATF6 UPR pathway is regulated by ORMDL3 expression in Ag-activated mast cells. Meanwhile, our western blot showed that p-eIF2α (phosphorylated by PERK) was increased by overexpression of ORMDL3 and decreased by its knockdown. However, qRT-PCR analysis revealed that the mRNA levels of PERK were not significantly changed upon alteration of ORMDL3 expression, suggesting that ORMDL3 might regulate eIF2α expression independent of PERK, but this needs to be further investigated. To verify the contribution of the ATF6 UPR pathway to the regulatory role of ORMDL3 on mast cells, we constructed an ATF6-KD cell model in conjunction with ORMDL3 overexpression in which varied level of ATF6 knockdown was achieved using different concentrations of doxycycline. We observed that downregulation of ATF6 mRNA and protein level by 70% using 100 ng/ml Dox reversed the impairment of degranulation as well as cytokine and chemokine production by ORMDL3 overexpression. Although ATF6 mRNA and protein level were downregulated by 15% and 25% respectively using 10 ng/ml Dox, it did not reverse the inhibition effect of ORMDL3 on mast cell degranulation. However, it did partially restart cytokine and chemokine production, suggesting that cytokine and chemokine production is more responsive to the ORMDL3/ATF6 pathway regulation than degranulation. These results suggest a potential link between the ORMDL3 regulated ATF6 UPR pathway and Ag-mediated mast cell activation.

Following this we then aimed to investigate the vital molecular mechanisms by which the ORMDL3/ATF6 UPR pathway regulates mast cell activation. ORMDL3 is considered to be an autophagy-related gene, given its role in mediating inflammation, ERS and UPR, which in turn are stimulating factors that induce autophagy (19). It was suggested that high levels of ORMDL3 in B cells induce ERS through ATF6 and results in autophagy (34). In endothelial cells, oxidized low-density lipoprotein (ox-LDL) upregulates ORMDL3 expression and subsequently promotes autophagy (35). In macrophages, the anomalous expression of ORMDL3 affects autophagy and contributes to the risk of inflammatory diseases (21).

Autophagy is a process that determines a cells fate in different ways (36); it can be regulated by any of the three branches of the UPR (ATF6, IRE1α, and PERK), although regulation is cell-specific and context-specific (12, 15–18). Interestingly, it is reported that autophagy plays a crucial role in mast cell degranulation. Conversion of LC3 I to LC3-II was found inherently induced in mast cells, and LC3-II localized in secretory granules of mast cells. Deletion of Atg7 has shown severe impairment of degranulation in BMMCs (29). Therefore, considering the following points that: there is a close relationship between ORMDL3 and autophagy; ORMDL3 regulates ERS-induced UPR; autophagy can be activated by the UPR pathway; autophagy is crucial to mast cell degranulation; we examined whether ORMDL3 may modulate autophagy through the ATF6 UPR pathway, thereby facilitating its regulatory role in mast cell activation. As expected, autophagy markers Beclin 1 and LC3B were shown to be increased in Ag-activated ORMDL3-OE cells, whereas they were decreased in ORMDL3-KD cells. Confocal microscopy was used to validate these results and demonstrated that ORMDL3-OE cells showed a strengthened formation of LC3B puncta, and ORMDL3-KD cells showed a decrease. ATF6 knockdown markedly reversed the upregulated autophagic activity by ORMDL3 overexpression, which confirmed that ORMDL3 regulates autophagy through ATF6. Furthermore, we demonstrated that inhibition of autophagy by 3-MA reversed the deficiency of degranulation and cytokine/chemokine production caused by ORMDL3 overexpression and an enhanced reversal was observed by ATF6 knockdown together with 3-MA treatment. Consequently, autophagy acts as a downstream effector of the ORMDL3/ATF6 UPR pathway.

The *in vivo* data corroborated the results obtained with MC/9 cells *in vitro*. PCA reaction is a commonly used measure of mast cell function *in vivo*. The reaction is induced by means of injecting antigen-specific IgE, which binds to FcεRI on tissue mast cells and results in mast cells sensitization. Twenty-four hours later, antigen is co-administered intravenously with Evans blue dye, resulting in localized degranulation of mast cells, secretion of vasoactive mediators and extravasation of Evans blue (37, 38). The Evans blue extravasation was enhanced in mice injected with ORMDL3 knockdown VLPs and/or ATF6 knockdown VLPs, whereas it was inhibited in mice injected with ORMDL3 overexpression VLPs. Based on these data, we confirmed that the ORMDL3-mediated ATF6 UPR pathway negatively regulates the degranulation of Ag-activated mast cells *in vivo*. One limitation of our study is that we did not use *Ormdl3*$^{-/-}$ mice. However, it is important to note that these mice have elevated serum levels of total sphingolipids, including ceramides (13), which binds to leukocyte mono-immunoglobulin-like receptor 3 (LMIR3) and inhibits mast cell activation (39, 40). Another limitation of the study is the use of single cell line (MC/9) for all the experiments. MC/9 cell line is derived from the fetal liver of a F1 mice. The size and morphology of MC/9 are similar to those of BMMCs. MC/9 expresses functional FcεRI and CD117. Its binding ability to mouse IgE is similar to that of mouse peritoneal mast cells. In our experiment, we successfully transduced MC/9 with ORMDL3 and ATF6 virus like particles (VLPs). We have tested mouse BMMCs for this investigation. It was practically difficult to obtain large number of cells required for this study. Moreover, we could not achieve satisfactory transduction efficiency of BMMCs which was critical for the investigation of the role of ORMDL3/ATF6 in mast cell activation.

In conclusion, our work supports the current hypothesis that ORMDL3 acts as a negative modulator of mast cell activation, further to this we have identified that the ATF6 UPR-autophagy pathway is critical in this response. A limitation of our study is that we did not identify the mechanism by which autophagy affects Ag-mediated mast cell activation. Although a previous study found that cellular ATP levels, which may be crucial for activation of kinases involved in mast cell function, were increased in Atg7$^{-/-}$ BMMCs (29), whether autophagy participates in mast cell function by alteration of cellular ATP levels is currently unknown and needs to be further investigated. Nevertheless, our findings provide evidence of the negative regulatory role of ORMDL3 on mast cell degranulation as well as cytokine and chemokine production. Additionally, we demonstrated that ORMDL3 selectively regulates the ATF6 UPR-autophagy signaling pathway, which is an important mechanism to link ORMDL3 to mast cell physiology providing a cellular and molecular explanation for the association between ORMDL3 and asthma pathogenesis.

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 animal study was reviewed and approved by the animal ethics committee of Shanghai General Hospital.

AUTHOR CONTRIBUTIONS

JL designed and performed experiments, analyzed and interpreted the data, and wrote the manuscript. MU and HJ designed experiments, analyzed and interpreted the data, and edited the manuscript. YTL, LLin, JW, XP, HL, YNL, and YG performed experiments. LLi designed experiments, supervised the work, contributed to study design and data interpretation, and edited the paper. All authors contributed to the article and approved the submitted version.

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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.2021.604974/full#supplementary-material

REFERENCES

1. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* (2012) 18:693–704. doi: 10.1038/nm.2755
2. Amin K. The role of mast cells in allergic inflammation. *Respir Med* (2012) 106:9–14. doi: 10.1016/j.rmed.2011.09.007
3. Gilfillan AM, Austin SJ, Metcalfe DD. Mast cell biology: introduction and overview. *Adv Exp Med Biol* (2011) 716:2–12. doi: 10.1007/978-1-4419-9533-9_1
4. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. *Nature* (2007) 448:470–3. doi: 10.1038/nature06014
5. James B, Milstien S, Spiegel S. ORMDL3 and allergic asthma: From physiology to pathology. *J Allergy Clin Immunol* (2019) 144:634–40. doi: 10.1016/j.jaci.2019.07.023
6. Das S, Miller M, Broide DH. Chromosome 17q21 Genes ORMDL3 and GSDMB in Asthma and Immune Diseases. *Adv Immunol* (2017) 135:1–52. doi: 10.1016/bs.ai.2017.06.001
7. Vicente CT, Revez JA, Ferreira MAR. Lessons from ten years of genome-wide association studies of asthma. *Clin Transl Immunol* (2017) 6:e165. doi: 10.1038/cti.2017.54
8. Miller M, Tam AB, Cho JY, Doherty TA, Pham A, Khorram N, et al. ORMDL3 is an inducible lung epithelial gene regulating metalloproteases, chemokines, OAS, and ATF6. *Proc Natl Acad Sci U S A* (2012) 109:16648–53. doi: 10.1073/pnas.1204151109
9. Ha SG, Ge XN, Bahaie NS, Kang BN, Rao A, Rao SP, et al. ORMDL3 promotes eosinophil trafficking and activation via regulation of integrins and CD48. *Nat Commun* (2013) 4:2479. doi: 10.1038/ncomms3479
10. Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, et al. Orm family proteins mediate sphingolipid homeostasis. *Nature* (2010) 463:1048–53. doi: 10.1038/nature08787
11. Kiefer K, Carreras-Sureda A, Garcia-Lopez R, Rubio-Moscardo F, Casas J, Fabrias G, et al. Coordinated regulation of the orosomucoid-like gene family expression controls de novo ceramide synthesis in mammalian cells. *J Biol Chem* (2015) 290:2822–30. doi: 10.1074/jbc.M114.595116
12. Ma X, Long F, Yun Y, Dang J, Wei S, Zhang Q, et al. ORMDL3 and its implication in inflammatory disorders. *Int J Rheum Dis* (2018) 21:1154–62. doi: 10.1111/1756-185x.13324
13. Debeuf N, Zhakupova A, Steiner R, Van Gassen S, Deswarte K, Fayazpour F, et al. The ORMDL3 asthma susceptibility gene regulates systemic ceramide levels without altering key asthma features in mice. *J Allergy Clin Immunol* (2019) 144:1648–59 e9. doi: 10.1016/j.jaci.2019.06.041
14. Chen AC, Burr L, McGuckin MA. Oxidative and endoplasmic reticulum stress in respiratory disease. *Clin Transl Immunol* (2018) 7:e1019. doi: 10.1002/cti2.1019
15. Cybulsky AV. Endoplasmic reticulum stress, the unfolded protein response and autophagy in kidney diseases. *Nat Rev Nephrol* (2017) 13:681–96. doi: 10.1038/nrneph.2017.129
16. Almanza A, Carlesso A, Chintha C, Creedican S, Doultsinos D, Leuzzi B, et al. Endoplasmic reticulum stress signalling - from basic mechanisms to clinical applications. *FEBS J* (2019) 286:241–78. doi: 10.1111/febs.14608
17. Hoyer-Hansen M, Jaattela M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ* (2007) 14:1576–82. doi: 10.1038/sj.cdd.4402200
18. Rashid HO, Yadav RK, Kim HR, Chae HJ. ER stress: Autophagy induction, inhibition and selection. *Autophagy* (2015) 11:1956–77. doi: 10.1080/15548627.2015.1091141
19. Wang M, Kaufman RJ. Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* (2016) 529:326–35. doi: 10.1038/nature17041
20. Zhang Y, Willis-Owen SAG, Spiegel S, Lloyd CM, Moffatt MF, Cookson W. The ORMDL3 Asthma Gene Regulates ICAM1 and Has Multiple Effects on Cellular Inflammation. *Am J Respir Crit Care Med* (2019) 199:478–88. doi: 10.1164/rccm.201803-0438OC
21. Kiefer K, Casas J, Garcia-Lopez R, Vicente R. Ceramide Imbalance and Impaired TLR4-Mediated Autophagy in BMDM of an ORMDL3-Overexpressing Mouse Model. *Int J Mol Sci* (2019) 20:1391. doi: 10.3390/ijms20061391
22. Cantero-Recasens G, Fandos C, Rubio-Moscardo F, Valverde MA, Vicente R. The asthma-associated ORMDL3 gene product regulates endoplasmic reticulum-mediated calcium signaling and cellular stress. *Hum Mol Genet* (2010) 19:111–21. doi: 10.1093/hmg/ddp471
23. Bugajev V, Halova I, Draberova L, Bambouskova M, Potuckova L, Draberova H, et al. Negative regulatory roles of ORMDL3 in the FcepsilonRI-triggered expression of proinflammatory mediators and chemotactic response in murine mast cells. *Cell Mol Life Sci* (2016) 73:1265–85. doi: 10.1007/s00018-015-2047-3
24. Grootjans J, Kaser A, Kaufman RJ, Blumberg RS. The unfolded protein response in immunity and inflammation. *Nat Rev Immunol* (2016) 16:469–84. doi: 10.1038/nri.2016.62
25. Greineisen WE, Maaetoft-Udsen K, Speck M, Balajadia J, Shimoda LM, Sung C, et al. Chronic Insulin Exposure Induces ER Stress and Lipid Body Accumulation in Mast Cells at the Expense of Their Secretory Degranulation Response. *PloS One* (2015) 10:e0130198. doi: 10.1371/journal.pone.0130198
26. Kritas SK, Saggini A, Cerulli G, Speziali A, Caraffa A, Antinolfi P. Asthma and Mast Cell Biology. *Eur J Inflamm* (2014) 12:261–5. doi: 10.1177/1721727X1401200205
27. Caslin HL, Kiwanuka KN, Haque TT, Taruselli MT, MacKnight HP, Paranjape A, et al. Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today. *Front Immunol* (2018) 9:868:868. doi: 10.3389/fimmu.2018.00868
28. Germic N, Frangez Z, Yousefi S, Simon HU. Regulation of the innate immune system by autophagy: neutrophils, eosinophils, mast cells, NK cells. *Cell Death Differ* (2019) 26:703–14. doi: 10.1038/s41418-019-0295-8
29. Ushio H, Ueno T, Kojima Y, Komatsu M, Tanaka S, Yamamoto A, et al. Crucial role for autophagy in degranulation of mast cells. *J Allergy Clin Immunol* (2011) 127:1267–76 e6. doi: 10.1016/j.jaci.2010.12.1078
30. Freitas R, Fraga CAM. NF-κB-IKKβ Pathway as a Target for Drug Development: Realities, Challenges and Perspectives. *Curr Drug Targets* (2018) 19:1933–42. doi: 10.2174/1389450119666180219120534
31. Chen J, Miller M, Unno H, Rosenthal P, Sanderson MJ, Broide DH. Orosomucoid-like 3 (ORMDL3) upregulates airway smooth muscle proliferation, contraction, and Ca(2+) oscillations in asthma. *J Allergy Clin Immunol* (2018) 142:207–18 e6. doi: 10.1016/j.jaci.2017.08.015
32. Hsu KJ, Turvey SE. Functional analysis of the impact of ORMDL3 expression on inflammation and activation of the unfolded protein response in human airway epithelial cells. *Allergy Asthma Clin Immunol* (2013) 9:4. doi: 10.1186/1710-1492-9-4
33. Miller M, Rosenthal P, Beppu A, Mueller JL, Hoffman HM, Tam AB, et al. ORMDL3 transgenic mice have increased airway remodeling and airway responsiveness characteristic of asthma. *J Immunol* (2014) 192:3475–87. doi: 10.4049/jimmunol.1303047
34. Dang J, Bian X, Ma X, Li J, Long F, Shan S, et al. ORMDL3 Facilitates the Survival of Splenic B Cells via an ATF6alpha-Endoplasmic Reticulum Stress-Beclin1 Autophagy Regulatory Pathway. *J Immunol* (2017) 199:1647–59. doi: 10.4049/jimmunol.1602124
35. Ma X, Qiu R, Dang J, Li J, Hu Q, Shan S, et al. ORMDL3 contributes to the risk of atherosclerosis in Chinese Han population and mediates oxidized low-density lipoprotein-induced autophagy in endothelial cells. *Sci Rep* (2015) 5:17194. doi: 10.1038/srep17194

36. Renz H. Autophagy: Nobel Prize 2016 and allergy and asthma research. *J Allergy Clin Immunol* (2017) 140:1548–9. doi: 10.1016/j.jaci.2017.03.021
37. Metcalfe DD, Peavy RD, Gilfillan AM. Mechanisms of mast cell signaling in anaphylaxis. *J Allergy Clin Immunol* (2009) 124:639–46; quiz 47-8. doi: 10.1016/j.jaci.2009.08.035
38. Bryce PJ, Falahati R, Kenney LL, Leung J, Bebbington C, Tomasevic N, et al. Humanized mouse model of mast cell-mediated passive cutaneous anaphylaxis and passive systemic anaphylaxis. *J Allergy Clin Immunol* (2016) 138:769–79. doi: 10.1016/j.jaci.2016.01.049
39. Matsukawa T, Izawa K, Isobe M, Takahashi M, Maehara A, Yamanishi Y, et al. Ceramide-CD300f binding suppresses experimental colitis by inhibiting ATP-mediated mast cell activation. *Gut* (2016) 65:777–87. doi: 10.1136/gutjnl-2014-308900
40. Izawa K, Yamanishi Y, Maehara A, Takahashi M, Isobe M, Ito S, et al. The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide. *Immunity* (2012) 37:827–39. doi: 10.1016/j.immuni.2012.08.018

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CASE REPORT
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Case Report: Refractory Chronic Spontaneous Urticaria Treated With Omalizumab in an Adolescent With Crohn's Disease

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Chronic spontaneous urticaria (CSU) is a mast cell-driven disease that is often associated with autoimmune or autoinflammatory conditions. Omalizumab is recommended in the treatment of refractory CSU in patients over 12 years of age who do not respond to four standard doses of antihistamines. Omalizumab blocks the mast cells' degranulation, thus interrupting the resulting inflammatory cascade driven by T-helper 2 (Th2) cytokines. The efficacy of omalizumab in controlling CSU and possible associated diseases has been studied in few patients so far. In particular, some case reports describe adults with CSU and concomitant inflammatory bowel diseases (IBD), such as Crohn's disease (CD) or ulcerative colitis (UC). Although the treatment of CD with anti-tumor necrosis factors-α (TNF-α) seems to be effective in controlling CSU, no cases of the utility of omalizumab in patients with both conditions have been described so far. At the moment, there is no evidence that the pathogenetic mechanisms underlying CD are linked to the same pathways that are inhibited by omalizumab for the treatment of CSU. We present the first pediatric case of refractory CSU and CD in which omalizumab led to CSU remission, even if the follow-up period was limited. In conclusion, our experience shows how CSU could be associated with CD and successfully treated with the monoclonal anti-IgE antibody in a patient on immunosuppressive therapy. However, more data is needed from a larger population.

Keywords: anti-IgE monoclonal antibody, chronic spontaneous urticaria, inflammatory bowel diseases, Crohn's disease, omalizumab, adolescent

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INTRODUCTION

Omalizumab is a recombinant monoclonal antibody (mAb)—direct against the Fcε portion of the immunoglobulin (Ig)E antibodies—that blocks interaction with the high-affinity receptors (FcεRI) expressed on the surface of target cells such as basophils and mast cells and that, consequently, blocks their release of several mediators (1).

Omalizumab acts mainly on a T-helper 2 (Th2) inflammation with a prominent role on mastocytes by inhibiting their degranulation and interrupting the resulting inflammatory cascade driven by Th2 cytokines (2). The efficacy of omalizumab has been proven in several diseases with a high level of evidence (i.e., allergic asthma, chronic urticaria, allergic rhinitis), medium level of evidence (i.e., nasal polyposis, facilitation of oral food allergen immunotherapy, facilitation of subcutaneous immunotherapy to aeroallergens, non-allergic asthma, allergic bronchopulmonary aspergillosis), and low level of evidence (i.e., mast cell activation syndrome, idiopathic anaphylaxis, atopic dermatitis, eosinophilic esophagitis, aspirin-exacerbated respiratory disease, asthma-chronic obstructive pulmonary disease overlap) (3).

In particular, the utility and safety of omalizumab in the treatment of severe allergic asthma has been known for many years. Indeed, it was approved by the United States Food and Drug Administration (FDA) in 2003, and 2 years later by the European Medicine Agency (EMA).

Moreover, the European Academy of Allergy and Clinical Immunology (EAACI), European Dermatology Forum (EDF), Global Allergy and Asthma European Network (GA2LEN), and World Allergy Organization (WAO) recommended the use of omalizumab in step three of the treatment for chronic spontaneous urticaria (CSU) in patients 12 years or older (3). CSU is defined by the daily appearance of wheals and/or angioedema, without an identified trigger, for a period lasting at least 6 weeks (3).

Autoimmunity seems to play a role of paramount importance in CSU, which is frequently associated with other autoimmune diseases, especially thyroiditis and celiac disease (4). Recently, few reports describe CSU associated with autoinflammatory gastrointestinal diseases, such as ulcerative colitis (UC) and Crohn's disease (CD), especially in adult patients (5–8).

We describe the first pediatric case of a girl with CD who developed refractory CSU and required treatment with omalizumab, leading to CSU remission.

CASE REPORT

We present the case of a Caucasian girl who has been suffering from CD since she was 12 years old. The colonoscopy revealed linear millimetric ulcerations on slightly hyperemic mucosa in the terminal ileum; the ileocecal valve presented rounded ulceration on the proximal edge; the mucosa of the whole colon up to the rectum was normal in appearance. The histological exam showed that, at the level of the terminal ileum and ileo-cecal valve, architecture of villi was within normal limits without an increase in intraepithelial T lymphocytes with pseudo-atrophic aspects and erosions of the epithelial lining. In the lamina propria, an increase in the inflammatory component, partly in the form of hyperplastic follicular lymphoid aggregates, partly in the neutrophilic and eosinophilic component, involved in some points the glandular structures without creating cryptic abscesses. There were no giant cells or granulomas. The cecum, the ascending-transverse-descending colon, the sigma and rectum were of normal morphology. At 12 years old, she started therapy with azathioprine and mesalazine without any disease relapse. At 17 years of age, she began to present episodes of angioedema, which were, in the beginning, isolated and, after 6 months, associated with urticaria. The patient had not undergone any treatment change for CD in the previous 5 years. In January 2019, she was referred to our Allergy Unit as urticarial episodes occurred daily (**Figure 1**) and persistently for more than 6 weeks despite being treated with second-generation non-sedating H1-antihistamines (sgAH1) up to 2 fold the approved doses. On the physical examination, she presented diffuse wheals, especially on the trunk and legs but sparing the face, in the absence of angioedema or other relevant clinical manifestations, including articular or musculoskeletal ones. Moreover, no clinical features of urticarial vasculitis were detected, which allowed us to rule it out. A full diagnostic work-up for chronic urticaria was performed (**Table 1**). She underwent a gastroenterology visit with esophagogastroduodenoscopy and colonoscopy with retrograde ileoscopy, which ruled out a relapse of CD. Moreover, the fecal calprotectin and the erythrocyte sedimentation rate (ESR) were in the normal range (**Table 1**). Therefore, she was diagnosed with CSU and angioedema in a patient with CD in remission.

We suggested maintaining the dose of sgAH1 twice a day and monitoring the disease activity by filling out the seven-day Urticaria Activity Score weekly (UAS7) as recommended by the EAACI/GA2LEN/EDF/WAO guideline (9). According to our center's clinical guidance and as recently explained in a

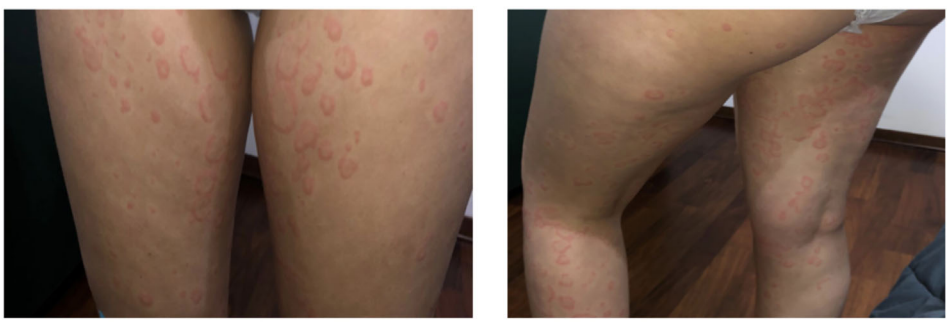

FIGURE 1 | Urticaria on the thighs and legs of the patient.

TABLE 1 | Laboratory workup.

Parameters, unit of measure	January 2019	February 2020	Normal range
CBC:			
- WBC, n/mm^3	5,310	4,980	4,000–10,000
- EOS, n/mm^3	63.7	81.2	30–350
AST, U/L	14	16	1–31
ALT, U/L	10	9	1–31
Creatinine, mg/dl	0.65	0.60	0.50–1.20
CRP, mg/dl	0.6	0.5	0–0.7
ESR, mm/hr	13	15	2–37
Specific IgE to common airway (mites, molds, animal epithelia and grass, weed, birch, olive, cypress pollens) and common food allergens (milk, egg, wheat, cod, tomato, soy, peanut), KU/l	<0.10	np	<0.10
HP-Abs U/ml	0.10	np	<0.30
TSH μIU/ml	3.13	3.01	0.35–4.94
fT4, pg/ml	8.8	8.5	6-12
TG-Ab, IU/ml	1	1	<4
TPO-Ab, IU/ml	1	1	<9
tTG-IgA, U/ml	0.4	1	<7
ANA	neg	neg	<1:80
C3, mg/dl	80	np	66–185
C4, mg/dl	35	np	15-52
C1-INH quantitative, g/l	0.22	np	0.15–0.33
C1-INH functional, %	100	np	70–100
Fecal calprotectin, μg/g	30	20	<70

ANA, antinuclear antibodies; ALT, alanine aminotransferase; AST, aspartate aminotransferase; C-reactive protein; CBC, complete blood count; C1-INH, C1 esterase inhibitor; CRP, C-reactive protein; EOS, eosinophils; ESR, erythrocytes sedimentation rate; fT4, free-thyroxine; HP-Abs, Helicobacter pylori-antibodies; IgE, Immunoglobulin E; neg, negative; np, not performed; TG-Ab, thyroglobulin-antibodies; TPO-antibodies, thyroid peroxidase-antibodies; TSH, thyroid-stimulating hormone; tTG-IgA, transglutaminase-Immunoglobulin A-antibodies; WBC, white blood cell.

published article by Sarti et al., we adjusted the dose of the sgAH1 therapy based on the patient's UAS7. Specifically, if the UAS7 was higher than 15 for at least 2 weeks, a step-up in therapy was performed. If it was 1–15, the sgAH1 dose was maintained. Finally, if it was 0 for at least 2 weeks, a step-down in therapy was performed (10). After 4 weeks, the patient was evaluated again through telemedicine; due to the low disease control, we suggested increasing the sgAH1 dose to three times a day (**Figure 2**). Taking into account the low control of urticaria despite the sgAH1 treatment at threefold, the approved doses and the occurrence of somnolence as a side effect of antihistamines at high doses, in June 2019, we started treatment with subcutaneous omalizumab (300 mg) every 4 weeks (**Figure 1**). The patient continued taking sgAH1 three times a day for the first two months of treatment with the monoclonal antibody. As her UAS7 progressively improved, the sgAH1 dose was tapered to two times a day and to once a day in September 2019 (**Figure 2**). After the sixth injection (November 2019), omalizumab was stopped for two months according to the therapeutic schedule approved in Italy. The patient continued to take only one dose of cetirizine until February 2020, when a relapse of urticaria's and angioedema's clinical manifestations occurred. For this reason, she was given a twofold increase in the standard dosage of her sgAH1 treatment. Also, to exclude a reactivation of CD, she underwent new tests (**Table 1**), which gave normal results. Because of the poor management of urticaria, sgAH1 was prescribed three times a day for a short period of time. Because of the poor tolerance to the latter therapy, in May 2020, we started the second cycle of treatment with subcutaneous omalizumab (300 mg) every 4 weeks (**Figure 2**). Through telemedicine, we decided to progressively reduce sgAH1 to twice a day and finally once a day from September 2020. In October 2020, she underwent the last omalizumab injection. From August to October 2020, the registered UAS7 was 0 (**Figure 2**). After one month from the last omalizumab injection, the urticaria and angioedema were under control with sgAH1 once a day, and UAS7 was still 0. Moreover, the patient received 12 injections of omalizumab without reporting any side effects.

DISCUSSION

In this case report, we highlighted the efficacy and safety of omalizumab in an adolescent with refractory CSU and CD in remission on immunosuppressive therapy. The use of monoclonal anti-IgE therapy in the immunosuppressed host is limited to a few case reports, including adult patients with the hyper-IgE syndrome (HIES) (11) and HIV infection (12) and an adolescent with a common variable immunodeficiency (CVI) (13). In particular, Bard et al. (11) reported the first case of a 26-year-old woman with HIES with severe recalcitrant eczematous dermatitis, which was successfully treated with high-dose monoclonal anti-IgE therapy. Moreover, Iemoli et al. (12) described a case of excellent tolerability and efficacy of omalizumab in the treatment of CSU in a 56-year-old HIV-positive man on antiretroviral therapy; in particular, the viral load remained undetectable, and the $CD4^+$ T cell counts improved. Recently, Comberiati et al. (13) reported the first case of a 19-year-old female with CVID treated successfully in terms of efficacy and safety with omalizumab for CSU after a non-effective trial with intravenous immunoglobulin at immunomodulatory dosage. The remission of cutaneous symptoms was obtained after the first omalizumab injection and persisted after the 12-month follow-up period.

As already described, CSU is a mast cell-driven disease (14). Two groups of mast cell degranulating signals have been described so far: IgE auto-antibodies to auto-allergens (type I autoimmunity) and IgG or IgM autoantibodies targeting activating mast-cell receptors (type II autoimmunity) (15). These two types of autoimmune mechanisms of skin mast cell degranulation are considered to be relevant causes of CSU in most patients (15). Indeed, CSU has been associated with numerous autoimmune diseases, including inflammatory bowel diseases (IBDs) (4). However, very few case reports of CSU or angioedema in adult patients associated with IBDs have been described so far (5–8).

Farkas et al. and Freeman (5, 6) described two cases of hereditary angioedema (HAE) associated with CD: two males aged 35 and 29. The particularity of the latter clinical case (6) was that the patient's mother suffered from the same diseases—HAE and CD—that are,

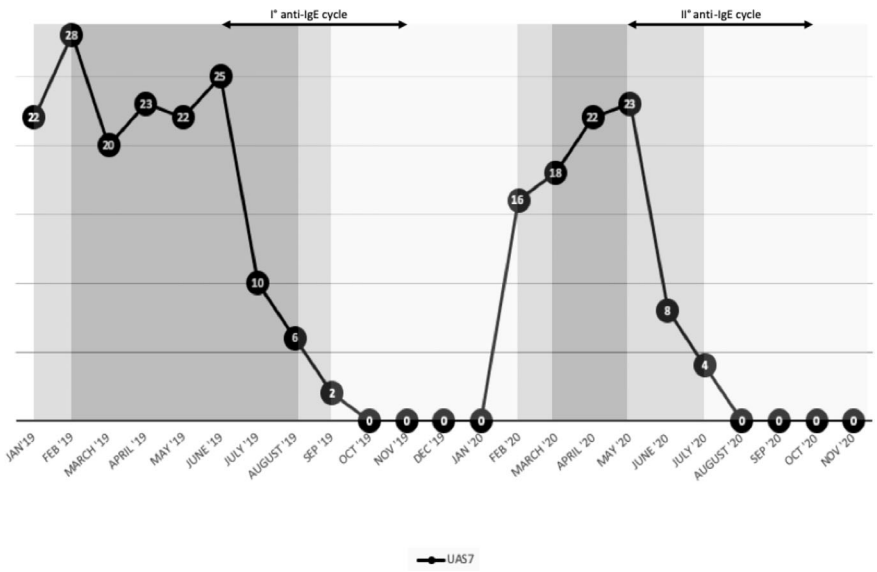

FIGURE 2 | Effect of second-generation not sedating H1-antihistamines and omalizumab on the clinical manifestation of chronic spontaneous urticaria described through 7-day Urticaria Activity Score (UAS7).

apparently, two unrelated conditions. Nevertheless, in this case, they appeared to be genetically linked. Mansueto et al. (16) reported the case of a 64-year-old man with CSU who presented signs and symptoms of subclinical CD, a manifestation that had never been previously described in these patients.

Currently, the action mechanism of omalizumab is not fully understood. It is known that it binds to free IgE, thus lowering free IgE levels and their receptors (17). In the literature, the use of omalizumab to treat CSU in patients with concomitant IBDs is limited to a few case reports. In particular, Witten et al. (18) described the successful use of omalizumab in a 23-year-old male with triple immune/autoinflammatory disease: CSU and angioedema associated with CD and familial Mediterranean fever (FMF). Grieco et al. (19) reported the case of a 49-year-old woman with UC who was under mesalazine treatment. She had autoimmune thyroiditis, chronic hypereosinophilia and CSU plus Besnier's prurigo treated with omalizumab with the resolution of urticaria and improvement of cutaneous clinical manifestations of prurigo.

The CSU and CD have a strong autoimmune involvement with an increase of proinflammatory cytokines (20). One common thread in the pathophysiology of both diseases is the imbalance in cytokine levels, in particular for IL-17 and TNF-α (21–23). Habal et al. (24) described the first case of CSU with angioedema coexistent with CD that was successfully treated with anti-TNF-α agents. Whether the CSU was cured due to the remission of the CD or because the TNF- α was a common cytokine in the pathogenetic pathway of the two diseases is difficult to understand. The authors hypothesized that given the similarity of cytokine derangements in CSU and CD, therapies that target the TNF- α could be effective in both conditions (24). In support of this hypothesis, cases of patients with refractory CSU, who were successfully treated with TNF-α inhibitors, are described in the literature (25, 26). Conversely, so far, no studies have concluded that anti-IgE therapy could improve both CSU and CD. Indeed, although CSU and CD are autoimmune diseases sharing the imbalance of some cytokines, when present in the same patient, they appear to be two concomitant diseases, and one is not the cause of the other.

In conclusion, even if the follow-up period of our case was limited and more data would be needed on more extensive populations, our experience shows how CSU could be associated with CD and successfully treated with monoclonal anti-IgE antibody even in a patient on immunosuppressive therapy.

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

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

SB, MG, GL, and LS conceptualized the work. SB, MG, FM, AG, and PL drafted the manuscript. SB and FM performed the

investigations and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Holgate S, Casale T, Wenzel S, Bousquet J, Deniz Y, Reisner C. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunol* (2005) 115(3):459–65. doi: 10.1016/j.jaci.2004.11.053
2. Chang TW, Chen C, Lin CJ, Metz M, Church MK, Maurer M. The potential pharmacologic mechanisms of omalizumab in patients with chronic spontaneous urticaria. *J Allergy Clin Immunol* (2015) 135(2):337–42.e2. doi: 10.1016/j.jaci.2014.04.036
3. Cardet JC, Casale TB. New insights into the utility of omalizumab. *J Allergy Clin Immunol* (2019) 143(3):923–6.e1. doi: 10.1016/j.jaci.2019.01.016
4. Kolkhir P, Borzova E, Grattan C, Asero R, Pogorelov D, Maurer M. Autoimmune comorbidity in chronic spontaneous urticaria: A systematic review. *Autoimmun Rev* (2017) 16:1196–208. doi: 10.1016/j.autrev.2017.10.003
5. Farkas H, Gyeney L, Nemesanszky E, Kaldi G, Kukán F, Masszi I, et al. Case Report Coincidence Of Hereditary Angioedema (Hae) With Crohn's Disease. *Immunol Investigations* (1999) 28(1):43–53. doi: 10.3109/08820139909022722
6. Freeman HJ. Hereditary angioneurotic edema and familial Crohn's disease. *Can J Gastroenterol* (2000) 14(4):337–9. doi: 10.1155/2000/828679
7. Naimeh LG, Muller BA. Chronic urticaria in a 17-year-old patient with a past history of bowel disease. *Ann Allergy Asthma Immunol* (2001) 86(5):511–6. doi: 10.1016/S1081-1206(10)62898-0
8. Liutu M, Kalimo K, Kalimo J, Kalimo H. Etiologic aspects of chronic urticaria *Int J Dermatol* (1998) 37(7):515–9. doi: 10.1046/j.1365-4362.1998.00328.x
9. Zuberbier T, Asero R, Bindslev-Jensen C, Walter Canonica G, Church MK, Giménez-Arnau AM, et al. EAACI/GALEN/EDF/WAO guideline: Management of urticaria. *Allergy: Eur J Allergy Clin Immunol* (2009) 64 (10):1427–23. doi: 10.1111/j.1398-9995.2009.02178.x
10. Sarti L, Barni S, Giovannini M, Liccioli G, Novembre E, Mori F. Efficacy and tolerability of the updosing of second-generation non-sedating H1 antihistamines in children with chronic spontaneous urticaria. *Pediatr Allergy Immunol* (2020) 32(1):153–60. doi: 10.1111/pai.13325
11. Bard S, Paravisini A, Aviles-Izquierdo JA, Fernandez-Cruz E, Sanchez-Ramon S. Eczematous Dermatitis in the Setting of Hyper-IgE Syndrome Successfully Treated With Omalizumab. *Arch DERMATOL* (2008) 144(12):1662–3. doi: 10.1001/archdermatol.2008.510
12. Iemoli E, Niero F, Borgonovo L, Cossu M, Piconi S. Successful Omalizumab treatment in HIV positive patient with chronic spontaneous urticaria: a case report. *Eur Ann Allergy Clin Immunol* (2017) 49(2):88–91.
13. Comberiati P, Costagliola G, Carli N, Legitimo A, D'Elios S, Consolini R, et al. Refractory chronic spontaneous urticaria treated with omalizumab in an adolescent with common variable immunodeficiency. *Front Immunol* (2019) 10:1700. doi: 10.3389/fimmu.2019.01700
14. Maurer M, Eyerich K, Eyerich S, Ferrer M, Gutermuth J, Hartmann K, et al. Urticaria: Collegium Internationale Allergologicum (CIA) Update 2020. *Int Arch Allergy Immunol* (2020) 181(5):321–33. doi: 10.1159/000507218
15. Kolkhir P, Church MK, Weller K, Metz M, Schmetzer O, Maurer M. Autoimmune chronic spontaneous urticaria: What we know and what we do not know. *J Allergy Clin Immunol* (2017) 139(6):1772–81.e1. doi: 10.1016/j.jaci.2016.08.050
16. Mansueto P, Carroccio A, Corsale S, Di Lorenzo G, Di Prima L, Pirrone G, et al. Chronic urticaria as a presenting symptom of Crohn's disease. *BMJ Case Rep* (2009) 2009:bcr08.2008.0781. doi: 10.1136/bcr.08.2008.0781
17. Kaplan AP, Giménez-Arnau AM, Saini SS. Mechanisms of action that contribute to efficacy of omalizumab in chronic spontaneous urticaria. *Allergy Eur J Allergy Clin Immunol* (2017) 72(4):519–33. doi: 10.1111/all.13083
18. Witten J, Siles R, Shen B, Yao Q. Triple Disease Combination: Familial Mediterranean Fever, Crohn's Disease, and Chronic Idiopathic Urticaria with Angioedema. *Inflamm Bowel Dis* (2015) 22(3):E12–3. doi: 10.1097/MIB.0000000000000702
19. Grieco T, Chello C, Faina V, Gomes VV, Alei L, Sernicola A, et al. Omalizumab therapy in a patient with cronic spontaneous urticaria, ulcerative colitis, hypereosinophilia and prurigo Besnier: a case report. *J Dermatol Treat* (2018) 29(sup3):10–3. doi: 10.1080/09546634.2018.1527990
20. Petagna L, Antonelli A, Ganini C, Bellato V, Campanelli M, Divizia A, et al. Pathophysiology of Crohn's disease inflammation and recurrence. *Biol Direct* (2020) 15(1):1–10. doi: 10.1186/s13062-020-00280-5
21. dos Santos JC, Azor MH, Nojima VY, Lourenço FD, Prearo E, Maruta CW, et al. Increased circulating proinflammatory cytokines and imbalanced regulatory T-cell cytokines production in chronic idiopathic urticaria. *Int Immunopharmacol* (2008) 8(10):1433–40. doi: 10.1016/j.intimp.2008.05.016
22. Strober W, Fuss IJ. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* (2011) 140(6):1756–67.e1. doi: 10.1053/j.gastro.2011.02.016
23. Peyrin-Biroulet L, Desreumaux P, Sandborn WJ, Colombel JF. Crohn's disease: beyond antagonists of tumour necrosis factor. *Lancet* (2008) 372 (9632):67–81. doi: 10.1016/S0140-6736(08)60995-2
24. Habal F, Huang V. Angioedema associated with Crohn's disease: Response to biologics. *World J Gastroenterol* (2012) 18(34):4787–90. doi: 10.3748/wjg.v18.i34.4787
25. Sand FL, Thomsen SF. Off-label use of TNF-alpha inhibitors in a dermatological university department: Retrospective evaluation of 118 patients. *Dermatol Ther* (2015) 28(3):158–65. doi: 10.1111/dth.12222
26. Wilson LH, Eliason MJ, Leiferman KM, Hull CM, Powell DL. Treatment of refractory chronic urticaria with tumor necrosis factor-alfa inhibitors. *J Am Acad Dermatol* (2011) 64(6):1221–2. doi: 10.1016/j.jaad.2009.10.043

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Human Mast Cell Line HMC1 Expresses Functional Mas-Related G-Protein Coupled Receptor 2

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The Mas-related G-protein-coupled receptor X2 (MRGPRX2) is prominently expressed by mast cells and induces degranulation upon binding by different ligands. Its activation has been linked to various mast cell-related diseases, such as chronic spontaneous urticaria, atopic dermatitis and asthma. Therefore, inhibition of MRGPRX2 activity represents a therapeutic target for these conditions. However, the exact pathophysiology of this receptor is still unknown. In vitro research with mast cells is often hampered by the technical limitations of available cell lines. The human mast cell types LAD2 and HuMC (human mast cells cultured from CD34+ progenitor cells) most closely resemble mature human mast cells, yet have a very slow growth rate. A fast proliferating alternative is the human mast cell line HMC1, but they are considered unsuitable for degranulation assays due to their immature phenotype. Moreover, the expression and functionality of MRGPRX2 on HMC1 is controversial. Here, we describe the MRGPRX2 expression and functionality in HMC1 cells, and compare these with LAD2 and HuMC. We also propose a model to render HMC1 suitable for degranulation assays by pre-incubating them with latrunculin-B (Lat-B). Expression of MRGPRX2 by HMC1 was proven by RQ-PCR and flowcytometry, although at lower levels compared with LAD2 and HuMC. Pre-incubation of HMC1 cells with Lat-B significantly increased the overall degranulation capacity, without significantly changing their MRGPRX2 expression, phenotype or morphology. The MRGPRX2 specific compound 48/80 (C48/80) effectively induced degranulation of HMC1 as measured by CD63 membrane expression and β-hexosaminidase release, albeit in lower levels than for LAD2 or HuMC. HMC1, LAD2 and HuMC each had different degranulation kinetics upon stimulation with C48/80. Incubation with the MRGPRX2 specific inhibitor QWF inhibited C48/80-induced degranulation, confirming the functionality of MRGPRX2 on HMC1. In conclusion, HMC1 cells have lower levels of MRGPRX2 expression than LAD2 or HuMC, but are attractive for *in vitro* research because of their high growth rate and stable phenotype. HMC1 can be used to study MRGPRX2-mediated degranulation after pre-incubation with Lat-B, which provides the opportunity to explore MPRGRX2 biology in mast cells in a feasible way.

Keywords: mast cell (MC), MRGPRX2, compound 48/80, qwf, HMC1, LAD2, HuMC, latrunculin

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INTRODUCTION

Mast cells are innate-type leukocytes that reside at barrier surfaces of the body, mainly the skin and mucosa. Here, they contribute to local immune responses induced by exogenous or physical triggers that disturb local tissue homeostasis (1). Mature mast cells harbor a wide array of surface membrane receptors that enable them to respond to many different triggers, provoking their effector functions (1). Depending on the type of stimulus, different types of activation can be induced, typically resulting in degranulation whereby different kinds of preformed mediators can be rapidly expelled (1, 2). Histamine, leukotrienes, prostaglandins and other vasoactive substances that are released by mast cells contribute to symptoms of anaphylaxis (3). Next to the rapid degranulation of preformed molecules, mast cells can initiate a slower pro-inflammatory response. This involves synthesis and secretion of cytokines and chemokines that subsequently activate neighboring cells and recruit and activate infiltrating immune cells (1). Furthermore, mast cells strongly interact with fibroblasts in wound healing responses, and mast cell-derived proteases are important in eradicating toxic venoms (4, 5). All the functional characteristics above illustrate the importance of mast cells in the control of variety of physiological and pathophysiological effects.

In modern medicine, mast cells have a mainly negative image due to their role in allergic disease. Consequently, the IgE-mediated route of mast cell activation has gained the most scientific attention in the past decades (6). More recently, it has become clear that other routes of mast cell activation can also lead to degranulation and, thereby, anaphylaxis. An intriguing G-protein coupled receptor termed the Mas-Related G-protein coupled Receptor X2 (MRGPRX2) was initially considered to be expressed exclusively on mast cells in humans (7). More recently, functional MRGPRX2 membrane surface expression has also been described on eosinophils and basophils, although no quantitative comparison was made with mast cells (8). It has also become clear that the expression and functionality of MRGPRX2 on human MC extracted from different bodily tissues can vary: skin mast cells appear to have the highest MRGPRX2 expression whereas lung mast cells do not express MRGPRX2 (9). MRGPRX2 has many ligands, including hormones and neuropeptides, small molecule drugs and venoms (7). It has extensively been proven that binding of one of these ligands to MRGPRX2 induces mast cell degranulation, but little to none cytokine production by mast cells (10, 11). However, the intracellular signaling cascade downstream of the receptor has not yet been fully elucidated.

Mast cell activation through MRGPRX2 presumably plays a major role in pseudo-allergic reactions induced by small molecule drugs, including neuromuscular blocking agents and insect venom (10, 12). However, it is unknown why not all individuals experience such hypersensitivity reactions upon administration of the mentioned MRGPRX2 ligands and, more importantly, how to prevent them. Next to hypersensitivity reactions to exogenous substances, MRGPRX2 activation has been linked to chronic spontaneous urticaria (13, 14), atopic dermatitis (15) and allergic asthma (16). Thereby, it forms a potentially interesting target for treatment of various mast cell related diseases. In order to develop effective therapies, the biological behavior of the receptor and its downstream intracellular mechanisms need to be elucidated. This requires profound insight into MRGPRX2 biology in mast cells, which could ideally be obtained from comprehensive *in vitro* approaches, where a standardized experimental setting is provided.

In vitro research with human mast cells is severely hampered by the fact that they typically display low proliferative activity. Moreover, the delicate nature of these cells is easily disturbed by physical triggers, including mechanical stress (17). Many researchers culture human mast cells (HuMC) from $CD34^+$ myeloid progenitor cells derived from buffy coats, cord blood or bone marrow to study human mast cell biology *in vitro* (18). These HuMC, which develop from progenitors cells by use of specific culture medium conditions, indeed resemble normal human mast cells closely and have been shown to express MRGPRX2 accordingly (19). Unfortunately, the method to differentiate them is time consuming and expensive. Furthermore, there is appears to be a limited time span in which they can be optimally used for experiments, e.g. at the age of 12-16 weeks (20).

To avoid these practical difficulties, a few human mast cell lines are available. Laboratory of Allergic Diseases type 2 cells (LAD2) were derived from CD34+ cells isolated from bone marrow aspirate of a patient with aggressive systemic mastocytosis without any detectable KIT mutation (21). LAD2 cells stably express IgE receptor type 1 (FcεR1), display a granular appearance, require stem cell factor (SCF) for survival and proliferate slowly (doubling time of 2 weeks). Furthermore, LAD2 cells have been proven to functionally express MRGPRX2 (22, 23). As such, the LAD2 cell line could be considered as reasonably resembling normal human mast cells. However, a main disadvantage for the use of LAD2 for *in vitro* research is their very slow proliferation rate, making massive expansion troublesome (21).

Another human mast cell line is Human Mast Cell 1 line (HMC1), that was derived from a patient with mast cell leukemia. HMC1 cells harbor either 1 or 2 activating mutations in KIT (dependent on the exact subtype of HMC1 line), rendering them independent of SCF for survival and making them highly proliferative with a doubling time of 2-3 days (24, 25). HMC1 displays a dedifferentiated phenotype as evidenced by marginal FcεR1 expression and a spindle-shaped hypogranular appearance. While they display only little release of histamine, tryptase and other typical mast cell mediators upon degranulation, HMC1 cells can produce large amounts of cytokines upon activation (24, 26). Although it is an immature, immortalized human mast cell line with consequential dissimilarities as compared with normal human mast cells, HMC1 could represent an attractive model for MRGPRX2 research, mainly due to its high *in vitro* proliferation rate. To our knowledge, only one other group has previously investigated the expression of MRGPRX2 in HMC1. In their paper, they

briefly described the functionality of HMC1 as nonexistent, although based on rather limited data (27).

It is thus clear that there is a need for a feasible *in vitro* mast cell model to study MRGPRX2 biology in this cell type. Although HMC1 have practical advantages over LAD2 and HuMC when used for *in vitro* studies, it should first be ensured whether they express and respond to MRGPRX2 and how this relates to LAD2 and HuMC. The goal of this study was to examine the expression and functionality of MRGPRX2 on HMC1, and compare this to LAD2 and HuMC. Moreover, we developed a method to improve HMC1 degranulation capacity which renders them a potentially useful model to functionally study MRGPRX2.

METHODS

Cell Culture

HMC1.2 cells [kindly provided by Dr. Butterfield, Mayo Clinic Rochester, Minnesota (25)], were cultured in Roswell Park Memorial Institute medium (RPMI, Lonza, Verviers, Belgium) supplemented with 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Lonza, Verviers, Belgium), 10% inactivated fetal calf serum (FCS), 50 µM β-mercapto-ethanol (Sigma-Aldrich, St. Louis, Missouri), and 1% antibiotics (penicillin/streptomycin, Lonza, Verviers, Belgium). We have used HMC1.2 cells, which harbor the G560V and D816V mutations in KIT. For the rest of this manuscript, the term 'HMC1' will be used. HMC1 cells were passaged 2-3 times a week and maintained at a density of 1-1.2*10^6 cells/ml.

LAD2 cells [kindly provided by Drs. Kirshenbaum and Metcalfe, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland (21)] were cultured in Stem Pro-34 medium, supplemented with 2.6% nutrient supplement (both Life Technologies, Grand Island, New York), 2 mmol/L ultraglutamine (Lonza, Verviers, Belgium), recombinant human stem cell factor (rhSCF) (100 ng/ml, R&D systems, Abbingdon, UK), and 1% antibiotics (penicillin/streptomycin). Cells were passaged once a week, preserving 50% old medium and adding 50% new medium with freshly added rhSCF (100 ng/ml).

Primary human mast cells (HuMC) were established from $CD34^+$ myeloid progenitors cells isolated from buffy coats of peripheral blood obtained from healthy blood bank donors, based on the protocol published by Folkerts et al. (28). First, peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats by ficoll density separation. Subsequently, lineage depletion (CD3/CD19/CD14) was conducted by magnetic-activated cell sorting with magnetic beads (Human Lineage Cell Depletion Kit; Miltenyi Biotec, Galdbach, Germany). The negative fraction, considered to be enriched for $CD34^+$ hematopoietic stem cells, was exposed to selection pressure using specific culturing conditions, enabling the progenitor cells to develop into HuMC. During the first month, cells were cultured in culture medium I, containing StemSpan medium (StemCell technologies, Vancouver, British Columbia, Canada) supplemented with 1% penicillin/streptomycin, recombinant human interleukin 6 (rhIL-6, 50 ng/mL, Peprotech, Rocky Hill, NJ), rhIL-3 (10 ng/mL, Peprotech), and rhSCF (100 ng/mL, Peprotech). Cells were kept at a density of $0.5\text{-}1*10^6$ cells/ml. With every passage, 50% of the old medium was preserved and 50% of new medium with freshly included rhSCF, rhIL-6 and rhIL-3 was added. Hereafter, culture medium I was gradually substituted by culture medium II by again adding 50% new medium to 50% preserved medium with every passage. Culture medium II contained Iscove's modified Dulbeccos medium with GlutaMAX-I (IMDM, Lonza, Verviers, Belgium), 50 µM β-mercapto-ethanol, 0.5% FCS, 1% Insulin-Transferrin-Selenium (Life Technologies, Carlsbad, CA), with 1% antibiotics (penicillin/streptomycin), with fresh addition of 100 ng/ml rhSCF and 50 ng/ml rhIL-6. HuMC were confirmed to have a specific mast cell phenotype after ~12 weeks in culture, by toluidine blue staining and flowcytometric analysis of FcεR1, KIT and MRGPRX2 expression (see below). HuMC aged 12-16 weeks were used for further experiments.

Receptor Profiling

Surface membrane expression of KIT (CD117), FcεRI and MRGPRX2 was assessed by flowcytometry on LAD2, HMC1, HuMC and PBMCs (as triple negative control). Hereto, $2*10^5$ cells were suspended in 500 µl of phosphate-buffered saline (PBS), followed by centrifugation at 200g (HuMC), 300g (HMC1 and LAD2) or 500g (PBMC) for 5 minutes. After removal of the supernatant, cell pellets were resuspended in 90 µl PBS. Subsequently, cells were stained by adding 5 µl monoclonal mouse-anti-human MRGPRX2-alexafluor488 antibody (R&D systems, Mineapolis U.S.), 1 µl monoclonal mouse-anti-human CD117-Pe-Cy7 antibody (Beckman Coulter, Brea, U.S.) and 2 µl monoclonal mouse-anti-human FcεRI-APC-A antibody (Biolegend, San Diego, U.S), followed by 15 minutes incubation at room temperature (RT) in the dark. Thereafter, cells were washed with 2 mL PBS, resuspended in 150 µl FACS flow buffer (1% FCS, 0.09% NaN_3, PBS) and analyzed on a flowcytometer (LSRII, Becton Dickinson, Franklin Lakes, New Jersey, U.S). The mean fluorescent intensity (MFI) for each antibody was determined by measuring at least $2*10^4$ single live cells. Per cell type, unstained and unstimulated stained conditions were always included. For each time point, the experiment was conducted two fold.

Toluidine Blue Staining

Firstly, cells were washed and resuspended in PBS in a concentration of $2*10^5$ cells/ml. In order to make cytospin slides, 50 µl of cell suspension was loaded into loading chambers, followed by centrifugation for 5 minutes at 14g using a cytofuge (Nordic Immunological laboratories, the Netherlands), thus transferring cells onto glass slides. Next, the cells were fixed with Mota's fixative for 10 minutes, which was then removed *via* indirect rinsing with deionized water. Subsequently, 2-3 droplets of toluidine blue dye were added onto the cells for 20 minutes, followed by indirect rinsing with deionized water. Cells were tapped dry and a second coverslip was placed over the stained cells, mounted with warmed gelatin

(Boom, Meppel, Netherlands). Stained slides were stored at 4 °C. The images were analyzed using an Axiovert microscope with AxioCAM MR5 (Zeiss, Oberkochen, Germany) and photographed at 100x magnification, within five days after the staining procedure.

HMC1 Degranulation Assay Using Latrunculin-B

HMC1 are recognized to display poor degranulation activity (26). To optimize HMC1 degranulation, we explored the effect of the zinc-finger toxic protein Latrunculin B (Lat-B) on degranulation induced by calcium ionophore A23187 (1μM, Sigma Aldrich, Missouri, U.S), since this has proven to be a potent general mast cell activator in our hands (29). Lat-B is a macrolide-type protein derived from the Red Sea sponge *Latrunculina magnifica* that influences actin in the cytoskeleton and thereby theoretically enhances degranulation capacity (30). Degranulation was determined by β-hexosaminidase release and CD63 expression, as described below. Degranulation assays were performed two to six fold for each time point.

MRGPRX2-Dependent Mast Cell Stimulation

Stimulation experiments were performed using the MRGPRX2 specific ligand compound 48/80 (C48/80, Sigma Aldrich, Missouri, U.S). For LAD2, a concentration range of 0.1-1.5-10 μg/ml C48/80 was used, while for HMC1 1-5-10-50-100 μg/ml of C48/80 was used. Based on the results found with LAD2 and HMC1, a concentration range of 0.1-1-10-50-100 μg/ml C48/80 was used to stimulate HuMC. As a positive control, cells were stimulated with A23187 (1 μM). Stimulation time ranged from 15 and 30 to 60 minutes and all stimulations were performed at 37°C. Due to a limited amount of cells, caused by the aforementioned slow proliferation rates of LAD2 and HuMC, not all concentrations and stimulation time-points could be tested for all three different types of mast cells.

To further prove the functionality and specificity of MRGPRX2 activation, additional inhibition experiments were conducted by pre-incubation with the MRGPRX2 specific antagonist QWF (Boc-Gln-D-Trp(Formyl)-Phe benzyl ester trifluoroacetate salt, Santa Cruz biotechnology, Texas, U.S (10, 31)) for 10 minutes, at concentrations of 10, 25 and 100 μM. An unstimulated condition containing an equal amount of the QWF diluent, dimethyl sulfoxide (DMSO), was included in every experiment as a control condition, not exceeding the maximal concentration of 0.28% DMSO during stimulation of the cells. Degranulation was assessed by β-hexosaminidase release and CD63 expression, as described below.

β-Hexosaminidase Release Assay

Mast cell degranulation was measured by β-hexosaminidase assay, essentially as previously described (29). The cells were diluted in PBS containing 1.5 μM Ca^{2+} and seeded in a 96-well plate at a concentration of $2*10^4$ cells/well. C48/80 or A23187, also diluted in PBS, were added in the indicated concentrations. After stimulation, the plate was centrifuged for 5 minutes at 300g. Hereafter, 50 μl of supernatant was transferred to another 96-wells plate (Nunc MaxiSorp™ flat-bottom) containing 50 μl 4 μM p-nitrophenyl N-acetyl- β-D-glucosamine (p-NAG, Sigma Aldrich, Missouri, U.S) in citrate buffer (pH 4.5). The cell pellet was lysed using 150 μl 0.1% Triton X solution (Sigma Aldrich, Missouri, U.S). Hereafter, 50 μl of cell lysate was transferred to another 96-wells plate containing 50 μl 4 μM pNAG solution. After incubation for 90 minutes at 37°C, 100 μl of glycine 400 mM was added to each well to end the reaction. Optical density (OD) values were measured at 405 and 620 nm, using ELISA plate reader (VersaMax microplate reader, Molecular Devices, San Jose, U.S.). The relative β-hexosaminidase release was calculated as follows:

$$\%\ \beta - \text{hexosaminidase release} = \frac{2x\ \Delta\text{supernatan}(\ = \text{OD supernatant} - \text{OD blank condition})}{(\Delta\text{supernatant} + (4 * \Delta\text{cell lysate})}$$

Surface Membrane CD63 Expression

Upregulation of CD63 expression was used as a measure for mast cell degranulation, as described previously (32). In brief, the cells were suspended in PBS with 1.5 μM Ca^{2+} to a concentration of $2x10^4$ cells in 500 μL per tube. The cells were stimulated as described above. Thereafter, the cells were fixed with 1% paraformaldehyde (PFA, Sigma Aldrich, Missouri, U.S), washed with FACS buffer and stained with 2 μl monoclonal Mouse-anti-Human CD63-APC antibody (ThermoFisher, Waltham, Massachusetts) and with 1 μl monoclonal mouse-anti-human CD117-PE-Cy7 as a positive control. Surface membrane expression of CD63 was measured using a flowcytometer (Canto II, Becton Dickinson, Franklin Lakes, New Jersey).

Because the percentage of CD63 positive cells on unstimulated mast cells could vary, especially for HMC1, a combination of CD63 expression plus increase in sideward scatter was used to determine the percentage of degranulated mast cells, and the relative increase of CD63 expression was calculated. **Figure S1** shows a representative example of flowcytometry results.

The x-fold increase of CD63 expression was calculated as follows:

$$\text{x} - \text{fold increase} = \frac{\text{percentage CD63 positive cells of total stimulated condition}}{\text{percentage CD63 positive cells of total unstimulated condition}}$$

Statistical Analysis

Statistical analysis was performed in GraphPad Prism version 5 or IBM SPSS statistics version 25. Per statistical analysis, data were checked for having a Gaussian distribution using a Shapiro-Wilk test in combination with a Q-Q plot. If data were normally distributed, a paired t-test was performed for the comparison of two groups, and a two-way ANOVA with Bonferroni post-hoc test for the comparison of multiple groups. When data had a

non-Gaussian distribution, Mann Whitney U tests were used to statistically compare continuous non-paired variables, and the Wilcoxon signed rank test for paired variables.

RESULTS

Expression of the Mast Cell Specific Receptors MRGPRX2, FcεRI and KIT

Surface membrane expression of MRGPRX2, FcεRI and KIT receptor was detected on all three different types of mast cells, albeit at different levels, with HuMC showing the highest and HMC1 showing the lowest expression level (**Figure 1**). All three receptors were hardly expressed by PBMC, confirming its use as negative control. Since MRGPRX2 surface expression by HMC1 was relatively low, we conducted real-time quantitative polymerase chain reaction (RQ-PCR) to confirm its expression. MRGPRX2 mRNA was expressed by HMC1 as well as HuMC (serving as positive control), while no MRGPRX2 mRNA was detected in PBMC (**Figure S2**).

Optimized HMC1 Degranulation Assay

Since HMC1 generally have a poor degranulation capacity, we first aimed at optimizing the degranulation assay for this cell type, in order to subsequently examine MRGPRX2 functionality. HMC1 cells displayed little granularity in comparison to LAD2 and HuMC (**Figure 2**), which might explain their limited immediate degranulation capacity (26). Pre-incubation of HMC1 cells with 2 μg/ml Lat-B for 24 hours approximately doubled the stimulatory effect of A23187 on degranulation, as shown by enhanced CD63 surface membrane expression as well as β-hexosaminidase release (**Figures 3A, B**).

Lat-B pre-incubation resulted in a marginal increase in MRGPRX2 and FcεR1 expression and a slight decrease in KIT expression on HMC1, but none of these changes were statistically significant (**Figures 4A–C**). Toluidine blue assessment revealed no gross morphological alterations of HMC1 upon incubation with Lat-B (**Figures 4D, E**).

MRGPRX2 Functionality

After establishing that incubation with Lat-B provided a suitable model for HMC1 degranulation, the potential of the MRGPRX2 specific agonist C48/80 to induce degranulation of HMC1 with and without Lat-B was investigated. C48/80 did induce surface membrane CD63 expression as well as β-hexosaminidase release by HMC1, which was enhanced by Lat-B pre-incubation (**Figures 5A, B**).

To further explore MRGPRX2 functionality, combined titration and time course experiments with C48/80 and the MRGPRX2 specific antagonist QWF were conducted. These experiments revealed that 100 times lower concentration of C48/80 was required to induce degranulation of LAD2, compared with HMC1; 1 μg/mL versus 100 μg/mL, respectively (**Figure 6**). Furthermore, C48/80-induced degranulation appeared optimal after 15-30 minutes of stimulation for LAD2, whereas at least 60 minutes stimulation was required for HMC1 (**Figure 6**). Unexpectedly, HuMC required higher concentrations of C48/80 than we anticipated on the basis of the previously observed MRGPRX2 expression (**Figure 1**), while the duration of stimulation did not influence the level of CD63 expression. Overall, HuMC and LAD2 reached much higher levels of CD63 surface membrane expression than HMC1.

To confirm the specificity of C48/80 for MRGPRX2, inhibition experiments were performed using the MRGPRX2-specific antagonist QWF to pre-incubate the cells. Indeed, C48/80-induced degranulation of HMC1 was significantly inhibited by QWF, as measured by CD63 surface membrane expression (**Figure 7A**) as well as β-hexosaminidase release (**Figure 7B**). The inhibitory effect of QWF displayed a dose-dependent manner (**Figure S3**). To check for nonspecific stimulation of β-hexosaminidase release or CD63 expression by the diluent DMSO or the inhibitor QWF, compound control experiments were performed with both substances in the highest concentrations used in this study. It was noted that only QWF appeared to increase CD63 surface membrane expression in itself (**Figure S4A**), whereas neither DMSO nor QWF induced any relevant β-hexosaminidase release (**Figure S4B**). To prevent bias

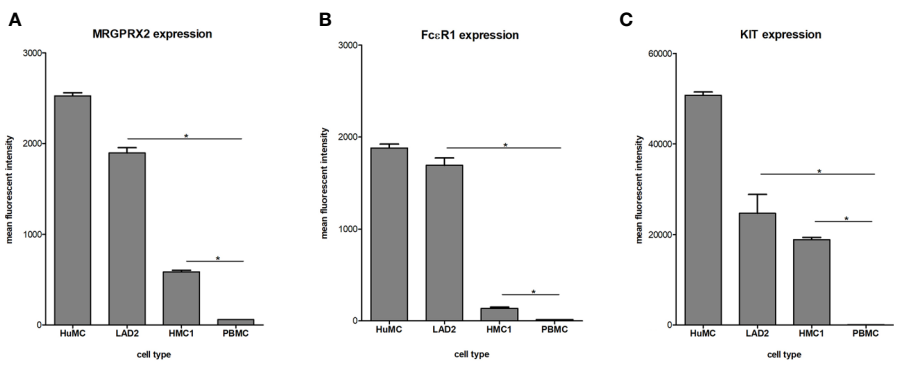

FIGURE 1 | Expression of MRGPRX2 **(A)** FcεR1 **(B)** and KIT **(C)** by different mast cell lines: HuMC, LAD2, HMC1. Receptor expression was assessed by triple staining and measured by flowcytometry. The mean fluorescent intensity (MFI) with SEM is shown of single, live population of HuMC (n=3) and LAD2, HMC1 and PBMCs (all n=6), stained with MRGPRX2-AF488, FcεRI-APC and cKIT-PE-Cy7. *p<0.05 for MFI compared with PBMC.

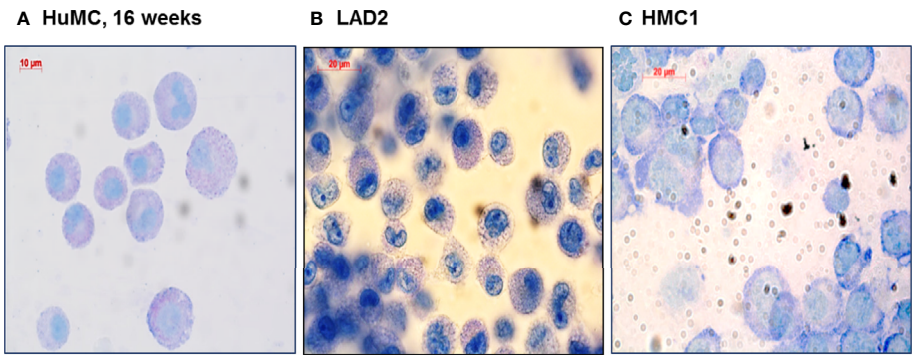

FIGURE 2 | Morphologic appearance of different mast cell lines: HuMC, LAD2, HMC1. Toluidine blue staining of HuMC **(A)** and LAD2 **(B)** show a typical granular pattern. HMC1 **(C)** shows a different morphology as compared to HuMC and LAD2, in particular, granules are absent.

by this nonspecific effect of QWF on CD63 expression, the concentration of QWF that did not induce more CD63 expression than the negative controls was selected as the maximal concentration used in inhibition experiments. However, this probably explains the fact that the effect of QWF on C48/80-induced CD63 expression was smaller than on β-hexosaminidase release as can be appreciated from **Figure 7**, although the biological explanation for this finding is lacking.

DISCUSSION

Here, for the first time to our knowledge, we have demonstrated that MRGPRX2 is functionally expressed by the HMC1 cell line. Since MRGPRX2 activation mainly induces mast cell degranulation and little to none cytokine production (11), a model was sought to enhance the degranulation by HMC1. Although these cells displayed diminished degranulation capacity compared with LAD2 and HuMC, we found HMC1 suitable for degranulation studies after pre-incubation with Lat-B.

The main advantage of HMC1 over other mast cell lines, is the fact that they have a 10-fold higher division rate than LAD2 and HuMC. Unfortunately, working with HMC1 also has several caveats, most importantly its poor degranulation capacity. Furthermore, its high constitutive KIT activity might have a negative influence on MGRPRX2-mediated activation (33). Until now, it was unclear whether HMC1 expressed MRGPRX2. In fact, one published study briefly described MRGPRX2 functionality on HMC1 as nonexistent, although this appeared to be based on somewhat unusual techniques and stimulation compounds for the investigation of MRGPRX2 functionality in human mast cells (cortistatin and bovine adrenal medulla docosapeptide) (27). However, some degree of MRGPRX2 expression could be demonstrated by realtime-PCR in this study, although the researchers did not use other techniques such as flowcytometry to confirm their findings (27).

Overall, HuMC theoretically form a more representative cell type to investigate MRGPRX2 biology *in vitro*. However, HuMC required much higher concentrations of C48/80 than we expected from the high levels of MRGPRX2 expression that were initially found. It must be noted that the HuMC which were used for the initial measurement of receptor expression (**Figures 1** and **S2**) were derived from different donors than the HuMC which were used for the stimulation assays (**Figure 6**).

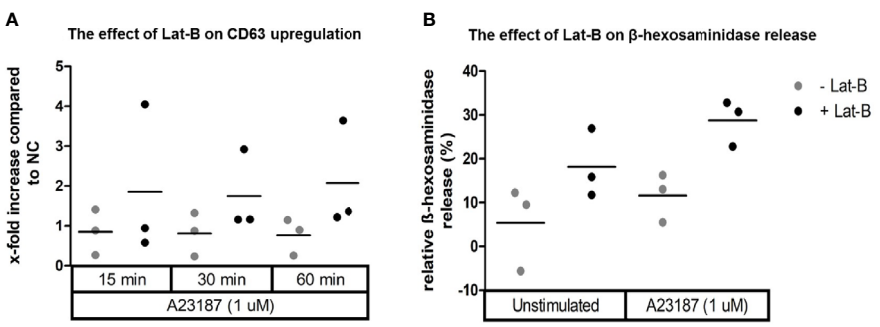

FIGURE 3 | Calcium ionophore A23187 induced degranulation of HMC1 with or without Lat-B. HMC1s pre-incubated with 2 µg/ml Lat-B for 24 hours showed an overall higher fold induction of CD63 expression upon stimulation with 1 µM A23187, as compared to HMC1 cells without pre-incubation **(A)**. The same effect was seen on β-hexosaminidase release **(B)**. No substantial differences were seen regarding the length of stimulation time.

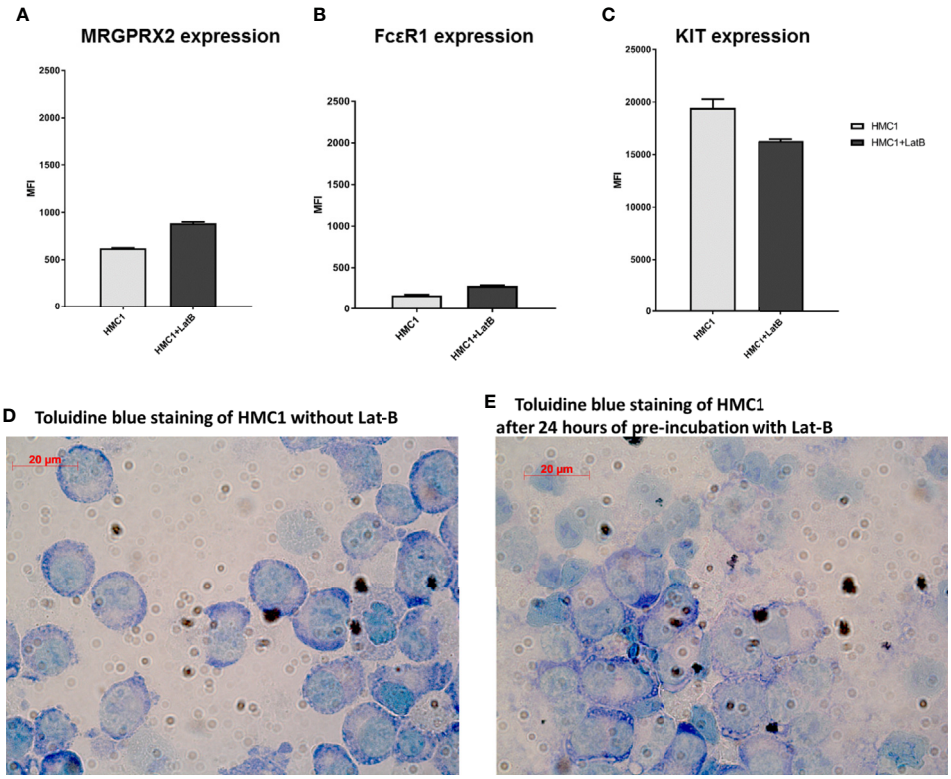

FIGURE 4 | Latrunculin-B does not induce phenotypical changes to HMC1 cells. The receptor expression of HMC1s with and without pre-incubation with 2 µg/ml Lat-B for 24 hours is shown (n=3, Mean Fluorescent Intensity with SEM). The expression of MRGRPX2 **(A)** and FcεRI **(B)** is slightly upregulated after pre-incubation with Lat-B, but not statistically significant. The expression of KIT is decreased after pre-incubation with Lat-B, but again not statistically significant **(C)**. The morphological appearance of HMC1s is not changed upon pre-incubation with Lat-B **(D, E)**.

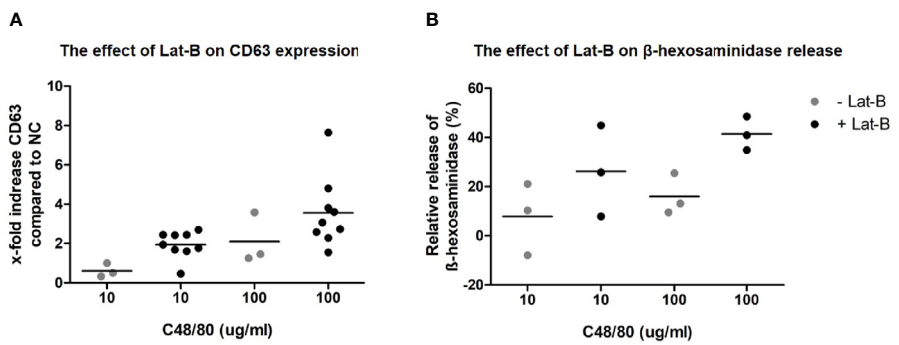

FIGURE 5 | C48/80 induced degranulation of HMC1 with and without Lat-B. **(A)** Stronger upregulation of CD63 in response to C48/80 stimulation was seen after pre-incubation with Lat-B, with a dose dependent effect. **(B)** The relative β-hexosaminidase release was increased after pre-incubation with Lat-B compared to without Lat-B, but not reaching statistical significance.

Upon determining the MRGPRX2 expression on the latter HuMC batches, we found a considerably lower MRGPRX2 expression than on the HuMC from the first two donors, using flowcytometry as well as RQ-PCR. The MRGPRX2 expression of unstimulated HuMC could vary up to a factor 10 between different donors, whereas the KIT expression was comparable across all donors (data not shown). Whether this can be influenced by tweaking the culture media or that it is merely an inter-individual difference needs to be further investigated. This inter-donor variability of MRGPRX2 expression shows that although resembling mature human mast cells, HuMC derived from buffy coats are an unpredictable system for studying MRGPRX2-mediated degranulation, potentially causing incompatible results of assays.

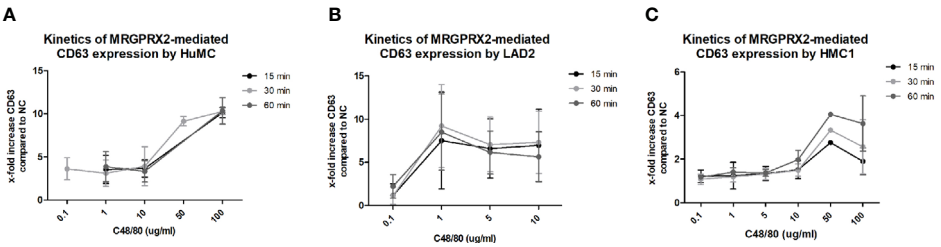

FIGURE 6 | Different kinetics of MRGPRX2 mediated degranulation of three mast cell lines: HMC1, LAD2, HuMC. The mean x-fold increase of CD63 expression upon stimulation with C48/80 is shown for all three cell lines at different concentrations of C48/80 and different time periods of stimulation. The error bars depict SEM. n=2 for HuMC and LAD2s and n=3 for HMC1s. **(A)** HuMCs of two donors were used. Both showed a similar pattern, with significant CD63 upregulation only for the highest concentrations of C48/80. There was no difference for different periods of stimulation. **(B)** LAD2s responded to much lower concentrations of C48/80, and 30 minutes proved the optimal period of stimulation. However, considerable variation was seen between the experiments. **(C)** HMC1s, pre-incubated with Lat-B, only showed CD63 upregulation upon stimulation with C48/80 at a concentration of 50 or 100 µg/ml for 60 minutes.

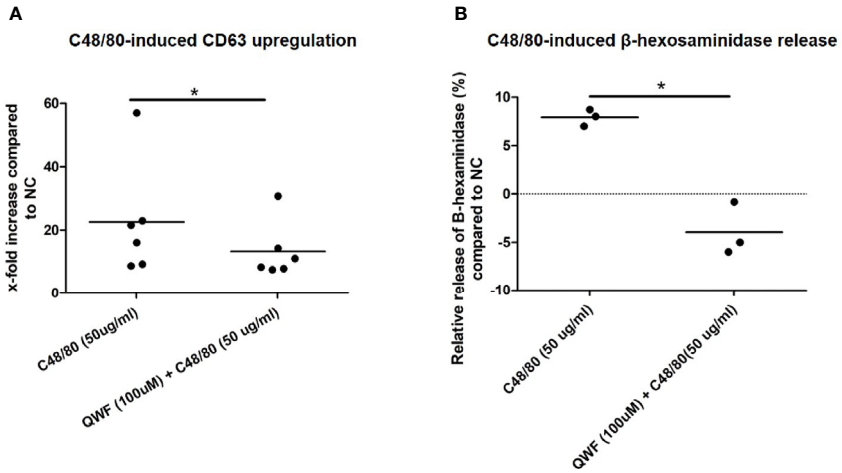

FIGURE 7 | QWF effectively inhibits C48/80 induced degranulation. **(A)** QWF effectively and significantly inhibits C48/80 induced degranulation of HMC1s, as measured by CD63 expression (*p 0.03, Wilcoxon rank test). **(B)** QWF effectively and significantly inhibits C48/80 induced degranulation of HMC1s, as measured by β-hexosaminidase release (*p 0.01). Relative increases compared with the negative control are shown, the dashed line thus represents the negative control condition.

To avoid differences in MRGPRX2 expression throughout experimental settings, the use of a phenotypically stable cell line such as HMC1 is more attractive, especially knowing that the degranulation capacity can be optimized with Lat-B. The effect of Lat-B on cell degranulation has been described before, and is presumed to be caused by its binding to monomeric actin, inhibiting F-actin polymerization and thereby disrupting micro-filament mediated processes (30). Disruption of this F-actin barrier is necessary to allow exocytosis of granules. F-actin disruption, followed by rapid cortical actin disassembly, is therefore an important feature of mast cell and basophil degranulation, as has been demonstrated for example by IgE-mediated degranulation in rat basophilic leukemia cell line (RBL) cells (34). Accordingly, Frigeri and Apgar showed augmentation of IgE-mediated degranulation by RBL cells after pre-incubation with Lat-B, with an increase in β-hexosaminidase release from 20% to 40% (35). Next to this, Smrz et al. found that SCF could solely induce degranulation of HuMC after incubation with Lat-B (36). This will probably not be of significance to HMC1, since they are independent of SCF due to the activating mutations in KIT which are the hallmark of this cell line.

Although we have attempted to exclude functional effects of Lat-B on mast cell behavior, it remains an artificial method which is different from the *in vivo* situation in various ways. Furthermore, the degranulation of HMC1 will never be an optimal representation of wild-type cell lines. For example, several endogenous and exogenous stimuli can influence MRGPRX2 activity in the human body, such as other cytokines and hormones (15). This might be different for HMC1 due to their immature phenotype and continuous KIT activity. Moreover, the kinetics of HMC1 degranulation are different compared with other mast cell types as was demonstrated here. Gaudenzio et al. previously demonstrated that the degranulation kinetics of HuMC can also vary according to different stimuli as well (11).

Dependent on the aim of a study and the stimuli used, researchers will have to choose the most suitable cell line based

on the characteristics of each cell type. HuMC theoretically form the most optimally representative cells for biological research, but since this is not a stable cell line, the phenotype and receptor expression can vary between donors. Furthermore, it is yet unknown to what extent the receptor expression fluctuates according to the maturation of the HuMC. LAD2 represents a more stable cell line, but is less useful for *in vitro* research due to its low proliferation rate.

In conclusion, HMC1 functionally expresses MRGPRX2, albeit at a lower level than LAD2 and HuMC. The use of HMC1 has the benefits of a phenotypically stable cell line with high proliferative activity, making them feasible to conduct extensive *in vitro* experiments. Incubation with the actin-disruptive macrolide Lat-B enhances the degranulation capacity of HMC1 without functionally changing their phenotype. Although HMC1 have different degranulation kinetics compared with wild-type mast cells, they form a feasible model for the investigation of mast cell degranulation and MRGPRX2 biology.

AUTHOR'S NOTE

All authors are members of the Academic Centre of Excellence for Allergic Diseases.

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.

AUTHOR CONTRIBUTIONS

MH, AS and WD created the concept of this study. AS, SM, BS and MS performed the experiments and created the figures. The research was discussed on multiple occasions with the whole team of authors throughout the study, adjusting the experiments where necessary. MH created the first draft of the manuscript. AS, SM, BS, PD, PH, MS and WD critically revised the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.625284/full#supplementary-material

Supplementary Figure 1 | Representative example of flowcytometry results for CD63 upregulation upon C48/80 induced degranulation of HMC. Degranulated mast cells are in Q2: they are CD63 positive, and have increase sideward scatter. **(A)**. For the negative control condition, only 0.290% of all cells are degranulated. **(B)**. After stimulation with C48/80, 25.7% of HMC1 are in Q2, thus considered degranulated.

Supplementary Figure 2 | Confirmation of MRGPRX2 RNA expression by HMC1. A clear mRNA expression of MRGPRX2 was shown to be present in HMC1 (n=3), but not in PBC (n=2), which were used as negative control. Mean with SEM is shown for CT values.

Supplementary Figure 3 | QWF dose-dependently inhibits C48/80 induced HMC1 degranulation. A titration of QWF doses was performed to identify the optimally inhibiting dose of QWF. Two representative experiments are shown. HMC1s, pre-incubated with Lat-B, were first incubated with QWF for 10 minutes and subsequently stimulated with C48/80 for 60 minutes. A dose-dependent decrease of QWF on CD63 expression was found.

Supplementary Figure 4 | Control conditions of the compounds used in this study. The effect of DMSO and QWF alone on the activation of HMC1 was investigated as an extra control. The highest concentrations used in the study were taken: 0.28% DMSO, and 100 µg/ml QWF. **(A)** Both DMSO and QWF did not induce relevant β-hexosaminidase release (n=3). **(B)** QWF appeared to induce a nonspecific CD63 upregulation although not statistically significant compared with the diluent control DMSO (n=6).

REFERENCES

1. Huber M, Cato ACB, Ainooson GK, Freichel M, Tsvilovskyy V, Jessberger R, et al. Regulation of the pleiotropic effects of tissue resident mast cells. *J Allergy Clin Immunol* (2019) 144(4S):S31–S45. doi: 10.1016/j.jaci.2019.02.004
2. Moon TC, Befus AD, Kulka M. Mast cell mediators: their differential release and the secretory pathways involved. *Front Immunol* (2014) 5:569. doi: 10.3389/fimmu.2014.00569
3. Metcalfe DD, Peavy RD, Gilfillan AM. Mechanisms of mast cell signaling in anaphylaxis. *J Allergy Clin Immunol* (2009) 124(4):639–46; quiz 47-8. doi: 10.1016/j.jaci.2009.08.035
4. Komi DEA, Khomtchouk K, Santa Maria PL. A Review of the Contribution of Mast Cells in Wound Healing: Involved Molecular and Cellular Mechanisms. *Clin Rev Allergy Immunol* (2020) 58(3):298–312. doi: 10.1007/s12016-019-08729-w
5. Metz M, Piliponsky AM, Chen CC, Lammel V, Abrink M, Pejler G, et al. Mast cells can enhance resistance to snake and honeybee venoms. *Science* (2006) 313(5786):526–30.
6. Caslin HL, Kiwanuka KN, Haque TT, Taruselli MT, MacKnight HP, Paranjape A, et al. Controlling Mast Cell Activation and Homeostasis: Work Influenced by Bill Paul That Continues Today. *Front Immunol* (2018) 9:868. doi: 10.3389/fimmu.2018.00868
7. McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* (2015) 519(7542):237–41. doi: 10.1038/nature14022
8. Wedi B, Gehring M, Kapp A. The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: Expression and function. *Allergy* (2020) 75(9):2229–42. doi: 10.1111/all.14213
9. Varricchi G, Pecoraro A, Loffredo S, Poto R, Rivellese F, Genovese A, et al. Heterogeneity of Human Mast Cells With Respect to MRGPRX2 Receptor Expression and Function. *Front Cell Neurosci* (2019) 13:299. doi: 10.3389/fncel.2019.00299
10. Porebski G, Kwiecien K, Pawica M, Kwitniewski M. Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2) in Drug Hypersensitivity Reactions. *Front Immunol* (2018) 9:3027. doi :10.3389/fimmu.2018.03027
11. Gaudenzio N, Sibilano R, Marichal T, Starkl P, Reber LL, Cenac N, et al. Different activation signals induce distinct mast cell degranulation strategies. *J Clin Invest* (2016) 126(10):3981–98. doi :10.1172/JCI85538
12. Che D, Rui L, Cao J, Wang J, Zhang Y, Ding Y, et al. Cisatracurium induces mast cell activation and pseudo-allergic reactions via MRGPRX2. *Int Immunopharmacol* (2018) 62:244–50. doi: 10.1016/j.intimp.2018.07.020
13. Vena GA, Cassano N, Di Leo E, Calogiuri GF, Nettis E. Focus on the role of substance P in chronic urticaria. *Clin Mol Allergy* (2018) 16:24. doi: 10.1186/s12948-018-0101-z
14. Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is

upregulated in the skin of patients with severe chronic urticaria. *J Allergy Clin Immunol* (2014) 134(3):622–33.e9. doi: 10.1016/j.jaci.2014.05.004
15. Wang Z, Babina M. MRGPRX2 signals its importance in cutaneous mast cell biology: Does MRGPRX2 connect mast cells and atopic dermatitis? *Exp Dermatol* (2020) 29(11):1104–11. doi: 10.1111/exd.14182
16. An J, Lee JH, Won HK, Kang Y, Song WJ, Kwon HS, et al. Clinical significance of serum MRGPRX2 as a new biomarker in allergic asthma. *Allergy* (2020) 75 (4):959–62. doi: 10.1111/all.14084
17. Fowlkes V, Wilson CG, Carver W, Goldsmith EC. Mechanical loading promotes mast cell degranulation via RGD-integrin dependent pathways. *J Biomech* (2013) 46(4):788–95. doi: 10.1016/j.jbiomech.2012.11.014
18. Yin Y, Bai Y, Olivera A, Desai A, Metcalfe DD. An optimized protocol for the generation and functional analysis of human mast cells from CD34(+) enriched cell populations. *J Immunol Methods* (2017) 448:105–11. doi: 10.1016/j.jim.2017.06.003
19. Elst J, Sabato V, Faber MA, Bridts CH, Mertens C, Van Houdt M, et al. MRGPRX2 and Immediate Drug Hypersensitivity: Insights from Cultured Human Mast Cells. *J Investig Allergol Clin Immunol* (2020) 0. doi: 10.18176/jiaci.0557
20. Schmetzer O, Valentin P, Smorodchenko A, Domenis R, Gri G, Siebenhaar F, et al. A novel method to generate and culture human mast cells: Peripheral CD34+ stem cell-derived mast cells (PSCMCs). *J Immunol Methods* (2014) 413:62–8. doi: 10.1016/j.jim.2014.07.003
21. Kirshenbaum AS, Akin C, Wu Y, Rottem M, Goff JP, Beaven MA, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk Res* (2003) 27(8):677–82. doi: 10.1016/S0145-2126(02)00343-0
22. Ding Y, Che D, Li C, Cao J, Wang J, Ma P, et al. Quercetin inhibits Mrgprx2-induced pseudo-allergic reaction via PLCgamma-IP3R related Ca(2+) fluctuations. *Int Immunopharmacol* (2019) 66:185–97. doi: 10.1016/j.intimp.2018.11.025
23. Lansu K, Karpiak J, Liu J, Huang XP, McCorvy JD, Kroeze WK, et al. In silico design of novel probes for the atypical opioid receptor MRGPRX2. *Nat Chem Biol* (2017) 13(5):529–36. doi: 10.1038/nchembio.2334
24. Nilsson G, Blom T, Kusche-Gullberg M, Kjellen L, Butterfield JH, Sundstrom C, et al. Phenotypic characterization of the human mast-cell line HMC-1. *Scand J Immunol* (1994) 39(5):489–98. doi: 10.1111/j.1365-3083.1994.tb03404.x
25. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk Res* (1988) 12 (4):345–55. doi: 10.1016/0145-2126(88)90050-1
26. Guhl S, Babina M, Neou A, Zuberbier T, Artuc M. Mast cell lines HMC-1 and LAD2 in comparison with mature human skin mast cells–drastically reduced levels of tryptase and chymase in mast cell lines. *Exp Dermatol* (2010) 19 (9):845–7. doi: 10.1111/j.1600-0625.2010.01103.x
27. Subramanian H, Kashem SW, Collington SJ, Qu H, Lambris JD, Ali H. PMX-53 as a dual CD88 antagonist and an agonist for Mas-related gene 2 (MrgX2) in human mast cells. *Mol Pharmacol* (2011) 31(6):1005–13. doi: 10.1124/mol.111.071472
28. Folkerts J, Gaudenzio N, Maurer M, Hendriks RW, Stadhouders R, Tam SY, et al. Rapid identification of human mast cell degranulation regulators using functional genomics coupled to high-resolution confocal microscopy. *Nat Protoc* (2020) 15(3):1285–310. doi: 10.1038/s41596-019-0288-6
29. Hermans MAW, Schrijver B, van Holten-Neelen C, Gerth van Wijk R, van Hagen PM, van Daele PLA, et al. The JAK1/JAK2- inhibitor ruxolitinib inhibits mast cell degranulation and cytokine release. *Clin Exp Allergy* (2018) 48(11):1412–20. doi: 10.1111/cea.13217
30. Spector I, Shochet NR, Kashman Y, Groweiss A. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* (1983) 219(4584):493–5. doi: 10.1126/science.6681676
31. Azimi E, Reddy VB, Shade KC, Anthony RM, Talbot S, Pereira PJS, et al. Dual action of neurokinin-1 antagonists on Mas-related GPCRs. *JCI Insight* (2016) 1(16):e89362. doi: 10.1172/jci.insight.89362
32. Groot Kormelink T, Arkesteijn GJ, van de Lest CH, Geerts WJ, Goerdayal SS, Altelaar MA, et al. Mast Cell Degranulation Is Accompanied by the Release of a Selective Subset of Extracellular Vesicles That Contain Mast Cell-Specific Proteases. *J Immunol* (2016) 197(8):3382–92. doi: 10.4049/jimmunol.1600614
33. Babina M, Wang Z, Artuc M, Guhl S, Zuberbier T. MRGPRX2 is negatively targeted by SCF and IL-4 to diminish pseudo-allergic stimulation of skin mast cells in culture. *Exp Dermatol* (2018) 27(11):1298–303. doi: 10.1111/exd.13762
34. Colin-York H, Li D, Korobchevskaya K, Chang VT, Betzig E, Eggeling C, et al. Cytoskeletal actin patterns shape mast cell activation. *Commun Biol* (2019) 2:93. doi: 10.1038/s42003-019-0322-9
35. Frigeri L, Apgar JR. The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. *J Immunol* (1999) 162 (4):2243–50.
36. Smrz D, Bandara G, Beaven MA, Metcalfe DD, Gilfillan AM. Prevention of F-actin assembly switches the response to SCF from chemotaxis to degranulation in human mast cells. *Eur J Immunol* (2013) 43(7):1873–82. doi: 10.1002/eji.201243214

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CASE REPORT
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Case Report: Omalizumab for Chronic Spontaneous Urticaria in Pregnancy

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Most chronic spontaneous urticaria (CSU) patients are female, and pregnancy can aggravate the disease activity of patients, but little is known about the efficacy and safety of omalizumab in pregnant CSU patients. We report two pregnant CSU patients treated with omalizumab and review the published information on omalizumab treatment during 11 pregnancies. The outcomes reported on patients with known pregnancies showed they had normal pregnancies and healthy babies as well as complete control of their CSU. The two new cases we reported support the view that omalizumab could be an effective and safe treatment option for pregnant and breastfeeding CSU patients. Further high-quality studies need to be carried out in order to obtain more information on the long-term efficacy and safety of the use of omalizumab during pregnancy in patients with chronic urticaria, including CSU.

Keywords: omalizumab, pregnancy, chronic spontaneous urticaria, wheals, angioedema

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INTRODUCTION

Chronic spontaneous urticaria (CSU) is a heterogeneous disorder with recurrent pruritic wheals and angioedema or both that markedly affects patients' quality of life (1, 2). The anti-IgE antibody omalizumab is used in CSU patients resistant to antihistamine treatment (3). Most CSU patients are female, and little is known about the efficacy and safety of omalizumab in pregnant CSU patients. We treated patient #1 and patient #2 at Peking University First Hospital with omalizumab and reviewed the published information on this treatment during pregnancy.

CASE DESCRIPTION

The first patient is a 33-year-old woman diagnosed with CSU and comorbid symptomatic dermographism 2 years ago. She had a history of spontaneously occurring recurrent pruritic

Abbreviations: CSU, Chronic Spontaneous Urticaria; UCT, Urticaria Control Test; DLQI, Dermatology Life Quality Index; CU-Q2ol, Chronic Urticaria Quality of Life Questionnaire.

wheals, most often on the extremities and trunk, that would last less than 24 h. As confirmed by provocation testing, patient #1 also developed wheals in response to rubbing of skin that happened in real life, and this was true even with minor triggers such as drying herself with a towel after showering. Patient #1 had not experienced angioedema. Treatment with first cetirizine 10mg/day and then, due to drowsiness, ebastine 10mg/day for several weeks did not reduce disease activity. As Patient #1 did not consent to the use of a higher than standard dosed antihistamine, we initiated treatment with omalizumab, in June 2020, at 300mg/month, which led to complete control as assessed by the use of the urticaria control test (UCT). The UCT assesses disease control in patients with CSU with four questions, each with five answer options (scored with 0–4 points), where a low total score indicates poor disease control and the maximum total score of 16 reflects complete control (4, 5). The UCT score of patient #1, 4 weeks after starting omalizumab, was 16. During the first 4 weeks, patient #1 also experienced a marked improvement of quality of life (QoL) impairment as assessed by the Dermatology Life Quality Index (DLQI) and the Chronic Urticaria QoL Questionnaire (CU-Q_2oL). For the DLQI and CU-Q_2oL, higher scores reflect higher QoL impairment, and a score of 0–1, for the DLQI, and of 23, for the CU-Q_2ol, indicated that there was no QoL impairment. A total of 4 weeks after the start of omalizumab application, the DLQI and CU-Q_2oL scores were 0 and 23, respectively. After 10 days of the third omalizumab application (August 2020), 10 weeks after the first application, patient #1 was found to be 10 weeks pregnant. She chose to stop omalizumab, continued with cetirizine treatment, 10 mg every 3 days, and has remained free of CU signs and symptoms since then.

The second patient is a 36-year-old woman with CSU. She had recurrent generalized wheals with pruritus (daily or almost daily for 5 years) that lasted for several hours each time and occurred without known triggers. Serum total IgE concentration was measured by a chemiluminescent immunoassay (ImmunoCAP; Thermo-Fisher Scientific, Sweden), and levels of 100 kU/L or greater were defined as increased. Thyroid autoantibodies, including serum anti-thyroid peroxidase antibody (anti-TPO IgG) and anti-thyroglobulin antibody (anti-TG IgG), were determined using an electrochemiluminescence immunoassay (Roche Elecsys-2010; Roche Diagnostics, U.S.) with normal reference ranges of 0–34 IU/ml and 0–115 IU/ml, respectively; the serum total IgE of Patient #2 was low at 19.3 kU/L, anti-TPO IgG was found to be elevated at 87 IU/ml, and anti-TG IgG was normal at 20 IU/ml). A biopsy taken from lesional skin of patient #2 showed a perivascular inflammatory infiltrate of lymphocytes and eosinophils, erythrocyte extravasation, and scant edema within the superficial and mid dermis, without fibrinoid deposits and leukocytoclasis, consistent with urticaria. Patient #2, after being diagnosed with CSU in June 2018, was treated with cyclosporine and was controlled well during the 10 months of treatment. In May 2019, CSU showed exacerbation, cyclosporine was stopped, and omalizumab treatment was started (UCT:1; DLQI:15; CU-Q2ol:80). Three days after the first application (300 mg), she was free of CSU signs and symptoms but only for 5 days. We increased the dose of a second application after 4 weeks to 450 mg, which led to complete recovery within 3 days for 3 months, at 450 mg/month (UCT:16; DLQI:0; CU-Q2ol:23). We then decreased the dose to 300 mg/month, and complete control was maintained by this treatment until January 2020, when patient #2 experienced severe exacerbation of CSU phenotype (UCT:1; DLQI:12; Cu-Q2ol:65) and was found to be 12 weeks pregnant. With the patient's informed consent, we increased the dose of omalizumab to 450 mg, and she regained complete control within 3 days. Two attempts to reduce to 300 mg/month failed, and 450 mg/month omalizumab treatment was maintained until August 2020, when patient #2 gave birth to a full-term healthy male infant (weight 3350g, length 50cm). Patient #2 is breastfeeding her child and is symptom-free with 450 mg/month omalizumab.

Patient #1 and Patient #2 have given written informed consent to the publication of their case details. The study was conducted according to the Declaration of Helsinki. It was approved by the Chinese Ethics Committee of Registering Clinical Trial (ChiECRCT20190131) and registered with the Chinese clinical trial registry (ChiCTR1900024869).

DISCUSSION

Taken together, both patients described herein became pregnant while on omalizumab treatment, both achieved complete response during pregnancy (albeit at higher than the standard dose in one patient), and both pregnancies were unproblematic.

At this time, only 11 pregnancies in CSU patients treated with omalizumab were reported in the literature (**Table 1** (6–11)). Similar to patients #1 and #2, almost all patients previously reported had normal pregnancies and healthy babies as well as complete control of their CSU. Of note, increasing the dose of omalizumab during pregnancy was only reported in one previous CSU patient. In this patient, the first CSU patient ever reported to receive omalizumab during pregnancy, disease activity while on omalizumab 150mg/month increased markedly, and intervals were shortened to every 15 days, which resulted in complete remission (11). Patient #2 was also given an increased dose during pregnancy, from 300mg to 450mg omalizumab per month, and is the first pregnant CSU patient reported to receive more than the licensed dosed omalizumab. Nonresponse to standard dosed omalizumab in CSU had not previously been linked to high body weight, and patient #2 was 70–80 kg (before, during, and after pregnancy) and 165 cm, i.e., not overweight. Omalizumab blood concentrations of patient #2 had not been assessed as no assay to do so was available to us and nonresponse to standard dosed omalizumab in CSU had not previously been linked to altered blood levels of omalizumab.

No evidence of a relationship between omalizumab exposure and increased risk of adverse events in pregnant patients and their infants was reported (12). Omalizumab can be an effective option for pregnant CSU patients and those who want to become pregnant.

TABLE 1 | Characteristics of reported cases of pregnant CU patients with omalizumab treatment.

	Title	Author	year	disease	number of patients	Age range	Timing of exposure	Omalizumab dose/interval	Evidence of efficacy	Evidence for safety								
										Maternal adverse events	Live births	Small for Gestational Age	Low birth weight	Preterm birth (<37wk)	Stillbirth/ fetal death (≥20wk)	Spontaneous abortion (<20wk)	congenital malformations	neonatal adverse outcomes
1	Our study	Our group	2020	CU	2	33,37	First Trimester	1/2:150-300mg/4 wk, 1/2:300-450mg/4 wk	complete control	0/2	1/1†	0/1	0/1	0/1	0/1	0/1	0/1	0/1
2	Omalizumab concentrations in pregnancy and lactation: A case study	Saito, J. et al.	2020	CSU	1	38	First Trimester	150mg/4 wk	complete control	0/1	1/1	0/1	0/1	0/1	0/1	0/1	skin defect, aortic aneurysm	0/1
3	Omalizumab as Third-Line Therapy for Urticaria During Pregnancy	Ensina, L. F. et al.	2017	CSU	2	29,32	First Trimester	1/2:150 mg/4 wk, 1/2:300mg twice with an interval of 12 wk	complete control	0/2	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
4	Omalizumab use during pregnancy for chronic spontaneous urticaria (CSU): report of two cases	González-Medina, M. et al.	2017	CSU	2	37,37	First Trimester	300 mg/4 wk	complete control	0/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
5	Omalizumab use during pregnancy for CIU: a tertiary care experience	Cuervo-Pardo L. et al.	2016	CSU	4	25-28	First Trimester	300 mg/4 wk	complete control	0/4	4/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
6	Successful and Safe Treatment of Chronic Spontaneous Urticaria with Omalizumab in a Woman during Two Consecutive Pregnancies	Ghazanfar, M. N. et.al.	2015	CSU	1	32	First Trimester	150mg/2wk, 300mg/4wk	complete control	0/1	2/2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
7	Effects of omalizumab in a patient with three types of chronic urticaria	Vieira Dos Santos R. et al.	2014	CSU ClndU	1	37	First Trimester	150mg/4wk, 150mg/2wk	complete control	0/1	1/1†	0/1	0/1	0/1	0/1	0/1	0/1	0/1

†The other patient was still pregnant. CU, Chronic Urticaria; CSU, Chronic Spontaneous Urticaria; ClndU, inducible urticaria.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

S-LL: performed data analysis and drafted the manuscript. MY: performed data analysis and prepared the manuscript. Z-TZ: designed the study and prepared the manuscript, reviewed the article critically for important intellectual content. MM: drafted the manuscript, reviewed the article critically for important intellectual content. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Agache I, Rocha C, Pereira A, Song Y, Alonso-Coello P, Solà I, et al. Efficacy and safety of treatment with omalizumab for chronic spontaneous urticaria: A systematic review for the EAACI Biologicals Guidelines. *Allergy* (2021) 76 (1):59–70. doi: 10.1111/all.14547
2. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA²LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticaria. *Allergy* (2018) 73 (7):1393–414. doi: 10.1111/all.13397
3. Zhao ZT, Ji CM, Yu WJ, Meng L, Hawro T, Wei JF, et al. Omalizumab for the treatment of chronic spontaneous urticaria: A meta-analysis of randomized clinical trials. *J Allergy Clin Immunol* (2016) 137(6):1742–50.e4. doi: 10.1016/j.jaci.2015.12.1342
4. Yu M, Chen Y, Liu B, Song XZZ. Validation of Chinese version of urticaria control test (UCT). *Chin J Dermatol* (2020) 53(7):52–7. doi: 10.35541/cjd.20191190
5. Weller K, Groffik A, Church MK, Hawro T, Krause K, Metz M, et al. Development and validation of the urticaria control test: a patient-reported outcome instrument for assessing urticaria control. *J Allergy Clin Immunol* (2014) 133(5):1365–72. doi: 10.1016/j.jaci.2013.12.1076
6. Saito J, Yakuwa N, Sandaiji N, Uno C, Yagishita S, Suzuki T, et al. Omalizumab concentrations in pregnancy and lactation: A case study. *J Allergy Clin Immunol Pract* (2020) 8(10):3603–4. doi: 10.1016/j.jaip.2020.05.054
7. Ensina LF, Cusato-Ensina AP, Camelo-Nunes IC, Solé D. Omalizumab as third-line therapy for urticaria during pregnancy. *J Investig Allergol Clin Immunol* (2017) 27(5):326–7. doi: 10.18176/jiaci.0179
8. González-Medina M, Curto-Barredo L, Labrador-Horrillo M, Giménez-Arnau A. Omalizumab use during pregnancy for chronic spontaneous urticaria (CSU): report of two cases. *J Eur Acad Dermatol Venereol* (2017) 31(5):e245–e6. doi: 10.1111/jdv.14034
9. Cuervo-Pardo L, Barcena-Blanch M, Radojicic C. Omalizumab use during pregnancy for CIU: a tertiary care experience. *Eur Ann Allergy Clin Immunol* (2016) 48(4):145–6. doi: 10.23822/EurAnnACI.1764-1489
10. Ghazanfar MN, Thomsen SF. Successful and safe treatment of chronic spontaneous urticaria with Omalizumab in a woman during two consecutive pregnancies. *Case Rep Med* (2015) 2015(2015):368053. doi: 10.1155/2015/368053
11. Vieira Dos Santos R, Locks Bidese B, Rabello de Souza J, Maurer M. Effects of omalizumab in a patient with three types of chronic urticaria. *Br J Dermatol* (2014) 170(2):469–71. doi: 10.1111/bjd.12628
12. Türk M, Carneiro-Leão L, Kolkhir P, Bonnekoh H, Buttgereit T, Maurer M. How to treat patients with chronic spontaneous urticaria with Omalizumab: questions and answers. *J Allergy Clin Immunol Pract* (2020) 8(1):113–24. doi: 10.1016/j.jaip.2019.07.021

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PD-L1 Blockade During Allergen Sensitization Inhibits the Synthesis of Specific Antibodies and Decreases Mast Cell Activation in a Murine Model of Active Cutaneous Anaphylaxis

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Programmed cell death ligand 1(PDL-1) is known for its inhibitory effect on the cellular immune response. Even though it is expressed on the surface of mast cells, its role in allergic diseases is unknown. We analyzed the effects of PD-L1 blockade in a murine model of active cutaneous anaphylaxis (ACA). C57BL/6 mice were sensitized and challenged with ovalbumin (OVA). Blood samples were collected to measure specific immunoglobulins. The mice were divided into six groups that underwent the active cutaneous anaphylaxis procedure. Group 1 (negative control) received 50 μl of phosphate-buffered saline (PBS) subcutaneously, and the other five groups were sensitized with 50 μg of OVA subcutaneously. Group 2 was the positive control, and the others received the anti-PD-L1 antibody or its isotype during sensitization (groups 3 and 4) or during the challenge (groups 5 and 6). All animals that underwent ACA on the ears with OVA and PBS were sacrificed, and the reaction was evaluated by extravasation of Evans blue (measured by spectrophotometry) and histological analysis of the collected fragments. Anti-PD-L1 blockade during the sensitization phase led to a reduction in specific IgE and IgG1 levels, allergic reaction intensity at the ACA site, and mast cell degranulation in the tissue. There was no significant biological effect of anti-PD-L1 administration on the challenge phase. PD-L1 blockade during allergen sensitization inhibited the synthesis of specific IgE and IgG1 and decreased mast cell activation in this murine model of anaphylaxis.

Keywords: PD-L1, anaphylaxis, murine model, active cutaneous anaphylaxis, inhibition

INTRODUCTION

Anaphylaxis is an immediate systemic hypersensitivity reaction induced by mast cell and basophil degranulation and is a medical emergency that can lead to death (1, 2). Studies have shown a growing incidence and mortality of anaphylactic reactions, especially those induced by drugs and food (2).

Animal models are essential to better understand the pathophysiological mechanisms involved in diseases and to evaluate the safety and efficacy of new therapies before starting clinical trials in humans. Anaphylaxis has been reproduced and analyzed in murine models thanks to the practicality of rearing, breeding, maintaining, and handling these animals and their availability, including knockout and transgenic models (3). Several animal models have been developed to study the mechanisms involved in allergic inflammation, including models of respiratory and food allergies and systemic and local anaphylaxis (3–8). Cutaneous anaphylaxis induced in animal models can be divided into active and passive anaphylaxis. In the active cutaneous anaphylaxis (ACA) model, mice are sensitized by receiving fractionated doses of the allergen, whereas in the passive cutaneous anaphylaxis (PCA) model, the animals are passively sensitized by receiving serum from other mice that were previously actively sensitized (9).

The levels of specific serum immunoglobulin E (IgE) and IgG1, which have anaphylactic functions in mice, and mast cell degranulation are often measured as markers of IgE-mediated allergic responses in animal models. Murine IgG1, present in the Th2 immune response, resembles human IgG4, and murine IgG2a, present in the Th1 immune response, has a similar function as human IgG1 (9). In the evaluation of mast cell degranulation, cells with preserved granules can be quantified by chloroacetate esterase (CAE) staining techniques, and spectrophotometric techniques are needed to measure the extravasation of dyes in tissues where there is increased vascular permeability (5, 10).

Recent studies have further clarified the factors that reduce the antitumor immune response, leading to the discovery of several molecules that act in the costimulatory and coinhibitory control pathways, called checkpoint pathways. A checkpoint pathway molecule that mediates tumor-induced immune suppression is the programmed cell death 1 (PD-1) protein. PD-1 is considered a member of the CD28 receptor family, while programmed cell death ligand 1 (PD-L1), one of its ligands, is a member of the B7 receptor family and is also known as CD274 (11, 12). PD-1 is expressed in the membrane of T and B lymphocytes, sending inhibitory signals into these cells when activated by its ligand PD-L1 or PD-L2, which are found in the membrane of dendritic cells and monocytes, but also in tumor cells (13, 14).

Physiologically, the PD-1/PD-L1 pathway works to control the degree of inflammation to prevent an exacerbated immune response with damage to normal tissue. There is marked expression of the PD-1 protein on the surface of activated T cells. When a T lymphocyte recognizes the antigen expressed by the MHC complex in the target cell, inflammatory cytokines are produced that induce the expression of PD-L1 in the tissue, which binds to and activates PD-1 in the T cell, inducing immune tolerance (15). In pathological processes, the activation of the PD-1 receptor by its ligands has an inhibitory effect on "exhausted" T lymphocytes against persistent chronic antigenic stimulation, as observed in tumors and chronic infections. The PD-1/PD-L1 pathway and its modulation are being widely studied in oncology, and the evolution of immunotherapy with antibodies against coinhibitory molecules in the treatment of cancer is one of the most successful therapeutic discoveries in recent years (16–18).

In addition to the inhibitory effect of PD-1 on lymphocytes, its binding to PD-L1 or PD-L2 leads to polarization of the immune response toward the Th2 or Th1 profile, respectively (19, 20). In an animal model of respiratory allergy, pulmonary dendritic cells express PD-L1 and PD-L2 after antigen recognition and activation. The PD-1/PD-L1 interaction produces a Th2 response with increased production of IL-4 and increased airway hyperresponsiveness (AHR). However, the PD-1/PD-L2 interaction initiates a Th1 response with increased expression of IFN-γ and, subsequently, a reduction in AHR (20).

A study in cultured mouse mast cells showed that these cells express several costimulatory and coinhibitory molecules in their membrane, including members of the B7 family, such as PD-L1 (21). Studies analyzing the expression of PD-L1 and its functions in human mast cells are lacking. Despite the many studies on the mechanisms of anaphylactic reactions, little is known about the coinhibitory pathway of the PD-1 receptor and its PD-L1 ligand in anaphylaxis. Exploring the coinhibitory pathway of immunoregulation in an anaphylaxis model could expand the range of treatment and prevention resources.

The objective of the present study was to evaluate the effects of blockade of the PD-L1 molecule on the sensitization and effector phases of ACA. We hypothesized that by blocking the PD-1/PD-L1 interaction, we could observe either an increase in the allergic reaction due to the lower inhibition of T lymphocytes, or even a decrease in the reaction due to weaker polarization of the immune response toward the Th2 profile.

MATERIALS AND METHODS

Animals

A total of 30 adult C57BL/6 mice aged 6 to 8 weeks were used, provided by Jackson Laboratory (Bar Harbor, Maine), which were reared according to the guidelines of the National Institutes of Health (NIH). The project was approved by the ethics committee of the two institutions involved in the project, the Dana-Farber Cancer Institute, Boston, Massachusetts (DFCI IRB 15-046) and the University of São Paulo Medical School (CEUA-FMUSP 1286-2019).

Experimental Design

The antigen was ovalbumin (OVA), and the adjuvant used was aluminum hydroxide, both from Sigma-Aldrich. The PD-L1 protein was blocked by the anti-PD-L1 antibody Ultra-LEAF^{TM} Purified anti-mouse CD274 (B7-H1, PD-L1) (BioLegend). The Ultra-LEAF^{TM} Purified Rat IgG2b antibody, κ isotype (BioLegend)

was used as a control for the intervention that would not activate or block the PD-L1 molecule.

We used the local anaphylaxis technique to evaluate the allergic inflammatory reaction, more specifically the ACA technique, where the sensitization and challenge phases are performed in the same animal. The sensitization protocol lasted 28 days and was performed subcutaneously (sc) at the base of the mouse's tail.

The analyzed mice were divided into six groups of five animals (**Table 1**). The animals in group 1 (negative control) received injections of 50 µl of phosphate-buffered saline (PBS) in the sensitization phase, whereas the animals in the other groups were sensitized with 50 µl of OVA at 1 µg/µl on days (D) 1, 7, 14, and 21. To evaluate the effects of PD-L1 blockade in both the allergic sensitization and the effector (challenge) phase, four of these groups were administered anti-PD-L1 antibody (groups 3 and 5) or its anti-PD-L1 isotype (groups 4 and 6) intraperitoneally at a dose of 200 µl per application. These antibodies were administered one day before each OVA application (D0, D6, D13, and D20) in groups 3 and 4 to evaluate sensitization or one day before challenge (D27) in groups 5 and 6 to evaluate the effector phase. Thus, the six groups were the negative control (PBS), positive control (OVA), anti-PD-L1 in the sensitization phase, anti-PD-L1 isotype in the sensitization phase, anti-PD-L1 in the challenge phase, and anti-PD-L1 isotype in the challenge phase (**Table 1** and **Figure 1**).

Blood samples were collected on D0, D13, and D27 from the ophthalmic plexus (300 µl/bleed) for measurement of specific IgE, IgG1, and IgG2a antibodies in plasma by indirect enzyme-linked immunosorbent assay (ELISA). On D28, the mice in group 1 (negative control) were challenged with 50 µl of PBS, and the animals in the other groups were injected with 10 µl of OVA at 5 µg/µl. On this occasion, mice from all groups also received 200 µl of 0.025% Evans blue intravenously. After 10 minutes, the animals were euthanized in a CO_2 gas chamber, and the reaction was evaluated by the extravasation of Evans blue measured by spectrophotometry and by histological analysis of collected fragments (**Figure 1**).

Measurement of Specific Antibodies

The specific IgE, IgG1, and IgG2a antibodies were measured in the plasma by indirect ELISA kits (Affymetrix and eBioscience). All five mice in each group had serum antibodies specific for OVA measured at D0, D14, and D28. The collected blood was centrifuged at 2000 rpm for 10 minutes, and the plasma was separated and frozen at -20°C. To quantify IgE, IgG1, and IgG2a, a microplate was coated with ovalbumin. After incubation and washing, the sera were added at a predetermined dilution. To develop the reaction, biotinylated detection antibody specific for IgE, IgG1, or IgG2a was added, followed by incubation and washing. Lastly, the developer solution containing streptavidin-peroxidase enzyme conjugate, substrate, and chromogen was added. The colorimetric reaction was read in a spectrophotometer at 450 nm. The results are expressed as mean absorbances and were compared with the standard provided by the kit in serial dilutions.

Active Cutaneous Anaphylaxis Reaction

The ACA assay was performed to evaluate the presence of a specific IgE with anaphylactic activity on the skin of sensitized mice. The animals received 10 µl of OVA at 5 µg/µl sc in the right ear. In the left ear, 10 µl of PBS was applied as a negative

TABLE 1 | Experimental groups according to substances and techniques used.

Experimental groups	Technique	Sensitization	Challenge	Antibody	
				Sensitization	Challenge
1 – Negative control	ACA	PBS	PBS	-	-
2 – Positive control		OVA	OVA	-	-
3 – Anti-PD-L1 during sensitization				Anti-PD-L1	-
4 – Anti-PD-L1 isotype during sensitization				Isotype	-
5 – Anti-PD-L1 during challenge				-	Anti-PD-L1
6 – Anti-PD-L1 isotype during challenge				-	Isotype

PBS, phosphate-buffered saline; OVA, ovalbumin; ACA, active cutaneous anaphylaxis; Anti-PD-L1, Ultra-LEAF™ Purified anti-mouse CD274 (B7-H1, PD-L1) (BioLegend, ref.:124318); isotype, Ultra-LEAF™ purified rat IgG2b antibody, κ isotype (BioLegend, ref: 400644).

FIGURE 1 | Timeline of the experimental protocol (29-day duration – from D0 to D28). Animals were sensitized with phosphate-buffered saline (PBS) or ovalbumin (OVA) on days 1, 7, 14 and 21 and challenged on D28, when euthanasia was also performed. * Blood collection for measurement of immunoglobulins (all groups); ↓ sensitization with PBS (group 1) or OVA (groups 2 to 6); ♦ anti-PD-L1 (during sensitization in group 3 or challenge in group 5); ⋄ anti-PD-L1 isotype (during sensitization in group 4 or challenge in group 6); ▫ euthanasia, active cutaneous anaphylaxis and skin biopsy (all groups).

control. Then 0.25% Evans blue was administered intravenously. After 10 minutes of reaction, the animals were sacrificed in a CO_2 chamber. The Evans blue that extravasated into the tissue was extracted after 12 hours in 700 µl of formamide at 63°C. Then, the absorbance of the solution composed of formamide and Evans blue was measured on a spectrophotometer at 620 nm. ACA was considered positive when the absorbance was greater than 0.150 nm (**Figure 2**).

Histological Analysis: Staining and Quantification of Mast Cells

CAE, which stains the granules of mast cells, was used to stain the tissues. The number of stained cells is lower when these cells are activated because degranulation occurs in this process. Tissue samples from the ear challenged with OVA were fixed in 10% buffered formalin and embedded in paraffin, ensuring a transverse orientation of all tissues, and then cut to a thickness of 4 mm. The slides were scanned using a Pannoramic 250 Flash II 3D scanner (Histech, Budapest, Hungary). The mast cells were quantified according to area (mm^2) using computer-generated image analysis (NIH Image*J* software, version 1.49v).

Statistical Analysis

Significant differences between the experimental groups were detected by one-way analysis of variance followed by the nonparametric Mann-Whitney test or the parametric Bonferroni test. P-values < 0.05 were considered significant. SPSS software was used for statistical analysis.

RESULTS

Specific IgE, IgG1, and IgG2a Antibodies With PD-L1 Blockade

Positive control and anti-PD-L1 isotype groups developed high levels of specific IgE for OVA since D13. At that moment, specific IgE from anti-PD-L1 group was comparable to control group (p>0.05). However, specific IgE levels from anti-PD-L1 group increased in D27, reaching higher levels than the negative control group, although they were lower than the positive control and anti-PD-L1 isotype groups (**Figure 3**).

Regarding specific IgG1, levels on D13 were still low and comparable between all groups. Nonetheless, serum specific IgG1 levels increased on D27 in the positive control and anti-PD-L1 isotype groups and this effect was blocked by anti-PD-L1 administration (**Figure 4**).

There was no difference in the serum IgG2a level between the four groups (**Figure 5**).

ACA With PD-L1 Blockade

The ACA reaction was considered positive, with significant extravasation of Evans blue, in groups 2 (OVA), 4 (anti-PD-L1 isotype during sensitization), 5 (anti-PD-L1 during challenge), and 6 (anti-PD-L1 isotype during challenge). Administration of the antibody anti-PD-L1 administered during sensitization (group 3) lead to inhibition of ACA, as shown in **Figure 6**.

Histology With PD-L1 Blockade

Histological evaluation was performed on two mice from each group. CAE staining of the tissue made it possible to visualize in red the granules of mast cells that were at rest, i.e., that had not degranulated. There were fewer tissue mast cells in the groups sensitized to OVA, but anti-PD-L1 administered at sensitization attenuated this effect (**Figures 7** and **8**).

DISCUSSION

Our study suggests that PD-L1 plays a crucial role in the activation of the Th2 immune response profile. In an animal model of OVA-induced allergy, PD-L1 blockade by anti-PD-L1 monoclonal antibody during sensitization decreased the specific immunoglobulins IgE and IgG1, as well as mast cell activation, as confirmed by ACA and by histology at the site of challenge. These effects were not observed when the blockade occurred only in the effector phase, suggesting that this pathway acts in the

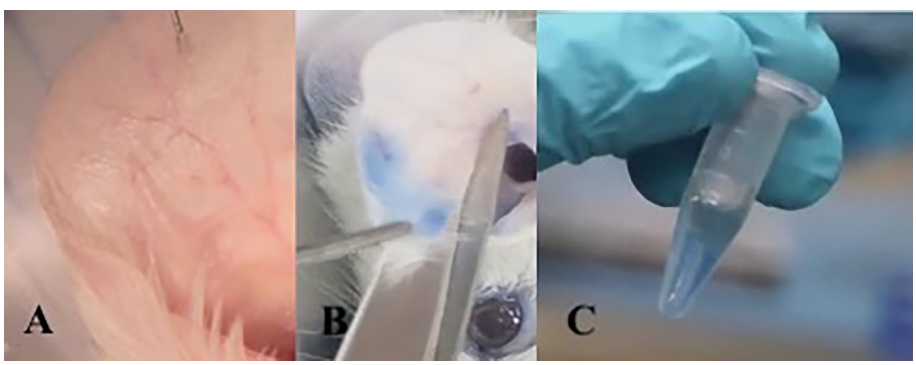

FIGURE 2 | Active cutaneous anaphylaxis technique: Intradermal injection into the ear with allergen diluted in PBS. **(A)** After 10 minutes of exposure to the allergen, the mouse is sacrificed and the ear is sectioned. **(B)** The ear fragment is kept in formamide at 63°C for 18 hours to extract Evans blue dye from the tissue **(C)**.

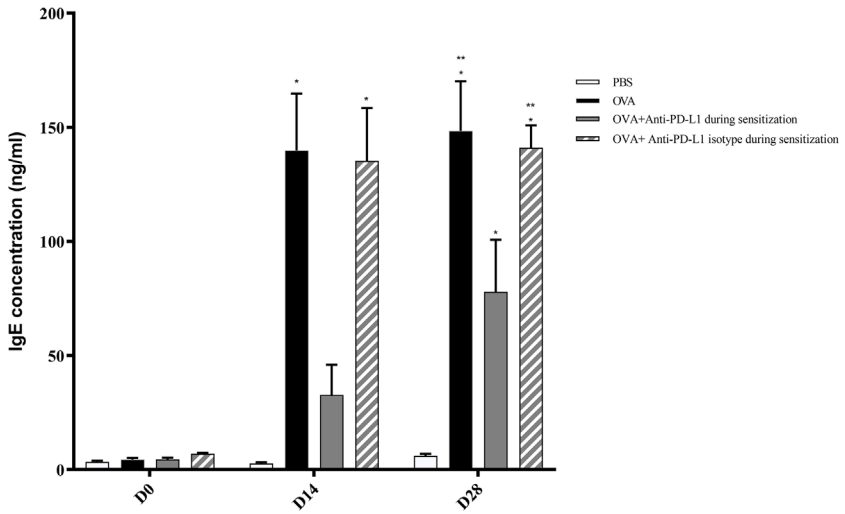

FIGURE 3 | Concentration of serum anti-OVA IgE in groups 1, 2, 3, and 4. There was an increase in anti-OVA IgE in groups 2 and 4 on D13 and D27 and in group 3 on D27. Group 3 (anti-PD-L1) had lower anti-OVA IgE than groups 2 and 4. ★ $p < 0.001$ compared to group 1 (PBS); ★★ $p < 0.001$ compared to group 3 (anti-PD-L1). PBS, phosphate-buffered saline; OVA, ovalbumin.

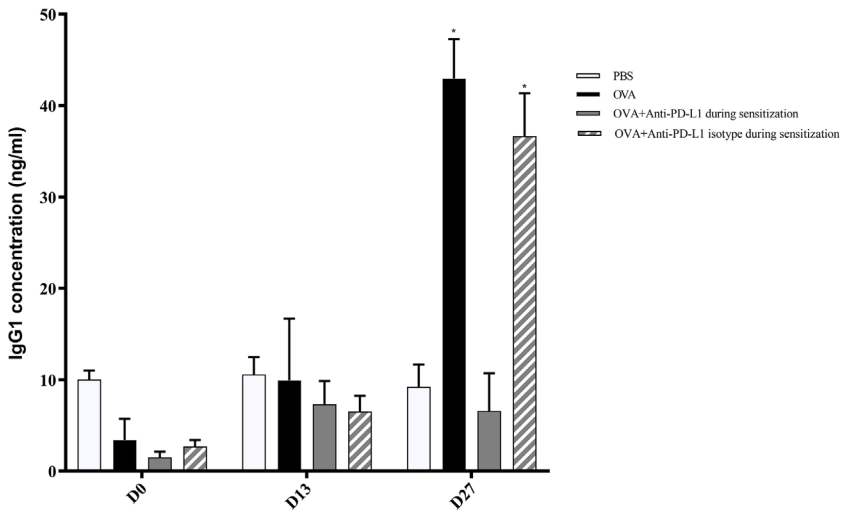

FIGURE 4 | Serum anti-OVA IgG1 concentration in groups 1, 2, 3, and 4. There was an increase in anti-OVA IgG1 in groups 2 and 4 on D27. ★ $p < 0.001$ compared to group 1 (PBS). PBS, phosphate-buffered saline; OVA, ovalbumin.

activation of Th2 cells and not directly in the activation of mast cells.

The PD-L1 receptor is strongly expressed in murine mast cells, but studies on its role in allergic processes are rare. To the best of our knowledge, this is the first study to evaluate the action of this protein in an allergy model focused on the immediate phase of the Gell & Coombs type I hypersensitivity reaction.

Some studies have attempted to elucidate the role of PD-1 pathways, induced by its binding to both PD-L1 and PD-L2, in murine models of allergic respiratory disease (19, 22–24). In a murine model of asthma, it was shown that PD-L1 was constitutively expressed in dendritic cells, macrophages, and B and T cells in the lungs of the animals and that this expression increased after challenge with ovalbumin. In turn, PD-L2 was poorly expressed in naïve dendritic cells, with a substantial increase after challenge. PD-L2 blockade at the time of allergen challenge, but not at sensitization, increased AHR and eosinophilia, in addition to IL-5 and IL-13 production, but reduced IFN-γ production in the lungs and lymph nodes. These effects were not observed with PD-L1 blockade, suggesting a

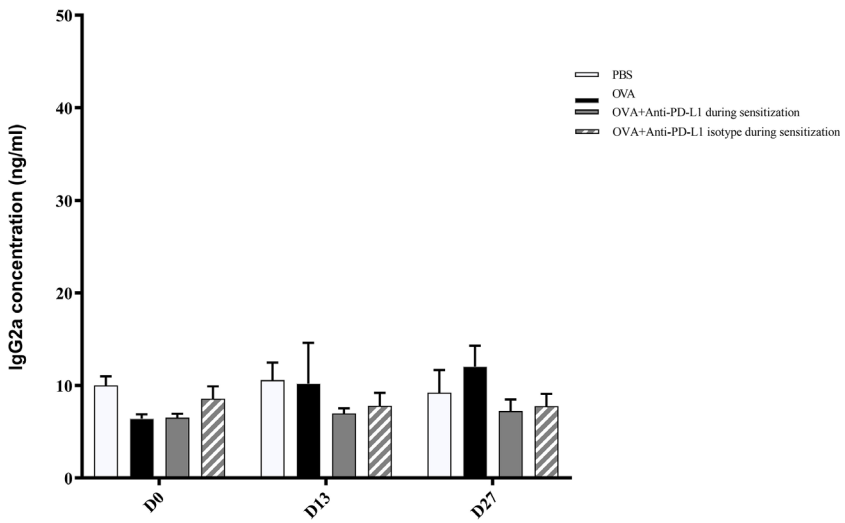

FIGURE 5 | Serum anti-OVA IgG2a concentration in groups 1, 2, 3, and 4. There was no difference in anti-OVA IgG2a between groups. PBS, phosphate-buffered saline; OVA, ovalbumin.

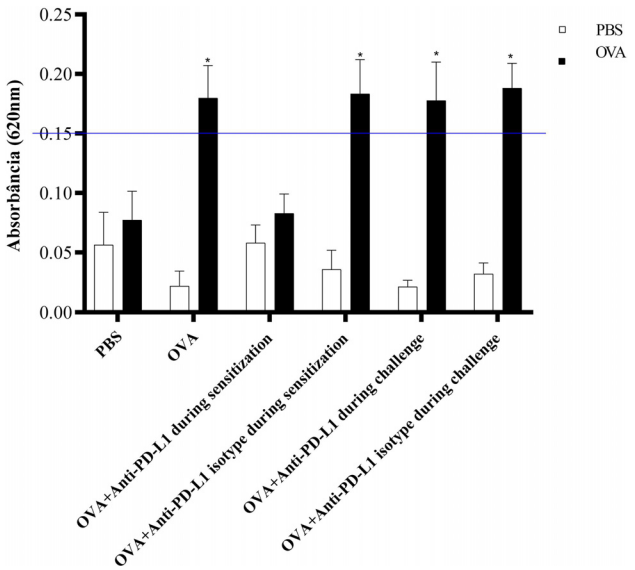

FIGURE 6 | Absorbance of Evans blue extravasation in active cutaneous anaphylaxis (ACA) of the six experimental groups. All animals underwent the ACA test in both ears. PBS was administered to the left ear (negative control – white column) and OVA to the right ear (allergen – black column). There was an increase in extravasation in the right ear of groups 2, 4, 5, and 6. There was no difference between the PBS and anti-PD-L1 groups during sensitization. ★ $p < 0.001$ compared to group 1 (PBS). PBS, phosphate-buffered saline; OVA, ovalbumin.

particular role for PD-L2 in the asthmatic response (22). In contrast, in a study assessing the impact of manipulation of the PD-L1 and PD-L2 pathways on the development of asthma, it was reported that blockade of the PD-L2 pathway led to increased airway inflammation, IL-4, and AHR. Blockade of the PD-L1 pathway led to a reduction in AHR and increased the production of IFN-γ (19). In another study, using an animal model of allergic conjunctivitis, PD-L2 antagonism in the induction phase led to increased Th2 cytokine levels in the supernatant of splenocytes in culture. In addition, anti-PD-L2 in the effector phase led to increased influx of eosinophils into the conjunctiva of the animals. These effects were not clearly seen with PD-1 blockade (23).

Activation by the PD-L1, but not PD-L2, pathway can induce $Foxp3^+$ regulatory T cells (25). The PD-1 pathway is indeed important for the action of Treg cells, as shown in a cockroach-induced asthma model. PD-1 blockade causes an increase in IL-4, IL-5 and IL-13, and a reduction in IL-10 in the bronchoalveolar lavage of challenged animals that have been subjected to Treg administration in the lungs (24). In a review article, based mainly on respiratory allergy models, the authors concluded that the PD-1/PD-L1 interaction seems to induce a Th2 response, with an increase in IL-4, whereas the PD-1/PD-L2 interaction induces a Th1 response with upregulated IFN-γ. Thus, it was suggested that the simultaneous expression of the PD-L1 and PD-L2 ligands could neutralize these effects and not cause any polarization (20).

However, in a more recent study in which different mice strains were subjected to another asthma model, blockade of the PD-1/PD-L1 pathway resulted in increased AHR, not by increasing Th2 activation but by increasing Th17. There were also varied effects on the different populations of helper T cells, and this effect also varied according to the mouse strain (26). We have not found experimental models in which the PD-L1/PD-L2 pathways have been evaluated in other allergic diseases mediated by IgE, such as systemic or cutaneous anaphylaxis.

In the present study, we blocked the interaction of PD-1/PD-L1 with anti-PD-L1, which may have resulted in weaker polarization toward Th2, with lower production of IgE and

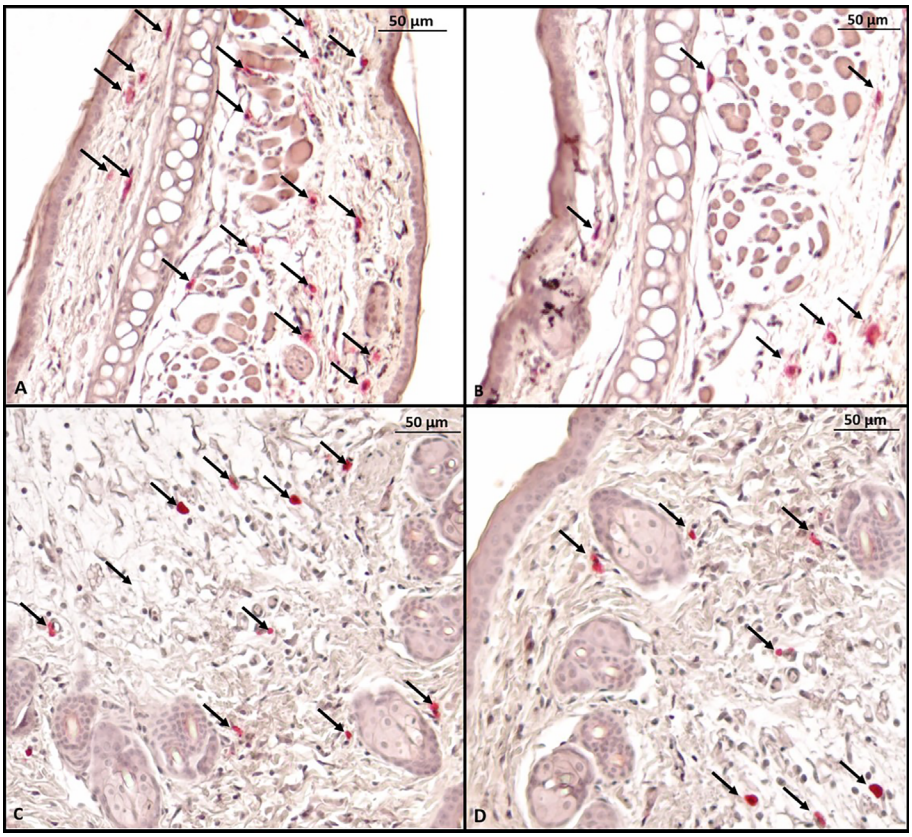

FIGURE 7 | Microscopy of a mouse ear fragment stained with chloroacetate esterase. Mast cells are indicated by black arrows. **(A)** Group 1 (phosphate-buffered saline); **(B)** Group 2 (Ovalbumine); **(C)** Group 3 (anti-PD-L1); **(D)** Group 4 (anti-PD-L1 isotype).

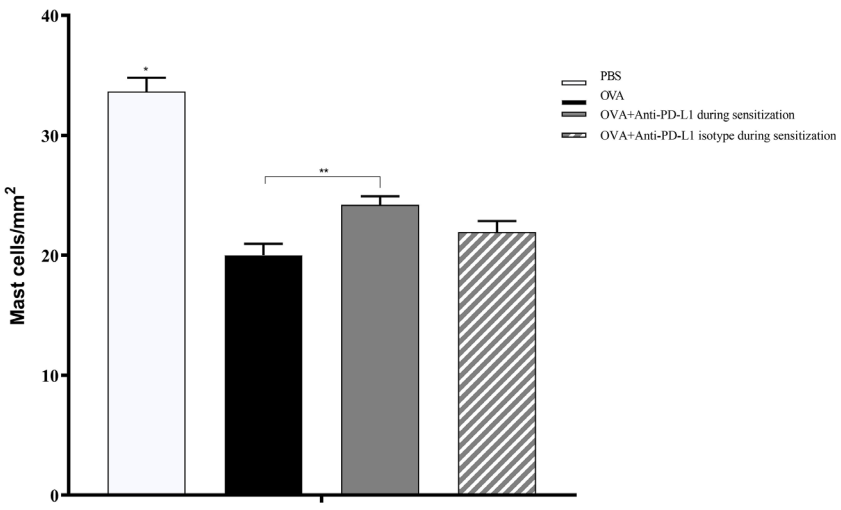

FIGURE 8 | Histological analysis with the number of mast cells stained with chloroacetate esterase in groups 1 to 4. The number of mast cells was higher in group 1 (PBS) than in the other groups, suggesting lower degranulation. Group 3 (anti-PD-L1) had a higher number of mast cells than group 2 (OVA). ★ $p < 0.0001$ compared to the other groups; ★★ $p < 0.01$ between groups 2 and 3. PBS, phosphate-buffered saline; OVA, ovalbumin.

IgG1. The serum level of specific IgE impacts mast cell sensitization, explaining the observation of a lower number of degranulated mast cells and lower allergic reaction in the group treated with anti-PD-L1. Nonetheless, impact of anti-PD-L1 in specific IgE levels on D27 was lower than on D13, suggesting a partial failure on the effect of this monoclonal antibody with repeated injections. It is possible that higher doses may be necessary in long-term treatment with repeated injections of anti-PD-L1.

Previous studies have led to interest in evaluating the role of these pathways in allergic diseases in humans. Recently, it was shown that in adults with mild asthma subjected to segmental bronchial allergen challenge, there is an increase in the expression of PD-1 and PD-L1 24 hours after the challenge, corroborating the role of this pathway in this Th2-profile disease (27). Increased expression of PD-1 in the cell membrane of a patient with chronic rhinosinusitis with nasal polyposis (28) and of both PD-1 and PD-L1 in individuals with allergic rhinitis (29) have also been recently demonstrated.

The PD-1/PD-L1 pathway is classically associated with the modulation of the immune response of T cells, so blocking this pathway has become a standard strategy for the treatment of some types of cancer. Contrary to the findings in murine models, it was suggested that PD-L1 blockade in cancer in humans could aggravate pre-existing allergic diseases. A recently published clinical case of a patient with lung cancer and probable asthma–chronic obstructive pulmonary syndrome overlap syndrome reported that this patient presented worsened lung condition when subjected to immunotherapy with anti-PD-L1 durvalumab. However, there was no evidence of worsened pulmonary function or increased in eosinophils, making it difficult to confirm that the monoclonal antibody caused the exacerbation of the allergic condition (30). We can speculate that blockade of this immunomodulatory pathway could result in reduced regulatory cell function, aggravating pre-existing inflammatory diseases. An active search for regulatory T cell induction is ongoing through allergen-specific immunotherapy for allergic diseases (31). A recent clinical trial with patients allergic to peach subjected to sublingual immunotherapy showed that the expression of PD-L1 by peripheral-blood monocytes increased in the treated group, suggesting a role for this pathway in allergen tolerance induction (32). This suggests that immunotolerance may also use the PD-L1 pathway, but the mechanisms have not been fully elucidated.

Our study has some limitations. We used a small number of animals for histological analysis, which may have biased the results. Moreover, although unlikely, it is possible that PD-L1 pathway has some direct action in mast cell activation independently of the sensitization phase that was not investigated. Future studies could evaluate the role of PD-L1 in the effector phase in a PCA model, administrating it to animals that were not previously sensitized with the allergen. In addition, although we performed ACA in both ears, only the ear that received the OVA challenge was biopsied. Although unlikely, it is possible that there was some variation in the density of mast cells in the tissue challenged with PBS.

Although stimulation of the PD-1/PD-L1 regulatory axis has the paradoxical functions of inhibiting the immune response and inducing the Th2 immune response, in our study the PD-L1 pathway was directly associated with the allergic response. Its blockade inhibited the synthesis of specific IgE and IgG1, mast cell degranulation, and vascular permeability, which suggests that the induction of the Th2 immune response by PD-L1 exceeded the inhibitory action of this receptor on T cells. The immunomodulation of this axis may represent a new preventive and therapeutic option in the treatment of allergic diseases such as anaphylaxis.

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 animal study was reviewed and approved by Dana-Farber Cancer Institute, Boston, Massachusetts (DFCI IRB 15-046)/ University of São Paulo Medical School (CEUA-FMUSP 1286-2019).

AUTHOR CONTRIBUTIONS

RB-S, JK, and PG-B initiated and coordinated the project. MA, MC, and PG-B designed the experiments. RB-S and MA conducted the experiments. All authors analyzed the data. RB-S, MA, and PG-B wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Sampson HA, Muñoz-Furlong A, Campbell RL, Adkinson NF, Bock SA, Branum A, et al. Second symposium on the definition and management of anaphylaxis: summary report–Second National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium. *J Allergy Clin Immunol* (2006) 117:391–7. doi: 10.1016/j.jaci.2005.12.1303
2. Simons FER, Ardusso LRF, Bilò MB, Dimov V, Ebisawa M, El-Gamal YM, et al. 2012 Update: World Allergy Organization Guidelines for the assessment and management of anaphylaxis. *Curr Opin Allergy Clin Immunol* (2012) 12:389–99. doi: 10.1097/ACI.0b013e328355b7e4
3. Oyoshi MK, Oettgen HC, Chatila TA, Geha RS, Bryce PJ. Food allergy: Insights into etiology, prevention, and treatment provided by murine models. *J Allergy Clin Immunol* (2014) 133:309–17. doi: 10.1016/j.jaci.2013.12.1045
4. Aun MV, Bonamichi-Santos R, Arantes-Costa FM, Kalil J, Giavina-Bianchi P. Animal models of asthma: utility and limitations. *J Asthma Allergy* (2017) 10:293–301. doi: 10.2147/JAA.S121092
5. Goose J, Blair AM. Passive cutaneous anaphylaxis in the rat, induced with two homologous reagin-like antibodies and its specific inhibition with disodium cromoglycate. *Immunology* (1969) 16:749–60.
6. Doyle E, Trosien J, Metz M. Protocols for the induction and evaluation of systemic anaphylaxis in mice. *Methods Mol Biol Clifton NJ* (2013) 1032:133–8. doi: 10.1007/978-1-62703-496-8_10
7. Evans H, Killoran KE, Mitre E. Measuring local anaphylaxis in mice. *J Vis Exp* (2014) 92:e52005. doi: 10.3791/52005
8. Ovary Z. Passive cutaneous anaphylaxis in the mouse. *J Immunol* (1958) 81:355–7.
9. Mountford AP, Fisher A, Wilson RA. The profile of IgG1 and IgG2a antibody responses in mice exposed to Schistosoma mansoni. *Parasite Immunol* (1994) 16:521–7. doi: 10.1111/j.1365-3024.1994.tb00306.x
10. Teshima R, Akiyama H, Akasaka R, Goda Y, Toyoda M, Sawada J. Simple spectrophotometric analysis of passive and active ear cutaneous anaphylaxis in the mouse. *Toxicol Lett* (1998) 95:109–15. doi: 10.1016/S0378-4274(98)00028-9
11. Murakami N, Riella LV. Co-inhibitory pathways and their importance in immune regulation. *Transplantation* (2014) 98:3–14. doi: 10.1097/TP. 0000000000000169
12. Brown GR, Hem V, Katz KS, Ovetsky M, Wallin C, Ermolaeva O, et al. Gene: a gene-centered information resource at NCBI. *Nucleic Acids Res* (2015) 43 (Database issue):D36–42. doi: 10.1093/nar/gku1055
13. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. *Immunol Rev* (2010) 236:219–42. doi: 10.1111/j.1600-065X.2010.00923.x
14. Mozaffarian N, Wiedeman AE, Stevens AM. Active systemic lupus erythematosus is associated with failure of antigen-presenting cells to express programmed death ligand-1. *Rheumatol Oxf Engl* (2008) 47:1335–41. doi: 10.1093/rheumatology/ken256
15. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, et al. PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front Pharmacol* (2017) 8:561. doi: 10.3389/fphar.2017.00561
16. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* (2012) 12:252–64. doi: 10.1038/nrc3239
17. Couzin-Frankel J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* (2013) 342:1432–3. doi: 10.1126/science.342.6165.1432
18. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. *Nat Rev Drug Discovery* (2015) 14:561–84. doi: 10.1038/nrd4591
19. Akbari O, Stock P, Singh AK, Lombardi V, Lee W-L, Freeman GJ, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing directions. *Mucosal Immunol* (2010) 3:81–91. doi: 10.1038/mi.2009.112
20. Singh AK, Stock P, Akbari O. Role of PD-L1 and PD-L2 in allergic diseases and asthma. *Allergy* (2011) 66:155–62. doi: 10.1111/j.1398-9995.2010.02458.x
21. Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, et al. Mast Cells Enhance T Cell Activation: Importance of Mast Cell Costimulatory Molecules and Secreted TNF. *J Immunol* (2006) 176:2238–48. doi: 10.4049/jimmunol.176.4.2238
22. Matsumoto K, Inoue H, Nakano T, Tsuda M, Yoshiura Y, Fukuyama S, et al. B7-DC regulates asthmatic response by an IFN-gamma-dependent mechanism. *J Immunol* (2004) 172:2530–41. doi: 10.4049/jimmunol.172.4.2530
23. Fukushima A, Yamaguchi T, Azuma M, Yagita H, Ueno H. Involvement of programmed deathligand 2 (PD-L2) in the development of experimental allergic conjunctivitis in mice. *Br J Ophthalmol* (2006) 90:1040–5. doi: 10.1136/bjo.2006.091314
24. McGee HS, Yagita H, Shao Z, Agrawal DK. Programmed Death-1 antibody blocks therapeutic effects of T-regulatory cells in cockroach antigen-induced allergic asthma. *Am J Respir Cell Mol Biol* (2010) 43:432–42. doi: 10.1165/rcmb.2009-0258OC
25. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci USA* (2008) 105:9331–6. doi: 10.1073/pnas.0710441105
26. McAlees JW, Lajoie S, Dienger K, Sproles AA, Richgels PK, Yang Y, et al. Differential control of CD4(+) T-cell subsets by the PD-1/PD-L1 axis in a mouse model of allergic asthma. *Eur J Immunol* (2015) 45:1019–29. doi: 10.1002/eji.201444778
27. Bratke K, Fritz L, Nokodian F, Geißler K, Garbe K, Lommatzsch M, et al. Differential regulation of PD-1 and its ligands in allergic asthma. *Clin Exp Allergy* (2017) 47:1417–25. doi: 10.1111/cea.13017
28. Kortekaas Krohn I, Bobic S, Dooley J, Lan F, Zhang N, Bachert C, et al. Programmed cell death-1 expression correlates with disease severity and IL-5 in chronic rhinosinusitis with nasal polyps. *Allergy* (2017) 72:985–93. doi: 10.1111/all.13136
29. Wen SL, Li F, Zhao F, Zuo JJ, Deng YQ, Zhang W, et al. Programmed cell death protein 1 and its ligands regulate immune balance in allergic rhinitis. *Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* (2020) 55:384–90. doi: 10.3760/cma.j.cn115330-20190618-00392
30. Wassmer F, Suehs C, Boissin C, Bourdin A, Charriot J. PD-L1 inhibitor-induced worsening of eosinophilic obstructive lung disease successfully rescued with anti-interleukin 5 receptor (IL-5R) therapy. *J Allergy Clin Immunol Pract* (2021) 9:567–9. doi: 10.1016/j.jaip.2020.08.02231
31. Celebi Sözener Z, Mungan D, Cevhertas L, Ogulur I, Akdis M, Akdis C. Tolerance mechanisms in allergen immunotherapy. *Curr Opin Allergy Clin Immunol* (2020) 20:591–601. doi: 10.1097/ACI.0000000000000693
32. Palomares F, Gomez F, Bogas G, Campo P, Perkins JR, Diaz-Perales A, et al. Immunological Changes Induced in Peach Allergy Patients with Systemic Reactioncs By Pru p 3 Sublingual Immunotherapy. *Mol Nutr Food Res* (2018) 62. doi: 10.1002/mnfr.201700669

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frontiers in Immunology

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Cold Agglutinins and Cryoglobulins Associate With Clinical and Laboratory Parameters of Cold Urticaria

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Mast cell-activating signals in cold urticaria are not yet well defined and are likely to be heterogeneous. Cold agglutinins and cryoglobulins have been described as factors possibly associated with cold urticaria, but their relevance has not been explained. We performed a single-center prospective cohort study of 35 cold urticaria patients. Cold agglutinin and cryoglobulin test results, demographics, detailed history data, cold stimulation test results, complete blood count values, C-reactive protein, total immunoglobulin E levels, and basal serum tryptase levels were analyzed. Forty six percent (n = 16) of 35 tested patients had a positive cold agglutinin test and 27% (n = 9) of 33 tested patients had a positive cryoglobulin test. Cold agglutinin positive patients, when compared to cold agglutinin negative ones, were mainly female (P = 0.030). No gender-association was found for cryoglobulins. A positive cold agglutinin test, but not a positive cryoglobulin test, was associated with a higher rate of reactions triggered by cold ambient air (P = 0.009) or immersion in cold water (P = 0.041), and aggravated by increased summer humidity (P = 0.007). Additionally, patients with a positive cold agglutinin test had a higher frequency of angioedema triggered by ingestion of cold foods or drinks (P = 0.043), and lower disease control based on Urticaria Control Test (P = 0.023). Cold agglutinin levels correlated with erythrocyte counts (r = –0.372, P = 0.028) and monocyte counts (r = –0.425, P = 0.011). Cryoglobulin concentrations correlated with basal serum tryptase levels (r = 0.733, P = 0.025) and cold urticaria duration (r = 0.683, P = 0.042). Results of our study suggest that cold agglutinins and cryoglobulins, in a subpopulation of cold urticaria patients, are linked to the course and possibly the pathogenesis of their disease.

Keywords: cold agglutinin, cryoglobulin, cold urticaria, mast cell, degranulation, clinical parameters, laboratory parameters, cold triggers

INTRODUCTION

Cold urticaria (ColdU) is a type of chronic inducible urticaria (CIndU) characterized by the occurrence of wheals and/or angioedema in response to cooling (1, 2). It is classified into: (a) cold contact urticaria, which is characterized by whealing in response to local cold stimulation tests (CSTs) on the forearm with an ice cube and/or Temp*Test*® technology, and (b) atypical ColdU, in which other provocation methods are needed to produce whealing (e.g., general body cooling) or local CSTs produce atypical responses. Cold-induced anaphylaxis may also occur (1). ColdU is confirmed by provocation testing, and disease activity is measured by trigger threshold(s) (3–5). According to the EAACI/GA^{2}LEN/EDF/WAO urticaria guideline, laboratory tests in ColdU are recommended only as an extended diagnostic approach to rule out other diseases, especially infections (3). In most patients, no underlying causes can be found (6).

ColdU is a mast cell-driven disease, where activating signals cause release of histamine from dermal mast cells (7–9). Histamine release in ColdU coincides with the onset of pruritus and swelling (10). The mast cell-activating signals in ColdU have not yet been well defined and are likely to be heterogeneous (1, 3). Autoantibodies are held to play a role in some patients with ColdU (1), and passive transfer studies found that localized ColdU can be induced in healthy human subjects by injecting them intradermally with serum of some ColdU patients (11). Further studies found that the serum factors that initiated ColdU depended on immunoglobulin E (IgE) (10, 12, 13), and rarely on immunoglobulin M (IgM) (14). A cold-inducible antigen, however, has not yet been identified (1).

Cold agglutinins (CAs) are cold-reactive antibodies that are able to agglutinate erythrocytes (15). Silpa-archa et al. found CAs in 40% of 20 studied ColdU subjects and reported "mainly low titers" (16). CAs were also described in a few ColdU case reports (17–19). The majority of CAs are IgM proteins (20), but few cases of immunoglobulin A (IgA) or immunoglobulin G (IgG) CAs have also been described (15, 21–23). The ability of CAs to agglutinate erythrocytes after binding to their cell surface can be attributed to the pentameric structure and large molecular size of IgM. Most CAs are directed against the Ii-blood group system of carbohydrate antigens (15). CAs are assessed semi-quantitatively by the CA titer, usually first measured at 4°C and defined as the inverse of the maximum serum dilution at which agglutination of donor erythrocytes is seen *in vitro*. The thermal amplitude is defined as the highest temperature at which the CA will react with its antigen (24). Interestingly, studies in hematology have shown that the pathogenicity of CAs depends more on the thermal amplitude, which can approach 37°C, than on the CA titer (15, 23, 25).

CAs have been mostly studied for their pathogenic role in cold-antibody autoimmune hemolytic anemias: (a) CA disease, a well-defined clonal B-cell lymphoproliferative disorder, and (b) CA syndrome, most frequently caused by *Mycoplasma pneumoniae*, Epstein-Barr virus, or aggressive lymphoma. Nearly all patients with CA disease have a CA titer ≥64 (**Table 1**) (15). At decreased ambient temperatures, cooling of peripheral skin sites (e.g., fingers, toes, ears, tip of the nose) allows binding of the CAs to erythrocytes in acral capillaries and their agglutination with subsequent possible cold-induced ischemic manifestations (e.g., acrocyanosis, Raynaud's phenomenon, rarely gangrene) (15, 21, 23). CA binding to erythrocytes activates the classical complement pathway and may cause hemolysis (15, 28). ColdU is a rare feature of CA disease (29). CAs are also found in a proportion of the adult population without any hemolysis or clinical symptoms. Such CAs are present in low titers (mostly <64), have low thermal amplitude, and are polyclonal (**Table 1**) (15). In a study of 14900 patients screened prior to a cardiopulmonary bypass procedure, 0.3% tested positive for CAs (30). Non-pathogenic CAs may be remnants of a primitive vertebrate immune system (15) and the product of either random rearrangement of the immunoglobulin gene segments in the bone marrow and/or produced as a result of molecular mimicry with structures on the surface of infectious agents (31).

Cryoglobulins (CGs) are antibodies that precipitate *in vitro* at temperatures below 37°C and dissolve after rewarming (26, 32, 33). Their precipitation *in vivo* can cause vasculitis of small and medium-sized vessels in the skin, joints, nerves and kidneys (26), and consequently signs and symptoms such as intermittent purpura, livedo, leg ulcers, acrocyanosis, Raynaud's phenomenon, arthralgias, and symptoms due to peripheral neuropathy (26, 34, 35). CGs have been detected in up to 10% of ColdU patients studied in 4 case series with 71-208 enrolled patients (36–39). Additionally, a few case reports of CG-positive ColdU patients have been published (40–43). CGs are divided into: (a) single-component monoclonal CGs, which contain only one immunoglobulin isotype (IgG or IgM, rarely IgA), and (b) mixed CGs, which are immune complexes composed of two different immunoglobulin isotypes, most commonly IgM and IgG (26, 27). Mixed CGs are often found secondary to chronic (viral, bacterial or

TABLE 1 | Cold agglutinin and cryoglobulin properties.

CA properties (15)	
CA disease	Anti-I (rarely anti-Pr or anti-IH) CA titer usually ≥64 at 4°C
CA syndrome	Anti-I or anti-i IgM or IgG
Normally occurring CAs	CA titer usually <64 at 4°C Low thermal amplitude Polyclonal
CG properties (26, 27)	
Single-component CGs	One Ig isotype (IgM or IgG, rarely IgA) Monoclonal Ig CG concentration often >2000 mg/L
Mixed CGs	Two different Ig isotypes (usually IgM and IgG) (a) Monoclonal Ig + polyclonal Ig or (b) polyclonal Ig
Normally occurring CGs	CG concentration <20 mg/L Mixed, polyclonal Ig

CA, cold agglutinin; CG, cryoglobulin; Ig, immunoglobulin.

Abbreviations: CA(s), cold agglutinin(s); ColdU, cold urticaria; CG(s), cryoglobulin(s); CR(s), cold-induced reaction(s); CRP, C-reactive protein; CST(s), cold stimulation test(s); CSTT, critical stimulation time threshold; CTT, critical temperature threshold; Ig, immunoglobulin; IgA, immunoglobulin A; IgE, immunoglobulin E; IgG, immunoglobulin G; IgM, immunoglobulin M; IQR, interquartile range; SD, standard deviation; UCT, Urticaria Control Test.

parasitic) infections (26). In studies performed by Sidana et al. (34), Dammacco et al. (35), and Costanzi and Coltman (43), ColdU was found in 2–4% of patients with CGs. Healthy individuals may have CGs at low concentrations (26, 27, 44). CGs are different from CAs (20, 45), although an IgM cryoprotein with both CA- and CG-properties has been described (46).

So far nothing is known about the impact of CAs and CGs on ColdU features on the molecular or clinical level, and no evaluation strategies have been proposed for CA-positive and CG-positive ColdU patients. In practice, referrals of these patients to hematologists and rheumatologists does not help guide further evaluation and treatment of ColdU. Consequently, we designed a comprehensive study with the aim to overcome these knowledge gaps. Detailed analysis of cold triggers has not yet been undertaken either and the effects of different relevant cold triggers on the clinical presentation of ColdU are still incompletely understood (1).

MATERIALS AND METHODS

Patients

Thirty five consecutive adult ColdU patients, who were referred to the Urticaria Center of Reference and Excellence (UCARE) at the University Clinic Golnik (47), were recruited. Their age ranged from 18 to 73 years (mean 41.4, SD ± 13.4). There were 66% (n = 23) women and 34% (n = 12) men (**Table 2**). All (n = 35) patients had ColdU based on their history and local CSTs were performed. Routine physical examination was also done. The study spanned through all seasons (from May 1, 2019 to March 31, 2020) and it was approved by the National Medical Ethics Committee of the Republic of Slovenia. Patients gave written informed consent.

Patient History

Detailed history data focusing on numerous clinical parameters were obtained in all (n = 35) patients. The age at ColdU onset ranged from 9 to 60 years (mean 33.5, SD ± 12.5), and 9% of patients had a pediatric-onset ColdU (≤18 years). The duration of ColdU ranged from 2 to 384 months (median 60, IQR 15–156). The following frequencies of predefined cold-induced reactions (CRs) were reported: itch in 94% (n = 33), wheals in 100% (n = 35), angioedema in 37% (n = 13), gastrointestinal symptoms in 17% (n = 6), respiratory symptoms in 40% (n = 14), and symptoms of reduced blood pressure in 29% (n = 10). Eleven percent (n = 4) of patients reported cold-induced gastrointestinal and respiratory symptoms, 3% (n = 1) experienced gastrointestinal symptoms

TABLE 2 | Characteristics of patients with a positive *vs*. negative cold agglutinin test.

Parameter	CA test			
	Total (*N* = 35)	**Negative (*N* = 19)**	**Positive (*N* = 16)**	***P* value (positive *vs*. negative)**
Female sex	23 (66)	**9 (47)**	**14 (88)**	**0.030***
Triggers				
Cold ambient air	28 (80)	**12 (63)**	**16 (100)**	**0.009***
Immersion in <25°C water	19 (54)	**7 (37)**	**12 (75)**	**0.041***
Higher summer humidity levels	15 (43)	**4 (21)**	**11 (69)**	**0.007***
Ingestion of cold foods/drinks	13 (37)	**4 (21)**	**9 (56)**	**0.043***
UCT score	12 (7–14)	**13 (9–16)**	**11 (7–12)**	**0.023**‡
CST results				
Positive ice cube test	21 (60)	11 (58)	10 (63)	1.000
Positive Temp*Test*® result	12 (34)	5 (26)	7 (44)	0.311
CSTT (s)	300 (30–300); *N* = 19	300 (98–300); *N* = 10	120 (30–300); *N* = 9	0.150
CTT (°C)	19 (15–25); *N* = 12	17 (16–25); *N* = 5	21 (14–24); *N* = 7	0.622
Laboratory findings				
Erythrocyte count (10^{12}/L)	4.7 ± 0.4	**4.9 ± 0.4**	**4.5 ± 0.3**	**0.005**†
Hemoglobin concentration (g/L)	142.6 ± 10.4	**148.4 ± 8.7**	**135.8 ± 7.8**	**<0.001**†
Hematocrit level (%)	41.7 ± 3.3	**43.2 ± 3.4**	**40.0 ± 2.5**	**0.004**†
Thrombocyte count (10^9/L)	265.9 ± 62.3	**246.4 ± 52.3**	**289.0 ± 66.9**	**0.042**†
Monocyte count (10^9/L)	0.5 ± 0.2	**0.6 ± 0.2**	**0.4 ± 0.2**	**0.002**†
Leukocyte count (10^9/L)	6.9 ± 1.7	7.3 ± 1.7	6.3 ± 1.5	0.070
Neutrophil count (10^9/L)	4.2 ± 1.3	4.5 ± 1.3	3.8 ± 1.2	0.121
Lymphocyte count (10^9/L)	1.9 ± 0.5	2.0 ± 0.5	1.9 ± 0.5	0.630
Eosinophil count (10^9/L)	0.2 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.324
Basophil count (10^9/L)	0.04 ± 0.03; *N* = 34	0.04 ± 0.03; *N* = 19	0.04 ± 0.03; *N* = 15	0.620
CRP (mg/L)	1.7 (0.5–3.4)	1.2 (0.5–3.2)	2.1 (0.7–4.5)	0.320
Basal serum tryptase (ng/mL)	5.5 (3.9–7.6); *N* = 34	6.1 (4.3–8.7); *N* = 18	5.3 (3.7–6.0)	0.352
Total IgE (IU/mL)	99 (36–207); *N* = 31	82 (24–257); *N* = 15	113 (50–201)	0.782
Mean daily Temp. on the day when blood was drawn	12.1 ± 7.3	**9.3 ± 6.6**	**12.1 ± 7.3**	**0.008**†

*Data are given as no. (%), mean ± SD, and median (IQR). If data was not obtained in all patients, patient numbers are displayed as "N" next to results. Statistical significance of differences between patient groups was calculated by *Fisher's Exact test, ‡Mann-Whitney U test, and †Independent-samples T test. Statistically significant P values are in bold. ColdU, cold urticaria; CA, cold agglutinin; CRP, C-reactive protein; CST, cold stimulation test; CSTT, critical stimulation time threshold; CTT, critical temperature threshold; IQR, interquartile range; N, number of patients; s, second; SD, standard deviation; UCT, Urticaria Control Test.*

and symptoms of reduced blood pressure, and 26% (n = 9) of patients had respiratory and hypotensive symptoms. Maximal duration of wheals ranged from 5 to 720 minutes (median 60, IQR 20–120), and the maximal duration of angioedema ranged from 20 to 720 minutes (median 60, IQR 30–120).

A special emphasis in history taking was given to the relevance of 6 predefined cold triggers and 3 predefined aggravating factors. Triggers of at least one CR were the following: cold ambient air in 80% (n = 28), immersion in <25°C water in 54% (n = 19), immersion in ≥25°C water in 20% (n = 7), localized contact with cold liquids in 60% (n = 21), contact with cold surfaces in 54% (n = 19), and ingestion of cold foods or beverages in 51% (n = 18) of patients. Factors that aggravated at least one CR were: wind in 63% (n = 22), increased humidity levels in the summer (e.g., morning humidity, rain, walking barefoot on grass) in 43% (n = 15), and increased non-summer humidity levels (e.g. rain in cold seasons) in 43% (n = 15) of patients.

Treatment efficacy with daily second-generation H_1-antihistamines in up to four times the licensed dose was graded as good in 23% (n = 8), moderate in 37% (n = 13), low in 9% (n = 3), no efficacy in 3% (n = 1), and unknown (not taken on a daily basis) in 29% (n = 10) of patients. Omalizumab treatment was required in 17% (n = 6) of patients. Nine percent (n = 3) of patients had at least one of the following cold-induced extracutaneous symptoms: fever, malaise, headache, eye redness, muscle pain, joint pain, or bone pain. Seventeen percent (n = 6) of patients also had chronic spontaneous urticaria, 6% (n = 2) symptomatic dermographism, 9% (n = 3) cholinergic urticaria, and 3% (n = 1) delayed pressure urticaria, but ColdU was a dominant disease in all these patients. Provocation tests for other CIndU types was done only in case of a suggestive history. Further history revealed the following comorbidities: at least one atopic disease (asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis) in 40% (n = 14), asthma in 34% (n = 12), thyroid disease in 17% (n = 6), Raynaud's phenomenon in 9% (n = 3), malignancy in 3% (n = 1), and autoimmune connective tissue disease in 3% (n = 1) of patients. Six percent (n = 2) of patients had a family history of ColdU.

Patient Questionnaires

All (n = 35) patients completed the Urticaria Control Test (UCT) questionnaire containing four questions to determine the level of disease control. UCT scores ranged from 3 to 16 (median 12, IQR 7–14) (**Table 2**). A score ≤11 indicates insufficient disease control, whereas a score ≥12 suggests adequate disease control (3, 4, 48).

Cold Stimulation Tests

Local CSTs on the volar forearm with an ice cube and a validated instrument Temp*Test*® were done in all (n = 35) patients. H_1-antihistamines and systemic glucocorticoids were discontinued at least 3 and 7 days prior to CSTs, respectively (3–5). A five-minute stimulation with a melting ice cube in a non-latex glove was done first. Skin response was assessed 10 minutes after the end of stimulation. If a positive reaction was observed (i.e., whealing on the cold stimulated area on the forearm), shorter stimulation times (30 seconds, 1 minute, 2 minutes, 3 minutes, 4 minutes) were used to determine the shortest time needed for whealing (i.e., critical stimulation time threshold, CSTT). A 5-minute stimulation with the Temp*Test*® and reading 10 minutes after the end of stimulation was used to determine the highest temperature that can induce whealing in a specific patient (i.e., critical temperature threshold, CTT).

Cold Agglutinin Test

Blood samples for CA analysis need to be kept at 37–38°C from sampling to separation of serum to avoid false low values and low sensitivity (49). To avoid this, all (n = 35) patients were referred to a dislocated Laboratory of Blood Transfusion Centre of Slovenia, where venous blood samples were collected and screened for the presence of CAs. The patients' serum was mixed with suspensions (0.9% sodium chloride) of group O and RhD positive erythrocytes obtained from 5 random blood donors, lightly shaken, incubated at 4°C for 2 hours, and scored for macroscopic agglutination. The test was reported positive if blood clot was readily visible. Blood donor erythrocytes that produced the strongest agglutination were used for further analyses with dilutions of patient's sera. The highest dilution able to agglutinate suspensions of erythrocytes at 4°C was recorded as the CA titer. Mean daily temperature on the day when blood for CA screening was drawn was obtained from the national weather service website (**Supplementary Table 1**).

Cryoglobulin Test

Screening for the presence of CGs was performed in 94% (n = 33) of patients at the Laboratory for Immunology of University Medical Centre Ljubljana. Patients were referred to this dislocated center to prevent improper sample collection and transport since it is crucial to maintain blood samples at 37 ± 2°C from the collection to the laboratory analysis (26). Blood samples were allowed to clot at 37°C for 2 hours and then centrifugated at 37°C. Sera were decanted in conical bottom test tubes and placed for 7 days at 4°C. After this incubation, cryoprecipitate was isolated by cold centrifugation and purified using 3 washes with centrifugation in cold phosphate-buffered saline to remove other serum proteins. Following the last wash, phosphate-buffered saline together with reagent for facilitating immune complex dissociation was added to the samples. These samples were then placed at 37°C for up to 1 hour to dissolve the precipitate for further analyses. The detected cryoprecipitate was confirmed by quantification with a spectrophotometer at 720 nm. A value below 100 mg/L was reported as a negative result, whereas values of 100 mg/L or higher were reported as precise CG concentrations. If the first stage was positive, the second qualitative stage was also done. It involved radial immunodiffusion for characterization of CG isotypes (IgG, IgM, IgA) based on the presence or absence of a precipitation ring (**Supplementary Table 2**). Mean daily temperature on the day when blood for CG screening was drawn was also obtained (**Supplementary Table 3**).

Other Laboratory Tests

Blood tests for most patients also included complete blood count with differential analysis, C-reactive protein (CRP), basal serum tryptase (ImmunoCAP 100 Thermo Fisher Scientific, Uppsala, Sweden), and total IgE (**Table 2**).

Statistical Analyses

Data from completed paper survey documents were transferred to an electronic databank and IBM SPSS Statistics version 27 was used for analysis. Numerical variables were first assessed for normality distribution using visualization and Shapiro-Wilk test of normality. Descriptive measures included proportions, median with the first and third quartile range, and mean with standard deviation. Categorical variables were assessed using the Fisher's Exact test. Numerical continuous variables with normal distribution were analyzed using parametric Independent-samples T test. Numerical variables that didn't meet parametric assumptions as well as ordinal variables were analyzed using Mann-Whitney U test. Relationships between continuous variables were assessed with Spearman's correlation. Spearman correlation coefficient was interpreted as follows: 0.20–0.39 weak, 0.40–0.59 moderate, and 0.60–0.79 strong. Tables and figures were employed to summarize the data.

RESULTS

Cold Stimulation Test Results

Sixty percent (n = 21) of patients had a positive ice cube test and 34% (n = 12) of them had a positive Temp*Test*® result (**Table 2**). All patients with negative skin tests reported wheals on cold exposure. All Temp*Test*®-positive patients also had a positive ice cube test, but only 57% (n = 12) of ice cube positive patients had a positive Temp*Test*® (P = 0.001). CSTT scores ranged from 30 to 300 seconds (median 300, IQR 30–300) and CTT scores ranged from 13 to 25°C (median 19, IQR 15—25) (**Table 2**). Cold contact urticaria (i.e., whealing on area on the forearm stimulated by an ice cube and/or Temp*Test*®) was diagnosed in 63% (n = 22) of patients. In one of these patients, local CSTs were negative at the point of enrollment, but the diagnosis was established based on past positive local CSTs. The rest of the patients (n = 13) had atypical forms of ColdU(1): 29% (n = 10) had systemic atypical ColdU, one had localized ColdU, and 6% (n = 2) had localized cold reflex urticaria.

Our Patient Cohort Has a High Frequency of CA-Positive and CG-Positive Patients

Almost half of our patients, 16 of 35 (46%), tested positive for CAs (**Table 2**), and their CA titers ranged from 1 to 16 (**Supplementary Table 1**). CGs were detectable in 27% (n = 9) of 33 tested patients (**Supplementary Table 2** and **Supplementary Table 3**). Two patients had single-component CGs of the IgG isotype, and 7 patients had mixed CGs. The maximum CG concentration was 189 mg/L (mean 152.0, SD ± 29.5) (**Supplementary Table 2**). Six percent (n = 2) of patients had a positive CA and CG test. No patients had a CA disease or a clinically manifested cryoglobulinemia.

CAs, but Not CGs, Are Linked to Female Gender

CA-positive patients were more often female than CA-negative patients (88% *vs*. 47%, P = 0.030) (**Table 2** and **Figure 1A**). No gender-association was found for CGs (**Supplementary Table 3**).

The Presence of CAs, but Not CGs, Influences the Patients' Real-Life Reactivity to Specific Cold Triggers

When CA-positive and CA-negative patients were compared, the former had a higher rate of reactions triggered by cold ambient air (100% *vs*. 63%, P = 0.009), immersion in <25°C water (75% *vs*. 37%, P = 0.041), or aggravated by increased humidity levels in the summer (69% *vs*. 21%, P = 0.007). They also had a higher rate of angioedema triggered by ingestion of cold foods or drinks (56% *vs*. 21%, P = 0.043) (**Table 2** and **Figure 1A**). No significant associations with specific cold triggers were found for CGs (**Supplementary Table 3**).

CAs, but Not CGs, Are Linked to Poor Disease Control

CA-positive patients had significantly lower UCT scores than CA-negative patients (median 11, IQR 7–12 *vs*. median 13, IQR 9–16; P = 0.023) (**Table 2** and **Supplementary Table 1**). Furthermore, CA titers weakly correlated with UCT scores (r = –0.359, P = 0.034) (**Table 3** and **Supplementary Table 1**). No significant differences in UCT scores were found between CG-positive and CG-negative patients (**Supplementary Table 3**).

A Positive CA test, but Not a Positive CG test, Was Associated With Specific Complete Blood Count Parameters

A positive CA test was linked to lower erythrocyte counts (P = 0.005) and lower hemoglobin concentrations (P <0.001) (**Table 2**; **Figure 1B**; **Supplementary Table 1**). CA titers correlated weakly with erythrocyte counts (r = –0.372, P = 0.028), moderately with hemoglobin concentrations (r = –0.557, P = 0.001), and moderately with hematocrit levels (r = –0.397, P = 0.018) (**Table 3**). CA-positive patients also had higher thrombocyte counts than CA-negative patients (P = 0.042) (**Figure 1B**), but CA titers did not significantly correlate with thrombocyte counts, albeit significance was borderline (r = 0.328, P = 0.054). Furthermore, CA-positive patients had lower monocyte counts when compared to CA-negative patients (P = 0.002) (**Table 2** and **Figure 1B**), and CA titers moderately correlated with monocyte counts as well (r = –0.425, P = 0.011) (**Table 3**). No significant associations with complete blood count parameters were found when CG-positive and CG-negative patients were compared (**Supplementary Table 3**).

CA Titers Might Be Subject to Seasonal Variations

We found a moderate correlation between CA titers and mean daily temperatures on the days when blood was drawn (r = 0.456, P = 0.006) (**Table 3** and **Supplementary Table 1**). No such associations were found for CG concentrations (**Table 3**).

CGs, but Not CAs, Are Linked to Basal Serum Tryptase Levels and ColdU Duration

CG concentrations strongly correlated with basal serum tryptase levels (r = 0.733, P = 0.025) and ColdU duration (r = 0.683, P = 0.042). CA titers were not linked to these features (**Table 3**).

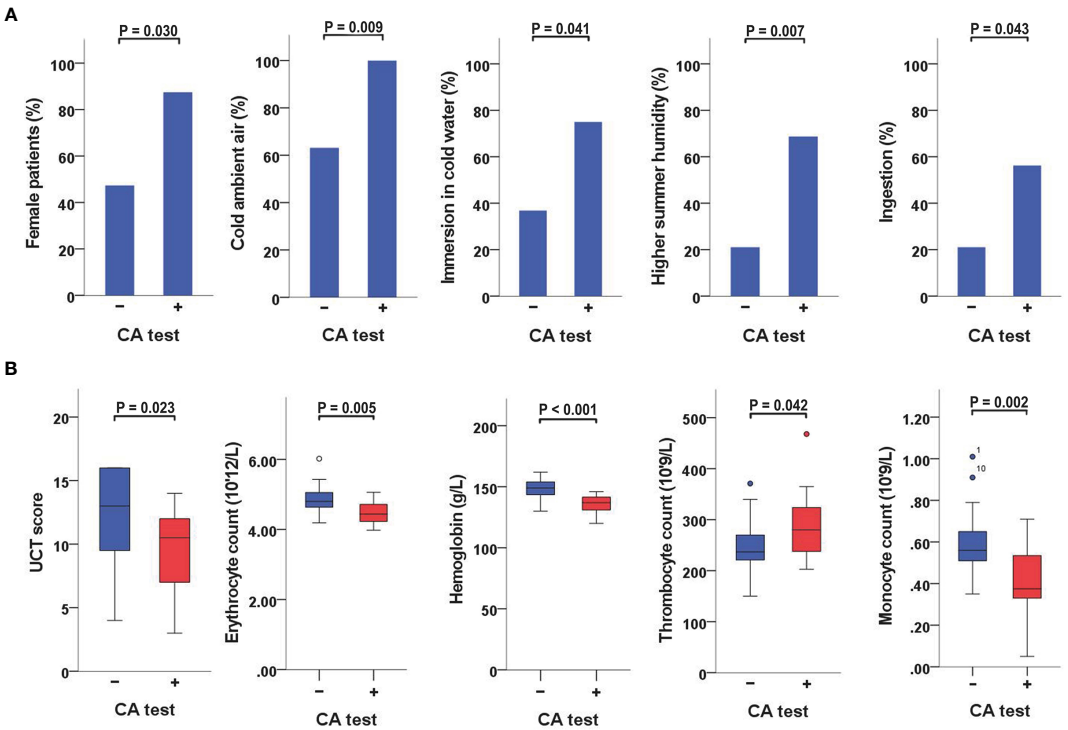

FIGURE 1 | Variables significantly linked to a positive CA-test **(A)** When CA-positive and CA-negative patients were compared, the former were more often female and had a higher rate of reactions triggered by cold ambient air or immersion in cold water, a higher rate of reactions aggravated by increased humidity levels in the summer, and a higher frequency of angioedema triggered by ingestion of cold foods or drinks. **(B)** When CA-positive and CA-negative patients were compared, the former also had lower UCT scores, lower erythrocyte counts, lower hemoglobin concentrations, higher thrombocyte counts, and lower monocyte counts. CA, cold agglutinin; UCT, Urticaria Control Test.

TABLE 3 | Correlations of cold agglutinin titers and cryoglobulin concentrations with patient characteristics.

Patient characteristic	CA titer *N* = 35		CG concentration *N* = 9	
	r	*P* value	r	*P* value
UCT score	**–0.359**	**0.034**	–0.059	0.880
Erythrocyte count	**–0.372**	**0.028**	0.059	0.881
Hemoglobin concentration	**–0.557**	**0.001**	0.000	1.000
Hematocrit level	**–0.397**	**0.018**	0.200	0.606
Monocyte count	**–0.425**	**0.011**	–0.017	0.966
Basal serum tryptase	–0.105	0.554	**0.733**	**0.025**
Mean daily Temp. on the day when blood was drawn	**0.456**	**0.006**	–0.663	0.073
ColdU duration	0.158	0.365	**0.683**	**0.042**

Statistically significant P values are in bold. CA, cold agglutinin; CG, cryoglobulin; ColdU, cold urticaria; N, number of patients; r, Spearman correlation coefficient; Temp., temperature; UCT, Urticaria Control Test.

DISCUSSION

In our study, a sizeable rate of ColdU patients tested positive for CAs and CGs. More importantly, patients who expressed these cold-reactive antibodies differed from those who did not in demographic, clinical and laboratory markers. Our results suggest that CAs and CGs, in a subpopulation of ColdU patients, are linked to the course and possibly the pathogenesis of their disease.

Our ColdU patients showed higher frequencies of positive CA and CG tests than previously reported (16, 36–39). There are several possible explanations for this discrepancy. CA titers in our patients were low (ranged from 1 to 16), and CG concentrations averaged 150 mg/L with a maximum of 189 mg/L. Earlier studies on ColdU did not report CA titers or CG concentrations and may have used higher thresholds for classifying patients as positive or used assays that differ from ours in sensitivity. Importantly, CAs and CGs may be underdiagnosed in ColdU due to improper sample handling (26, 49), and we very likely avoided false negative results by having blood samples drawn at tertiary centers specialized in CA and CG analysis. Other explanations include differences in patient populations and times of sampling. Our study population entailed patients with several different forms of ColdU, and 37% had atypical types of ColdU including systemic atypical ColdU (n = 10), localized ColdU (n = 1), and localized cold reflex urticaria (n = 2). Although we did not detect differences in CAs or CGs between ColdU subtypes (data not shown), possibly due to the low number of patients, such differences may exist. None of the previous ColdU studies

report on sampling times, and our study showed a correlation between CA titers and mean daily ambient temperatures on the days when blood for CAs was drawn. CAs, in patients with ColdU, may get depleted in cold seasons due to reactions to cold ambient air. Bendix et al. analyzed CA titers of 276 healthy blood donors in January and July and observed no seasonal variations in CA titers (50). This may suggest that lower CA levels in cold seasons may be linked to depletion of CAs in ColdU.

CA titers in our ColdU were significantly linked to a demographic (i.e., female gender), clinical (i.e., reactivity to cold ambient air, UCT scores), and laboratory features (i.e., lower erythrocyte counts, hemoglobin levels, hematocrit levels, and monocyte counts). Studies in hematology have shown that CA titers <64 do not necessarily indicate that CAs are not pathogenically relevant at 4°C (51). Even fatal autoimmune hemolytic anemia was reported in a patient with a CA titer 16 (52). Agglutination and lysis of erythrocytes are not related directly to the CA titer and rather appear to depend on the density of CA receptors on the erythrocyte surface (23).

Our CA-positive patients were significantly more often female, which is consistent with previous reports (30, 50, 53). Furthermore, higher CA titers were linked to a female gender. Both may be explained by the presence of two X chromosomes, which carry immune response genes, or hormonal factors that influence CA expression (54).

In our study, a positive CA test was associated with a higher rate of reactions triggered by cold ambient air or immersion in cold water and aggravated by increased summer humidity. This suggests that CA-positive patients are more sensitive to cold exposure, and that CAs in the skin might get activated by convective cooling due to moving currents (e.g., immersion in cold water), and by rapid evaporative cooling (e.g., vaporization of water directly from the skin in humid environments).

A positive CA test was also linked to a higher frequency of angioedema triggered by the ingestion of cold foods or beverages. The mechanism remains unexplained. Interestingly, I-antigen was detected in the gastric mucosa of animals (23), and Innet et al. described a CA-positive patient who experienced intermittent anemia and preferred to drink warm liquids (55). The fact that CA-positive patients react to cold ambient air and more severely (angioedema due to cold foods/drinks) may explain our finding that they also have lower disease control.

Our study does not explain why some patients with ColdU have CAs. It is known that infections, for example with *Mycoplasma pneumoniae* or *Streptococcus pneumoniae*, can cause cold agglutinin disease (15, 21, 49, 56, 57). These bacteria display I-related antigens on their surface and can trigger CA production (31), and ColdU has often been linked to preceding infections (1). Up to one third of ColdU patients have been reported to benefit from treatment with doxycycline, and 19% showed full remission (58), but the mechanisms for such improvements have not yet been explained. Furthermore, doxycycline is also effective against *Mycoplasma pneumoniae* (59) and *Streptococcus pneumoniae* (60).

Since our patients' serum was tested for agglutination of group O erythrocytes, their CAs are likely auto-anti-I (54). Pruzanski and Katz (23) described the biological diversity of CAs almost 40 years ago, which not only agglutinate erythrocytes, but also have complex interactions with other cells expressing the Ii-antigen (i.e., granulocytes, monocytes, macrophages, lymphocytes, thrombocytes, fibroblasts). Interestingly, higher CA titers in our patients correlated with lower erythrocyte and monocyte counts. It is therefore tempting to speculate that CAs in ColdU get activated when the skin is cooled and bind to cells that express the Ii-antigens (like in CA disease), thereby reducing their numbers. These cells may then activate complement and generate C3a and C5a, which are potent mast cell activators (28).

Defined cutoff levels of CGs, which would aid in the interpretation of our results, do not yet exist for ColdU. Our CG-positive patients were referred to a rheumatologist for further evaluation, but none of them received a diagnosis of a rheumatologic disease. Nonetheless, we found that higher CG levels in our ColdU patients were strongly associated with longer ColdU duration and higher basal serum tryptase levels. The latter suggests that CGs may promote mast cell degranulation, as serum tryptase is derived from mast cells and ColdU is a mast-cell driven disease (61). Costanzi and Coltman demonstrated that ColdU activity can be passively transferred to normal recipients with IgG or IgG-IgM CGs obtained from sera of patients with ColdU and cryoglobulinemia (43). All of our CG-positive ColdU patients had the IgG CG-isotype, and 7 of 9 also had the IgM CG-isotype.

Only 6% (n = 2) of studied patients had both, a positive CA and CG test. This suggests that having one type of cold protein does not present a risk for having another. Furthermore, our study showed that CAs and CGs are linked to different sets of markers of ColdU.

The major strengths of our study are that it was performed at a single center allowing good comparability of results, and that we obtained detailed history from all patients. However, it also has limitations: (a) we analyzed a relatively low number of patients, which did not allow for meaningful subgroup analyses, for example of patients with typical *vs*. atypical ColdU, (b) CA and CG tests were each performed in specialized laboratories and not repeated, (c) the thermal amplitude of CAs was not determined, and (d) we did not perform the same laboratory tests in a control group of healthy, age-matched individuals who may have low-titer, low-thermal amplitude CAs and low-concentration CGs in their serum (**Table 1**).

Despite these limitations, this is the first study of CAs and CGs in ColdU to show their association with clinical features, and the fact that such links exist suggests relevance. Our results, therefore, encourage further studies on CAs and CGs in ColdU to explore their function, their use as markers and their impact on treatment responses.

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 National Medical Ethics Committee of the Republic of Slovenia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MB initiated the study. MB and MK evaluated the patients. All authors substantially contributed to study design, analysis, interpretation of data, and manuscript development. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Maltseva N, Borzova E, Fomina D, Bizjak M, Terhorst-Molawi D, Kosnik M, et al. Cold urticaria what we know and what we do not know. *Allergy* (2021) 76:1077–94. doi: 10.1111/all.14674
2. Maurer M, Fluhr JW, Khan DA. How to Approach Chronic Inducible Urticaria. *J Allergy Clin Immunol Pract* (2018) 6:1119–30. doi: 10.1016/j.jaip.2018.03.007
3. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA(2)LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73:1393–414. doi: 10.1111/all.13397
4. Maurer M, Zuberbier T, Siebenhaar F, Krause K. Chronic Urticaria - What Does the New Guideline Tell Us? *J Dtsch Dermatol Ges* (2018) 16:584–93. doi: 10.1111/ddg.13531
5. Magerl M, Altrichter S, Borzova E, Gimenez-Arnau A, Grattan CE, Lawlor F, et al. The Definition, Diagnostic Testing, and Management of Chronic Inducible Urticarias - The Eaaci/Ga(2) LEN/EDF/UNEV Consensus Recommendations 2016 Update and Revision. *Allergy* (2016) 71:780–802. doi: 10.1111/all.12884
6. Krause K, Zuberbier T, Maurer M. Modern Approaches to the Diagnosis and Treatment of Cold Contact Urticaria. *Curr Allergy Asthma Rep* (2010) 10:243–9. doi: 10.1007/s11882-010-0121-3
7. Murphy GF, Austen KF, Fonferko E, Sheffer AL. Morphologically Distinctive Forms of Cutaneous Mast Cell Degranulation Induced by Cold and Mechanical Stimuli: An Ultrastructural Study. *J Allergy Clin Immunol* (1987) 80:603–11. doi: 10.1016/0091-6749(87)90015-7
8. Lawlor F, Kobza Black A, Breathnach AS, McKee P, Sarathchandra P, Bhogal B, et al. A Timed Study of the Histopathology, Direct Immunofluorescence and Ultrastructural Findings in Idiopathic Cold-Contact Urticaria Over a 24-H Period. *Clin Exp Dermatol* (1989) 14:416–20. doi: 10.1111/j.1365-2230.1989.tb02601.x
9. Church MK, Kolkhir P, Metz M, Maurer M. The Role and Relevance of Mast Cells in Urticaria. *Immunol Rev* (2018) 282:232–47. doi: 10.1111/imr.12632
10. Kaplan AP, Gray L, Shaff RE, Horakova Z, Beaven MA. In Vivo Studies of Mediator Release in Cold Urticaria and Cholinergic Urticaria. *J Allergy Clin Immunol* (1975) 55:394–402. doi: 10.1016/0091-6749(75)90078-0
11. Sherman WB, Seebohm PM. Passive Transfer of Cold Urticaria. *J Allergy* (1950) 21:414–24. doi: 10.1016/0021-8707(50)90017-7
12. Houser DD, Arbesman CE, Ito K, Wicher K. Cold Urticaria. *Immunol Stud Am J Med* (1970) 49:23–33. doi: 10.1016/s0002-9343(70)80110-3
13. Akiyama T, Ushijima N, Anan S, Takahashi I, Yoshida H. A Case of Cold Urticaria Due to a Serum Factor Belonging to the IGE Class. *J Dermatol* (1981) 8:139–43. doi: 10.1111/j.1346-8138.1981.tb02580.x
14. Wanderer AA, Maselli R, Ellis EF, Ishizaka K. Immunologic Characterization of Serum Factors Responsible for Cold Urticaria. *J Allergy Clin Immunol* (1971) 48:13–22. doi: 10.1016/0091-6749(71)90050-9
15. Berentsen S. New Insights in the Pathogenesis and Therapy of Cold Agglutinin-Mediated Autoimmune Hemolytic Anemia. *Front Immunol* (2020) 11:590. doi: 10.3389/fimmu.2020.00590
16. Silpa-archa N, Kulthanan K, Pinkaew S. Physical Urticaria: Prevalence, Type and Natural Course in a Tropical Country. *J Eur Acad Dermatol Venereol* (2011) 25:1194–9. doi: 10.1111/j.1468-3083.2010.03951.x
17. Torok L, Borka I, Szabo G. Waldenstrom's Macroglobulinaemia Presenting With Cold Urticaria and Cold Purpura. *Clin Exp Dermatol* (1993) 18:277–9. doi: 10.1111/j.1365-2230.1993.tb02188.x
18. Juhlin L. Cold Urticaria With Persistent Weals. *Br J Dermatol* (1981) 104:705–7. doi: 10.1111/j.1365-2133.1981.tb00759.x
19. Kramer DW, Perilstein PK. Case Report of Cold Sensitivity With Cold Hemagglutinins. *Angiology* (1951) 2:283–92. doi: 10.1177/000331975100200406
20. Stone MJ. Heating Up Cold Agglutinins. *Blood* (2010) 116:3119–20. doi: 10.1182/blood-2010-07-297523
21. McNicholl FP. Clinical Syndromes Associated With Cold Agglutinins. *Transfus Sci* (2000) 22:125–33. doi: 10.1016/s0955-3886(00)00033-3
22. Silberstein LE, Berkman EM, Schreiber AD. Cold Hemagglutinin Disease Associated With IgG Cold-Reactive Antibody. *Ann Intern Med* (1987) 106:238–42. doi: 10.7326/0003-4819-106-2-238
23. Pruzanski W, Katz A. Cold Agglutinins–Antibodies With Biological Diversity. *Clin Immunol Rev* (1984) 3:131–68.
24. Berentsen S. Complement Activation and Inhibition in Autoimmune Hemolytic Anemia: Focus on Cold Agglutinin Disease. *Semin Hematol* (2018) 55:141–9. doi: 10.1053/j.seminhematol.2018.04.002
25. Garratty G, Petz LD, Hoops JK. The Correlation of Cold Agglutinin Titrations in Saline and Albumin With Haemolytic Anaemia. *Br J Haematol* (1977) 35:587–95. doi: 10.1111/j.1365-2141.1977.tb00623.x
26. Kolopp-Sarda MN, Miossec P. Cryoglobulins: An Update on Detection, Mechanisms and Clinical Contribution. *Autoimmun Rev* (2018) 17:457–64. doi: 10.1016/j.autrev.2017.11.035
27. Kallemuchikkal U, Gorevic PD. Evaluation of Cryoglobulins. *Arch Pathol Lab Med* (1999) 123:119–25. doi: 10.1043/0003-9985(1999)123<0119:EOC>2.0.CO;2
28. Baines AC, Brodsky RA. Complementopathies. *Blood Rev* (2017) 31:213–23. doi: 10.1016/j.blre.2017.02.003
29. Lauchli S, Widmer L, Lautenschlager S. Cold Agglutinin Disease–the Importance of Cutaneous Signs. *Dermatology* (2001) 202:356–8. doi: 10.1159/000051682
30. Jain MD, Cabrerizo-Sanchez R, Karkouti K, Yau T, Pendergrast JM, Cserti-Gazdewich CM. Seek and You Shall Find–But Then What do You do? Cold Agglutinins in Cardiopulmonary Bypass and a Single-Center Experience With Cold Agglutinin Screening Before Cardiac Surgery. *Transfus Med Rev* (2013) 27:65–73. doi: 10.1016/j.tmrv.2012.12.001
31. Potter KN. Molecular Characterization of Cold Agglutinins. *Transfus Sci* (2000) 22:113–9. doi: 10.1016/s0955-3886(00)00031-x
32. Silva F, Pinto C, Barbosa A, Borges T, Dias C, Almeida J. New Insights in Cryoglobulinemic Vasculitis. *J Autoimmun* (2019) 105:102313. doi: 10.1016/j.jaut.2019.102313
33. Basile U, Gulli F, Gragnani L, Pocino K, Napodano C, Miele L, et al. Different Biochemical Patterns in Type II and Type III Mixed Cryoglobulinemia in HCV Positive Patients. *Dig Liver Dis* (2018) 50:938–43. doi: 10.1016/j.dld.2018.03.028

34. Sidana S, Rajkumar SV, Dispenzieri A, Lacy MQ, Gertz MA, Buadi FK, et al. Clinical Presentation and Outcomes of Patients With Type 1 Monoclonal Cryoglobulinemia. *Am J Hematol* (2017) 92:668–73. doi: 10.1002/ajh.24745
35. Dammacco F, Sansonno D, Piccoli C, Tucci FA, Racanelli V. The Cryoglobulins: An Overview. *Eur J Clin Invest* (2001) 31:628–38. doi: 10.1046/j.1365-2362.2001.00824.x
36. Koeppel MC, Bertrand S, Abitan R, Signoret R, Sayag J. Urticaria Caused by Cold. 104 Cases. *Ann Dermatol Venereol* (1996) 123:627–32.
37. Doeglas HM, Rijnten WJ, Schroder FP, Schirm J. Cold Urticaria and Virus Infections: A Clinical and Serological Study in 39 Patients. *Br J Dermatol* (1986) 114:311–8. doi: 10.1111/j.1365-2133.1986.tb02822.x
38. Neittaanmaki H. Cold Urticaria. Clinical Findings in 220 Patients. *J Am Acad Dermatol* (1985) 13:636–44. doi: 10.1016/s0190-9622(85)70208-3
39. Yee CSK, El Khoury K, Albuhairi S, Broyles A, Schneider L, Rachid R. Acquired Cold-Induced Urticaria in Pediatric Patients: A 22-Year Experience in a Tertiary Care Center (1996-2017). *J Allergy Clin Immunol Pract* (2019) 7:1024–31.e3. doi: 10.1016/j.jaip.2018.10.025
40. Ito A, Kazama T, Ito K, Ito M. Purpura With Cold Urticaria in a Patient With Hepatitis C Virus Infection-Associated Mixed Cryoglobulinemia Type III: Successful Treatment With Interferon-Beta. *J Dermatol* (2003) 30:321–5. doi: 10.1111/j.1346-8138.2003.tb00394.x
41. Miralles Lopez JC, Lopez Andreu FR, Sanchez-Gascon F, Lopez Rodriguez C, Negro Alvarez JM. Cold Urticaria Associated With Acute Serologic Toxoplasmosis. *Allergol Immunopathol (Madr)* (2005) 33:172–4. doi: 10.1157/13075702
42. Hauptmann G, Lang JM, North ML, Oberling F, Mayer G, Lachmann PJ. Lymphosarcoma, Cold Urticaria, IgG1 Monoclonal Cryoglobulin and Complement Abnormalities. *Scand J Haematol* (1975) 15:22–6. doi: 10.1111/j.1600-0609.1975.tb01051.x
43. Costanzi JJ, Coltman CAJr. Kappa Chain Cold Precipitable Immunoglobulin G (IgG) Associated With Cold Urticaria. I. Clinical Observations. *Clin Exp Immunol* (1967) 2:167–78.
44. Maire MA, Mittey M, Lambert PH. The Presence of Cryoprecipitable Immunoglobulins in Normal Human Sera may Reflect Specific Molecular Interactions. *Autoimmunity* (1989) 2:155–64. doi: 10.3109/08916938909019952
45. Berentsen S, Randen U, Tjonnfjord GE. Cold Agglutinin-Mediated Autoimmune Hemolytic Anemia. *Hematol Oncol Clin North Am* (2015) 29:455–71. doi: 10.1016/j.hoc.2015.01.002
46. Zago-Novaretti M, Khuri F, Miller KB, Berkman EM. Waldenström's Macroglobulinemia With an IgM Paraprotein That is Both a Cold Agglutinin and a Cryoglobulin and has a Suppressive Effect on Progenitor Cell Growth. *Transfusion* (1994) 34:910–4. doi: 10.1046/j.1537-2995.1994.341095026980.x
47. Maurer M, Metz M, Bindslev-Jensen C, Bousquet J, Canonica GW, Church MK, et al. Definition, Aims, and Implementation of GA(2) Len Urticaria Centers of Reference and Excellence. *Allergy* (2016) 71:1210–8. doi: 10.1111/all.12901
48. Weller K, Groffik A, Church MK, Hawro T, Krause K, Metz M, et al. Development and Validation of the Urticaria Control Test: A Patient-Reported Outcome Instrument for Assessing Urticaria Control. *J Allergy Clin Immunol* (2014) 133:1365–72. doi: 10.1016/j.jaci.2013.12.1076
49. Berentsen S, Roth A, Randen U, Jilma B, Tjonnfjord GE. Cold Agglutinin Disease: Current Challenges and Future Prospects. *J Blood Med* (2019) 10:93–103. doi: 10.2147/JBM.S177621
50. Bendix BJ, Tauscher CD, Bryant SC, Stubbs JR, Jacob EK. Defining a Reference Range for Cold Agglutinin Titers. *Transfusion* (2014) 54:1294–7. doi: 10.1111/trf.12453
51. Garratty G. A New Mechanism for Immune Destruction of Red Blood Cells? *Transfusion* (2010) 50:274–7. doi: 10.1111/j.1537-2995.2009.02564.x
52. Mandigers C, Keuning J, Booij A. A Patient With Fatal Cold Haemagglutinins. *Netherlands J Med* (1996) 49:209–11. doi: 10.1016/0300-2977(96)00051-4
53. Dube VE, Zuckerman L, Philipsborn HFJr. Variation of Cold Agglutinin Levels. *Vox Sang* (1978) 34:71–6. doi: 10.1111/j.1423-0410.1978.tb03725.x
54. Dube VE, Tanaka M, Chmiel J, Anderson B. Effect of ABO Group, Secretor Status and Sex on Cold Hemagglutinins in Normal Adults. *Vox Sang* (1984) 46:75–9. doi: 10.1111/j.1423-0410.1984.tb00055.x
55. Innet LM, Lester JL,3, Tait N. Cold Agglutinins: Preoperative Diagnosis Leads to an Uneventful Perfusion. *Perfusion* (1995) 10:343–5. doi: 10.1177/026765919501000510
56. Gertz MA. Cold Hemolytic Syndrome. *Hematol Am Soc Hematol Educ Program* (2006) 19–23. doi: 10.1182/asheducation-2006.1.19
57. Berentsen S. How I Manage Patients With Cold Agglutinin Disease. *Br J Haematol* (2018) 181:320–30. doi: 10.1111/bjh.15109
58. Gorczyza M, Schoepke N, Krause K, Hawro T, Maurer M. Patients With Chronic Cold Urticaria may Benefit From Doxycycline Therapy. *Br J Dermatol* (2017) 176:259–61. doi: 10.1111/bjd.14820
59. Pereyre S, Goret J, Bebear C. Mycoplasma Pneumoniae: Current Knowledge on Macrolide Resistance and Treatment. *Front Microbiol* (2016) 7:974. doi: 10.3389/fmicb.2016.00974
60. Cunha BA, Baron J, Cunha CB. Similarities and Differences Between Doxycycline and Minocycline: Clinical and Antimicrobial Stewardship Considerations. *Eur J Clin Microbiol Infect Dis* (2018) 37:15–20. doi: 10.1007/s10096-017-3081-x
61. Akin C. Mast Cell Activation Syndromes. *J Allergy Clin Immunol* (2017) 140:349–55. doi: 10.1016/j.jaci.2017.06.007

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Lower IgA Levels in Chronic Spontaneous Urticaria Are Associated With Lower IgE Levels and Autoimmunity

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Background: The pathogenesis of chronic spontaneous urticaria (CSU) is still insufficiently understood. Recent findings suggest that immunoglobulins, in particular IgE but also IgA, play a role in the development of CSU.

Objective: Our aim was to assess differences in clinical and laboratory markers between CSU patients with and without lower levels of serum IgA and IgE.

Methods: We analyzed the data of 606 patients with CSU by dividing them into four groups based on their IgA and IgE levels. The groups were compared for their spectrum of symptoms, disease activity, concomitant autoimmunity and routine laboratory markers. Autoreactivity was assessed by basophil activation test (BAT). Moreover, IgE-anti-thyroid peroxidase (TPO) was measured.

Results: Of the patients with lower IgE levels, 66.5% also had lower IgA levels ($r=0.316$, $p<0.001$). Patients with lower IgA and lower IgE levels showed a higher prevalence of recurrent angioedema ($p=0.03$, $p=0.04$) and concomitant autoimmunity ($p=0.006$, $p<0.001$). Autoreactivity was also found more frequently in patients with lower IgA and lower IgE levels ($p=0.003$, $p<0.001$). Reduced basophil counts were linked to both, lower IgA and lower IgE levels ($p<0.001$), whereas low eosinophil counts were primarily present in patients with lower IgE levels ($p=0.04$, $p<0.001$). Patients with elevated IgE-anti-TPO levels had lower IgA ($p=0.007$) and IgE levels ($p=0.001$).

Conclusion: Lower IgA levels in CSU are linked to lower IgE levels and features of autoimmune urticaria. Our findings encourage to screen CSU patients for serum IgA and IgE levels and to further assess their role as disease biomarkers.

Keywords: immunoglobulin A, immunoglobulin E, chronic spontaneous urticaria, autoreactivity, autoimmune disease, basophils, eosinophils

INTRODUCTION

Chronic spontaneous urticaria (CSU) is a common disease that affects around 1% of the general population (1). It is characterized by the appearance of pruritic wheals, angioedema or both for more than six weeks without a specific and definite triggering factor (2).

The symptoms of CSU are primarily caused by the activation and degranulation of skin mast cells (3). The specific mechanisms that lead to this activation are still not clarified entirely. Based on recent pathomechanistic studies and different response profiles to anti-immunoglobulin E (IgE) treatment, the concept of two different types of autoimmunity in CSU was established (4). In type I autoimmunity, or autoallergy, patients have IgE-antibodies against different autoantigens, such as thyroid peroxidase (TPO) (5), interleukin-24 (6) or double stranded DNA (7) that crosslink the IgE on mast cells and basophils and thus lead to degranulation. Type IIb autoimmunity is characterized by mast cell-activating IgG-antibodies, for example against IgE (8) or its high-affinity receptor FcεRI (9), that induce histamine release of mast cells and basophils. Patients with type IIb autoimmunity are characterized by a positive autologous serum skin test (ASST), serum autoreactivity in basophil activation test (BAT) or basophil histamine release assay (BHRA) and a positive immunoassay for IgG-antibodies against FcεRI or IgE (10). However, recent findings show that only in 8% of patients with type IIb autoimmune CSU all three elements are present simultaneously (11).

Low levels of IgE in patients with CSU have been linked to type IIb autoimmunity (11). Also, total levels of IgE are linked to the response to treatment with omalizumab, a therapeutic anti-IgE-antibody, with non-responders to this third-line treatment showing lower levels of IgE (12). Patients with type IIb autoimmune CSU also respond slower to omalizumab treatment (13). In contrast, CSU patients with type I autoimmunity and high IgE levels show better and faster responses to omalizumab, respectively (14, 15).

Currently, little is known about the role of immunoglobulin A (IgA) in CSU. Selective immunoglobulin A-deficiency (SIgAD) is the most common primary immunodeficiency. It is defined as a serum IgA level of less than 0.07 g/l with normal levels of serum immunoglobulin G (IgG) and immunoglobulin M (IgM) in patients older than four years in whom other causes of hypogammaglobulinemia have been excluded (16). Patients with SIgAD have a higher risk of autoimmune diseases (17), as do patients with CSU (18). Furthermore, in patients with SIgAD the prevalence of inflammatory skin diseases including CSU is higher (19). Interestingly, Frossi et al. found SIgAD in patients with CSU to be linked to the presence of other autoimmune diseases and signs of type IIb autoimmune CSU, such as positive ASST and BAT results (20). However, only four patients (all of them with exceptionally low IgA levels) were included in this study. Therefore, further work on the importance of IgA in CSU in a bigger cohort is required.

We hypothesized that lower levels of IgA are linked to features of type IIb autoimmune CSU including lower IgE. To test our hypothesis, we measured total serum IgA and IgE levels in more than 600 CSU patients and assessed those with lower levels of one or both for their clinical and laboratory features.

Abbreviations: ANA, Anti-nuclear antibodies; ANCA, Anti-neutrophil cytoplasmic antibodies; ASST, Autologous serum skin test; BAT, Basophil activation test; BSA, Bovine serum albumin; CSU, Chronic spontaneous urticaria; DLQI, Dermatology life quality index; EDTA, Ethylenediaminetetraacetic acid; FBS, Fetal bovine serum; IgA, Immunoglobulin A; IgE, Immunoglobulin E; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IQR, Interquartile range; Md, Median; PBS, phosphate-buffered saline; SIgAD, Selective immunoglobulin A-deficiency; TBS, Tris-buffered saline; TPO, Thyroid peroxidase; UAS, Urticaria activity score.

METHODS

Patients

We analyzed the data of 606 patients with CSU treated at the Urticaria Reference and Excellence Center (UCARE) (21) at Charité-Universitätsmedizin Berlin. All patients provided oral and written informed consent that included the publication of their pseudonymized data. For affected children (n=10), parents provided written informed consent. The study was approved by the Ethics Committee of Charité-Universitätsmedizin Berlin, Germany.

The patients were divided into four groups based on their IgA and IgE levels: 1) lower IgA and lower IgE levels ($IgA^{lower}IgE^{lower}$), 2) lower IgA and normal or elevated IgE levels ($IgA^{lower}IgE^{n/hi}$), 3) lower IgE and normal or elevated IgA levels ($IgA^{n/hi}IgE^{lower}$) and 4) normal or elevated IgA and IgE levels ($IgA^{n/hi}IgE^{n/hi}$). Based on the median IgA value of 1.84 g/l in this cohort of 606 CSU patients, we classified patients who had a serum IgA level of <1.84 g/l as having lower IgA levels. For lower IgE levels, the cut-off of <40 kU/l was used as this had previously been reported to be clinically relevant in CSU patients (11, 22).

All patients with complete routine diagnostic data on immunoglobulin levels A, E, G and M, were included in the study (n=523). Additionally, to increase group sizes, partially missing immunoglobulin values were determined in retrospect from frozen sera of patients who were known to have either lower IgA or lower IgE levels (n=83).

Patients treated with anti-IgE-antibodies (omalizumab) or immunosuppressives were not included in the study. Moreover, patients were asked, if possible, to stop taking oral corticosteroids and antihistamines, starting four weeks before the analysis, to ensure reliable results.

Clinical Markers

Disease activity was measured by the 7-day once-daily urticaria activity score (UAS7) that is based on wheal number and itch severity documented by the patient (minimum: 0, maximum: 42) (2). To assess health-related quality of life impairment, the dermatology life quality index (DLQI) was applied (minimum: 0, maximum: 30) (23). Furthermore, the spectrum of symptoms (wheals with or without angioedema) and the prevalence of concomitant diseases, especially autoimmunity, were assessed.

Routine Laboratory Markers

Aside from serum levels of IgA and IgE, different serologic markers were assessed in all patients including C-reactive

protein, leukocyte, basophil and eosinophil count, IgG and IgM levels.

Assessment of Autoimmunity

Patients were screened for the presence of comorbid autoimmune disease and elevated levels of anti-neutrophil cytoplasmic antibodies (ANCA), (cANCA ≥10 U/ml, pANCA ≥5 U/ml), anti-nuclear antibodies (ANA) (≥1:160), rheumatoid factor IgM (≥20U/ml), circulating immune complexes (≥55 µg/ml), IgG-anti-TPO (≥35 kU/l) or thyroid stimulating hormone-receptor-antibodies (≥2 U/l).

Assessment of Autoreactivity

Autoreactivity was assessed by an indirect BAT to screen for autoantibodies in the patients' serum. Moreover, IgE-anti-TPO as a potential relevant autoallergen in CSU was measured (5).

The BAT was performed with fresh healthy donor basophils and frozen patient serum that was thawed and diluted in phosphate-buffered saline (PBS) (final concentration of 20%). 50 µl of heparinized whole blood, taken from the same healthy donor for all experiments, was incubated with 50 µl of 20% serum for 15 minutes at 37°C and with 5% CO_2 in a 96 well plate. As a positive control, 50 µl PBS containing 2.5 µg/ml mouse anti-human IgE HP6029 and 2.5 µg/ml mouse anti-human IgE HP6061 (both SouthernBiotech, Birmingham, AL) was used. PBS functioned as negative control. After washing the samples with 150 µl PBS + 2mM ethylenediaminetetraacetic acid (EDTA; Invitrogen, Carlsbad, CA) the plate was centrifuged at 340 x g for 10 minutes at 4°C. The supernatant was aspirated with a vacuum pump and the cells were stained with antibodies against CD3 (BD Biosciences, Franklin Lakes, NJ, REF#560365), CD193 (BD Biosciences, Franklin Lakes, NJ, REF#558208), CD63 (BD Biosciences, Franklin Lakes, NJ, REF#561982) and CD203c (Beckman Coulter, Brea, CA, REF#IM3575) at 1:42 each in FACS buffer (1% bovine serum albumin [BSA; Serva, Heidelberg, Germany] in PBS) supplemented with 2.4% Kiovig (Baxter AG, Vienna, Austria). The cells were lysed with 200 µl of red blood cell lysis buffer (BioLegend, San Diego, CA) for five minutes. After centrifugation, the supernatant was discarded and the pellet was resuspended in 250 µl MACS buffer (1% BSA + 2 mM EDTA in PBS). Samples were then measured by flow cytometry (MACS Quant, Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowJo (v.10.6.1, BD Biosciences, Franklin Lakes, NJ). The gating strategy used here is shown in the **Supplemental Figure 1**. The BAT was considered positive, if more than 7.77% of the total basophils were both CD63 and CD203c positive. As cut-off, the 95th percentile of double positive cells induced by control sera (n=51) was used.

CSU serum levels of IgE-anti-TPO were measured in an enzyme-linked immunosorbent assay. The plate was coated overnight with 2 µg/ml of MHE-18 (BioLegend, San Diego, CA). After blocking with 1% fetal bovine serum (FBS, Biochrom, Berlin, Germany) in tris-buffered saline (TBS), plates were incubated with serum (diluted 1:5 in TBS, overnight, at 4°C), followed by biotinylated human-TPO (in.vent Diagnostica GmbH, Hennigsdorf, Germany). For detection, streptavidin-HRP (ThermoFisher Scientific, Waltham, MA) and ECL Prime (GE Healthcare, Chicago, IL) were used. Between each step, intensive washing with TBS containing 0.05% Tween 20 was performed. Chimeric human IgE-anti-TPO was used as positive control and standard. It was obtained from the supernatant of SP-2/Sp1.4 transfected mouse myeloma cells (kindly provided by Sandra McLachlan, Thyroid Autoimmune Disease Unit, Cedars-Sinai Medical Center and University of California Los Angeles) grown in 2 mM L-Glutamine, 10% FBS gold IMEM-medium, 10 µg/ml Streptomycin and 100 U/ml Penicillin (all Sigma-Aldrich, Deisenhofen, Germany), as described before (5). IgE-anti-TPO levels >1.09 ng/ml were considered as elevated. As cut-off, the 95th percentile of IgE-anti-TPO levels measured in healthy control sera (n=14) was used.

Statistical Analysis

Statistical analysis was performed with the Statistical Package for the Social Science (IBM SPSS version 27; IBM Corp, New York, NY) and figures were created using GraphPad Prism (version 9.0.0; GraphPad Software, San Diego, CA). Normal distribution was tested with Kolmogorov Smirnov test. As the data was not normally distributed, the median (Md) and interquartile range (IQR) were used. For comparison of continuous variables, the Mann-Whitney-U test was performed when comparing two groups and the Kruskal-Wallis test (one-way ANOVA) was applied when comparing more than two groups. Correlation was assessed using Spearman-Rho test for variables that were not normally distributed. Binary variables were analyzed using Pearson Chi-square test. Post hoc analysis involved pairwise comparisons using the z-test of two proportions with a Bonferroni correction. Statistical significance was indicated by $p \leq 0.05$.

RESULTS

Lower IgA Levels Are Linked to Lower IgE and IgG Levels and Higher IgM Levels

In our study cohort the median age of patients was 43 years, ranging from 13 to 82 years. More female (n=461) than male patients (n=145) with CSU were included in the cohort. The median disease duration was two years and the median age at diagnosis 36 years. During the examination period, 4.7% of patients (n=17) received low to medium doses of oral corticosteroids and 23.4% (n=88) received antihistamines (**Table 1**).

More than a third of CSU patients showed lower IgE levels (n=224, 37.0%). Of these patients, 66.5% (n=149) were classified as having lower IgA levels (**Table 1**). IgA and IgE levels were significantly correlated (r=0.316, p<0.001) (**Figure 1A**) and patients with lower IgA (<1.84 g/l) also had significantly lower IgE levels (md=42.50 kU/l *vs.* md=111.00 kU/l, p<0.001) (**Figure 1B**).

Moreover, IgG levels were particularly lower in patients with lower IgA levels. The difference was significant between $IgA^{lower}IgE^{lower}$ patients compared to $IgA^{n/hi}IgE^{lower}$ patients

TABLE 1 | Main clinical and laboratory features of the patients.

	Lower IgA and lower IgE levels (IgA^{lower} IgE^{lower})	Lower IgA and normal or elevated IgE levels (IgA^{lower} $IgE^{n/hi}$)	Lower IgE and normal or elevated IgA levels ($IgA^{n/hi}$ IgE^{lower})	Normal or elevated IgA and IgE levels ($IgA^{n/hi}$ $IgE^{n/hi}$)	Signifi-cance level
	n=149 (24.6%)	**n=154 (25.4%)**	**n=75 (12.4%)**	**n=228 (37.6%)**	
Female patients, % (n)	86.6 (129)	78.6% (121)	73.3 (55)	68.4 (156)	**p=0.001**
Age at diagnosis in years, md (IQR)	40.00 (26.00-49.60)	31.92 (23.00-42.00)	41.00 (27.25-50.00)	37.00 (26.42-50.00)	**p=0.001**
Disease duration in years, md (IQR)	1.58 (1.00-5.00)	2.08 (1.00-7.00)	2.00 (1.00-6.00)	2.14 (1.00-8.00)	p=0.16
Antihistamine treatment*, % (n)	23.7 (23)	23.7 (22)	25.5 (14)	22.1 (29)	p=0.97
Systemic corticosteroid treatment*, % (n)	3.2 (3)	7.9 (7)	5.7 (3)	3.1 (4)	p=0.35
IgA in g/l (0.7-4.0), md (IQR)	1.27 (0.87-1.53)	1.42 (1.13-1.61)	2.37 (1.98-3.16)	2.47 (2.14-3.10)	**p<0.001**
IgE in kU/l (0.0-100.0), md (IQR)	14.60 (6.37-23.60)	122.50 (64.00-241.00)	17.20 (8.54-27.70)	160.00 (89.95-287.00)	**p<0.001**
IgG in g/l (7.0-16.0), md (IQR)	9.05 (7.81-10.80)	9.57 (8.51-10.52)	10.05 (8.83-11.74)	10.24 (8.77-12.07)	**p<0.001**
IgM in g/l (0.4-2.3), md (IQR)	1.05 (0.69-1.48)	0.99 (0.67-1.40)	0.98 (0.71-1.30)	0.97 (0.67-1.37)	p=0.33
UAS7 (0-42), md (IQR)	19.00 (10.00-27.00)	18.00 (12.00-25.00)	17.00 (9.00-29.00)	16.00 (8.00-23.00)	p=0.14
DLQI (0-30), md (IQR)	9.00 (4.00-13.00)	9.50 (6.00-15.00)	6.00 (3.00-12.50)	6.00 (3.00-11.00)	**p=0.01**
Presence of angioedema, % (n)	49.7 (73)	33.8 (50)	34.3 (24)	35.1 (74)	**p=0.01**
C-reactive protein in mg/l, md (IQR)	2.00 (1.00-5.40)	1.90 (0.70-6.45)	2.25 (0.90-5.25)	2.50 (1.00-5.60)	p=0.65
Leukocyte count/nl, md (IQR)	6.16 (5.15-7.47)	6.61 (5.17-7.85)	5.59 (4.78-7.01)	6.31 (5.43-7.58)	p=0.12
Eosinophil count/nl, md (IQR)	0.09 (0.06-0.17)	0.17 (0.09-0.29)	0.13 (0.08-0.19)	0.16 (0.10-0.24)	**p<0.001**
Basophil count/nl, md (IQR)	0.01 (0.00-0.02)	0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.02 (0.01-0.04)	**p<0.001**
IgE-anti-TPO in ng/ml, md (IQR)	1.01 (0.80-1.17)	0.91 (0.73-1.16)	1.00 (0.83-1.13)	0.92 (0.76-1.08)	p=0.26

DLQI, Dermatology life quality index; IgA, Immunoglobulin A; IgE, Immunoglobulin E; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IQR, Interquartile range; Md, Median; TPO, Thyroid peroxidase; UAS, Urticaria activity score.
**Treatment with antihistamines or systemic corticosteroids (prednisone, median 7 mg/d, range 1-40 mg/d) at the time of immunoglobulin assessment or in the 7 days before.*
Reference values for serum immunoglobulin classes are indicated in brackets. For clinical scores minimum and maximum values are presented. Significances for differences between the groups were measured by Kruskal-Wallis test for continuous variables and Pearson Chi-square test for binary variables. Statistically significant correlations are written in boldface.

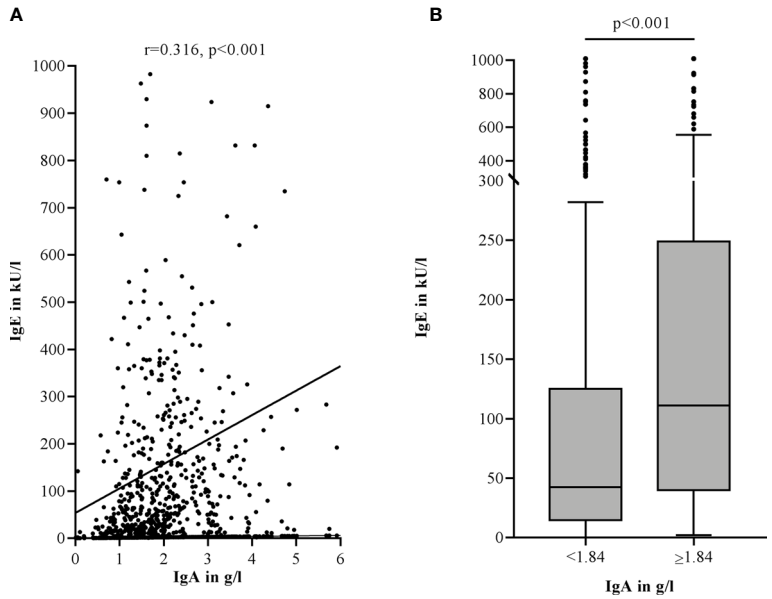

FIGURE 1 | Lower IgA levels are correlated with lower total IgE levels in the serum of CSU patients (Spearman-Rho) **(A)**. Lower IgA levels are associated with lower total IgE levels. Boxes are displayed as median and interquartile range. The whiskers indicate the range. For statistical significance, a Mann-Whitney-U test was performed **(B)**. IgA levels >6 g/l **(A)** and IgE levels >1000 kU/l **(A, B)** are not shown in this graphic.

(p=0.002) and $IgA^{n/hi}IgE^{n/hi}$ patients (p<0.001) (**Figure 2A**). IgG levels also correlated significantly with IgA (r=0.280, p<0.001) and less strongly with IgE levels (r=0.104, p=0.01) (**Figure 2B**).

IgM levels revealed no significant differences between the groups but were especially high in $IgA^{lower}IgE^{lower}$ patients (**Table 1**) and showed a weak negative correlation with IgA levels (r=-0.090, p=0.03).

Lower IgA and IgE Levels Are Associated With Female Gender, High Rates of Angioedema and Lower Basophil and Eosinophil Counts

Levels of IgA were lower in women (md=1.76 g/l) compared to men (md=2.12 g/l) (p<0.001), as were levels of IgE (md=60.90 kU/l *vs.* 118.00 kU/l, p<0.001). The proportion of females was significantly higher in $IgA^{lower}IgE^{lower}$ patients compared to $IgA^{n/hi}IgE^{n/hi}$ patients (86.6% *vs.* 68.4%, p<0.001). With a median age of 32 years at diagnosis, $IgA^{lower}IgE^{n/hi}$ patients were significantly younger compared to all other groups ($IgA^{lower}IgE^{lower}$: p=0.006, $IgA^{n/hi}IgE^{lower}$: p=0.009, $IgA^{n/hi}IgE^{n/hi}$: p=0.002) (**Table 1**).

The prevalence of recurrent angioedema was highest in $IgA^{lower}IgE^{lower}$ patients. Here, nearly half of the patients, namely 49.7% showed angioedema. The difference was significant compared to $IgA^{lower}IgE^{n/hi}$ patients (33.8%, p=0.03) and $IgA^{n/hi}IgE^{n/hi}$ patients (35.1%, p=0.04) (**Table 1**).

Quality of life impairment, as assessed by DLQI was highest in patients with lower levels of IgA. The difference was significant for $IgA^{lower}IgE^{n/hi}$ patients (md=9.5) compared to $IgA^{n/hi}IgE^{n/hi}$ patients (md=6.0, p=0.01). The disease activity did not show significant differences between the groups as assessed by UAS7 (**Table 1**). However, for both UAS7 and DLQI, a negative correlation with IgA levels was found (UAS7: r=-0.095, p=0.04; DLQI: r=-0.173, p=0.005). Of note, the correlations increased when only including patients with concomitant recurrent angioedema (UAS7: r=-0.242, p=0.001; DLQI: r=-0.291, p=0.003) and the correlations completely disappeared when excluding patients with concomitant autoimmune phenomena (n=248).

Basophil counts were lowest in $IgA^{lower}IgE^{lower}$ patients. The differences were significant compared to $IgA^{lower}IgE^{n/hi}$ patients (p<0.001) and $IgA^{n/hi}IgE^{n/hi}$ patients (p<0.001) (**Figure 3A**). Eosinophil counts were mainly linked to lower IgE levels as shown by significantly lower eosinophil numbers in $IgA^{lower}IgE^{lower}$ and $IgA^{n/hi}IgE^{lower}$ patients compared to $IgA^{lower}IgE^{n/hi}$ patients (p<0.001, p=0.04) (**Figure 3B**).

Lower IgA and IgE Levels Are Linked to High Rates of Concomitant Autoimmune Diseases, Autoreactivity and High Levels of IgE-anti-TPO

Four of ten CSU patients (248 of 594, 41.8%) had one or more comorbid autoimmune disease and/or elevated levels of specific relevant autoantibodies. The most common comorbid autoimmune diseases were Hashimoto's thyroiditis and Graves' disease. Positivity to ANA, ANCA, rheumatoid factor IgM or circulating immune complexes was associated mostly to lower IgE levels ($IgA^{lower}IgE^{lower}$ and $IgA^{n/hi}IgE^{lower}$), whereas the prevalence of thyroid autoimmune diseases was especially high in patients with both lower IgA and lower IgE levels (**Table 2**). In total, the proportion of patients with autoimmune diseases and/

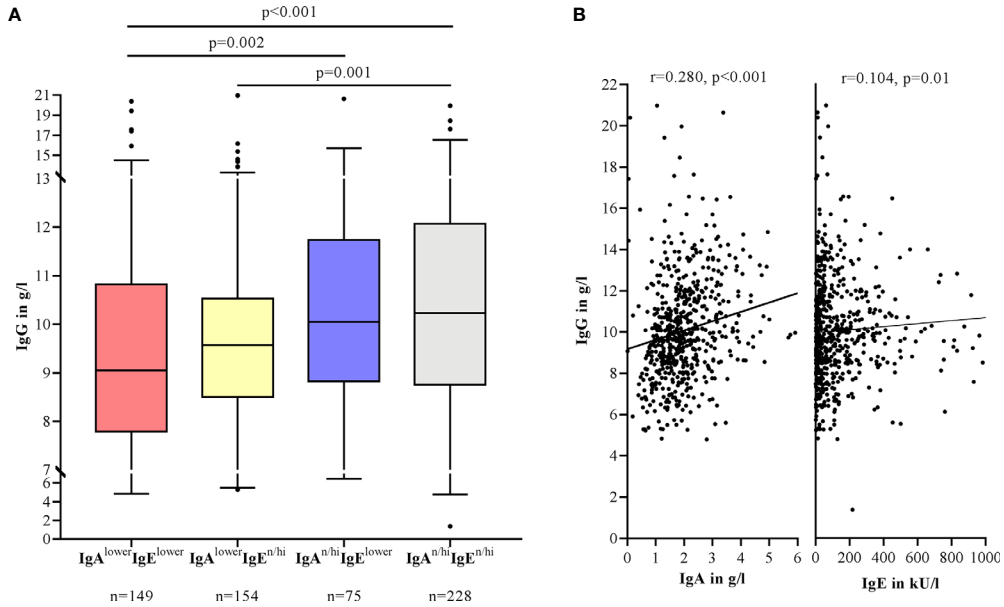

FIGURE 2 | Lower IgG levels are associated with lower IgA levels in the serum of CSU patients. Boxes are displayed as median and interquartile range. The whiskers indicate the range. For statistical significance, a Kruskal-Wallis test was performed **(A)**. Correlation of IgG with IgA and IgE levels by Spearman-Rho. IgA levels >6 g/l and IgE levels >1000 kU/l are not shown in this graphic **(B)**.

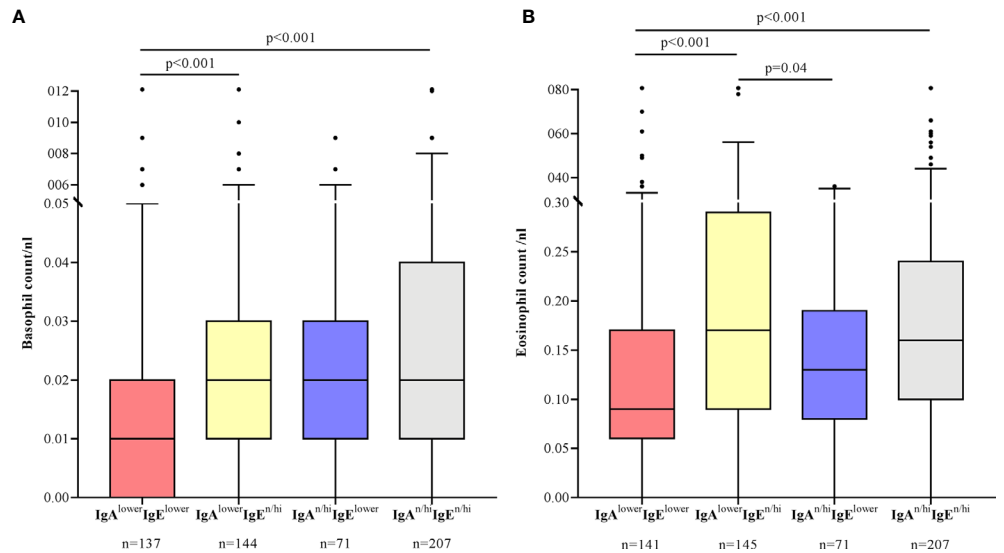

FIGURE 3 | Basophil counts are lowest in CSU patients with lower IgA and IgE levels **(A)**, whereas eosinophil counts are low in CSU patients with lower IgE levels with or without lower IgA levels **(B)**. Boxes are displayed as median and interquartile range. The whiskers indicate the range. For statistical significance, a Kruskal-Wallis test was performed. Basophil counts >0.12/nl and eosinophil counts >0.8/nl are not shown in this graphic.

or elevated autoantibodies was therefore highest in patients with lower IgE levels (**Figure 4**).

The BAT as a marker of type IIb autoimmunity was positive in 16.6% of CSU patients (n=500), with highest percentages of activated basophils (CD63 and CD203c positivity) in patients with lower IgA and IgE levels ($IgA^{lower}IgE^{lower}$). The difference in activation rates was significant compared to all other groups (p=0.006, p=0.005, p<0.001) (**Figure 5A**). This effect was independent of the prevalence of autoimmune diseases as the exclusion of all patients with autoimmune phenomena (n=275 remaining) still revealed highest rates in the $IgA^{lower}IgE^{lower}$ group compared to all other groups (p=0.04, p<0.001, p=0.009).

Both IgA and IgE levels showed a significant negative correlation with the percentage of activated cells (IgA: r=-0.208,

TABLE 2 | Prevalence of autoimmune phenomena in patients with CSU sorted by type.

	Type	Criteria	IgA^{lower} IgE^{lower}	IgA^{lower} $IgE^{n/hi}$	$IgA^{n/hi}$ IgE^{lower}	$IgA^{n/hi}$ $IgE^{n/hi}$	Significance level
A	Clinically diagnosed autoimmune disease	Patient history confirmed by treating physician	23.5% (n=35)	14.3% (n=22)	14.7% (n=11)	11.0% (n=25)	**p=0.01**
B	ANA, ANCA, rheumatoid factor IgM and/or circulating immune complexes	Positivity of ≥1 of the following	37.0% (n=54)	28.4% (n=42)	34.7% (n=25)	24.7% (n=55)	p=0.06
	ANCA	cANCA ≥10 U/ml, pANCA ≥5 U/ml	6.0% (n=7)	5.6% (n=7)	5.7% (n=3)	4.0% (n=7)	p=0.85
	ANA	≥1:160	38.7% (n=36)	29.1% (n=25)	39.5% (n=17)	24.6% (n=29)	p=0.09
	Rheumatoid factor IgM	≥20U/ml	4.2% (n=6)	6.3% (n=9)	0.0% (n=0)	3.6% (n=8)	p=0.17
	Circulating immune complexes	≥55 µg/ml	11.8% (n=15)	5.6% (n=7)	16.4% (n=10)	8.2% (n=16)	p=0.08
C	Thyroid antibodies + clinical phenotype	Positivity of ≥1 of the following + clinic of hypo- or hyperthyroidism	16.4% (n=24)	7.4% (n=11)	8.1% (n=6)	7.0% (n=16)	**p=0.01**
	IgG-anti-TPO	≥35 kU/l	35.0% (n=49)	12.8% (n=18)	16.7% (n=12)	21.4% (n=48)	**p<0.001**
	Thyroid stimulating hormone-receptor-antibodies	≥2 U/l	7.3% (n=10)	12.9% (n=18)	4.2% (n=3)	5.5% (n=12)	p=0.15
	Autoimmune phenomena	**A** and/or **B** and/or **C**	55.1% (n=81)	36.0% (n=54)	50.0% (n=37)	34.1% (n=76)	**p<0.001**

ANA, Anti-nuclear antibodies; ANCA, Anti-neutrophil cytoplasmic antibodies, IgA, Immunoglobulin A; IgE, Immunoglobulin E; IgG, Immunoglobulin G; IgM, Immunoglobulin M; TPO, Thyroid peroxidase.
Significances for differences between the groups were measured by Pearson Chi-square test. Statistically significant correlations are written in boldface.

p<0.001; IgE: r=-0.218, p<0.001) (**Figure 5B**). Positive BAT rates were also found more often in $IgA^{lower}IgE^{lower}$ patients (32.6%) compared to $IgA^{lower}IgE^{n/hi}$ patients (14.2%, p=0.003) and $IgA^{n/hi}IgE^{n/hi}$ patients (7.1%, p<0.001). There was a weak correlation between UAS7 and the percentage of activated cells (r=0.106, p=0.04).

IgE-anti-TPO levels were measured in 399 patients. Although quantitative IgE-anti-TPO levels did not markedly differ between the four groups (**Table 1**), patients with elevated IgE-anti-TPO levels had significantly lower IgA (md=1.72 g/l *vs.* 1.85 g/l, p=0.007) (**Figure 6A**) and IgE levels (md=39.25 kU/l *vs.* 75.40 kU/l, p=0.001) (**Figure 6B**) and IgE-anti-TPO showed a weak negative correlation with IgE levels (r=-0.120, p=0.02). Nearly half of the patients with elevated levels of IgG-anti-TPO or thyroid stimulating hormone-receptor antibodies (n=107), also had elevated IgE-anti-TPO levels (n=52, 48.6%).

DISCUSSION

This is the first study on IgA levels in a sizeable cohort of CSU patients. Lower IgA and lower IgE levels often appear together and the combination of both is linked to features of autoreactivity (e.g. positive BAT and elevated IgE-anti-TPO levels) and the appearance of autoimmune phenomena.

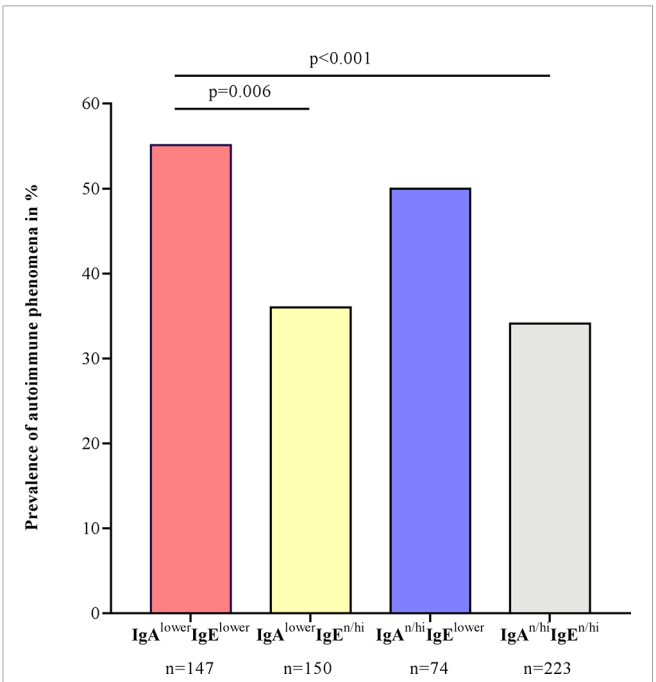

FIGURE 4 | Autoimmune phenomena are more prevalent in CSU patients with lower serum IgE levels. Autoimmune phenomena were defined as the presence of clinically diagnosed autoimmune diseases and/or elevated levels of ANCA, ANA, rheumatoid factor IgM or circulating immune complexes and/ of elevated levels of IgG-anti-TPO or thyroid stimulating hormone-receptor-antibodies, if they were in accordance with the clinical symptoms. For statistical significance, the z-test of two proportions with a Bonferroni correction was performed.

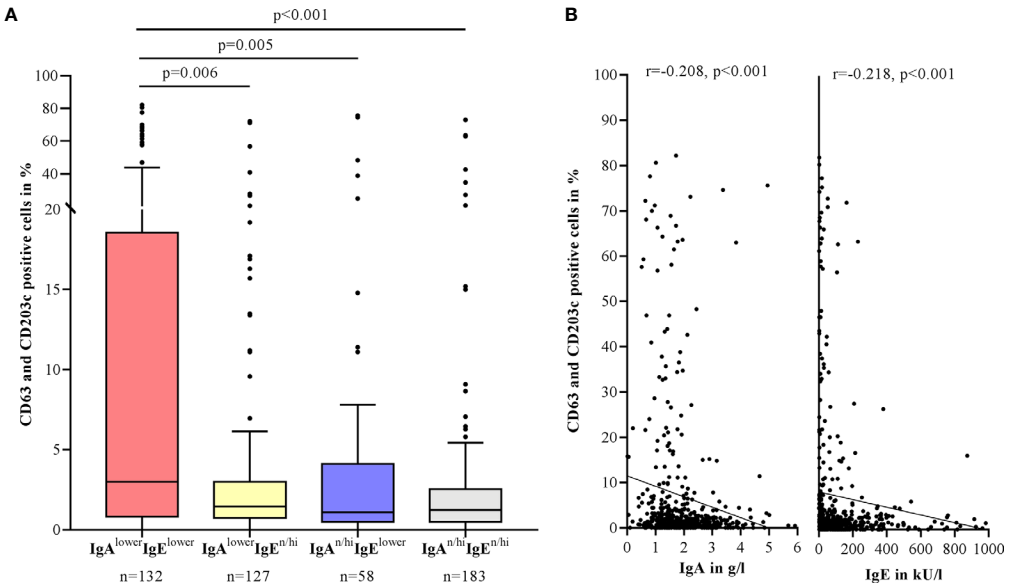

FIGURE 5 | BAT positivity is associated with lower serum IgA and IgE levels in CSU patients. Percentage of CD63 and CD203c positive cells in the BAT after stimulation of healthy basophils with patient serum. Boxes are displayed as median and interquartile range. The whiskers indicate the range. For statistical significance, a Kruskal-Wallis test was performed **(A)**. IgA and IgE levels correlate negatively with the percentage of activated basophils by Spearman-Rho. IgA levels >6 g/l and IgE levels >1000 kU/l are not shown in this graphic **(B)**.

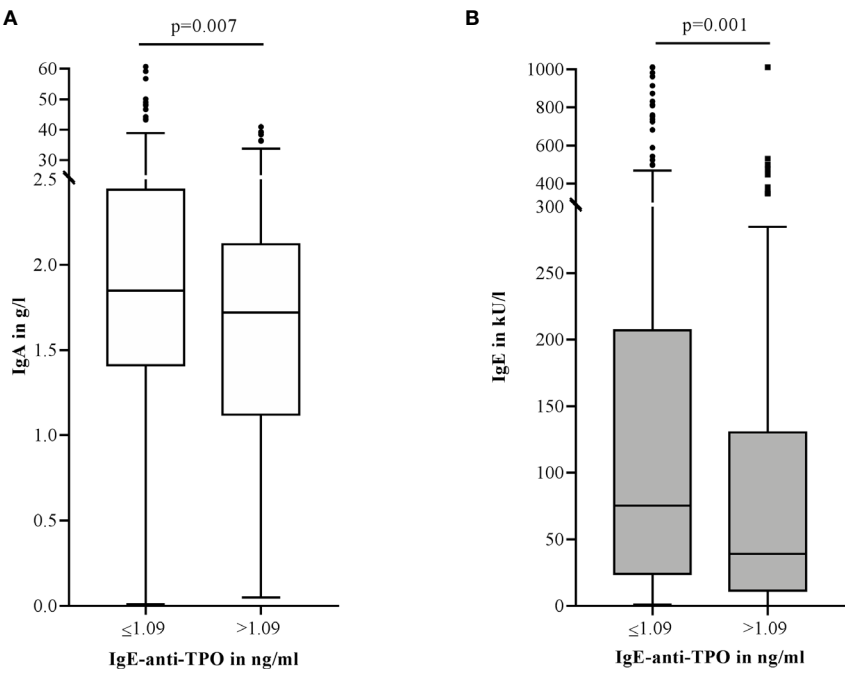

FIGURE 6 | Elevated IgE-anti-TPO levels (>1.09 ng/ml, n=120) in CSU patients are associated with lower serum IgA **(A)** and IgE **(B)** levels compared to patients with normal IgE-anti-TPO levels (≤1.09 ng/ml, n=279). Boxes are displayed as median and interquartile range. The whiskers indicate the range. For statistical significance, a Mann-Whitney-U test was performed. IgA levels >6 g/l and IgE levels >1000 kU/l are not shown in this graphic.

More than half of the patients with lower IgE levels also showed reduced IgA levels, indicating that these two types of immunoglobulins are linked to each other. This is supported by observations in common variable immunodeficiency, which frequently presents with very low levels of IgA in combination with reduced levels of IgE (24). Interestingly, patients with both lower IgA and lower IgE levels also had reduced IgG levels but increased IgM levels as compared to other groups. An explanation for this finding could be a B-cell defect resulting in an impaired antibody class switch with imbalanced immunoglobulin production in CSU. This has been reported for patients with SIgAD, who showed increased levels of serum IgM as a compensatory mechanism. The increased IgM levels might protect patients against severe infections, as SIgAD patients with normal IgM levels developed severe infections more often (25).

However, it remains to be clarified whether lower IgA and IgE levels were present in patients before the development of their urticaria and were thus involved in the pathogenesis of the disease, or whether the imbalanced immunoglobulins were caused by the disease itself.

A functional role of lower IgA in the pathogenesis of CSU could be attributed to the signaling through FcαRI. In a murine IgE-mediated model of asthma it was shown that stimulation of FcαRI with serum IgA inhibits FcεRI-induced degranulation of mast cells by IgE based on an antagonism between IgA and IgE *via* their respective receptors (26). In humans, FcαRI is expressed on immune cells such as eosinophils, neutrophils and monocytes but not on mast cells. Serum IgA in humans is supposed to negatively regulate immune cell activation and degranulation *via* FcαRI (27, 28). One could hypothesize that lower IgA in CSU would lead to increased activation of eosinophils, neutrophils and monocytes resulting in inflammation and autoimmunity.

The quality of life was impaired the greatest in patients with lower IgA levels ($IgA^{lower}IgE^{lower}$ and $IgA^{lower}IgE^{n/hi}$), whereas the CSU disease activity did not differ significantly. We attribute the effect on quality of life to higher rates of concomitant conditions, such as autoimmune phenomena and recurrent angioedema in patients with lower IgA and lower IgE levels. Rates of recurrent angioedema, for instance, were highest in patients with lower IgA and lower IgE levels. Patients with positive ASST results have been found to be more likely to develop angioedema (29). This fits our observation of frequent autoreactivity in patients with lower IgA and IgE levels. Of further interest, Bond et al. reported a link between SIgAD and hereditary angioedema (30).

In our study we found that basophil counts seem to be influenced especially by the combination of lower IgA and lower IgE levels, whereas eosinophil counts are related predominantly to the levels of IgE. Earlier reports found that basopenia is linked to severe, antihistamine-resistant CSU and type IIb autoimmunity (31), which fits our results that patients with lower IgA and IgE levels show more type IIb autoimmunity. It is hypothesized that basopenia is caused by the recruitment of circulating basophils into the wheals (8, 32). Recent findings also indicate a connection between eosinopenia and type IIb autoimmunity, high disease activity, poor response to treatment and lower IgE levels (33). This is in line with the results of our study. Also, it was shown that some CSU patients can benefit from anti-interleukin-5-targeted treatment with mepolizumab (34) or reslizumab (35) which affect recruitment, activation and survival of eosinophils. Beyond this, concomitant lower IgA levels were found in some patients with eosinophil deficiency (36). The exact role of eosinophils in the pathogenesis of CSU, however, remains unclear (37).

Our data imply that lower IgA and lower IgE levels taken together are more sensitive in diagnosing type IIb autoimmunity in CSU patients than lower IgE levels alone. The connection between lower levels of serum IgE and type IIb autoimmunity that we found has been described previously (38, 39). However, the reason for this remains unknown. One hypothesis is that IgE complexes with anti-IgE-antibodies and is therefore not measurable in these patients (40).

Recently, IgM- and IgA-antibodies against the IgE receptor FcεRI were found in addition to IgG-antibodies, which are characteristic for type IIb autoimmunity in CSU (41). Although only IgM-antibodies could be linked to type IIb autoimmunity so far, based on our results it would be of interest to measure IgA-antibodies in a CSU cohort.

The prevalence of SIgAD was shown to be increased in patients with autoimmune diseases and the other way around (42). Abolhassani et al. proposed that this effect is mediated by a reduced mucosal defence in patients with SIgAD. Thus, antigens from the environment could enter the ciruclation more easily and result in the development of autoreactive antibodies *via* induction of molecular mimicry and cross-reaction with self-antigens (43). In our study only three patients showed signs of SIgAD (meaning an IgA level <0.07 g/l), but it is reasonable to think that lower normal levels of IgA, although not as low as in SIgAD might have a similar, less strong effect. Another explanation for the association of lower IgA levels with autoreactivity may be the presence of IgG- or IgE-anti-IgA-antibodies (44). Since patients with reduced IgA levels are more likely to develop autoreactive antibodies, this could result in the maturation of autoantibodies that are directed against IgE leading to decreased serum IgE levels.

We also found that high IgE-anti-TPO levels, a common autoantibody in type I autoimmunity in CSU, are connected principally to lower total IgE levels. This stands in contrast to earlier findings suggesting that type I autoimmunity in CSU is connected to high levels of IgE (45, 46). Our results, instead, indicate that lower IgE levels may be linked to features of both, type I autoimmunity, as well as type IIb autoimmunity. Of note, we did not assess other specific IgE autoantibodies besides IgE-anti-TPO. As the levels of specific IgE autoantibodies may be more relevant for the diagnosis of type I autoimmunity as compared to the levels of total IgE, future studies should screen for different IgE autoantibodies in order to distinguish between types of autoimmunity in CSU.

A limitation of our study is the classification into lower *vs.* normal and elevated IgA levels based on a median IgA level of

1.84 g/l in our cohort. Whether the distribution of IgA values differs in other populations, needs to be studied. Nevertheless, our results emphasize that not only very low IgA levels are of importance in CSU patients, but also that levels on the lower range of the normal value should be evaluated.

Further limitations in the design of our study are the gender and age differences between the four groups (**Table 1**). The fact that women's IgA and IgE levels are lower has been found previously (47–49). Therefore, it has to be considered whether sex is a confounding factor in our study. Especially when investigating the prevalence of autoimmune phenomena this could be of relevance, since these are known to be increased in women. But when only looking at the male patients (n=145) in our study population, autoimmune phenomena, although not statistically significant, were also associated with lower levels of IgE (47.4% in $IgA^{lower}IgE^{lower}$ and 45.0% in $IgA^{n/hi}IgE^{lower}$ patients compared to 31.3% in $IgA^{lower}IgE^{n/hi}$ and 27.1% in $IgA^{n/hi}IgE^{n/hi}$ patients).

In addition, $IgA^{lower}IgE^{n/hi}$ were younger at the time of diagnosis compared to all other groups. IgA levels usually rise with the age (47, 50), which could explain why younger patients had lower IgA levels. However, this effect is not seen when looking at patients who had lower IgE levels on top ($IgA^{lower}IgE^{lower}$, md=40 years). This may be attributed to IgE levels, that often drop with higher age, possibly due to the decrease of sensitization (51). Further work on the effect of age on the combined role of IgA and IgE levels is needed.

To conclude, our results show that reduced immunoglobulin levels are common in CSU and associated with autoimmunity. Our findings implicate that especially the combination of lower serum IgA and IgE levels is of relevance in the diagnostic workup of CSU patients, as it is connected to higher rates of type IIb autoimmunity, according to BAT results, as well as more angioedema and lower basophil counts. Lower IgE, independent of IgA values, is connected to lower eosinophil counts, higher levels of IgE-anti-TPO and autoimmune phenomena, whereas lower IgA is connected to lower IgG levels and decreased quality of life. The pathomechanisms underlying the decrease in immunoglobulin levels and their interaction in CSU (e.g. impaired class switch) are still not well understood and further work on the role of both immunoglobulins as diagnostic biomarkers is required. The assessment of serum IgA and IgE levels is cost-effective and easy-to-perform. It may be useful in diagnosing subgroups of CSU (e.g. type IIb autoimmunity). Also, it may help to identify appropriate and optimal therapies for each patient based on a precision medicine approach, such as anti-IgE-, anti-interleukin-5-targeted therapies or immunosuppressives, if CSU first-line-treatment fails.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethikkomission, Ethikausschuss am Campus Charité - Mitte, Charité - Universitätsmedizin Berlin. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MS and KK substantially contributed to the conception and design of the study, performed analyses and interpretation of the data and drafted the manuscript. PK contributed to the statistical analyses and interpretation of the data. MS and Y-KX performed the cellular assays. JS and SF substantially contributed to the development and interpretation of laboratory tests. MMa and MMe contributed to interpretation of the data. JS, SF, PK, Y-KX, FS, SA, MMa, and MMe provided critical input to the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.657211/full#supplementary-material

Supplementary Figure 1 | Gating strategy of the basophil activation test by flow cytometry. Relationships between the plots are indicated by arrows. Representative plots of basophils from a positive and negative control as well as after treatment with an exemplary CSU serum are shown.

REFERENCES

1. Fricke J, Ávila G, Keller T, Weller K, Lau S, Maurer M, et al. Prevalence of Chronic Urticaria in Children and Adults Across the Globe: Systematic Review With Meta-Analysis. *Allergy* (2020) 75(2):423–32. doi: 10.1111/all.14037
2. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA(2)LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73 (7):1393–414. doi: 10.1111/all.13414
3. Church MK, Kolkhir P, Metz M, Maurer M. The Role and Relevance of Mast Cells in Urticaria. *Immunol Rev* (2018) 282(1):232–47. doi: 10.1111/imr.12632
4. Kolkhir P, Church MK, Weller K, Metz M, Schmetzer O, Maurer M. Autoimmune Chronic Spontaneous Urticaria: What We Know and What We do Not Know. *J Allergy Clin Immunol* (2017) 139(6):1772–81. doi: 10.1016/j.jaci.2016.08.050
5. Altrichter S, Peter HJ, Pisarevskaja D, Metz M, Martus P, Maurer M. Ige Mediated Autoallergy Against Thyroid Peroxidase–a Novel Pathomechanism of Chronic Spontaneous Urticaria? *PLoS One* (2011) 6(4):e14794. doi: 10.1371/journal.pone.0014794
6. Schmetzer O, Lakin E, Topal FA, Preusse P, Freier D, Church MK, et al. Il-24 is a Common and Specific Autoantigen of IgE in Patients With Chronic Spontaneous Urticaria. *J Allergy Clin Immunol* (2018) 142(3):876–82. doi: 10.1016/j.jaci.2017.10.035
7. Hatada Y, Kashiwakura J, Hayama K, Fujisawa D, Sasaki-Sakamoto T, Terui T, et al. Significantly High Levels of anti-dsDNA Immunoglobulin E in Sera and the Ability of dsDNA to Induce the Degranulation of Basophils From Chronic Urticaria Patients. *Int Arch Allergy Immunol* (2013) 161 Suppl 2:154–8. doi: 10.1159/000350388
8. Grattan CE, Francis DM, Hide M, Greaves MW. Detection of Circulating Histamine Releasing Autoantibodies With Functional Properties of anti-IgE in Chronic Urticaria. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (1991) 21 (6):695–704. doi: 10.1111/j.1365-2222.1991.tb03198.x
9. Fiebiger E, Maurer D, Holub H, Reininger B, Hartmann G, Woisetschlager M, et al. Serum IgG Autoantibodies Directed Against the Alpha Chain of Fc Epsilon RI: A Selective Marker and Pathogenetic Factor for a Distinct Subset of Chronic Urticaria Patients? *J Clin Invest* (1995) 96(6):2606–12. doi: 10.1172/JCI118325
10. Konstantinou GN, Asero R, Ferrer M, Knol EF, Maurer M, Raap U, et al. EAACI Taskforce Position Paper: Evidence for Autoimmune Urticaria and Proposal for Defining Diagnostic Criteria. *Allergy* (2013) 68(1):27–36. doi: 10.1111/all.12056
11. Schoepke N, Asero R, Ellrich A, Ferrer M, Gimenez-Arnau A, EHG C, et al. Biomarkers and Clinical Characteristics of Autoimmune Chronic Spontaneous Urticaria: Results of the PURIST Study. *Allergy* (2019) 74 (12):2427–36. doi: 10.1111/all.13949
12. Deza G, Bertolin-Colilla M, Pujol RM, Curto-Barredo L, Soto D, Garcia M, et al. Basophil FcepsilonRI Expression in Chronic Spontaneous Urticaria: A Potential Immunological Predictor of Response to Omalizumab Therapy. *Acta Dermato-Venereol* (2017) 97(6):698–704. doi: 10.2340/00015555-2654
13. Gericke J, Metz M, Ohanyan T, Weller K, Altrichter S, Skov PS, et al. Serum Autoreactivity Predicts Time to Response to Omalizumab Therapy in Chronic Spontaneous Urticaria. *J Allergy Clin Immunol* (2017) 139(3):1059–61. doi: 10.1016/j.jaci.2016.07.047
14. Weller K, Ohanyan T, Hawro T, Ellrich A, Sussman G, Koplowitz J, et al. Total IgE Levels are Linked to the Response of Chronic Spontaneous Urticaria Patients to Omalizumab. *Allergy* (2018) 73(12):2406–8. doi: 10.1111/all.13586
15. Asero R. Chronic Spontaneous Urticaria Treated With Omalizumab: What Differentiates Early From Late Responders? *Eur Ann Allergy Clin Immunol* (2021) 53(1):47–8. doi: 10.23822/EurAnnACI.1764-1489.147
16. Conley ME, Notarangelo LD, Etzioni A. Diagnostic Criteria for Primary Immunodeficiencies. Representing Pagid (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). *Clin Immunol (Orlando Fla)* (1999) 93(3):190–7. doi: 10.1006/clim.1999.4799
17. Ludvigsson JF, Neovius M, Hammarstrom L. Association Between IgA Deficiency & Other Autoimmune Conditions: A Population-Based Matched Cohort Study. *J Clin Immunol* (2014) 34(4):444–51. doi: 10.1007/s10875-014-0009-4
18. Kolkhir P, Borzova E, Grattan C, Asero R, Pogorelov D, Maurer M. Autoimmune Comorbidity in Chronic Spontaneous Urticaria: A Systematic Review. *Autoimmun Rev* (2017) 16(12):1196–208. doi: 10.1016/j.autrev.2017.10.003
19. Magen E, Masalha A, Waitman DA, Kahan N, Viner I, Klassov L, et al. Prevalence of Dermatologic Diseases Among Patients With Selective Immunoglobulin A Deficiency. *Allergy Asthma Proc* (2017) 38(1):70–7. doi: 10.2500/aap.2017.38.4018
20. Frossi B, De Carli S, Bossi F, Pucillo C, De Carli M. Co-Occurrence of Chronic Spontaneous Urticaria With Immunoglobulin A Deficiency and Autoimmune Diseases. *Int Arch Allergy Immunol* (2016) 169(2):130–4. doi: 10.1159/000445058
21. Maurer M, Metz M, Bindslev-Jensen C, Bousquet J, Canonica GW, Church MK, et al. Definition, Aims, and Implementation of GA(2) Len Urticaria Centers of Reference and Excellence. *Allergy* (2016) 71(8):1210–8. doi: 10.1111/all.12901
22. Montjoye L, Darrigade AS, Giménez-Arnau A, Herman A, Dumoutier L, Baeck M. Correlations Between Disease Activity, Autoimmunity and Biological Parameters in Patients With Chronic Spontaneous Urticaria. *Eur Ann Allergy Clin Immunol* (2021) 53(2):55–66. doi: 10.23822/EurAnnACI.1764-1489.132
23. Lennox RD, Leahy MJ. Validation of the Dermatology Life Quality Index as an Outcome Measure for Urticaria-Related Quality of Life. *Ann Allergy Asthma Immunol* (2004) 93(2):142–6. doi: 10.1016/S1081-1206(10)61466-4
24. Lawrence MG, Palacios-Kibler TV, Workman LJ, Schuyler AJ, Steinke JW, Payne SC, et al. Low Serum Ige Is a Sensitive and Specific Marker for Common Variable Immunodeficiency (Cvid). *J Clin Immunol* (2018) 38(3):225–33. doi: 10.1007/s10875-018-0476-0
25. Aghamohammadi A, Cheraghi T, Gharagozlou M, Movahedi M, Rezaei N, Yeganeh M, et al. Iga Deficiency: Correlation Between Clinical and Immunological Phenotypes. *J Clin Immunol* (2009) 29(1):130–6. doi: 10.1007/s10875-008-9229-9
26. Pasquier B, Launay P, Kanamaru Y, Moura IC, Pfirsch S, Ruffié C, et al. Identification of FcalphaRI as an Inhibitory Receptor That Controls Inflammation: Dual Role of FcRgamma Itam. *Immunity* (2005) 22(1):31–42. doi: 10.1016/S1074-7613(04)00377-2
27. Ben Mkaddem S, Rossato E, Heming N, Monteiro RC. Anti-Inflammatory Role of the IgA Fc Receptor (CD89): From Autoimmunity to Therapeutic Perspectives. *Autoimmun Rev* (2013) 12(6):666–9. doi: 10.1016/j.autrev.2012.10.011
28. van Gool MMJ, van Egmond M. Iga and Fcαri: Versatile Players in Homeostasis, Infection, and Autoimmunity. *Immunotarg Ther* (2020) 9:351–72. doi: 10.2147/ITT.S266242
29. Nettis E, Dambra P, D'Oronzio L, Cavallo E, Loria MP, Fanelli M, et al. Reactivity to Autologous Serum Skin Test and Clinical Features in Chronic Idiopathic Urticaria. *Clin Exp Dermatol* (2002) 27(1):29–31. doi: 10.1046/j.0307-6938.2001.00962.x
30. Bond WRJr., Herrod HG, Duberstein LE. Hereditary Angioedema: Association With IgA Deficiency and Otolaryngologic Disorders. *Laryngoscope* (1981) 91(3):416–21. doi: 10.1288/00005537-198103000-00012
31. Grattan CE, Walpole D, Francis DM, Niimi N, Dootson G, Edler S, et al. Flow Cytometric Analysis of Basophil Numbers in Chronic Urticaria: Basopenia is Related to Serum Histamine Releasing Activity. *Clin Exp Allergy* (1997) 27 (12):1417–24. doi: 10.1111/j.1365-2222.1997.tb02986.x
32. Grattan CE, Dawn G, Gibbs S, Francis DM. Blood Basophil Numbers in Chronic Ordinary Urticaria and Healthy Controls: Diurnal Variation, Influence of Loratadine and Prednisolone and Relationship to Disease Activity. *Clin Exp Allergy* (2003) 33(3):337–41. doi: 10.1046/j.1365-2222.2003.01589.x
33. Kolkhir P, Church MK, Altrichter S, Skov PS, Hawro T, Frischbutter S, et al. Eosinopenia, in Chronic Spontaneous Urticaria, is Associated With High Disease Activity, Autoimmunity, and Poor Response to Treatment. *J Allergy Clin Immunol Pract* (2020) 8(1):318–25. doi: 10.1016/j.jaip.2019.08.025
34. Magerl M, Terhorst D, Metz M, Altrichter S, Zuberbier T, Maurer M, et al. Benefit of Mepolizumab Treatment in a Patient With Chronic Spontaneous Urticaria. *J Dtsch Dermatol Ges* (2018) 16(4):477–8. doi: 10.1111/ddg.13481
35. Maurer M, Altrichter S, Metz M, Zuberbier T, Church MK, Bergmann KC. Benefit From Reslizumab Treatment in a Patient With Chronic Spontaneous

Urticaria and Cold Urticaria. *J Eur Acad Dermatol Venereol* (2018) 32(3): e112–3. doi: 10.1111/jdv.14594
36. Gleich GJ, Klion AD, Lee JJ, Weller PF. The Consequences of Not Having Eosinophils. *Allergy* (2013) 68(7):829–35. doi: 10.1111/all.12169
37. Altrichter S, Frischbutter S, Fok JS, Kolkhir P, Jiao Q, Skov PS, et al. The Role of Eosinophils in Chronic Spontaneous Urticaria. *J Allergy Clin Immunol* (2020) 145(6):1510–6. doi: 10.1016/j.jaci.2020.03.005
38. De Swerdt A, Van Den Keybus C, Kasran A, Cadot P, Neyens K, Coorevits L, et al. Detection of Basophil-Activating IgG Autoantibodies in Chronic Idiopathic Urticaria by Induction of CD 63. *J Allergy Clin Immunol* (2005) 116(3):662–7. doi: 10.1016/j.jaci.2005.04.042
39. Cho CB, Stutes SA, Altrich ML, Ardoin SP, Phillips G, Ogbogu PU. Autoantibodies in Chronic Idiopathic Urticaria and Nonurticarial Systemic Autoimmune Disorders. *Ann Allergy Asthma Immunol* (2013) 110(1):29–33. doi: 10.1016/j.anai.2012.10.020
40. Abd El-Azim M, Abd El-Azim S. Chronic Autoimmune Urticaria: Frequency and Association With Immunological Markers. *J Investigational Allergol Clin Immunol* (2011) 21(7):546–50.
41. Altrichter S, Zampeli V, Ellrich A, Zhang K, Church MK, Maurer M. Igm and IgA in Addition to IgG Autoantibodies Against Fcεriα are Frequent and Associated With Disease Markers of Chronic Spontaneous Urticaria. *Allergy* (2020) 75(12):3208–15. doi: 10.1111/all.14412
42. Singh K, Chang C, Gershwin ME. Iga Deficiency and Autoimmunity. *Autoimmun Rev* (2014) 13(2):163–77. doi: 10.1016/j.autrev.2013.10.005
43. Abolhassani H, Gharib B, Shahinpour S, Masoom SN, Havaei A, Mirminachi B, et al. Autoimmunity in Patients With Selective IgA Deficiency. *J Investigational Allergol Clin Immunol* (2015) 25(2):112–9.
44. Freeman GL. Syndromes Associated With Eosinopenia. *Allergy* (1998) 53 (3):331–3. doi: 10.1111/j.1398-9995.1998.tb03901.x
45. Shin YS, Suh DH, Yang EM, Ye YM, Park HS. Serum Specific Ige to Thyroid Peroxidase Activates Basophils in Aspirin Intolerant Urticaria. *J Korean Med Sci* (2015) 30(6):705–9. doi: 10.3346/jkms.2015.30.6.705
46. Sánchez J, Sánchez A, Cardona R. Causal Relationship Between Anti-TPO Ige and Chronic Urticaria by In Vitro and In Vivo Tests. *Allergy Asthma Immunol Res* (2019) 11(1):29–42. doi: 10.4168/aair.2019.11.1.29
47. Gonzalez-Quintela A, Alende R, Gude F, Campos J, Rey J, Meijide LM, et al. Serum Levels of Immunoglobulins (Igg, IgA, IgM) in a General Adult Population and Their Relationship With Alcohol Consumption, Smoking and Common Metabolic Abnormalities. *Clin Exp Immunol* (2008) 151(1):42–50. doi: 10.1111/j.1365-2249.2007.03545.x
48. Jarvis D, Luczynska C, Chinn S, Burney P. The Association of Age, Gender and Smoking With Total IgE and Specific Ige. *Clin Exp Allergy* (1995) 25 (11):1083–91. doi: 10.1111/j.1365-2222.1995.tb03255.x
49. Omenaas E, Bakke P, Elsayed S, Hanoa R, Gulsvik A. Total and Specific Serum IgE Levels in Adults: Relationship to Sex, Age and Environmental Factors. *Clin Exp Allergy* (1994) 24(6):530–9. doi: 10.1111/j.1365-2222.1994.tb00950.x
50. Weber-Mzell D, Kotanko P, Hauer AC, Goriup U, Haas J, Lanner N, et al. Gender, Age and Seasonal Effects on IgA Deficiency: A Study of 7293 Caucasians. *Eur J Clin Invest* (2004) 34(3):224–8. doi: 10.1111/j.1365-2362.2004.01311.x
51. Amaral AFS, Newson RB, Abramson MJ, Antó JM, Bono R, Corsico AG, et al. Changes in IgE Sensitization and Total IgE Levels Over 20 Years of Follow-Up. *J Allergy Clin Immunol* (2016) 137(6):1788–95. doi: 10.1016/j.jaci.2015.09.037

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Cryoglobulins, Cryofibrinogens, and Cold Agglutinins in Cold Urticaria: Literature Review, Retrospective Patient Analysis, and Observational Study in 49 Patients

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Introduction: Cryoproteins, such as cryoglobulins, cryofibrinogens and cold agglutinins, precipitate at low temperatures or agglutinate erythrocytes and dissolve again when warmed. Their pathogenetic and diagnostic importance in cold urticaria (ColdU) is unclear. In this study, we aimed to characterize the prevalence of cryoproteins in patients with ColdU.

Methods: We conducted 3 analyses: i) a systematic review and meta-analysis of published data using an adapted version of the Joanna Briggs Institute's critical appraisal tool for case series, ii) a retrospective analysis of 293 ColdU patients treated at our Urticaria Center of Reference and Excellence (UCARE) from 2014 to 2019, and iii) a prospective observational study, from July 2019 to July 2020, with 49 ColdU patients as defined by the EAACI/GA2LEN/EDF/UNEV consensus recommendations.

Results: Our systematic review identified 14 relevant studies with a total of 1151 ColdU patients. The meta-analyses showed that 3.0% (19/628), 1.1% (4/357) and 0.7% (2/283) of patients had elevated levels of cryoglobulins, cryofibrinogens and cold agglutinins, respectively. Our retrospective analyses showed that cryoproteins were assessed in 4.1% (12/293) of ColdU patients. None of 9 ColdU patients had cryoglobulins, and one of 5 had cold agglutinins. In our prospective study, none of our patients had detectable cryoglobulins (0/48) or cryofibrinogens (0/48), but 4.3% (2/46) of patients had cold agglutinins (without any known underlying autoimmune or hematological disorder).

Conclusion: Our investigation suggests that only very few ColdU patients exhibit cryoproteins and that the pathogenesis of ColdU is driven by other mechanisms, which remain to be identified and characterized in detail.

Keywords: cold urticaria, cryoglobulins, cryofibrinogens, cold agglutinins, cryoproteins

INTRODUCTION

Cold urticaria (ColdU) is a subtype of chronic inducible urticaria, where wheals, angioedema or both are evoked by exposure to low temperatures (1). A reliable medical history and the results of cold stimulation tests (CSTs) such as the ice cube test or the Temp*Test*® establish the diagnosis (2).

Two different classification approaches can be found in the published literature. The classification of ColdU based on CSTs differentiates between typical ColdU, with wheals in the CSTs, and atypical ColdU, without wheals or atypical wheals in the CSTs (3). In the other approach, the etiology-based classification, a hereditary and an acquired form are distinguished, the latter being further subdivided into primary and secondary. Secondary acquired ColdU can be caused by hematologic diseases, infections, malignancies, cryoproteins (CPs) or other diseases (4). The pathophysiology of ColdU is not well understood, and autoallergic and autoimmune mechanisms, temperature-sensitive receptors, neurogenic signals, and CPs have been speculated to play a role (5, 6).

The term cryoprotein or cold protein usually includes cryoglobulins, cryofibrinogens, cold agglutinins, and sometimes other proteins. Cryoglobulins (CGs) are immunoglobulins that precipitate *in vitro* at low temperatures and dissolve when warmed (7). CGs can be divided into monoclonal (Type I) and mixed (Type II and III) immunoglobulins (8) and can cause various cold-related diseases with joint, nerve, and kidney involvement, weakness, palpable purpura, Raynaud's phenomenon, skin rashes and ulcers (9). Various infections, especially hepatitis C, immune diseases and hematological disorders have been described as possible triggers for the occurrence of CGs (9, 10). Cryofibrinogens (CFs), which only precipitate in plasma, can occur independently or in association with CGs. Malignancies, infections and autoimmune diseases are known to induce the occurrence of CFs (11, 12). CFs can cause skin manifestations such as Raynaud's phenomenon and purpura as well as arterial and venous thromboses (11). Cryoglobulinemia and cryofibrinogenemia, i.e. the presence of the respective CP, are distinct from cryoglobulinemic or cryofibrinogenic diseases or syndromes, i.e. resulting disease entities (11, 13). Healthy individuals may have CGs (14, 15) or CFs (11).

Cold agglutinins (CAs), also known as cold(auto)antibodies, are mostly type M immunoglobulins that agglutinate erythrocytes at low temperatures and may lead to complement activation (16).

As of now, the role and relevance of CPs in the pathogenesis of ColdU remain ill-defined and many questions need to be answered. There are several published reports on the prevalence of CPs in patients with ColdU, including many case reports and small case series. These have not been systematically analyzed at the present time, and the rates of CP-positive ColdU patients, thus, are not known. Also, it is largely unknown how often ColdU patients are assessed for CPs and what drives the decision to do so.

Abbreviations: CA(s), Cold agglutinin(s); CF(s), Cryofibrinogens; CG(s), Cryoglobulin(s); ColdU, Cold Urticaria; CP(s), Cryoprotein(s); CST(s), Cold stimulation test(s); UCARE, Urticaria Center of Reference and Excellence.

To answer these questions and address these unmet needs, we used a three-tiered approach. First, we performed a systematic review and meta-analysis of published reports on the rates of ColdU patients who tested positive for CGs, CFs, and CAs. Second, we retrospectively assessed how often close to 300 patients with ColdU treated at our Urticaria Center of Reference and Excellence (UCARE) (17) were assessed for these CPs and why. Finally, we prospectively measured CPs in almost 50 consecutive patients with ColdU. The overall aim of our report is to provide more clarity on the prevalence, role and relevance of CPs in ColdU.

MATERIALS AND METHODS

Systematic Review and Meta-Analysis of Published Reports

On June 30, 2020, we conducted a systematic PubMed literature search with the terms ("cold urticaria") AND ("cryoglobulin*" OR "cryofibrinogen*" OR "cold agglutinin*") in accordance with the PRISMA guidelines (18) and included all published studies between 1980 and 2019 in German, English, Spanish and French. Additionally, we manually checked references in key publications on ColdU (3, 19–21), since CPs are often only reported as part of the patient description in the full text.

To be eligible, the studies had to have measured CPs in patients with ColdU diagnosed according to established criteria such as a typical patient history or positive CSTs. Accordingly, the occurrence of CPs was not allowed to be a study exclusion criterion. We excluded studies that exclusively dealt with secondary or hereditary ColdU forms and case series with fewer than 5 patients in order to avoid bias through selective reporting and publishing.

The study selection was carried out independently by two researchers based on predefined criteria. After excluding all non-relevant studies based on title and abstract screening, we procured the full texts and contacted the authors for additional information, as necessary and possible. Patient characteristics and results of CP testing were extracted independently by both researchers using a survey table. Information on inclusion criteria for the respective studies, reasons for incomplete laboratory results and reported secondary diagnoses were also collected to identify selective reporting. Where applicable, the accordance of the term "cryoglobulinemia" in the respective study with the definition used in this review was ensured. The study quality and the bias risk were then independently assessed by two researchers using a specifically adapted version of the Joanna Briggs Institute's critical appraisal tool for case series (22) (see **Table 1**). Finally, the average percentage of CP-positive patients, i.e. patients who tested positive for CGs, CFs, or CAs, was calculated, weighted according to the respective study size.

Retrospective Analyses of ColdU Patients

We retrospectively searched the database of all chronic urticaria patients diagnosed with ColdU who presented at the UCARE at Charité-Universitätmedizin Berlin, Germany, between 2014 and 2019 for information on CPs. Collected data included patient

TABLE 1 | Quality assessment of the studies identified through the systematic literature review.

	1 Clear Inclusion criteria	2 Clear measurement of diagnosis	3 Clear demographics	4 If applicable: Clear reasons for not testing	5 Type of cold protein described	6 Report of secondary causes	Points (max 6)
Neittaanmäki 1985 (23)	Yes	Yes	2/3	No	Yes	Yes	4.6
Wanderer et al., 1986 (4)	Yes	Yes	2/3	All tested	Yes	Yes	5.6
Doeglas et al., 1986 (24)	Yes	Yes	2/3	All tested	Yes	No	4.6
Henquet et al., 1992 (25)	Yes	Yes	2/3	No	Yes	No	3.6
Husz et al., 1994 (26)	Yes	Yes	2/3	All tested	Yes	N/A	5.6
Koeppel et al., 1996 (27)	Yes	Yes	2/3	No	Yes	cumulative	4.6
Möller et al., 1996 (28)	Yes	Yes	2/3	No	No	No	2.6
Santaolalla Montoya et al., 2002 (29)	No	Yes	2/3	Yes ("bad conservation")	Yes	N/A	4.6
Tosoni et al., 2003 (30)	No	Yes	2/3	All tested	Yes	Yes	4.6
Alangari et al., 2004 (31)	Yes	Yes	3/3	No	Yes	cumulative	5
Katsarou-Katsari et al., 2008 (32)	Yes	Yes	3/3	No	Yes	Yes	6
Stepaniuk et al., 2018 (33)	Yes	Yes	1/3	Not for all patients	Yes	N/A	4.3
Yee et al., 2019 (34)	Yes	Yes	2/3	No	Yes	No	3.6
Kulthanan et al., 2019 (35)	Yes	Yes	3/3	Yes ("retrospective")	Yes	N/A	6

1. Was there clear criteria for inclusion?

2. Was the condition (ColdU) measured in a standard, reliable way for all participants included?

3. Was there clear reporting of the demographics of the participants in the study? (age, age of disease onset and ColdU duration) (1/3 point per item)

4. Did the study have all patients tested? If not: Did the study provide information about the reasons?

5. Was the outcome parameter (cold protein) clearly defined?

6. Were secondary causes of positive cryoproteins clearly reported? [N/A (not applicable) and cumulative reporting count as 1 point].

age, laboratory workup, type of diagnosis, onset of ColdU and other diagnoses. Patient data were analyzed and reported anonymously in accordance with data protection regulations, and stored in a MS Excel Version 2019 based database.

Prospective Assessment of ColdU Patients

Between July 2019 and July 2020, we assessed 49 patients out of 60 consecutive patients with ColdU treated at our UCARE. Of the patients originally seen during the study period (N = 60), 4 patients were excluded because the diagnosis of ColdU was not confirmed or was questionable; one patient was excluded because he no longer had active disease. In 6 patients, a sufficient amount of blood could not be obtained due to organizational reasons, patient-related reasons or laboratory difficulties. There was no control group. Our study was approved by the local ethics committee (reference EA1/069/19), and all patients provided informed consent. Inclusion criteria were 1) diagnosed ColdU as defined by the 2016 EAACI/GA^{2}LEN/EDF/UNEV consensus recommendations as the "recurrence of itchy wheals and/or angioedema […] reproducible in response to […] cold exposure" (1), 2) disease duration of 6 weeks or longer, 3) no intake of H1 antihistamines within 3 days and/or glucocorticosteroids within 7 days prior to CSTs and blood sampling. ColdU was diagnosed in all of our patients based on their typical history and CSTs. For CSTs, the Temp*Test* 4.0® and the ice cube test with 5 minutes of cold application and reading after 10 minutes were used (36). ColdU was classified as "typical" in patients who developed a wheal at the site of the CST within 10 minutes after cold exposure and as "atypical" in patients who did not (3).

We obtained and analyzed patient demographics and the course and severity of the disease including systemic reactions to cold exposure. We also assessed patients for family history for ColdU, cold-associated complaints like wheals, pruritus or angioedema and comorbidities such as atopic diseases, infections, malignancies, connective tissue disorders, thyroid diseases. Data were collected and pseudonymously entered in an MS Excel Version 2019 based database.

Laboratory Workup

CP analyses were conducted at the central laboratory of the Charité (Labor Berlin, CGs and CFs) and the Institute of Transfusion Medicine (CAs). All peripheral-venous blood samples were collected in a standardized way with prewarmed tubes and transported directly to the laboratory, ensuring a constant transport temperature of 37°C. Since temperature deviations are a confounding factor for the analysis, a rapid and standardized transport procedure for all samples was implemented.

CAs were determined in 6ml EDTA and 10ml native venous blood, and plasma and sediment were separated at 37°C. Subsequently, samples were analyzed for CAs of blood group system I/i using foreign adult and umbilical vein erythrocytes. Reaction strength at room temperature (20°C) was evaluated by one to two observers depending on agglutination strength in categories ranging from negative (no agglutination), within physiological range (mild to moderate agglutination), borderline (more severe agglutination) to pathological/positive (massive and lumpy agglutination). At the same time, patient erythrocytes were examined with the direct Coombs test, in which in most cases only complement adhesion is expected, since any CAs detach from the erythrocytes again upon recirculation.

CFs were determined using immunoprecipitation in 2 ml EDTA, and CGs were analyzed from 2 ml serum. After centrifugation, the clear plasma or serum was examined for the formation of precipitates for 72 hours at 4°C. If precipitates or turbidity were

evident, the supernatant was warmed to 37°C and, if dissolved, stored a second time at 4°C. Only in case of repeated dissolution and formation of precipitates, the sample was considered positive for CGs or CFs. Cryoprecipitates in the serum were considered as positive CGs and cryoprecipitates in the EDTA as the sum of positive CGs and positive CFs, so that both tests were always performed simultaneously for differentiation. No further differentiation or quantification of the samples was performed.

Statistical Methods

The quantitative variables reported in this study were summarized using median, range (Min, Max) and interquartile range (IQR) using R Version 3.6.3 (37).

RESULTS

Literature Review: The Studies to Date Show a Wide Scatter of Results, but on Average the Frequency of Positive CPs in ColdU Patients was Low

Our systemic review identified 71 publications of potential relevance (**Figure 1**), of which 68 were excluded after reviewing the title, abstract or full-text. In addition, we found 11 relevant publications by checking the references of key publications, resulting in a total of 14 studies from the years 1985 to 2019 that were evaluated in this review (**Table 2**). Most reports (11 of 14) had 4 or more points out of possible 6 on the quality score and were therefore considered to be of medium or high quality (**Table 1**).

CPs had been determined in all 14 studies in patient cohorts ranging from 9 to 208 ColdU patients. In 0 to 28.6% (average summarized for all studies 3.0%) of the ColdU patients, positive CGs had been detected. This was reported to be associated with secondary disease in two of these patients, one of whom had lymphosarcoma (23) and one of whom had chronic lymphocytic leukemia (4). CFs had been determined in 4 studies in patient cohorts ranging from 5 to 208 ColdU patients. In 0 to 20% (summarized for all studies 0.7%) of the ColdU patients, positive CFs had been detected. CAs had been analyzed in 9 studies ranging from 7 to 208 patients. 0 to 21.4% (summarized for all studies: 1.1%) positive CAs were reported.

Retrospective Study: In Routine Clinical Practice, Very Few ColdU Patients Were Tested for CPs, and the Rate of Positive Tests Was Low

Between 2014 and 2019, 293 patients with various forms of ColdU were seen at our UCARE. In 12 of these patients, CPs were determined. CGs were determined in 9 patients, CA were determined in 5 patients, both CG and CA were analyzed in 2 patients. CFs were not determined in any of the patients. The main reasons for CP determination were the high disease severity (in 9 of 12 patients) and suspicion of autoimmunity or hematological disease (in 3 of 12 patients). We found no anomalies in the 9 patients tested for CGs, but one of the 5 ColdU patients tested for CAs showed a positive result. However, this patient is known to have Raynaud's syndrome, which may be associated with the presence of CAs.

Prospective Study: Rates of CP- Positive ColdU Patients Were Very Low

Of the 49 analyzed ColdU patients, 73.5% (N = 36) were female and 26.5% (N = 13) were male, the median age was 40 years (IQR: 40 – 53 years). 69.5% (N = 34) were diagnosed with typical ColdU. Patient characteristics are described in **Table 3** and anonymized data of these patients is described in the supplementary material.

None of 48 patients tested positive for CGs or CFs, and 2 of 46 patients (4.3%) tested positive for CAs. The two patients with positive CAs were female and aged 30 and 16 years, respectively. Both reported having experienced pruritus, wheals and angioedema within the 12 months prior to study inclusion. One woman had been diagnosed with classic acquired (ice cube test negative,

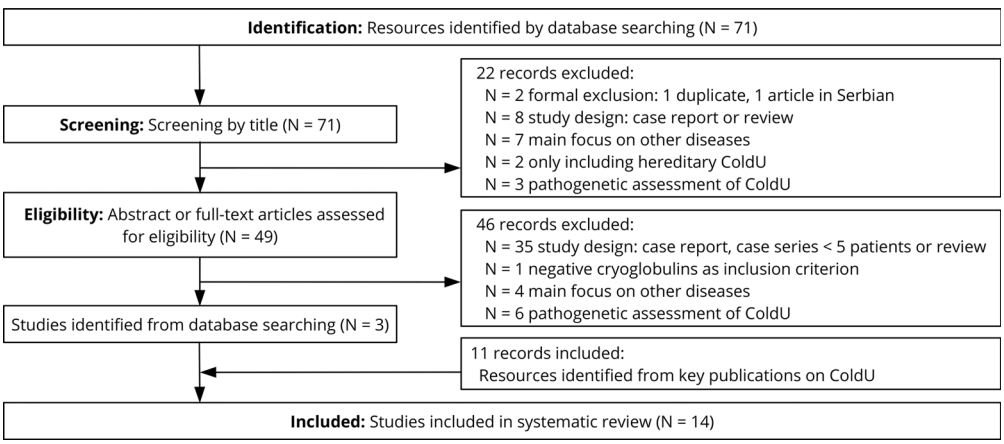

FIGURE 1 | Study selection process during the systematic literature review.

TABLE 2 | Overview of the existing literature on ColdU and the rate of positive cryoproteins.

Author, Year, Country[1], Reference	ColdU forms	N in study (M/F)	N tested: CG / CA / CF	Age[2]	Age of disease onset[2]	ColdU duration[2]	CG: %	CA: %	CF: %
Neittaanmäki, 1985, FIN (25)	All forms	220(81/139)	208 / 208 / 208	N/A	M 25.1y (R 1-74y)	M 6.3y (R 3w-37y)	2: 1.0%	0 : 0%	0: 0%
Wanderer, 1986, USA (4)	Primary, secondary, atypical	50(23/27)	50 / - / -	N/A	M 17.8y (R 3-63y)	M 4.8y (R 3m-22y)	2: 4.0%	N/T	N/T
Doeglas, 1986, NLD, (28)	No combined cold contact- and cholinergic-heat urticaria	39(14/25)	39 / 39 / -	Mdn 36y (R 10-71y)	N/A	Mdn 5y (R 4m–35y)	4: 10.3%	0: 0%	N/T
Henquet, 1992, NLD (52)	All + cold induced cholinergic urticaria	30(10/20)	18 / 14 / 5	N/A	M 26.2y (R 9-58y)	Mdn 2y (R 0-20y)	0: 0%	3 "slightly": 21.4%	1 "slightly": 20%
Husz, 1994, HUN (53)	Cold contact urticaria	42(14/28)	42 / 42 / -	12-63y	N/A	4m - 5y	0: 0%	0: 0%	N/T
Koeppel, 1996, FRA (34)	Superficial and deep cold urticaria	104(41/63)	72 / - / 56	N/A	M 33.5y (R 1-74y)	M 57m (R 5d-62y)	4: 5.6%	N/T	1: 1.8%
Möller, 1996, DEU (31)	All forms	56(25/31)	34 / -	M 41.0y ± SD 15.6 (R 5-72y)	N/A	M 7.9y ±SD 5.8	(1: 2.9%)*		N/T
Santaolalla, 2002, ESP (30)	Probably all forms, children's hospital	12(4/8)	9 / 7 / -	N/A	M 12.75y	M 3.5y (R 10m-8y)	0: 0%	0: 0%	N/T
Tosoni, 2003, ITA (29)	ColdU where hydroxizine and cetirizine therapy was not fitting, only positive CST	14(4/10)	14 / - / -	M 30.4y (R 11-50y)	N/A	M 48.9m (R 7-102m)	4: 28.6%	N/T	N/T
Alangari, 2004, USA (54)	Age onset <18	30 (13/17)	19 / 17 / -	Mdn 12.0y (R 2.0-19y)	Mdn 7.0y (R 0.5-14.5y)	Mdn 3.2y (R 0.5-13.5y)	0: 0%	1: 5.9%	N/T
Katsarou-Katsari, 2008, GRC (36)	No familiar ColdU	62(30/32)	50 / - / -	M 41.5y ± SD 15.6 (R 20-75y)	M 32.5 ±SD 16.5 (R 4-65y)	M 5.6y ±SD 3.5	2: 4%	N/T	N/T
Stepaniuk, 2018, CAN (55)	No combined urticaria	50(15/35)	16 / 15 / -	Mdn 28.5y (R 2-62y)	N/A	N/A	0: 0%	0: 0%	N/T
Yee, 2018, USA (35)	Acquired ColdU, age <19J	415(210/205)	71 / - / -	N/A	Mdn 8.0y (IQR: 4.6-12y)	N/A	1: 1.4%	N/T	N/T
Kulthanan, 2019, THA (56)	All forms, age >18J	27(6/21)	20 / 15 / 14	M 37.5y ±SD 15.0	M 34.8y ±SD 16.5	M 8.0y ± 5.6 (6 patients)	0: 0%	0: 0%	0: 0%
Summary[3]	**Different forms**	**1151 (490/661)**	**628 / 357 / 283**				**19: 3.0% (R 0-28.6%)**	**4: 1.1% (R 0-21.4%)**	**2: 0.7% (R 0-20%)**

M, Male; F, Female; N, number of patients; CST, Cold Stimulation Test; d, day(s); m, month(s); y, year(s); CG, cryoglobulins; CA, cold agglutinins; CF, cryofibrinogens; CP, cryoprotein(s); N/A, information not available; N/T, Not tested; SD, Standard Deviation; R, Range; IQR, Interquartile Range; M, Mean; Mdn, Median.

[1]country code according to ISO 3166.

[2]if median and mean were given, only median was mentioned in this table.

[3]including only studies with available data, calculation: sum of positive tests in all studies divided by the total number of patients tested in all studies.

**This study was not included in the summary of cryoproteins.*

Temp*Test* positive) and the other with atypical ColdU (IceCubeTest and Temp*Test* negative). The classic acquired ColdU patient also reported cold-induced anaphylaxis and breathing difficulties in response to cold exposure, suffered from hypothyroidism and has experienced a systemic reaction after a hymenoptera sting. The atypical ColdU patient reported no further diseases or systemic reactions. Information on the clinical differences between patients with physiological, elevated, and undetectable CAs is shown in **Table 3**. We could not detect major differences between patients with physiological and undetectable CAs.

DISCUSSION

In this study, we showed that the frequency of positive CPs in ColdU patients was low with all three methods used. Moreover,

TABLE 3 | Demographic characteristics of 49 ColdU patients included at Charité Berlin between July 2019 and July 2020.

		Overall (N = 49)	Negative CAs (N = 22)	Physiologic CAs (N = 22)	Positive CAs (N = 2)
Age, Median [Min, Max]		40.0 [14.0, 82.0]	44.5 [17.0, 67.0]	39.0 [14.0, 82.0]	23.0 [16.0, 30.0]
Gender	Female	36 (73.5%)	15 (68.2%)	16 (72.7%)	2 (100%)
	Male	13 (26.5%)	7 (31.8%)	6 (27.3%)	0 (0%)
Ethnicity	Caucasian	46 (93.9%)	22 (100%)	21 (95.5%)	2 (100%)
	Latino/Hispanic	1 (2.0%)	0 (0%)	1 (4.5%)	0 (0%)
	Middle East	2 (4.1%)	0 (0%)	0 (0%)	0 (0%)
Age of onset of the disease, Median [Min, Max]		31.0 [0, 81.0]	28.0 [0, 64.0]	34.5 [10.0, 81.0]	21.0 [12.0, 30.0]
Time since onset of symptoms in months, Median [Min,Max]		60.0 [3.00, 600]	95.0 [4.00, 600]	37.0 [3.00, 480]	26.0 [4.00, 48.0]
Cold-induced reactions ≤ 12 months					
Pruritus		49 (100%)	22 (100%)	22 (100%)	2 (100%)
Wheals		40 (81.6%)	18 (81.8%)	18 (81.8%)	2 (100%)
Angioedema		28 (57.1%)	12 (54.5%)	13 (59.1%)	2 (100%)
Cold stimulation tests	Ice Cube Test positive	31 (63.3%)	14 (63.6%)	15 (68.2%)	0 (0%)
	Temp*Test* positive	33 (67.3%)	15 (68.2%)	15 (68.2%)	1 (50.0%)
Cryoproteins					
Cryoglobulins	Negative	48 (98.0%)	–	–	–
	Not determined*	1 (2.0%)	–	–	–
Cold agglutinins	Positive	2 (4.1%)	–	–	–
	Negative	22 (44.9%)	–	–	–
	Within physiological range	22 (44.9%)	–	–	–
	Not determined*	3 (6.1%)	–	–	–
Cryofibrinogens	Negative	48 (98.0%)	–	–	–
	Not determined*	1 (2.0%)	–	–	–
Diagnosis					
Typical cold urticaria		34 (69.4%)	16 (72.7%)	16 (72.7%)	1 (50.0%)
Atypical cold urticaria		3 (6.1%)	2 (9.1%)	0 (0%)	1 (50.0%)
Probably atypical cold urticaria		10 (20.4%)	3 (13.6%)	6 (27.3%)	0 (0%)
Cholinergic cold urticaria		1 (2.0%)	1 (4.5%)	0 (0%)	0 (0%)
Cold-induced pruritus		1 (2.0%)	0 (0%)	0 (0%)	0 (0%)
Cold-induced anaphylaxis		23 (46.9%)	11 (50.0%)	10 (45.5%)	1 (50.0%)
First-degree relatives with ColdU		3 (6.1%)	1 (4.5%)	1 (4.5%)	0 (0%)
Past medical History					
Raynaud's syndrome		7 (14.3%)	3 (13.6%)	2 (9.1%)	0 (0%)
Lip cyanosis after cold exposure		7 (14.3%)	5 (22.7%)	0 (0%)	0 (0%)
Asthma bronchiale		6 (12.2%)	3 (13.6%)	3 (13.6%)	0 (0%)
Allergic rhinitis/conjunctivitis, atopic dermatitis		20 (40.8%)	10 (45.5%)	8 (36.4%)	0 (0%)
Current or previous malignancies		1 (2.0%)	0 (0%)	1 (4.5%)	0 (0%)
Thyroid disorders		12 (24.5%)	5 (22.7%)	5 (22.7%)	1 (50.0%)
Connective tissue disorders		2 (4.1%)	1 (4.5%)	1 (4.5%)	0 (0%)
Systemic reaction after hymenoptera sting		7 (14.3%)	3 (13.6%)	2 (9.1%)	1 (50.0%)

**Sample not suited for analysis.*

N, number of patients; CAs, Cold agglutinins.

ColdU patients were rarely assessed for CPs in routine clinical practice and testing positive was rarely linked to clinical features or consequences. Our results discourage routine clinical testing of ColdU patients for CPs and call for further characterization of the role and relevance of CPs in ColdU patients who test positive.

Comparison with Previous Literature

When compared to previous publications, the findings of our literature review are consistent with the review by Alain Claudy, with whose dataset we calculated a proportion of 2% positive CGs in ColdU patients (20). Contradictory results with a proportion of 20% positive CGs in ColdU patients were reported in the review by Houser et al. (38), which was cited in the teaching book by Czarnetzki (39). In order to summarize all publications until 1970, Houser et al. divided the number of publications with ColdU patients and cryoglobulinemia by the total number of publications with ColdU patients. Since the majority of the included studies are case reports, their result can be explained by the distortion due to a presumably high reporting and publication bias.

The frequency of positive CGs in the studies identified through our systematic literature review ranged between 0% and 28.6%, with strikingly high values in Doeglas (24), 10.3%, and Tonsoni (30), 28.6%. The high percentage in Doeglas et al. can be plausibly explained by the different study design, as it is the only study with a measurement of CPs in patients at 3-6 different points in time. The high proportion in Tosoni et al. of 4 patients with slightly positive CGs (28.6%) can only be partly attributed to the small number of cases (N = 14) and remains largely unexplained.

Limitations

When evaluating the literature on CPs and ColdU, limitations on several levels must be considered: On the one hand, distortion by

a reporting bias should be considered, meaning that purely negative laboratory tests and cases with negative CGs may not be reported or published. This effect is reflected in the fact that the proportion of positive CGs in the case reports is much higher and decreases with increasing sample size.

The heterogeneity of the studies included in this review is a further potential constraint when summarizing the literature (see **Table 1**). Two studies (29, 30) did not clearly report their inclusion criteria and one (28) did not differentiate between the different CPs (28). Moreover, 7 studies did not explain the reasons for their incomplete laboratory results. Different authors included and excluded different forms of ColdU, while others did not define criteria at all. None of the papers reported on the handling of blood samples or on the laboratory procedure used, which could lead to distortions due to different measurement standards (7) and reference values (40) in different countries, as a survey of 137 European laboratories showed (41). Moreover, most publications did not report on possible underlying diseases in CP-positive patients, so that we could not distinguish between primary and secondary cryoproteinemia.

The validity of the prospective and retrospective study might be limited by the specific patient selection at our UCARE: Patients who had already been seen by a specialist before study enrollment may have been given a different diagnosis or referred directly to other departments, such as hematology, as a result. Additionally, because this study was conducted at a tertiary hospital, patients with particularly severe ColdU may have been primarily enrolled in the study. In addition, due to the study design, we could not reconstruct the exact blood collection and transport logistics in the retrospective analysis.

Nonetheless, this study analyzed a substantial number of well-characterized ColdU patients with quality-controlled sampling and transport, and only 5 other studies known to us had included similar or higher numbers of patients (4, 23, 27, 32, 34).

Explanatory Approaches for the Low Frequency of CPs and the Large Scatter of Results of Previous Publications

Our results raise the question of why, on the one hand, such disparate results were reported in the literature and why, on the other hand, we observed such a low frequency of CPs.

First, according to a theory by Wanderer (42), many laboratory results on CP could be false negatives and the actual true frequency of positive CP in ColdU patients might be much higher. However, this theory is contradicted by the fact that all studies known to us involving a large number of patients reported a very low percentage of positive CP [such as (23, 27, 34)].

More likely, therefore, is the theory that there are two manifestations of ColdU: a CP-negative and a much rarer CP-positive form. This theory is supported by the numerous case reports of patients suffering from CG-positive ColdU and additionally other diseases such as infections (38, 43–50) or hematologic disorders/malignancies (5, 51–55). In these patients, the underlying disease could have caused cryoproteinemia and as a consequence ColdU. It is possible that the underlying disease may also be diagnosed later, as Polliack and Lugassy found out (56). Furthermore, it is conceivable that the symptoms and the clinical course of CP-positive and CP-negative ColdU differ, although to our knowledge there have been no publications on this subject to date.

Another explanatory approach is that the presence of CP in a patient and the occurrence of ColdU could be completely independent of each other. To verify this theory, a comparison with the frequency of positive CPs in the general population would be helpful, but we could not find any published data on this. Furthermore, it seems possible that a third disease has caused both cryoglobulinemia and ColdU in CP-positive patients. In this case, CPs might have a diagnostical, but only an indirect pathogenical relation to ColdU.

Implications

Overall, we have found that CPs are rare in ColdU patients, suggesting that physicians may limit the measurement of CPs even more to patients with a clinical suspicion of secondary ColdU. In addition to cost savings, this automatically leads to fewer false positive findings and fewer unnecessary blood samples taken from patients. It remains to be clarified to what extent positive CPs have a diagnostic and therapeutic benefit for ColdU patients or to what extent they influence the course of the disease or the patients' symptoms. Furthermore, we recommend the development of an international standardized protocol and defined threshold values for CP detection to reduce false negative and false positive results. The pathomechanism of ColdU with and without CPs continues to remain unclear and requires further research.

Conclusion

In summary, both through our literature review and through the analysis of our patient data, we could show that only few ColdU patients exhibit CPs and that the pathogenesis of ColdU might mainly be driven by other mechanisms, which remain to be identified and characterized in detail.

DATA AVAILABILITY STATEMENT

The raw data was generated at Charité — Universitätsmedizin Berlin, Germany. All data shown in the study is in the **Supplementary Material**, further data supporting the findings of this study is available from the corresponding author on request.

ETHICS STATEMENT

Ethical approval and consent details: Ethical approval was obtained from the ethics committee of the Charité – Universitätsmedizin Berlin, number EA1/069/19. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KG performed the statistical analysis and drafted the manuscript. DA, KK, and SA were involved in patient recruitment and proof-reading of the manuscript. MM and MB were involved in study planning and proof-reading of the manuscript. DT-M has planned the study, coordinated the study, collected patient data, was involved in statistical analysis and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.675451/full#supplementary-material

REFERENCES

1. Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA^{2}LEN/EDF/WAO Guideline for the Definition, Classification, Diagnosis and Management of Urticaria. *Allergy* (2018) 73:1393–414. doi: 10.1111/all.13397
2. Siebenhaar F, Weller K, Mlynek A, Magerl M, Altrichter S, dos SRV, et al. Acquired Cold Urticaria: Clinical Picture and Update on Diagnosis and Treatment. *Clin Exp Dermatol* (2007) 32:241–5. doi: 10.1111/j.1365-2230.2007.02376.x
3. Maltseva N, Borzova E, Fomina D, Bizjak M, Terhorst-Molawi D, Košnik M, et al. Cold Urticaria - What We Know and What We do Not Know. *Allergy* (2020). doi: 10.1111/all.14674
4. Wanderer AA, Grandel KE, Wasserman SI, Farr RS. Clinical Characteristics of Cold-Induced Systemic Reactions in Acquired Cold Urticaria Syndromes: Recommendations for Prevention of This Complication and a Proposal for a Diagnostic Classification of Cold Urticaria. *J Allergy Clin Immunol* (1986) 78:417–23. doi: 10.1016/0091-6749(86)90027-8
5. Hauptmann G, Lang JM, North ML, Oberling F, Mayer G, Lachmann PJ. Lymphosarcoma, Cold Urticaria, IgG1 Monoclonal Cryoglobulin and Complement Abnormalities. *Scand J Haematol* (1975) 15:22–6. doi: 10.1111/j.1600-0609.1975.tb01051.x
6. Costanzi JJ, Coltman CA. Kappa Chain Cold Precipitable Immunoglobulin G (Igg) Associated With Cold Urticaria. I. Clinical Observations. *Clin Exp Immunol* (1967) 2:167–78.
7. Kolopp-Sarda M-N, Miossec P. Cryoglobulins: An Update on Detection, Mechanisms and Clinical Contribution. *Autoimmun Rev* (2018) 17:457–64. doi: 10.1016/j.autrev.2017.11.035
8. Brouet J-C, Clauvel J-P, Danon F, Klein M, Seligmann M. Biologic and Clinical Significance of Cryoglobulins: A Report of 86 Cases. *Am J Med* (1974) 57:775–88. doi: 10.1016/0002-9343(74)90852-3
9. Trejo O, Ramos-Casals M, García-Carrasco M, Yagüe J, Jiménez S, De La Red G, et al. Cryoglobulinemia: Study of Etiologic Factors and Clinical and Immunologic Features in 443 Patients From a Single Center. *Med (Baltimore)* (2001) 80:252–62. doi: 10.1097/00005792-200107000-00004
10. Ferri C, Zignego AL, Pileri SA. Cryoglobulins. *J Clin Pathol* (2002) 55:4–13. doi: 10.1136/jcp.55.1.4
11. Michaud M, Pourrat J. Cryofibrinogenemia. *J Clin Rheumatol Pract Rep Rheum Musculoskelet Dis* (2013) 19:142–8. doi: 10.1097/RHU.0b013e318289e06e
12. Moiseev S, Luqmani R, Novikov P, Shevtsova T. Cryofibrinogenaemia-a Neglected Disease. *Rheumatol Oxf Engl* (2017) 56:1445–51. doi: 10.1093/rheumatology/kew379
13. Roccatello D, Saadoun D, Ramos-Casals M, Tzioufas AG, Fervenza FC, Cacoub P, et al. Cryoglobulinaemia. *Nat Rev Dis Primer* (2018) 4:11. doi: 10.1038/s41572-018-0009-4
14. Maire MA, Mittey M, Lambert PH. The Presence of Cryoprecipitable Immunoglobulins in Normal Human Sera may Reflect Specific Molecular Interactions. *Autoimmunity* (1989) 2:155–64. doi: 10.3109/08916938909019952
15. Romaszko JP, Tridon A, Jouanel P, Subtil E, Dibet P, Betail G. [Detection and Analysis of Cryoglobulins: Comparative Study of a Population of Patients and One of Healthy Controls]. *Pathol Biol (Paris)* (1993) 41:525–9.
16. Felber N. Cold Agglutinin Disease. *Postgrad Med* (1976) 60:89–94. doi: 10.1080/00325481.1976.11714476
17. Maurer M, Metz M, Bindslev-Jensen C, Bousquet J, Canonica GW, Church MK, et al. Definition, Aims, and Implementation of GA(2) Len Urticaria Centers of Reference and Excellence. *Allergy* (2016) 71:1210–8. doi: 10.1111/all.12901
18. Moher D, Liberati A, Tetzlaff J, Altman DGPRISMA Group. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. *J Clin Epidemiol* (2009) 62:1006–12. doi: 10.1016/j.jclinepi.2009.06.005
19. Kulthanan K, Hunnangkul S, Tuchinda P, Chularojanamontri L, Weerasubpong P, Subchookul C, et al. Treatments of Cold Urticaria: A systematic Review. *J Allergy Clin Immunol* (2019) 143:1311–31. doi: 10.1016/j.jaci.2019.02.005
20. Claudy A. Cold Urticaria. *J Investig Dermatol Symp Proc* (2001) 6:141–2. doi: 10.1046/j.0022-202x.2001.00028.x
21. Dressler C, Werner RN, Eisert L, Zuberbier T, Nast A, Maurer M. Chronic Inducible Urticaria: A systematic Review of Treatment Options. *J Allergy Clin Immunol* (2018) 141:1726–34. doi: 10.1016/j.jaci.2018.01.031
22. Moola S, Munn Z, Tufanaru C, Aromataris E, Sears K, Sfetcu R, et al. "Chapter 7: Systematic Reviews of Etiology and Risk," In: *Joanna Briggs Institute Reviewer's Manual*. The Joanna Briggs Institute. Available at: https://reviewersmanual.joannabriggs.org/.
23. Neittaanmäki H. Cold Urticaria. Clinical Findings in 220 Patients. *J Am Acad Dermatol* (1985) 13:636–44. doi: 10.1016/s0190-9622(85)70208-3
24. Doeglas HM, Rijnten WJ, Schröder FP, Schirm J. Cold Urticaria and Virus Infections: A Clinical and Serological Study in 39 Patients. *Br J Dermatol* (1986) 114:311–8. doi: 10.1111/j.1365-2133.1986.tb02822.x
25. Henquet CJM, Martens BPM, Van Vloten WA. Cold Urticaria : A Clinico-Therapeutic Study in 30 Patients; With Special Emphasis on Cold Desensitization. *Cold Urticaria Clin-Ther Study 30 Patients Spec Emphas Cold Desensitization* (1992) 2:75–7.
26. Husz S, Tóth-Kása I, Kiss M, Dobozy A. Treatment of Cold Urticaria. *Int J Dermatol* (1994) 33:210–3. doi: 10.1111/j.1365-4362.1994.tb04956.x
27. Koeppel MC, Bertrand S, Abitan R, Signoret R, Sayag J. [Urticaria Caused by Cold. 104 Cases]. *Ann Dermatol Venereol* (1996) 123:627–32.
28. Möller A, Henning M, Zuberbier T, Czarnetzki-Henz BM. [Epidemiology and Clinical Aspects of Cold Urticaria]. *Hautarzt Z Dermatol Venerol Verwandte Geb* (1996) 47:510–4. doi: 10.1007/s001050050461
29. Santaolalla Montoya M, Martínez Molero MI, Santaolalla San Juana F, Baeza ML, Alonso Lebrero E, Zapatero Remón L. [Cold Urticaria: Review of 12 Cases]. *Allergol Immunopathol (Madr)* (2002) 30:259–62. doi: 10.1016/s0301-0546(02)79134-9
30. Tosoni C, Lodi-Rizzini F, Bettoni L, Toniati P, Zane C, Capezzera R, et al. Cinnarizine is a Useful and Well-Tolerated Drug in the Treatment of Acquired Cold Urticaria (Acu). *Eur J Dermatol EJD* (2003) 13:54–6.

31. Alangari AA, Twarog FJ, Shih M-C, Schneider LC. Clinical Features and Anaphylaxis in Children With Cold Urticaria. *Pediatrics* (2004) 113:e313–7. doi: 10.1542/peds.113.4.e313
32. Katsarou-Katsari A, Makris M, Lagogianni E, Gregoriou S, Theoharides T, Kalogeromitros D. Clinical Features and Natural History of Acquired Cold Urticaria in a Tertiary Referral Hospital: A 10-Year Prospective Study. *J Eur Acad Dermatol Venereol JEADV* (2008) 22:1405–11. doi: 10.1111/j.1468-3083.2008.02840.x
33. Stepaniuk P, Vostretsova K, Kanani A. Review of Cold-Induced Urticaria Characteristics, Diagnosis and Management in a Western Canadian Allergy Practice. *Allergy Asthma Clin Immunol* (2018) 14:85. doi: 10.1186/s13223-018-0310-5
34. Yee CSK, El Khoury K, Albuhairi S, Broyles A, Schneider L, Rachid R. Acquired Cold-Induced Urticaria in Pediatric Patients: A 22-Year Experience in a Tertiary Care Center (1996-2017). *J Allergy Clin Immunol Pract* (2019) 7:1024–31.e3. doi: 10.1016/j.jaip.2018.10.025
35. Kulthanan K, Tuchinda P, Chularojanamontri L, Kiratiwongwan R. Cold Urticaria: Clinical Features and Natural Course in a Tropical Country. *Allergy Asthma Immunol Res* (2019) 11:538–47. doi: 10.4168/aair.2019.11.4.538
36. Młynek A, Magerl M, Siebenhaar F, Weller K, Vieira Dos Santos R, Zuberbier T, et al. Results and Relevance of Critical Temperature Threshold Testing in Patients With Acquired Cold Urticaria. *Br J Dermatol* (2010) 162:198–200. doi: 10.1111/j.1365-2133.2009.09441.x
37. R Core Team. R: A Language and Environment for Statistical Computing. (2018).
38. Houser DD, Arbesman CE, Ito K, Wicher K. Cold Urticaria. Immunologic Studies. *Am J Med* (1970) 49:23–33. doi: 10.1016/s0002-9343(70)80110-3
39. Czarnetzki BM. *Urticaria*. Berlin Heidelberg: Springer-Verlag (1986). doi: 10.1007/978-3-642-70313-3
40. Motyckova G, Murali M. Laboratory Testing for Cryoglobulins. *Am J Hematol* (2011) 86:500–2. doi: 10.1002/ajh.22023
41. Vermeersch P, Gijbels K, Mariën G, Lunn R, Egner W, White P, et al. A Critical Appraisal of Current Practice in the Detection, Analysis, and Reporting of Cryoglobulins. *Clin Chem* (2008) 54:39–43. doi: 10.1373/clinchem.2007.090134
42. Wanderer AA. A Potential New Therapy for Cold Urticaria and Chronic Idiopathic Urticaria. *J Allergy Clin Immunol* (2007) 119:517. doi: 10.1016/j.jaci.2006.10.041
43. Zucchi E, Cavallieri F, Giovannini G, Antonelli F, Mascia MT, Bedin R, et al. Post-Infectious Sensory Neuropathy With anti-GT1a and GQ1b Antibodies Associated With Cold Urticaria. *J Clin Neurosci Off J Neurosurg Soc Australas* (2018) 56:175–7. doi: 10.1016/j.jocn.2018.06.056
44. Miralles López JC, López Andreu FR, Sánchez-Gascón F, López Rodríguez C, Negro Alvarez JM. Cold Urticaria Associated With Acute Serologic Toxoplasmosis. *Allergol Immunopathol (Madr)* (2005) 33:172–4. doi: 10.1157/13075702
45. Morais-Almeida M, Marinho S, Gaspar A, Arêde C, Loureiro V, Rosado-Pinto J. Cold Urticaria and Infectious Mononucleosis in Children. *Allergol Immunopathol (Madr)* (2004) 32:368–71. doi: 10.1016/s0301-0546(04)79270-8
46. Ito A, Kazama T, Ito K, Ito M. Purpura With Cold Urticaria in a Patient With Hepatitis C Virus Infection-Associated Mixed Cryoglobulinemia Type III: Successful Treatment With Interferon-Beta. *J Dermatol* (2003) 30:321–5. doi: 10.1111/j.1346-8138.2003.tb00394.x
47. Lin RY, Schwartz RA. Cold Urticaria and HIV Infection. *Br J Dermatol* (1993) 129:465–7. doi: 10.1111/j.1365-2133.1993.tb03179.x
48. Barranco Sanz P, López Serrano C. [Cold Urticaria Associated With Serologic Markers of Hepatitis B and Cryoglobulinemia]. *Allergol Immunopathol (Madr)* (1987) 15:167–9.
49. Tyson CJ, Czarny D. Cold-Induced Urticaria in Infectious Mononucleosis. *Med J Aust* (1981) 1:33–5. doi: 10.5694/j.1326-5377.1981.tb135285.x
50. Wands JR, Perrotto JL, Isselbacher KJ. Circulating Immune Complexes and Complement Sequence Activation in Infectious Mononucleosis. *Am J Med* (1976) 60:269–72. doi: 10.1016/0002-9343(76)90436-8
51. Morrison JG, Hull PR, Fourie E. Erythema Elevatum Diutinum, Cryoglobulinaemia, and Fixed Urticaria on Cooling. *Br J Dermatol* (1977) 97:99–104. doi: 10.1111/j.1365-2133.1977.tb15435.x
52. Rawnsley HM, Shelley WB. Cold Urticaria With Cryoglobulinemia in a Patient With Chronic Lymphocytic Leukemia. *Arch Dermatol* (1968) 98:12–7. doi: 10.1001/archderm.98.1.12
53. Thiers H, Moulin G. Urticaria Caused by Cold and Cryoglobulinemia Revealing a Myeloma. *Bull Soc Fr Dermatol Syphiligr* (1969) 76:578–81.
54. Chodirker WB, Komar RR. Angioimmunoblastic Lymphadenopathy in a Child With Unusual Clinical and Immunologic Features. *J Allergy Clin Immunol* (1985) 76:745–52. doi: 10.1016/0091-6749(85)90681-5
55. Török L, Borka I, Szabó G. Waldenström's Macroglobulinaemia Presenting With Cold Urticaria and Cold Purpura. *Clin Exp Dermatol* (1993) 18:277–9. doi: 10.1111/j.1365-2230.1993.tb02188.x
56. Polliack A, Lugassy G. Autoimmunity and Auto-Immune Syndromes Associated With and Preceding the Development of Lymphoproliferative Disorders. *Leukemia* (1992) 6(Suppl 4):152–4.

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CASE REPORT
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Case Report and Review of the Literature: Bullous Skin Eruption After the Booster-Dose of Influenza Vaccine in a Pediatric Patient With Polymorphic Maculopapular Cutaneous Mastocytosis

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Vaccination is a well-known trigger for mast cell degranulation in subjects affected by mastocytosis. Nevertheless, there is no exact standardized protocol to prevent a possible reaction after a vaccine injection, especially for patients who have already presented a previous vaccine-related adverse event, considering that these patients frequently tolerate future vaccine doses. For this reason, we aim to share our experience at Meyer Children's University Hospital in Florence to raise awareness on the potential risk for future vaccinations and to discuss the valuable therapeutic strategies intended to prevent them, taking into account what is proposed by experts in literature. We describe the case of an 18-month-old female affected by a polymorphic variant of maculopapular cutaneous mastocytosis that presented an extensive bullous cutaneous reaction 24 hours after the second dose (booster dose) of inactivated-tetravalent influenza vaccine, treated with a single dose of oral corticosteroid therapy with betamethasone (0.1 mg/kg) and an oral antihistamine therapy with oxatomide (1 mg/kg/daily) for a week, until resolution. To the best of our knowledge, in the literature, no documented case of reaction to influenza vaccine in maculopapular cutaneous mastocytosis is described. Subsequently, the patient started a background therapy with ketotifen daily (0.05 mg/kg twice daily), a non-competitive H1-antihistamine, and a mast cell stabilizer (dual activity). A non-standardized pharmacological premedication protocol with an H1-receptor antagonist (oxatomide, 0.5 mg/kg) administered 12 hours before the immunizations, and a single dose of betamethasone (0.05 mg/kg) together with another dose of oxatomide (0.5 mg/kg) administered 2 hours before the injections was followed to make it possible for the patient to continue with the scheduled vaccinations. Indeed, no reactions were

subsequently reported. Thus, in our experience, a background therapy with ketotifen associated with a premedication protocol made by two doses of oxatomide and a single dose of betamethasone was helpful to make possible the execution of the other vaccines. We suggest how in these children, it could be considered the idea of taking precaution when vaccination is planned, regardless of the kind of vaccine and if a dose of the same vaccine was previously received. However, international consensus needs to be reached to manage vaccinations in children with mastocytosis and previous adverse reactions to vaccines.

Keywords: vaccination, cutaneous mastocytosis, prevention, premedication, adverse reaction, pediatrics

INTRODUCTION

The term "mastocytosis" defines a various group of disorders characterized by an increase of mast cells in cutaneous tissue and different organs such as bone marrow, spleen, liver, lymph nodes, and gastrointestinal tract (1, 2). The organs most frequently involved are skin and bone marrow (2, 3). Mast cells' uncontrolled proliferation is mainly associated with gaining function mutations in c-KIT, the gene that encodes for a tyrosine kinase receptor expressed by mast cells (4, 5). Disease manifestations are mostly linked to mast-cell-derived mediators' release, especially histamine, and include pruritus, flushing, gastrointestinal signs and symptoms, and rarely anaphylaxis (6, 7). The typical Darier's sign refers to the development of a wheal-and-flare reaction after a few minutes of scratching or rubbing a cutaneous lesion, due to the release of vasoactive mediators (8).

The current classification of mastocytosis, recently updated by World Health Organization in 2016, identifies three major disease groups: cutaneous mastocytosis, systemic mastocytosis, and mast-cell sarcoma (9, 10).

The classification of cutaneous mastocytosis is subcategorized based on the macroscopic features of the skin lesions, their distribution, and onset into three groups: maculopapular cutaneous mastocytosis (MPCM), formerly known as urticaria pigmentosa (furtherly sub-dived in monomorphic and polymorphic types), diffuse cutaneous mastocytosis (DCM), and mastocytoma of the skin (3, 9).

In children, mastocytosis is commonly limited to the skin, has a favorable prognosis, and improves spontaneously by the mid-teenage years. On the contrary, adult mastocytosis is usually systemic and may have a chronic or aggressive course (7, 9, 11).

The most frequent type of pediatric mastocytosis is MPCM, specifically the polymorphic variant, followed by the cutaneous isolated mastocytoma and then the diffuse cutaneous mastocytosis (9). Mastocytosis can appear at all ages but, it is possible to identify two incidence peaks: early childhood (65%) and young adulthood (35%). Focusing on the pediatric age, about 15% are connatal forms, 30% appear in the first 6 months of life, and the others between 2 and 15 years (7).

Patients affected by mastocytosis present a greater risk than the general population for anaphylaxis. The main cause of anaphylaxis in pediatric mastocytosis is idiopathic, followed by some stimuli such as foods, medications, and Hymenoptera venoms. The latter ones represent a rare cause of anaphylaxis in pediatric mastocytosis, while they represent the most common triggers in adult patients. In pediatric mastocytosis, the risk of anaphylaxis is related to the extent and the density of lesions. Elevated basal serum tryptase levels, systemic disease, KIT D816V mutation, and previous episodes of anaphylaxis represent other suggested risk factors. For this reason, it can be considered reasonable to provide these children with extensive disease and/or previous anaphylaxis with an emergency autoinjector of epinephrine (12, 13).

Vaccination is a well-known trigger for mast cell degranulation in subjects affected by mastocytosis. Nevertheless, there is no exact standardized protocol to prevent a possible reaction after a vaccine injection, especially for patients who have already presented a previous vaccine-related adverse event, considering that these patients frequently tolerate future vaccine doses. For this reason, we aim to share our experience at Meyer Children's University Hospital in Florence to raise awareness on the potential risk for future vaccinations and to discuss the valuable therapeutic strategies intended to prevent them, taking into account what is proposed by experts in literature.

CASE REPORT

A female child of 18 months, affected by a polymorphic variant of MPCM, was presented to our attention following a significant diffuse bullous cutaneous reaction 24 hours after the injection of the second dose (booster dose) of the inactivated tetravalent influenza vaccine **(Figure 1)**.

Before this event, the patient did not present any cutaneous reactions to the injection of the other vaccinations scheduled in Italy for this age group, although fever was frequently reported after most of these immunizations (**Table 1**).

Hence, the patient was addressed to our center with the primary purpose of continuing the scheduled vaccinations in a hospital clinical setting. In her family history, no other cases of mastocytosis were reported. The older sister was affected by atopic dermatitis, and the mother had a history of reaction to penicillins. No other significant disorders, except for mastocytosis, were mentioned in her personal medical history.

At the age of 8 months, a first isolated mastocytoma appeared on the neck followed by more lesions, heterogeneous in shape

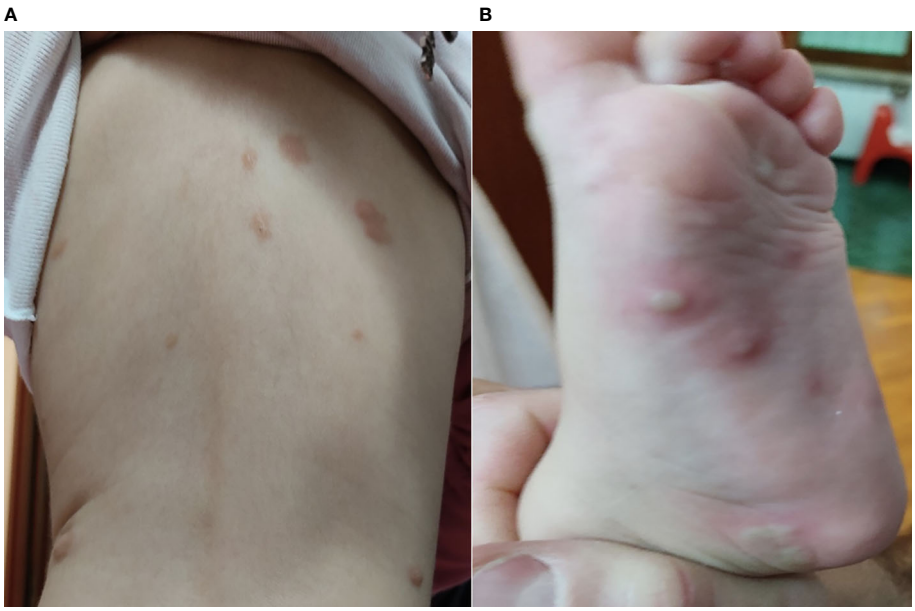

FIGURE 1 | **(A)** Initial bullous skin eruption involving the back of the patient after 24 hours from the second dose of the influenza vaccine; **(B)** Bullous lesions presented on the left foot of the patient after 24 hours from the second dose of the influenza vaccine.

TABLE 1 | Vaccines received by the patient in accordance to the Italian scheduled vaccinations.

Vaccines	Number of doses	Adverse clinical manifestations
Hexavalent vaccine	3	Fever*
Meningococcal B vaccine	3	Fever*
Rotavirus vaccine	3	Fever*
Meningococcal C vaccine	1	Fever*
Pneumococcal vaccine	2	Fever*

**Fever arisen 6–8 hours after each dose. Influenza vaccine is highly recommended in Italy. For children under 9 years of age who have not previously been vaccinated against influenza, a second dose should be administered after an interval of at least 4 weeks.*

and size, brownish-yellowish, on the head, chest, back, and legs. Sometimes, blisters occurred within the existing lesions. The diagnosis of polymorphic MPCM was made at the age of 9 months, after a dermatological evaluation that pointed out the characteristic skin lesions. During the physical exam, a gentle rubbing of a chest lesion revealed a prominent Darier's sign associated with flushing of the face, neck, and chest. The serum tryptase level subsequently evaluated was within normal limits at 4.1 mcg/L. No skin biopsy was performed.

At the age of 13 months, the patient received her first dose of influenza vaccine, and only a 2-day fever with no cutaneous involvement was reported. One month later, the patient received the second dose of inactivated-tetravalent influenza vaccine, and after 24 hours from the injection, she presented an extensive bullous skin eruption involving the face, head, chest, shoulder, back, hands, and feet (**Figure 1**). No blisters were present before the immunization. Moreover, she showed significant irritability and generalized pruritus. The adverse reaction was promptly treated with a single dose of oral betamethasone (0.1 mg/kg) and an oral antihistamine therapy with oxatomide (1 mg/kg/daily) for a week, with the complete resolution of the lesions.

After 6 days from the resolution of the clinical manifestations, another evaluation of serum tryptase level was made, showing results within normal limits (2 mcg/L). After this adverse reaction, parents refused the next Italian scheduled vaccination with the third dose of 13-valent Pneumococcal conjugate vaccine (PCV13). The patient was later addressed by our Allergy Unit thanks to the parents' desire to catch up with the scheduled vaccination in a hospital clinical setting.

After a clinical evaluation of the patient, we planned the third dose of the PCV13 after 2 weeks from the first visit. Until that day, the patient assumed a background therapy with ketotifen (0.05 mg/kg twice daily), a non-competitive H1-antihistamine, and a mast cell stabilizer (dual activity). Furthermore, 12 hours before the injection, the patient assumed oxatomide (0.5 mg/kg), and 2 hours before the injection, she assumed the same dose of oxatomide together with a single dose of betamethasone (0.05 mg/kg). We decided not to perform a patch test to evaluate a possible delayed reaction to a component of the vaccine because of the possibility of provoking mastocytes degranulation. After 2 hours of observation from the injection, the patient did not present any cutaneous or systemic clinical manifestations. The patient was discharged with instructions to continue the therapy with ketotifen (0.05 mg/kg twice daily).

Afterward, the patient continued the same background therapy with ketotifen until the varicella vaccination, planned after 1 month. Similarly to the previous vaccination, the patient assumed oxatomide (0.5 mg/kg) 12 hours before the immunization and the same dose of oxatomide together with a single dose of betamethasone (0.05 mg/kg) 2 hours before the immunization. After the immunization, no adverse reactions were presented, including fever.

DISCUSSION

MPCM, the most common form of mastocytosis seen in childhood, is subdivided into two variants: polymorphic and monomorphic (9).

In the polymorphic variant, lesions are larger and asymmetric, and they have a typical distribution that affects the head, neck, and extremities. The lesions can be red, brown, or yellow and appear flat or elevated with sharp or indistinct margins. Darier's sign, pruritus, and dermographism are usually present, while blistering may occur, especially in children under 3 years old. Serum tryptase levels are usually in the normal range; rarely patients with pronounced lesions can present increased level that usually decreases within 1 or 2 years (8, 14). The prognosis is excellent, and usually, lesions resolve by adolescence (2).

On the other side, the monomorphic variant is typical of adult age. The lesions are usually smaller, round, and similar. In these patients, serum tryptase levels can be persistently increased over time, and the disease tends to persist into adulthood with a higher risk of developing systemic mastocytosis (3, 8, 14).

Mastocytoma is the second most common lesion in the pediatric population. It usually occurs in the form of a single lesion, yellow-brownish, with the frequent formation of pomphoid lesions, the possible formation of bullous skin eruptions, and regional or diffuse flushing after the mechanical stimulation of the lesion. It has a dynamic pattern and can increase by dimensions (but not number) and usually regress by mid-teenage years (14, 15). According to Hartmann et al., a maximum of three compatible lesions still should qualify as mastocytoma, while more than three lesions should be considered as attributable to a case of MPCM (3). The diagnosis is based exclusively on clinical manifestations, and serum tryptase levels are usually within the normal range (3, 14).

DCM is characterized by diffuse infiltration of the dermis with mast cells, which results in generalized erythema and diffuse papules associated with pachydermia, darker skin, and accentuation of hair follicles "peau d'orange" (16). Dermographism and blistering are characterizing signs of disease (3, 8). Diagnosis is made by skin biopsy (16). DCM can resolve by adolescence, but sometimes progress toward systemic mastocytosis can be seen, usually accompanied by hepatomegaly, splenomegaly, and bone marrow involvement (3, 8).

With a complete inspection of the skin accompanied by Darier's sign evaluation, the physical examination is critical to have an initial approach to the disease. A skin biopsy that shows evidence of compatible histological findings can confirm the diagnosis, even if, in the absence of them, a diagnosis cannot be ruled out (17). As concerns the laboratory test, a significant role is played by the serum level of tryptase. In cutaneous mastocytosis and isolated mastocytoma, serum levels of tryptase are usually within the standard limit. If a tryptase serum level >20 mcg/L is detected, without signs and symptoms of systemic involvement, the value should be considered in first place associated with a release of mediators by cutaneous mast cells. The evaluation of serum levels is indeed recommended after 24 hours from the resolution of a clinical event caused by mast cells degranulation. Anyway, periodic control of tryptase should be considered in order to rule out a progression of disease (18). The study of c-KIT mutation should be carried out in selected patients (8), while a bone marrow biopsy should be requested if systemic clinical manifestations, especially organomegaly, in conjunction with elevation of tryptase level are present (9, 19).

Multiple pathways have been described in the activation and degranulation of mast cells. One important signaling pathway is induced by the high-affinity receptor for IgE, and it is involved in immediate allergic reactions. Other triggers for mast cell activation are known, and they involve certain cytokines, immune complexes, complement proteins, drugs (e.g., non-steroidal anti-inflammatory drugs and opioids), radiocontrast media, products of bacteria, or parasites. Physical factors such as heat, cold, friction, stress, and physical effort can induce mast cell activation and act as cofactors in allergic and anaphylactic reactions (20, 21). In our experience, we indeed avoided these known cofactors of mast cell degranulation. The vaccine was kept out of the fridge for 2 hours to bring it to room temperature, and the injection was carefully carried out as not to rub the skin. The patient was quiet, and we knew that she was not assuming other medications, except for the ones that we prescribed, and that she did not have histamine liberators food.

Vaccines may act as triggers in patients with mastocytosis, especially children. Indeed, some substances contained in vaccines, such as dextran, gelatin, and polymyxin B, may be responsible for this adverse reaction (22). However, literature does not specifically describe the pathway associated with mastocyte degranulation after immunization.

An Italian study of 102 patients, including 35 children and 67 adults with mastocytosis, demonstrated no relationship between types of vaccines and risk of mast cell degranulation after immunization. Seven children in the study presented reactions after the immunization. Subsequently, some of these children tolerated the same vaccine components, strengthening the hypothesis on the possible role of variable triggers in determining reactions to vaccines in cases of mastocytosis (**Table 2**) (22).

Parente et al., supposed that mast cell activation could be induced by a component in vaccines that acts like a superantigen, which unspecifically binds to human IgE, leading to mast cell degranulation. Furthermore, they speculated that the development of IgG or IgM after the first dose of vaccine,

TABLE 2 | Clinical data about patients from the analyzed studies on vaccination and mastocytosis.

Authors	Patients with reactions/Total sample	Variant of Mastocytosis/Age of Diagnosis (years)	Eliciting Vaccine (number of doses received)	Reaction/Time interval	Therapy	Subsequent Vaccines/ Premedication
Zanoni et al. (22)	7/102 (35 children and 67 adults)	Mastocytoma/0.5	Hexavalent (1)	Urticaria and angioedema/20 min	Unknown symptomatic treatment	DTaP, IPV, HB, Hib, MMRV/not available
		Mastocytoma/0.4	Hexavalent (2)	Local and facial flushing/20 min		Hexavalent/not available
		Mastocytoma/2	MenC (1)	Fever and gastrointestinal clinical manifestations/ 24 h		PCV/not available
		Mastocytoma/0.6	Hexavalent (3)	Injection site reaction and fever/8 h		
		MPCM/0.2	PCV (2), MenC (1)	Fever/8 h		
		MPCM/0.5	HPV (1)	Hives on arm and nasal obstruction/12 h		
		DCM/0.4	Hexavalent (1, 2) PCV (1, 2)	Hives and itch on trunk and febrile convulsions/12 h		
Parente et al. (6)	4/72 children	MPCM/0.3	Hexavalent (1)	Bullous skin reaction/6–12 h	Oral antihistamine	Other mandatory vaccines/oral antihistamines
		Mastocytoma/2	Hexavalent (1)	Diffuse urticaria/1–4 h	Oral antihistamine	Other mandatory vaccines
		Mastocytoma/4	Hexavalent (1)	Diffuse urticaria/1–4 h	Oral antihistamine	Other mandatory vaccines
		DCM/0.1	Hexavalent (1)	Bullous skin reaction and mild bronchospasm/ 6–12 h	Nebulized epinephrine and oral antihistamine	Other mandatory vaccines/oral antihistamines
Bankova et al. (16)	1 child	DCM	DTaP IPV HiB PCV Rotavirus	Confluent blisters on the back, abdomen, and upper arms/a day later	5-day course of low-dose oral steroids	Unknown/oral and topical sodium cromolyn
Johansen et al. (23)	3/35 children	MPCM	DTaP IPV HiB PCV Rotavirus	Skin flushing, itch, blisters, gastrointestinal clinical manifestations/hours	Oral antihistamine	
		MPCM	DTaP	Skin flushing and pruritus/minutes	Oral antihistamine	
		MPCM	All vaccines	Fever/hours	Acetaminophen	

Hexavalent, diphtheria–tetanus toxoid–acellular pertussis (DTaP)–hepatitis B (HB)–inactivated polio vaccine (IPV)–Haemophilus influenzae B vaccine (HiB); h, hours; HPV, human papilloma virus vaccine; IPV, inactivated polio vaccine; min, minutes; Men C, meningococcal C vaccine; MMRV, measles, mumps, rubella, varicella vaccine; PCV, pneumococcal vaccine.

which neutralizes the superantigens before they interact with IgE, could explain the lack of reaction to the booster vaccination.

This possible role of variable triggers in determining reactions to vaccines in cases of mastocytosis is not deeply analyzed in the literature. Additionally, when a specific trigger was identified, its role in the underlying mechanisms of reactions and cofactors could not be explained. For that reason, in the future, it will be of great interest to analyze the role of possible cofactors in cases of reactions to vaccines in patients with mastocytosis.

Parente et al., in their study of the evaluation of vaccination safety in 72 pediatric patients with mastocytosis, concluded that vaccine reactions in mastocytosis are usually mild and do not generally occur with boosters (as confirmed by Hussain et al.) (**Table 2**) (6, 9). After the first dose of the hexavalent vaccine, four patients presented cutaneous reactions. All four of the children who presented a vaccine-related reaction received the subsequent vaccinations and boosters in a monitored hospital setting. Among them, the two patients who presented a previous bullous reaction assumed oral antihistamines beginning 48 hours before vaccine administration. Moreover, the patient affected by DCM was on a continuous antihistamine treatment. None of the four children presented a further reaction to the subsequent two boosters of hexavalent or the other mandatory vaccines, which may demonstrate a potential role for antihistaminic treatment in preventing further reaction. Hence, Parente et al., propose to limit a prolonged observing time to 2 hours, only in case of administration of the first dose of vaccine (6). In our experience, considering the significant reaction that occurred on the booster dose of a previous vaccine and in contrast to scientific literature that defines how adverse events usually occur with the first dose of vaccine, we found it difficult to stigmatize reaction to vaccination in mastocytosis, and so we decided to extend the 2-hours observation time for all the vaccines administered (6). Parents should be trained to identify the warning signs of an early adverse reaction and taught how to use an epinephrine autoinjector when necessary (12, 22). In the analyzed study by Parente et al., hexavalent vaccines (and therefore, polyvalent formulation) seem to be associated with a higher risk of reaction (6). Nevertheless, there is no significant overwhelming scientific evidence in the literature to suggest avoiding polyvalent formulation. Taking into account ministerial Italian recommendations (24) and other sources of information (16, 22), would suggest avoiding the co-administration of multiple vaccines (22) and not the use of polyvalent vaccines, also for practical reasons, including the maximization of the adherence to scheduled vaccinations. We decided to administer varicella vaccine in a single injection, instead of measles, mumps, rubella, and varicella vaccine polyvalent formulation, only for the wish of patient's family, that was still frightened and distrustful of vaccines. It is indeed very important that families regain their trust in vaccinations, especially among these kinds of patients and in the anti-vaccination movement era (25).

A recent retrospective study of 35 patients evaluated vaccination reactions in pediatric patients with MPCM (**Table 2**) (23). The study's conclusions are similar to previous ones and highlight that vaccination reactions in mastocytosis tend to be mild, so scheduled vaccinations should be continued (23).

Bankova et al., highlight the importance of precautions with procedures and adequate premedication to reduce the risk of severe complications. The authors describe a case of generalized bullous cutaneous reaction in a 5-month-old boy affected by DCM after the scheduled 4-month vaccinations (**Table 2**). In this case, the patient was treated with a low-dose oral steroid for 5 days, which was indicated by the authors as the preferred therapy for cases with blister formation according to indications reported in the literature. Subsequently, the patient presented two additional episodes of blistering associated with vaccination and viral illness but, these episodes became milder, thanks to the initiation of a regimen of oral and topical sodium cromolyn (16).

CONCLUSION

The lack of specific guidelines to follow in case of vaccination reaction in cutaneous mastocytosis represents a significant issue to face. The lack of predictive elements of the risk of reaction makes it impossible to define its level for future vaccinations, even if the literature states that these patients frequently tolerate future vaccine doses. Furthermore, there is a lack of consensus on which therapy is most appropriate. In addition, there are no criteria to define a stratification of risk that allows us to identify who is the most deserving patient to receive it. It would be very important to collect extensive multicentric data with the aim to compare different schemes of premedication to establish the most efficient protocol in the most suitable patient, but this is made difficult by the rarity of pathology.

In this case report, we are far from establishing the best therapeutic strategy to adopt, but we took into account and shared information collected from the literature to define a non-standardized pharmacological and procedural protocol, which we adopted in our experience. Thus, a background therapy with ketotifen associated with a premedication protocol made by two doses of oxatomide and a single dose of betamethasone was helpful to make possible the execution of the other vaccines. Though, as previously underlined, since the incidence of future reactions appears unpredictable, the child might have tolerated the subsequent vaccination without premedication, but we did not consider this a deterrent to not perform a premedication therapy considering the significant role of premedication described in literature in these patients to prevent and control adverse events and the patient's family fear to execute the other mandatory vaccinations, especially so shortly after a relevant reaction. It is indeed important to highlight as well the significant role played by the premedication therapy to make the patient's family trust vaccinations again, considering that the patient subsequently did not present in the two vaccinations executed in our center any kind of clinical manifestations.

The choice to use betamethasone in our premedication protocol can be justified by the long-time latency between vaccine injection and clinical manifestations, so to control any delayed reactions. We avoided all the possible stimuli of reaction, and we adopted specific

measures of care, as explained in literature (16, 21, 22). Finally, we suggest how in these children, it could be considered the idea of taking precaution when vaccination is planned, regardless of the kind of vaccine and if a dose of the same vaccine was previously received. However, international consensus needs to be reached to manage vaccinations in children with mastocytosis and previous adverse reactions to vaccines.

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

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

CF and FM conceptualized the work. DS, MG, TO, FAP, SR, and FM drafted the manuscript. DS, MG, TO, SB, FAP, GL, CC, LS, LL, CF, CA, SR, and FM performed the investigations and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Castells M, Metcalfe DD, Escribano L. Diagnosis and Treatment of Cutaneous Mastocytosis in Children. *Am J Clin Dermatol* (2011) 12:259–70. doi: 10.2165/11588890-000000000-00000
2. Wiechers T, Rabenhorst A, Schick T, Preussner LM, Förster A, Valent P, et al. Large Maculopapular Cutaneous Lesions Are Associated With Favorable Outcome in Childhood-Onset Mastocytosis. *J Allergy Clin Immunol* (2015) 136:1581–90.e3. doi: 10.1016/j.jaci.2015.05.034
3. Hartmann K, Escribano L, Grattan C, Brockow K, Carter MC, Alvarez-Twose I, et al. Cutaneous Manifestations in Patients With Mastocytosis: Consensus Report of the European Competence Network on Mastocytosis; the American Academy of Allergy, Asthma & Immunology; and the European Academy of Allergology and Clinical Immunology. *J Allergy Clin Immunol* (2016) 137:35–45. doi: 10.1016/j.jaci.2015.08.034
4. Arase N, Wataya-Kaneda M, Murota H, Nakagawa Y, Yamaoka T, Itoi-Ochi S, et al. Genotype and Phenotype Analysis of Patients With Pediatric Cutaneous Mastocytosis, Especially Wild-Type KIT Patients. *J Dermatol* (2020) 47:426–9. doi: 10.1111/1346-8138.15266
5. Bodemer C, Hermine O, Palmérini F, Yang Y, Grandpeix-Guyodo C, Leventhal PS, et al. Pediatric Mastocytosis Is a Clonal Disease Associated With D816V and Other Activating c-KIT Mutations. *J Invest Dermatol* (2010) 130:804–15. doi: 10.1038/jid.2009.281
6. Parente R, Pucino V, Magliacane D, Petraroli A, Loffredo S, Marone G, et al. Evaluation of Vaccination Safety in Children With Mastocytosis. *Pediatr Allergy Immunol Off Publ Eur Soc Pediatr Allergy Immunol* (2017) 28:93–5. doi: 10.1111/pai.12647
7. Paoloni G, Berti I. Il Bambino Con Mastocitosi. *Medico e Bambino* (2011) 30:155–9.
8. Klaiber N, Kumar S, Irani A-M. Mastocytosis in Children. *Curr Allergy Asthma Rep* (2017) 17:80. doi: 10.1007/s11882-017-0748-4
9. Hussain SH. Pediatric Mastocytosis. *Curr Opin Pediatr* (2020) 32:531–8. doi: 10.1097/MOP.0000000000000922
10. Valent P, Akin C, Hartmann K, Nilsson G, Reiter A, Hermine O, et al. Advances in the Classification and Treatment of Mastocytosis: Current Status and Outlook Toward the Future. *Cancer Res* (2017) 77:1261–70. doi: 10.1158/0008-5472.CAN-16-2234
11. Siebenhaar F, Weller K, Blume-Peytavi U, Maurer M. [Childhood-Onset Mastocytosis]. *Hautarzt Z Dermatol Venerol Verwandte Geb* (2012) 63:104–9. doi: 10.1007/s00105-011-2201-2
12. Brockow K, Jofer C, Behrendt H, Ring J. Anaphylaxis in Patients With Mastocytosis: A Study on History, Clinical Features and Risk Factors in 120 Patients. *Allergy* (2008) 63:226–32. doi: 10.1111/j.1398-9995.2007.01569.x
13. Broesby-Olsen S, Carter M, Kjaer HF, Mortz CG, Møller MB, Kristensen TK, et al. Pediatric Expression of Mast Cell Activation Disorders. *Immunol Allergy Clin North Am* (2018) 38:365–77. doi: 10.1016/j.iac.2018.04.009
14. Ferreira S, Fernandes I, Cabral R, Machado S, Lima M, Selores M. Cutaneous Manifestations in Mastocytosis: Update. *Acta Médica Port* (2020) 33:275–81. doi: 10.20344/amp.12189
15. Leung AKC, Lam JM, Leong KF. Childhood Solitary Cutaneous Mastocytoma: Clinical Manifestations, Diagnosis, Evaluation, and Management. *Curr Pediatr Rev* (2019) 15:42–6. doi: 10.2174/1573396315666181120163952
16. Bankova LG, Walter JE, Iyengar SR, Lorenzo ME, Hornick JL, Castells MC. Generalized Bullous Eruption After Routine Vaccination in a Child With Diffuse Cutaneous Mastocytosis. *J Allergy Clin Immunol Pract* (2013) 1:94–6. doi: 10.1016/j.jaip.2012.08.008
17. Heide R, Beishuizen A, Groot HD, Hollander JCD, Doormaal JJV, Monchy JGRD, et al. Mastocytosis in Children: A Protocol for Management. *Pediatr Dermatol* (2008) 25:493–500. doi: 10.1111/j.1525-1470.2008.00738.x
18. Platzgummer S, Bizzaro N, Bilò MB, Pravettoni V, Cecchi L, Sargentini V, et al. Recommendations for the Use of Tryptase in the Diagnosis of Anaphylaxis and Clonal Mastcell Disorders. *Eur Ann Allergy Clin Immunol* (2020) 52:51–61. doi: 10.23822/EurAnnACI.1764-1489.133
19. Carter MC, Clayton ST, Komarow HD, Brittain EH, Scott LM, Cantave D, et al. Assessment of Clinical Findings, Tryptase Levels, and Bone Marrow Histopathology in the Management of Pediatric Mastocytosis. *J Allergy Clin Immunol* (2015) 136:1673–9.e3. doi: 10.1016/j.jaci.2015.04.024
20. Brockow K, Plata-Nazar K, Lange M, Nedoszytko B, Niedoszytko M, Valent P. Mediator-Related Symptoms and Anaphylaxis in Children With Mastocytosis. *Int J Mol Sci* (2021) 22:2684. doi: 10.3390/ijms22052684
21. Hudson A, Finlayson L. Diffuse Cutaneous Bullous Mastocytosis and Disseminated Intravascular Coagulation Postvaccination: A Case Report. *J Cutan Med Surg* (2016) 20:596–9. doi: 10.1177/1203475416661312
22. Zanoni G, Zanotti R, Schena D, Sabbadini C, Opri R, Bonadonna P. Vaccination Management in Children and Adults With Mastocytosis. *Clin Exp Allergy J Br Soc Allergy Clin Immunol* (2017) 47:593–6. doi: 10.1111/cea.12882
23. Johansen ML, Lawley LP. Assessing Vaccination Reactions in Pediatric Patients With Maculopapular Cutaneous Mastocytosis. *Pediatr Dermatol* (2021) 38:502–3. doi: 10.1111/pde.14492
24. Istituto Superiore di Sanità. Guida Alle Controindicazioni Alle Vaccinazioni. Available at: http://www.salute.gov.it/portale/documentazione/p6_2_2_1.jsp?lingua=italiano&id=2759 (Accessed March 28, 2021).
25. Sollai S, Iacopelli J, Giovannini M, Prato M, Galli L, de Martino M, et al. Use of Specific Immunoglobulins and Vaccines for the Management of Accidental Needlestick Injury in the Child: A Practical Review in the Anti-Vaccination Movement Era. *J Chemother* (2016) 28:355–8. doi: 10.1080/1120009X.2016.1173318

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Novel Insights on MRGPRX2-Mediated Hypersensitivity to Neuromuscular Blocking Agents And Fluoroquinolones

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Neuromuscular blocking agents (NMBAs) like atracurium and rocuronium as well as fluoroquinolones (FQs) cause mast cell-mediated anaphylaxis by activating Mas-related G protein-coupled receptor X2 (MRGPRX2), but many questions remain unanswered. Here, we address three of them, namely whether primary human mast cells show similar activation by these drugs as murine mast cells and mast cell lines, how sugammadex protects from atracurium-induced MRGPRX2-mediated mast cell activation, and why some but not all patients treated with rocuronium develop anaphylaxis. We used peripheral blood-derived cultured mast cells from healthy donors and patients, assessed mast cell activation and degranulation by quantifying intracellular calcium and CD63 expression, respectively, and made use of MRGPRX2-silencing, *via* electroporation with Dicer-substrate small interfering RNAs, and single cell flow cytometric analyses. Atracurium, ciprofloxacin, and levofloxacin activated and degranulated primary human mast cells, but only MRGPRX2-positive and not MRGPRX2-negative or -silenced mast cells. Sugammadex attenuated the atracurium-induced and MRGPRX2-mediated activation and degranulation of human mast cells by reducing free atracurium levels. The mast cells of patients with IgE-independent anaphylaxis to rocuronium were similar, in their MRGPRX2 expression and function, to those of patients with IgE-mediated anaphylaxis. These findings further improve our understanding of the role and relevance of MRGPRX2-driven mast cell activation in anaphylactic reactions to NMBAs and FQs and may help to improve their prediction, prevention, and treatment.

Keywords: MRGPRX2, IgE, mast cell, drug, anaphylaxis, CD63, flow cytometry, rocuronium

INTRODUCTION

The activation of Mas-related G protein-coupled receptor X2 (MRGPRX2) on mast cells (MCs) is held to be a major pathway in the pathogenesis of IgE-independent immediate drug hypersensitivity reactions (IDHRs) that occur in response to neuromuscular blocking agents (NMBAs) such as atracurium and rocuronium as well as fluoroquinolones (FQs) such as moxifloxacin and ciprofloxacin. NMBAs and FQs can cause perioperative anaphylaxis, and adverse reactions to these drugs administered during general anesthesia may be very severe and life-threatening, with a mortality rate of up to 9% (1–7).

As of today, many questions on the role of MRGPRX2 in NMBA- and FQ-induced anaphylaxis remain unanswered and need to be addressed. These include, but are not limited to, 1) whether findings on MRGPRX2 activation by NMBAs and FQs from mouse models or genetically modified cell lines can be extrapolated to humans, 2) what the precise role and mechanisms of action of sugammadex, a NMBA reversal agent, are in atracurium-induced and MRGPRX2-induced adverse reactions (8), and 3) why some but not all patients treated with NMBAs experience MRGPRX2-related IDHRs.

At present, most of the information on the effects of NMBAs and FQs on MRGPRX2 are derived from studies performed with MRGPRX2-expressing neoplastic cells, like the LAD-2 MC line, or with the mouse orthologue, MRGPRB2. It is clear that there are differences between these cellular models and primary human MCs. LAD-2 cells, for example, are more responsive to MRGPRX2-agonists than human peripheral blood-derived cultured MCs (PBCMCs) (9, 10), and some NMBAs are less potent activators of MRGPRX2 than MRGPRB2 (11). This makes it difficult, at present, to assess NMBAs and FQs for their MRGPRX2-activating capacity and potency in the human system and to predict the risk that comes with their use. What is needed are results from studies with primary human cells. Such studies, until very recently, were not possible due to the lack of suitable tools, models and assays.

Sugammadex is a modified γ-cyclodextrin designed as a selective relaxant-binding agent that acts by encapsulating rocuronium as an inclusion complex and removing it from the neuromuscular junction (12). Although sugammadex does not reverse atracurium-induced neuromuscular block (13), it has recently been shown to attenuate atracurium-induced MRGPRX2-dependent MC activation in LAD-2 cells, when used at molar excess (14). Currently, the underlying mechanisms of this effect are unclear. The prevention of atracurium-induced MC activation may be caused by atracurium encapsulation, i.e. the elimination of free atracurium, by a direct inhibition *via* sugammadex, or by inhibitory effects of sugammadex-atracurium complexes. Addressing this gap of knowledge may guide the development of more effective approaches for the use of sugammadex for the prevention of NMBA-induced IDHRs.

Although MCs in all humans are held to express MRGPRX2, very few patients develop IDHRs when treated with NMBAs such as rocuronium. The reasons for this are unknown, but may include genetic polymorphisms and mutations resulting in an augmented responsiveness of MRGPRX2, distinct receptor binding sites, differences in MRGPRX2 signalosome, epigenetic modifications, post-transcriptional modifications resulting in synthesis of MRGPRX2 variants, temporarily or constitutively varying surface expressions and the influence of co-factors (15) (10). As recently suggested by Chompunud et al. (10), one way to address the role of MRGPRX2 mutations is to compare MCs for MRGPRX2 expression and function between patients who experienced IDHRs and had a positive skin test rocuronium, with and without rocuronium-specific IgE and/or positive BAT rocuronium (16).

Recently, we and others developed tools, models and assays that allow for the investigation of the three questions at hand. These include a primary human MC model in which silencing of MRGPRX2 *via* DsiRNA electroporation is coupled to flow cytometric analysis (17). In this model, the introduction of *MRGPRX2*-targeting DsiRNA (MRGPRX2-DsiRNA) almost completely mitigates intracellular calcium elevations and/or degranulation in response to MRGPRX2 ligands such as the opiate morphine, the FQ moxifloxacin, and the NMBA rocuronium (17). Here, we made use of these novel instruments to address important and unanswered questions on the role of MRPRX2 in NMBA- and FQ-induced IDHRs.

MATERIALS AND METHODS

Generation of Human Peripheral Blood-Derived Cultured Mast Cells (PBCMCs)

Human PBCMCs were generated as described previously (18). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from blood samples of healthy donors or rocuronium-hypersensitive patients using Histopaque. Next, $CD34^+$ progenitors were isolated from PBMCs (EasySep Human CD34 Selection Kit, Stemcell Technologies) and cultured, for 4-5 weeks, in serum-free methylcellulose-based medium (MethoCult SF H4236, Stemcell Technologies) supplemented with penicillin (100 units/mL, Life Technologies), streptomycin (100 µg/mL, Life Technologies), low-density lipoprotein (LDL, 10 µg/mL, Stemcell Technologies), 2-mercaptoethanol (55 µmol/L, Life Technologies), stem cell factor (SCF, 100 ng/mL, Miltenyi Biotec), interleukin-3 (IL-3, 100 ng/mL, PeproTech) and interleukin-6 (IL-6, 50 ng/mL, Miltenyi Biotec). PBCMCs harbor a $MRGPRX2^+$ and a $MRGPRX2^-$ subpopulation (**Supplementary Figure 1**). Each PBCMC culture yielded 75 ± 5% $MRGPRX2^+$ cells (n=15).

Assessment of Mast Cell Activation by Intracellular Calcium Staining

For intracellular calcium staining, PBCMCs at a concentration of $5x10^5$ cells/mL, were loaded with 1 µM Fluo-4 AM (ThermoFisher

Abbreviations: BAT, Basophil activation test; DsiRNA, dicer small interference RNA; FcεRI, High affinity receptor for IgE; FMO, fluorescence minus one; FQ(s), fluoroquinolone(s); IDHR(s), immediate drug hypersensitivity reaction(s); IDT(s), intradermal test(s); IgE, Immunoglobulin E; IL, interleukin; LDL, low-density lipoprotein; MC(s), mast cell(s); MRGPRX2, mas-related G protein coupled receptor X2; NMBA(s), neuromuscular blocking agent(s); PBCMC(s), peripheral blood cultured mast cell(s); PBMC(s), peripheral blood mononuclear cell(s); S-A-Cx, sugammadex-atracurium inclusion complex; SCF, stem cell factor; SEM, standard error of the mean; sIgE, specific IgE antibody; SPT(s), skin prick test(s).

Scientific) for 45 min at 37°C, washed with phosphate-buffer saline (PBS, ThermoFisher Scientific), and resuspended in 300 µL pre-warmed (37°C) Tyrode's buffer (Sigma-Aldrich). The intensity of Fluo-4 AM was measured at a single cell level for 50 seconds without stimulation. Next, the cells were stimulated with Tyrode's buffer as negative control, substance P (74 µM, Sigma Aldrich) as positive control for the MRGPRX2 pathway, anti-FcεRI as a positive control for the IgE-pathway (2.5 µg/mL, ThermoFisher Scientific), succinylcholine (Celocurine®, CSP Benelux), atracurium (Tracrium®; Aspen Pharma), ciprofloxacin (Bayer) or levofloxacin (Fresenius Kabi), followed by immediate further reading for 2 min. Dose-response experiments (**Supplementary Figure 2**) were done with succinylcholine (27.7 µM, 277 µM, 1384 µM, 2768 µM and 5536 µM), atracurium (10.8 µM, 108 µM, 538 µM, 1076 µM and 2152 µM), ciprofloxacin (15.1 µM, 151 µM, 755 µM and 1510 µM) and levofloxacin (13.8 µM, 138 µM, 692 µM, 1384 µM and 2767 µM). Optimal stimulation concentrations were 5536 µM for succinylcholine, 2152 µM for atracurium, 755 µM for ciprofloxacin (1510 µM appeared to be cytotoxic) and 2767 µM for levofloxacin. Combining the staining of MRGPRX2 and intracellular calcium comprised first staining of the membrane expression of MRGPRX2 for 20 min at 4°C, after which the cells were washed with PBS and stained with Fluo-4 AM according to the protocol described above.

Assessment of Mast Cell Degranulation by CD63 Up-Regulation

For CD63 measurements, PBCMCs, defined as $CD117^+$ and $CD203c^+$ cells, were dissolved in pre-warmed (37°C) Tyrode's buffer at a concentration of $5x10^5$ cells/mL. Next, 100 µL of the cells were stimulated with 100 µL tyrode buffer, anti-FcεRI, substance P, succinylcholine, atracurium, ciprofloxacine or levofloxacin, for 3 and 20 min at 37°C. Reactions were stopped by placing the cells on ice. Subsequently, supernatants were removed by centrifugation (500 x g, 4°C, 5 min). Cells were stained with anti-human CD117-APC (clone 104D2, BD Biosciences), anti-human CD203c-PECy7 (clone NP4D6, eBioscience), anti-human MRGPRX2-PE (clone K125H4, BioLegend) and anti-human CD63-FITC (clone H5C6, BD Biosciences) for 20 min at 4°C. Next, cells were fixed with 1 mL Phosflow Lyse/Fix buffer (BD Biosciences) for 20 min. Finally, cells were washed and resuspended in PBS with 0.1% sodium azide and measured using flow cytometry.

MRGPRX2 Silencing by DsiRNA Electroporation

PBCMCs at a concentration of $1x10^6$ cells/mL were washed twice in cold serum-free Opti-MEM I medium (Gibco Invitrogen) and resuspended in 200 µL of the same medium. Cells were transferred to a 4.0-mm electroporation cuvette (Cell Projects), and 1 µM pool of two DsiRNA against *MRGPRX2* at a 1:1 ratio or a non–targeting control DsiRNA (Integrated DNA Technologies, Catalog #:51-01-14-03) were added to the cuvette (Duplex sequences: DsiRNA 1: 5'-GGCAUUCAGUGGUUCCUAAUAU UAT-3' and 3'-AACCGUAAGUCACCAAGGA UUAUAAUA-5', DsiRNA 2: 5'GUUACGUGUUCCA CAGAAUAAAATA-3' and 3'-UUCA AUGCACAAGGUGUCUUAUUUUAU-5'). A square wave protocol (500 V, 5 ms, 0 gap, 1 pulse) was used to electroporate the cells (Gene Pulser Xcell™ device, Bio-Rad Laboratories). Immediately after electroporation, cells were transferred to 5 mL of IMDM medium (Thermofischer Scientific) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) (pre-heated at 37°C) and incubated for 20 min at 37°C and 5% CO_2. Thereafter, cells were centrifuged and transferred to IMDM medium with SCF and IL-6. Five days after electroporation, a repeated analysis of MRGPRX2 expression and functionality of the PBCMCs was performed as described above.

Sugammadex-Atracurium Inclusion Complex (S-A-Cx) Experiments

To investigate the effects of sugammadex on MRGPRX2-mediated activation of MCs induced by atracurium, cells were stimulated with atracurium and sugammadex (Bridion®; MSD), alone or together (designated as S-A-Cx 1, 2 and 3), in equimolar concentrations (atracurium: 538 µM, 1076 µM and 2152 µM; sugammadex: 551 µM, 1102 µM and 2204 µM). S-A-Cx 1 consisted of 538 µM atracurium and 551 µM sugammadex, S-A-Cx 2 of 1076 µM atracurium and 1102 µM sugammadex, and S-A-Cx 3 of 2152 µM atracurium and 2204 µM sugammadex. Based on the known affinity of sugammadex for atracurium, we estimated the remaining free atracurium concentrations of S-A-Cx 1, 2, and 3 to be 263 µM, 407 µM, and 612 µM, respectively. Therefore, outcomes from the S-A-Cx analyses were compared with results obtained in cells exposed to the corresponding free atracurium concentration. The activation and degranulation were studied using both intracellular calcium staining and CD63-upregulation, according to the protocol described above.

In Vivo and *Ex Vivo* Testing for Responses to Rocuronium

We assessed eight patients with previous IDHRs and rocuronium hypersensitivity was documented by skin testing, as previously described (19). Their PBCMCs were investigated for MRGPRX2 levels and function. Skin tests were done with rocuronium (Esmeron®; Merck Sharp and Dohme, Brussels, Belgium), saline buffer as a negative control and histamine as a positive control (10 mg/mL; HAL Allergy Benelux NV, Haarlem, The Netherlands), first as skin prick tests (SPTs) and, if negative, by intradermal tests (IDTs). The maximal non-irritating rocuronium concentration was 10 mg/mL for SPTs and 0.05 mg/mL for IDTs. Rocuronium was diluted immediately before use. SPTs with a wheal ≥ 3 mm with surrounding erythema after 15 minutes were considered positive. For IDTs, injection of 0.02 mL was performed, and reactions were read after 20-30 minutes. IDT responses with a wheal surrounded by an erythema ≥ 8 mm (or doubling compared to injection bleb) were considered positive. The direct basophil activation test (BAT) for rocuronium using the patients' basophils was performed as described (20). Results were expressed as the net percentages of $CD63^+$ basophils, and the threshold of positivity was set at 4% (20). Total IgE and specific IgE (sIgE) to morphine were quantified by the FEIA ImmunoCAP system (Phadia Thermo Fisher, Uppsala, Sweden) according to the manufacturer's instructions (21). As shown in **Table 1**, patients were stratified by the results of their rocuronium basophil test and/or levels of specific IgE as

positive (i.e. IgE-dependent, patients 1-4) and negative (i.e. non IgE-dependent and probably MRGPRX2-mediated, patients 5-8). MRGPRX2 expression and functionality of their PBCMCs were compared between the two groups (16).

PBCMCs of rocuronium-hypersensitive patients were functionally studied using intracellular calcium staining and CD63 upregulation as described above. PBCMCs were stimulated with tyrode buffer, substance P (74 μM), anti-FcεRI (0.5, 2.5 and 5.0 μg/mL), amoxicillin (1370 μM, GSK), morphine hydrochloride (50 μM, 250 μM and 500 μM, Sterop), or rocuronium bromide (1.64 μM, 16.4 μM, 164 μmM, 1640 μM; Esmeron®, Organon). Dose response testing was performed as previously described (17).

Flow Cytometric Analyses

Flow cytometric analyses were performed on a calibrated FACSCalibur (BD Immunocytometry systems) with argon-ion lasers (488nm and 633nm), for the intracellular calcium measurements, or on a calibrated FACSCanto II flow cytometer (BD Immunocytometry Systems, San Jose, CA) equipped with three lasers (405 nm, 488 nm and 633 nm), for the CD63 measurement. Correct compensation settings were performed using BD CompBeads (BD Biosciences). Flow cytometric data were analyzed using Kaluza Analysis 2.1 software (Beckman Coulter) and FCS6 Express 6 flow research edition (*de novo* software, Glendale, California). A fluorescence minus one (FMO) sample was used to set a marker between positive and negative cells according to the 99th percentile. The results of the calcium measurements were expressed as fold increase against the basal intensity. Results of CD63 measurements were expressed as the net value of percentages of positive cells, i.e. the percentage of CD63$^+$ cells in stimulated cells minus the percentage of CD63$^+$ cells in resting cells. Al least 500 PBCMCs were counted per sample.

Statistical Analysis

GraphPad Prism version 7 software was used for data analysis and paired Student's t-tests were performed. Results are expressed as mean ± SEM. A P-value of < 0.05 was considered significant. The "n" in the figures denotes the total number of different donors used.

TABLE 1 | Outcome of rocuronium skin and basophil activation tests as well as levels of rocuronium-specific IgE in patients with immediate hypersensitivity reactions to rocuronium.

Patient	Rocuronium response assessed			Total IgE
	Skin test positivity	BAT	sIgE*	
1	SPT	pos	neg/0.5	46.5
2	SPT	pos	0.3/3.2	58
3	IDT (0.01 mg/mL)	NR	0.4/2.1	157
4	IDT (0.05 mg/mL)	NR	10/92.7	1118
5	SPT	neg	neg	213
6	IDT (0.05 mg/mL)	neg	neg	193
7	IDT (0.05 mg/mL)	neg	neg	26
8	IDT (0.05 mg/mL)	neg	neg	455

*Skin tests were done with rocuronium (10 mg/mL), saline buffer as a negative control and histamine as a positive control (10 mg/mL), first as skin prick tests and, if negative, by intradermal tests. SPT, skin prick test; IDT, intradermal test; BAT, basophil activation test; sIgE, specific IgE; pos, positive test; neg, negative test; NR, non-responder; total IgE is expressed as kU/L; * data provided are for specific IgE, in kUA/L, to rocuronium/morphine, with thresholds for positivity: > 0.13 kUA/L (21)/>0.35 kUA/L. Specific IgE to morphine is a marker for sensitization to tertiary and quaternary ammonium structures (21–23).*

Ethical Considerations

Our study was approved by the Ethical Committee of the Antwerp University Hospital (Belgium B300201837509). All participants gave written informed consent.

RESULTS

Ciprofloxacin, Levofloxacin, and Atracurium, but Not Succinylcholine, Induce Activation and Degranulation of PBCMCs *via* MRGPRX2

The FQs ciprofloxacin and levofloxacin potently activated PBCMCs, with a rapid increase in intracellular calcium (**Figure 1A** and **Supplementary Figure 3**). They also induced degranulation, with upregulated surface expression of the lysosomal degranulation marker CD63 (**Figure 1B**). Of the two NMBAs tested, atracurium, but not succinylcholine, induced significant activation of PBCMCs as demonstrated by increase in intracellular calcium (**Figure 1A** and **Supplementary Figure 3**), and degranulation, i.e. upregulation of CD63 (**Figure 1B**). EC50 values are displayed in **Table 2**.

Only PBCMCs that expressed MRGPRX2, but not MRGPRX2-negative PBCMCs, showed significant activation by ciprofloxacin, levofloxacin, and atracurium, as assessed by calcium flux (**Figure 2A** and **Supplementary Figure 4**) as well as degranulation (**Figure 2B**). Moreover, degranulation of MRGPRX2-positive PBCMCs was most pronounced in cells with the highest surface expression of MRGPRX2 (**Figure 2B**). Silencing of MRGPRX2 in PBCMCs, by treatment with MRGPRX2-DsiRNA, reduced MRGPRX2 expression by more than 70% and prevented PBCMCs activation and degranulation by ciprofloxacin, levofloxacin, and atracurium (**Figure 3** and **Supplementary Figures 5**–**7**).

Sugammadex Attenuates Atracurium-Induced Activation and Degranulation of PBCMCs

Atracurium dose-dependently activated and degranulated PBCMCs, *via* MRGPRX2, whereas sugammadex did not cause PBCMC activation or degranulation (**Figure 4** and **Supplementary Figure 4**). Complexing of atracurium with sugammadex, at a 1:1 molar ratio, markedly reduced, but did not completely eliminate the activation and degranulation of PBCMCs (**Figure 4**). Degranulation by free atracurium, induced at the concentrations achieved by complexing with sugammadex, was comparable to that induced by the sugammadex-atracurium complexes (**Figure 5**).

Rocuronium Causes Similar Activation and Degranulation of PBCMCs From Rocuronium-Hypersensitive Patients Who Have Specific IgE and Those Who *Do Not*

PBCMCs obtained from patients with rocuronium hypersensitivity, i.e. a history of rocuronium-induced IDHR, showed similar levels of activation and degranulation in patients who had specific IgE and/

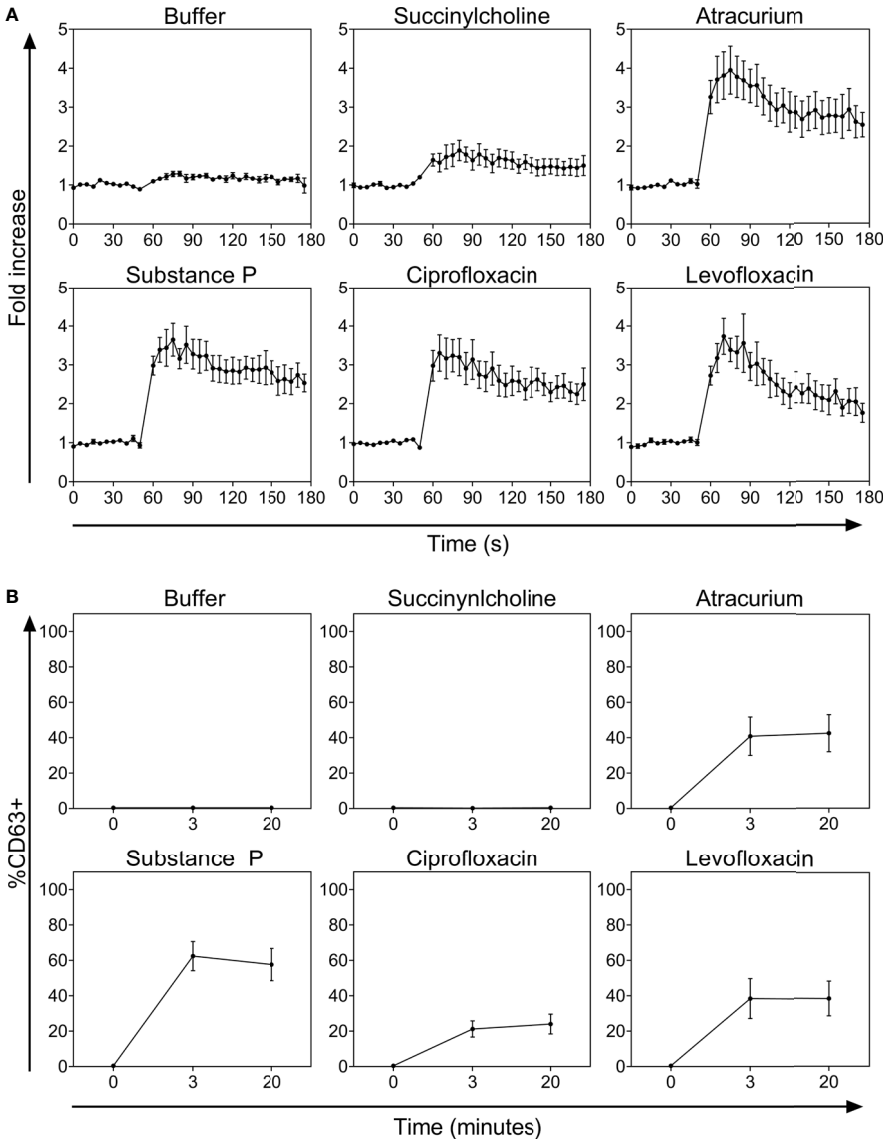

FIGURE 1 | **(A)** Calcium staining and **(B)** time curves of CD63 up-regulation after incubation with buffer, the natural ligand of MRGPRX2 substance P (74 µM), succinylcholine (5536 µM), atracurium (2152 µM), ciprofloxacin (755 µM) or levofloxacin (2767 µM). In all experiments, n=5. Fold increase is calculated against the basal intensity. s, seconds.

TABLE 2 | EC50 values.

	EC50 (µM)
Atracurium	391 µM
Ciprofloxacin	319 µM
Levofloxacin	1557 µM

or a positive BAT to rocuronium as compared to those who did not (**Figures 6** and **7**). Rocuronium-hypersensitive patients with and without specific IgE and/or a positive BAT to rocuronium had similar rates of MRGPRX2-positive cells, 65 ± 11% and 63 ± 4%, respectively (**Supplementary Figure 8**).

DISCUSSION

Here, we answer three important questions on the role and relevance of the MRGPRX2 pathway in MC-dependent immediate hypersensitivity reactions to NMBAs and FQs (11, 15, 24, 25). We show 1) that atracurium, ciprofloxacin and levofloxacin degranulate human MCs *via* MRGPRX2, 2) that sugammadex attenuates atracurium-induced and MRGPRX2-mediated MC degranulation by reducing free atracurium, and 3) that rocuronium-hypersensitive patients with and without sensitization to this NMBA show similar mast cell MRGPRX2 expression and function. These findings help our understanding

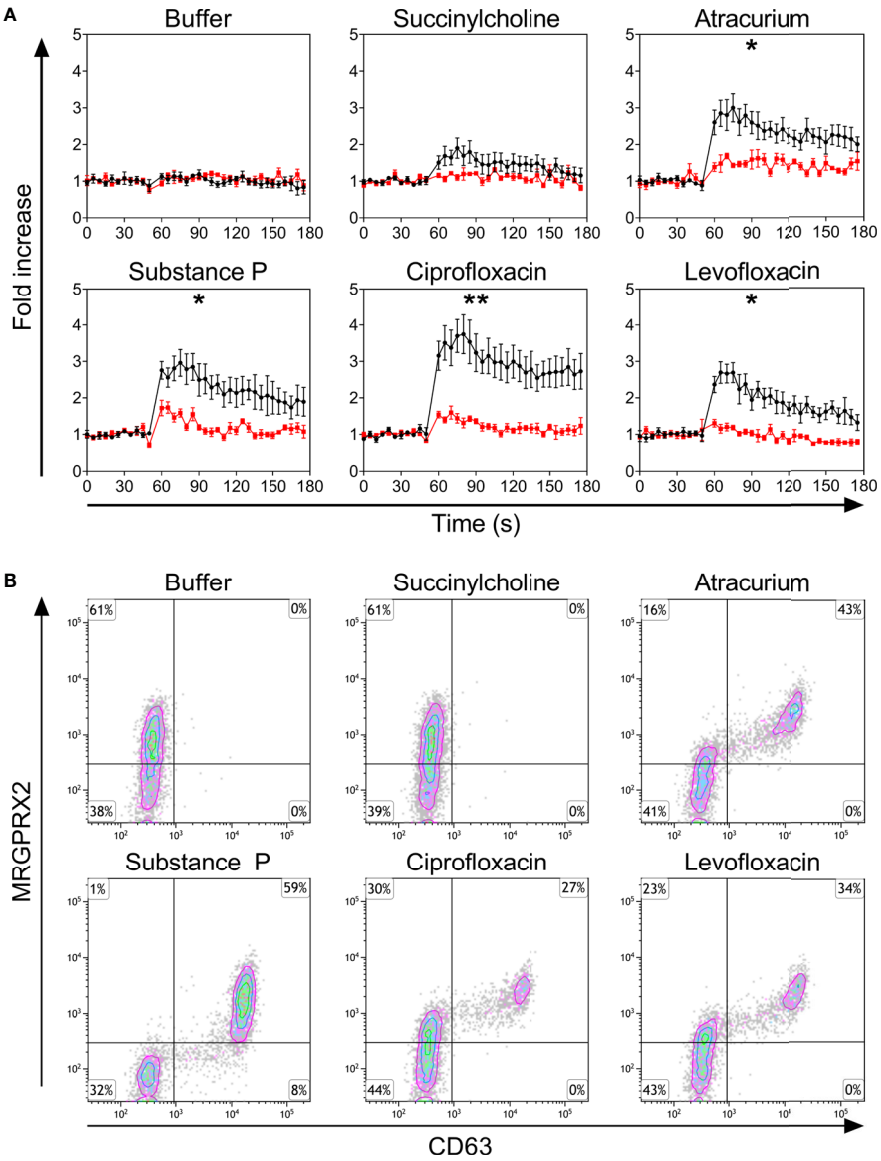

FIGURE 2 | **(A)** Calcium imaging in the MRGPRX2$^+$ (black) and MRGPRX2$^-$ (red) subpopulations and **(B)** up-regulation of surface CD63. **(A)** Calcium staining and **(B)** representative plots for CD63 up-regulation after incubation with buffer, the natural ligand of MRGPRX2 substance P (74 μM), succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM). For the representative plot, PBCMCs were incubated for 3 min. In all experiments, n=5. Fold increase is calculated against the basal intensity. Area under the curves were compared using a paired student t-test, $p < 0.05$*, $p < 0.01$**. s, seconds.

and may improve the prevention of anaphylactic reactions to NMBAs and FQs.

Our results of studies with primary human MCs show that degranulation in response to atracurium, ciprofloxacin and levofloxacin is restricted to the MRGPRX2$^+$ subpopulation and that these responses can be effectively mitigated by silencing of MRGPRX2. In contrast, succinylcholine did not degranulate human MCs. These observations are in line with the results of previous studies with murine cells and human MC lines (11, 14, 25, 26). Our results also confirm that selective MRGPRX2 silencing in primary human MCs *via* electroporation of DsiRNA against *MRGPRX2* enables studying the effects of receptor activation on both calcium mobilization and degranulation (17). As shown previously, this silencing approach has a high transfection efficiency and results in markedly downregulated cell surface levels of MRGPRX2 (17). Combined with the novel flow cytometric technique we used in this study, this approach enables analyses at the single cell level, which allows for the identification and characterization of cellular subsets in non-homogeneous populations, such as PBCMCs with MRGPRX2 positive and negative subsets. This also explains why traditional mediator release tests, which only provide averaged results across all cells,

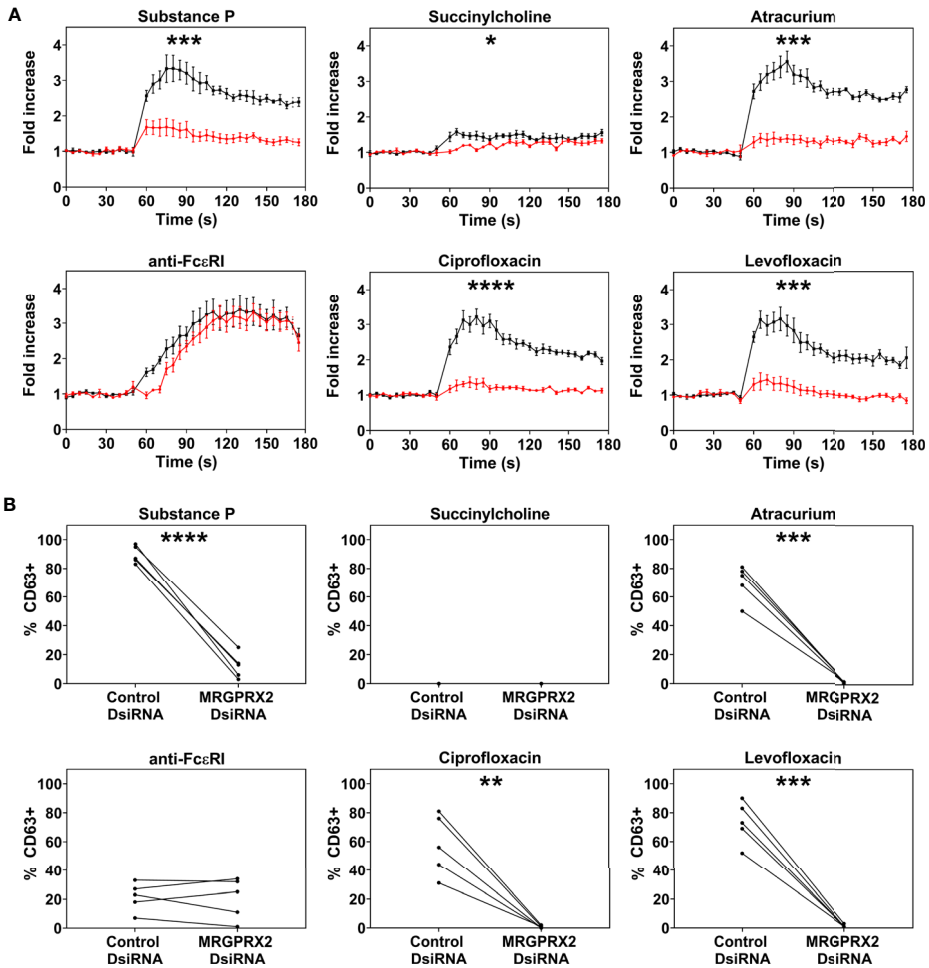

FIGURE 3 | Effect of MRGPRX2 silencing on PBCMC functionality after *MRGPRX2*-specific DsiRNA electroporation. PBCMCs were electroporated with a negative control DsiRNA (black) or a *MRGPRX2*-specific DsiRNA (red). **(A)** Effect of the silencing on the calcium levels and **(B)** CD63 up-regulation after 3 min of incubation. Incubation with substance P (74 μM), the natural agonist of MRGPRX2, anti-FcεRI (2.5 μg/mL), succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM). In all experiments, n=5. Area under the curves were compared using a paired student t-test, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, $p < 0.0001$****. Note that the effect of MRGPRX2 silencing align with the differences observed between MRGPRX2$^+$ and MRGPRX2$^-$ cells as shown in **Figure 1B** and **Supplementary Figure 4**. As shown previously, MRGPRX2 silencing has no significant effect on anti-FcεRI-dependent intracellular calcium signalling and degranulation (17). s, seconds.

are of limited use for the characterization of MRGPRX2-mediated responses in human MCs.

How does sugammadex, a NMBA reversal agent, prevent atracurium-induced and MRGPRX2-induced adverse reactions? Earlier work with leukemic LAD-2 cells demonstrated that co-incubation of atracurium with sugammadex, in molar excess, can inhibit atracurium-induced MRGPRX2-mediated calcium mobilization and degranulation (14). Our findings from studies with primary human PBCMCs confirm that this is indeed the case. More importantly, our study shows that sugammadex reduces MRGPRX2 responses by sequestration of free atracurium. Sugammadex-atracurium complexes, by themselves, are not inhibitory. While this is interesting, mechanistically, it may be of limited clinical relevance, as recent reports suggest that sugammadex does not stop atracurium-induced basophil and MC degranulation, once initiated (14, 19). This argues against the use of sugammadex in patients with ongoing atracurium-mediated perioperative anaphylaxis (27).

That NMBAs induced MRGPRX2-related IDHRs occur only in some patients and not most or all has been suggested to be due to higher levels of MRGPRX2 expression or susceptibility to activation in the former (10, 15). Our findings suggest that this is probably not the case. The mast cells of rocuronium-hypersensitive patients without specific IgE to rocuronium and a negative BAT, i.e. patients with IgE-independent IDHRs to rocuronium, had the same levels of MRGPRX2 expression and the same susceptibility to MRGPRX2 activation as rocuronium-sensitized patients, i.e. patients with IgE-dependent IDHRs to rocuronium (16). Further

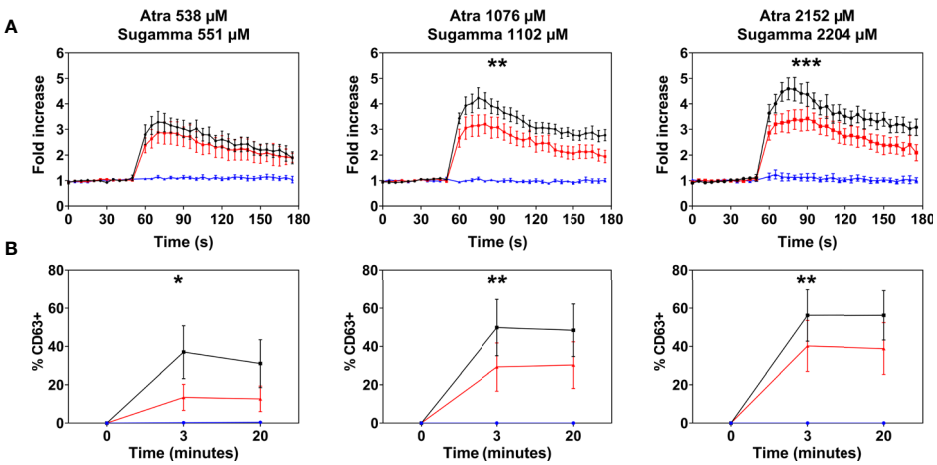

FIGURE 4 | Effect of sugammadex on PBCMC activation induced by atracurium. **(A)** Intracellular calcium staining or **(B)** CD63 upregulation of PBCMCs after incubation with atracurium (538 µM, 1076 µM or 2152 µM) (black), sugammadex (551 µM, 1102 µM or 2204 µM) (blue) or the S-A-Cx in equimolar concentrations (red). In all experiments, n=5. Area under the curves were compared using a paired student t-test, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***, $p < 0.0001$***. s, seconds.

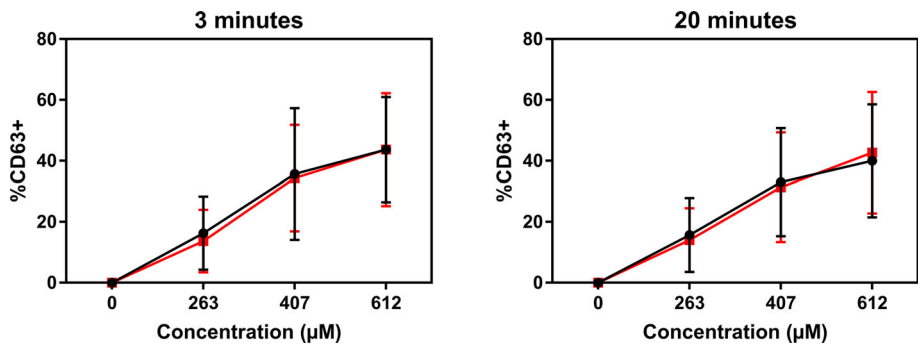

FIGURE 5 | Sugammadex-atracurium complexes (S-A-Cx) and corresponding free atracurium show similar mast cell degranulation. CD63 upregulation of PBCMCs after incubation for 3 or 20 minutes with the S-A-Cx (red) or the corresponding estimated concentrations of free atracurium (black) (263 µM, 407 µM or 612 µM). In all experiments, n=3.

studies aimed at identifying those at risk of rocuronium IDHRs should focus on alternative mechanisms (15). Our results come with two caveats. First, we analyzed patient PBCMCs rather than skin mast cells (there are some differences) (10), and second, our conclusions are based on the assumption that positive sIgE and BAT results are indicative for an IgE-dependent reaction and that the absence of sIgE and a negative BAT point to a MRGPRX2-dependent reaction. There is, currently, no validated assay to test patients for MRGPRX2-dependent anaphylaxis. Exclusion of other mechanisms, i.e. IgE-mediated activation, by sIgE and BAT is the best approach to identifying patients with probably MRGPRX2-dependent reactions (16, 28). Unlike MCs, resting circulation basophils barely express MRGPRX2 (29, 30) and have repeatedly been shown not to respond in a non-specific manner to potent MRGPRX2 agonists such as opiates (e.g. morphine, codeine and pholcodine) (31, 32), FQs (e.g. moxifloxacin, ciprofloxacin, levofloxacin) (6, 33) and certain NMBAs (e.g. atracurium, mivacurium) (3, 34, 35). Positive CD63-BAT results together with sIgE and wortmannin inhibition experiments point to patients with specific sensitization and IgE-dependent reactions (32, 33, 36).

Our study has several strengths and limitations. Its major strength is that we assessed MRGPRX2, across all three questions we addressed, with novel and powerful models and techniques including primary human MCs generated from peripheral blood, silencing of MRGPRX2 *via* DsiRNA electroporation, and single cell flow cytometric analyses. Its limitations include the relatively low number of patients analyzed, which did not allow for meaningful comparisons of subpopulations of patients, owed largely to the rare occurrence of NMBA-mediated IDHRs. Another limitation of our approach, as in many *in vitro* and *ex vivo* experiments, lies in the necessity of supra-therapeutic (sometimes near toxic) stimulation concentrations.

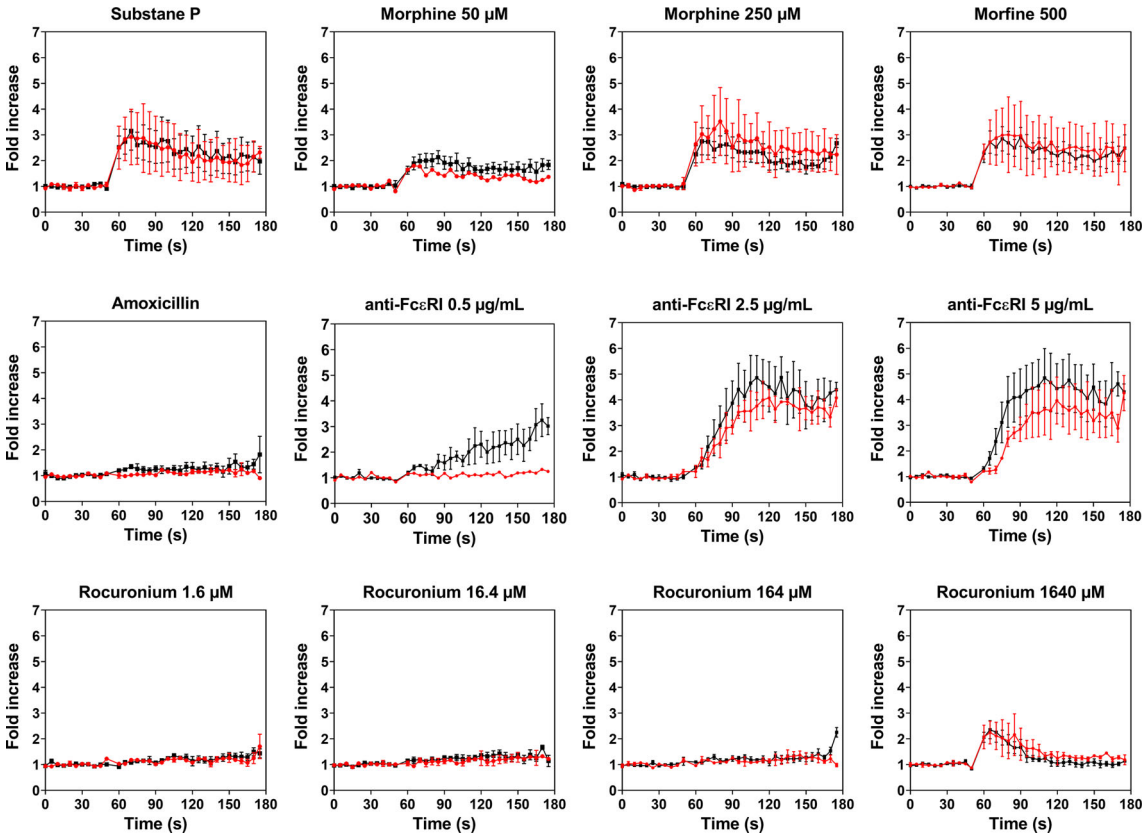

FIGURE 6 | Calcium imaging of PBCMCs derived from patients with IgE-mediated (red) and non IgE-mediated, i.e. probably MRGPRX2-mediated, rocuronium hypersensitivity (black). Incubation with buffer, the positive control substance P (74 µM), the natural agonist of MRGPRX2, anti-FcεRI (0.5 µg/mL, 2.5 µg/mL or 5 µg/mL), amoxicillin (1370 µM), morphine (50 µM, 250 µM or 500 µM), or rocuronium (1.6 µM, 16.4 µM, 164 µM or 1640 µM). Fold increase is calculated against the basal intensity. N= 2 or 4. s, seconds.

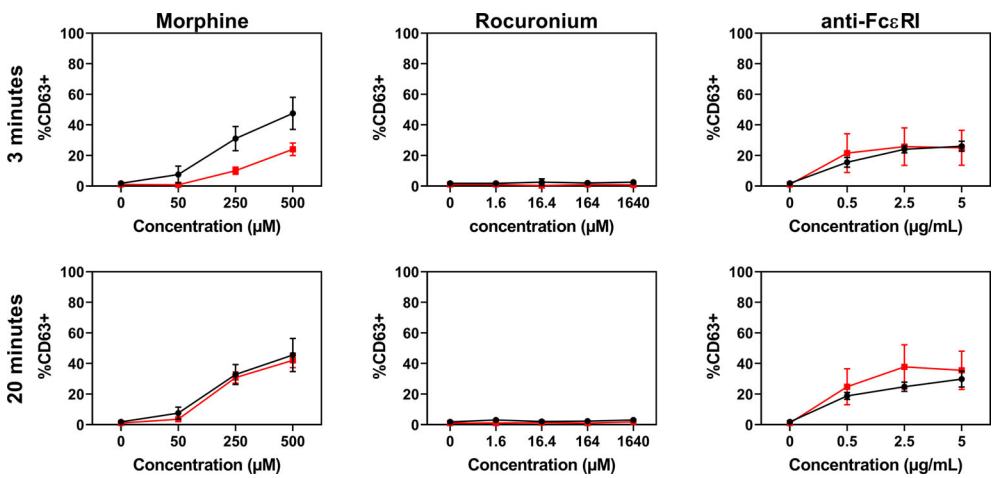

FIGURE 7 | Dose-response curves for CD63 expression in PBCMCs of patients with IgE-mediated (red) and non IgE-mediated, i.e. probably MRGPRX2-mediated, rocuronium hypersensitivity (black). Dose-response curves of CD63 up-regulation after 3 min of stimulation or after 20 min of stimulation. N=4.

Taken together, this is the first study to show, in primary human MCs, that the NMBA atracurium as well as the FQs ciprofloxacin and levofloxacin induce degranulation *via* MRGPRX2, that sugammadex attenuates atracurium-induced MRGPRX2-activation and downstream MC degranulation by reducing free atracurium, and that MCs of rocuronium-hypersensitive patients with IgE-independent IDHRs to this NMBA are not different in their MRGPRX2 expression and function as compared to patients with IgE-dependent IDHRs. We also conclude that the use of PBCMCs together with *MRGPRX2*-targeted silencing can contribute to answer further questions on the role and relevance of MRGPRX2 in IDHRs and beyond (37). Our results should encourage such studies, as a better understanding of the MRGPRX2 pathway is needed to improve the prevention and treatment of anaphylactic reactions to NMBAs and FQs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article are available on request from the corresponding author.

ETHICS STATEMENT

Our study was approved by the Ethical Committee of the Antwerp University Hospital (Belgium B300201837509). All participants gave written informed consent. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JE performed all experiments and wrote the paper. The experiments were performed under supervision of CB, CM and MH who also contributed to the experimental design. The electroporation experiments were performed under supervision of VT, EL and DC-D. VS and DE coordinated and supervised the project and wrote the paper. All authors contributed to the article and approved the submitted version.

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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.2021.668962/full#supplementary-material

Supplementary Figure 1 | Representative plot for the MRGPRX2 expression on PBCMCs. Peripheral blood cultured mast cells (PBCMCs) are defined as $CD117^+CD203c^+$ cells. PBCMCs harbor two subpopulations: cells with surface expression of MRGPRX2 ($MRGPRX2^+$) and cells without expression of MRGPRX2 ($MRGPRX2^-$).

Supplementary Figure 2 | Dose-response curves for changes in intracellular calcium and CD63 expression in PBCMCs. **(A)** Dose-response curves of intracellular calcium levels. **(B)** Dose-response curves of CD63 up-regulation after 3 min of stimulation or **(C)** after 20 min of stimulation. Attempts to increase the ciprofloxacin concentration revealed to be cytotoxic.

Supplementary Figure 3 | Representative plots for intracellular calcium imaging in PBCMCs. PBCMCs were, after 50 sec, stimulated with buffer, substance P (74 μM), the natural agonist of MRGPRX2, succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM).

Supplementary Figure 4 | Representative plot for intracellular calcium imaging in $MRGPRX2^+$ **(A)** and $MRGPRX2^-$ **(B)** subpopulations. PBCMCs were, after 50 sec, stimulated with buffer, substance P (74 μM), the natural agonist of MRGPRX2, succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM).

Supplementary Figure 5 | Silencing of the MRGPRX2-receptor after electroporation with *MRGPRX2*-specific DsiRNA. A comparison of the surface expression of MRGPRX2 or FcεRI between PBCMC electroporated with control DsiRNA or DsiRNA specific for *MRGPRX2*. In all experiments, n=5. $p < 0.001$***. As shown earlier, the maximal expression of FcεRI remains unaltered in MRGPRX2 silenced cells (17).

Supplementary Figure 6 | Representative plots of CD63 up-regulation after silencing of *MRGPRX2*. Cells were electroporated with a negative control **(A)** or *MRGPRX2*-specific DsiRNA **(B)**. Thereafter, cells were incubated with buffer, substance P (74 μM), anti-FcεRI (2.5 μg/mL), succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM). MRGPRX2 silencing has no significant effect on anti-FcεRI-dependent intracellular calcium signalling (17).

Supplementary Figure 7 | Representative plots of CD63 up-regulation after silencing of *MRGPRX2*. Cells were electroporated with a negative control **(A)** or target specific DsiRNA **(B)**. Thereafter, cells were incubated for 3 minutes with buffer, substance P (74 μM), anti-FcεRI (2.5 μg/mL), succinylcholine (5536 μM), atracurium (2152 μM), ciprofloxacin (755 μM) or levofloxacin (2767 μM). MRGPRX2 silencing has no significant effect on anti-FcεRI degranulation (17).

Supplementary Figure 8 | MRGPRX2 expression on PBCMCs cultured from patients with an IgE-dependent and non IgE-mediated, i.e. probably MRGPRX2-mediated, rocuronium hypersensitivity reaction. Peripheral blood cultured mast cells (PBCMCs) are defined as $CD117^+CD203c^+$ cells. PBCMCs harbor two subpopulations: cells with surface expression of MRGPRX2 ($MRGPRX2^+$) and cells without expression of MRGPRX2 ($MRGPRX2^-$).

REFERENCES

1. Di Leo E, Delle Donne P, Calogiuri GF, Macchia L, Nettis E. Focus on the Agents Most Frequently Responsible for Perioperative Anaphylaxis. *Clin Mol Allergy* (2018) 16:16. doi: 10.1186/s12948-018-0094-7
2. Leysen J, Bridts CH, De Clerck LS, Vercauteren M, Lambert J, Weyler JJ, et al. Allergy to Rocuronium: From Clinical Suspicion to Correct Diagnosis. *Allergy* (2011) 66:1014–9. doi: 10.1111/j.1398-9995.2011.02569.x
3. Uyttebroek AP, Sabato V, Leysen J, Bridts CH, De Clerck LS, Ebo DG. Flowcytometric Diagnosis of Atracurium-Induced Anaphylaxis. *Allergy* (2014) 69:1324–32. doi: 10.1111/all.12468

4. Van Gasse AL, Elst J, Bridts CH, Mertens C, Faber M, Hagendorens MM, et al. Rocuronium Hypersensitivity: Does Off-Target Occupation of the MRGPRX2 Receptor Play a Role? *J Allergy Clin Immunol Pract* (2019) 7:998–1003. doi: 10.1016/j.jaip.2018.09.034
5. Uyttebroek AP, Sabato V, Bridts CH, De Clerck LS, Ebo DG. Moxifloxacin Hypersensitivity: Uselessness of Skin Testing. *J Allergy Clin Immunol Pract* (2015) 3:443–5. doi: 10.1016/j.jaip.2014.12.012
6. Van Gasse AL, Sabato V, Uyttebroek AP, Elst J, Faber MA, Hagendorens MM, et al. Immediate Moxifloxacin Hypersensitivity: Is There More Than Currently Meets the Eye? *Allergy* (2017) 72:2039–43. doi: 10.1111/all.13236
7. Jones SC, Budnitz DS, Sorbello A, Mehta H. US-Based Emergency Department Visits for Fluoroquinolone-Associated Hypersensitivity Reactions. *Pharmacoepidemiol Drug Saf* (2013) 22:1099–106. doi: 10.1002/pds.3499
8. Elst J, Sabato V, Ebo DG. MRGPRX2 Activation in Mast Cells by Neuromuscular Blocking Agents and Other Agonists: Modulation by Sugammadex by Nithya a. Fernandopulle Et Al. *Clin Exp Allergy* (2021) 51(7):976–7. doi: 10.1111/cea.13837
9. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. Neuropeptides Activate Human Mast Cell Degranulation and Chemokine Production. *Immunology* (2008) 123:398–410. doi: 10.1111/j.1365-2567.2007.02705.x
10. Chompunud Na Ayudhya C, Amponnawarat A, Roy S, Oskeritzian CA, Ali H. MRGPRX2 Activation by Rocuronium: Insights From Studies With Human Skin Mast Cells and Missense Variants. *Cells* (2021) 10(1):156. doi: 10.3390/cells10010156
11. McNeil BD, Pundir P, Meeker S, Han L, Undem BJ, Kulka M, et al. Identification of a Mast-Cell-Specific Receptor Crucial for Pseudo-Allergic Drug Reactions. *Nature* (2015) 519:237–41. doi: 10.1038/nature14022
12. Bom A, Hope F, Rutherford S, Thomson K. Preclinical Pharmacology of Sugammadex. *J Crit Care* (2009) 24:29–35. doi: 10.1016/j.jcrc.2008.10.010
13. de Boer HD, van Egmond J, van de Pol F, Bom A, Driessen JJ, Booij LH. Time Course of Action of Sugammadex (Org 25969) on Rocuronium-Induced Block in the Rhesus Monkey, Using a Simple Model of Equilibration of Complex Formation. *Br J Anaesth* (2006) 97:681–6. doi: 10.1093/bja/ael240
14. Fernandopulle NA, Zhang S, Soeding PF, Mackay GA. MRGPRX2 Activation in Mast Cells by Neuromuscular Blocking Agents and Other Agonists: Modulation by Sugammadex. *Clin Exp Allergy* (2020) 51(5):685–95. doi: 10.1111/cea.13801
15. Porebski G, Kwiecien K, Pawica M, Kwitniewski M. Mas-Related G Protein-Coupled Receptor-X2 (MRGPRX2) in Drug Hypersensitivity Reactions. *Front Immunol* (2018) 9:3027. doi: 10.3389/fimmu.2018.03027
16. Ebo DG, van der Poorten ML, Elst J, Van Gasse AL, Mertens C, Bridts C, et al. Immunoglobulin E Cross-Linking or MRGPRX2 Activation: Clinical Insights From Rocuronium Hypersensitivity. *Br J Anaesth* (2020) 126(1):e27–9. doi: 10.1016/j.bja.2020.10.006
17. Elst J, Sabato V, Faber MA, Bridts CH, Mertens C, Van Houdt M, et al. MRGPRX2 and Immediate Drug Hypersensitivity: Insights From Cultured Human Mast Cells. *J Investig Allergol Clin Immunol* (2020) 30:0. doi: 10.18176/jiaci.0557
18. Cop N, Decuyper II, Faber MA, Sabato V, Bridts CH, Hagendorens MM, et al. Phenotypic and Functional Characterization of *In Vitro* Cultured Human Mast Cells. *Cytometry B Clin Cytom* (2017) 92:348–54. doi: 10.1002/cyto.b.21399
19. Leysen J, Bridts CH, De Clerck LS, Ebo DG. Rocuronium-Induced Anaphylaxis is Probably Not Mitigated by Sugammadex: Evidence From an *In Vitro* Experiment. *Anaesthesia* (2011) 66:526–7. doi: 10.1111/j.1365-2044.2011.06729.x
20. Ebo DG, Bridts CH, Hagendorens MM, Mertens CH, De Clerck LS, Stevens WJ. Flow-Assisted Diagnostic Management of Anaphylaxis From Rocuronium Bromide. *Allergy* (2006) 61:935–9. doi: 10.1111/j.1398-9995.2006.01094.x
21. Ebo DG, Venemalm L, Bridts CH, Degerbeck F, Hagberg H, De Clerck LS, et al. Immunoglobulin E Antibodies to Rocuronium: A New Diagnostic Tool. *Anesthesiology* (2007) 107:253–9. doi: 10.1097/01.anes.0000270735.40872.f2
22. Fisher MM, Baldo BA. Immunoassays in the Diagnosis of Anaphylaxis to Neuromuscular Blocking Drugs: The Value of Morphine for the Detection of Ige Antibodies in Allergic Subjects. *Anaesth Intensive Care* (2000) 28:167–70. doi: 10.1177/0310057X0002800207
23. Laroche D, Chollet-Martin S, Leturgie P, Malzac L, Vergnaud MC, Neukirch C, et al. Evaluation of a New Routine Diagnostic Test for Immunoglobulin E Sensitization to Neuromuscular Blocking Agents. *Anesthesiology* (2011) 114:91–7. doi: 10.1097/ALN.0b013e31820164d2
24. Navinés-Ferrer A, Serrano-Candelas E, Lafuente A, Muñoz-Cano R, Martín M, Gastaminza G. MRGPRX2-Mediated Mast Cell Response to Drugs Used in Perioperative Procedures and Anaesthesia. *Sci Rep* (2018) 8:11628. doi: 10.1038/s41598-018-29965-8
25. Liu R, Hu S, Zhang Y, Che D, Cao J, Wang J, et al. Mast Cell-Mediated Hypersensitivity to Fluoroquinolone is MRGPRX2 Dependent. *Int Immunopharmacol* (2019) 70:417–27. doi: 10.1016/j.intimp.2019.02.001
26. Azimi E, Reddy VB, Shade KC, Anthony RM, Talbot S, Pereira PJS, et al. Dual Action of Neurokinin-1 Antagonists on Mas-Related Gpcrs. *JCI Insight* (2016) 1:e89362. doi: 10.1172/jci.insight.89362
27. Garvey LH, Dewachter P, Hepner DL, Mertes PM, Voltolini S, Clarke R, et al. Management of Suspected Immediate Perioperative Allergic Reactions: An International Overview and Consensus Recommendations. *Br J Anaesth* (2019) 123:e50–64. doi: 10.1016/j.bja.2019.04.044
28. Ebo DG, Bridts CH, Mertens CH, Sabato V. Principles, Potential, and Limitations of *Ex Vivo* Basophil Activation by Flow Cytometry in Allergology: A Narrative Review. *J Allergy Clin Immunol* (2020) 2163:219–26. doi: 10.1016/j.jaci.2020.10.027
29. Sabato V, Elst J, Van Houdt M, Bridts C, Mertens C, Ebo DG. Surface Expression of MRGPRX2 on Resting Basophils: An Area of Controversy. *Allergy* (2020) 75:2421–2. doi: 10.1111/all.14252
30. Elst J, Sabato V, Hagendorens MM, van Houdt M, Faber MA, Bridts CH, et al. Measurement and Functional Analysis of the Mas-Related G Protein-Coupled Receptor MRGPRX2 on Human Mast Cells and Basophils. *Methods Mol Biol* (2020) 2163:219–26. doi: 10.1007/978-1-0716-0696-4_18
31. Van Gasse AL, Hagendorens MM, Sabato V, Bridts CH, De Clerck LS, Ebo DG. IgE to Poppy Seed and Morphine are Not Useful Tools to Diagnose Opiate Allergy. *J Allergy Clin Immunol Pract* (2015) 3:396–9. doi: 10.1016/j.jaip.2014.12.002
32. Leysen J, De Witte L, Sabato V, Faber M, Hagendorens M, Bridts C, et al. IgE-Mediated Allergy to Pholcodine and Cross-Reactivity to Neuromuscular Blocking Agents: Lessons From Flow Cytometry. *Cytometry B Clin Cytom* (2013) 84:65–70. doi: 10.1002/cyto.b.21074
33. Aranda A, Mayorga C, Ariza A, Doña I, Rosado A, Blanca-Lopez N, et al. *In Vitro* Evaluation of IgE-Mediated Hypersensitivity Reactions to Quinolones. *Allergy* (2011) 66:247–54. doi: 10.1111/j.1398-9995.2010.02460.x
34. Monneret G, Benoit Y, Debard AL, Gutowski MC, Topenot I, Bienvenu J. Monitoring of Basophil Activation Using CD63 and CCR3 in Allergy to Muscle Relaxant Drugs. *Clin Immunol* (2002) 102:192–9. doi: 10.1006/clim.2001.5156
35. Abuaf N, Rajoely B, Ghazouani E, Levy DA, Pecquet C, Chabane H, et al. Validation of a Flow Cytometric Assay Detecting *In Vitro* Basophil Activation for the Diagnosis of Muscle Relaxant Allergy. *J Allergy Clin Immunol* (1999) 104:411–8. doi: 10.1016/S0091-6749(99)70386-6
36. Ebo DG, Baldo BA, Van Gasse AL, Mertens C, Elst J, Sermeus L, et al. Anaphylaxis to Sugammadex-Rocuronium Inclusion Complex: An IgE-Mediated Reaction Due to Allergenic Changes at the Sugammadex Primary Rim. *J Allergy Clin Immunol Pract* (2020) 8:1410–5.e3. doi: 10.1016/j.jaip.2019.11.018
37. Kühn H, Kolkhir P, Babina M, Düll M, Frischbutter S, Fok JS, et al. Mas-Related G Protein-Coupled Receptor X2 and Its Activators in Dermatologic Allergies. *J Allergy Clin Immunol* (2021) 147(2):456–69. doi: 10.1016/j.jaip.2019.11.018

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Annexin A1 Mimetic Peptide Ac_{2-26} Modulates the Function of Murine Colonic and Human Mast Cells

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Mast cells (MCs) are main effector cells in allergic inflammation and after activation, they release stored (histamine, heparin, proteases) and newly synthesized (lipid mediators and cytokines) substances. In the gastrointestinal tract the largest MC population is located in the lamina propria and submucosa whereas several signals such as the cytokine IL-4, seem to increase the granule content and to stimulate a remarkable expansion of intestinal MCs. The broad range of MC-derived bioactive molecules may explain their involvement in many different allergic disorders of the gastrointestinal tract. Annexin A1 (AnxA1) is a 37 KDa glucocorticoid induced monomeric protein selectively distributed in certain tissues. Its activity can be reproduced by mimetic peptides of the N-terminal portion, such as Ac_{2-26}, that share the same receptor FPR-L1. Although previous reports demonstrated that AnxA1 inhibits MC degranulation in murine models, the effects of exogenous peptide Ac_{2-26} on intestinal MCs or the biological functions of the Ac_{2-26}/FPR2 system in human MCs have been poorly studied. To determine the effects of Ac_{2-26} on the function of MCs toward the possibility of AnxA1-based therapeutics, we treated WT and IL-4 knockout mice with peptide Ac2-26, and we examined the spontaneous and compound 48/80 stimulated colonic MC degranulation and cytokine production. Moreover, *in vitro*, using human mast cell line HMC-1 we demonstrated that exogenous AnxA1 peptide is capable of interfering with the HMC-1 degranulation in a direct pathway through formyl peptide receptors (FPRs). We envisage that our results can provide therapeutic strategies to reduce the release of MC mediators in inflammatory allergic processes.

Keywords: mast cell (MC), compound 48/80, human MC line (HMC-1), colon explant culture, annexin A1, IL-4, FPRs

INTRODUCTION

Mast cells (MCs) have been identified as the main effector cells for allergic inflammation. They are distributed in specific sites such as skin, blood vessels, respiratory and intestinal mucosa, contributing to host defenses. The classical and most effective mechanism for MC activation is cross-linking of cell-surface bound IgE to its high-affinity receptor (FcεRI) by allergen in acute allergic reactions. After activation, MCs are able to release newly synthesized (lipid mediators and cytokines) and stored (histamine, heparin, proteases) substances which are contained in their cytoplasmic lipid bodies and granules (1–3). In mice, the ability of MCs to respond to particular stimuli, can be modulated by cytokines, growth factors, and other microenvironmental signals. The heterogeneity of MC subpopulations depends on the anatomical site in which they reside, allowing improved flexibility and diversity of responsiveness (4). Considering that MCs complete the differentiation and maturation in target tissues in the presence of local trophic factors such as IL-9, IL-10, IL-3, IL-4, IL-33, CXCL12 (5), the absence of some of these signals could determine differences on the biological activity of these cells.

The normal gastrointestinal tract (GI) contains the largest MC population in the lamina propria of the mucosa and in the submucosa. This amount can increase up to tenfold in the course of many intestinal diseases. Several findings support that the function of MCs in the GI is not limited to the antigen-reactive effector cells, playing a central role in the control of the barrier fitness and the transport properties of the intestinal epithelium (6). Mucosal MCs can respond to both IgE/antigen-dependent and non-IgE-dependent stimulation by a mechanism depending on the release of bioactive mediators into adjacent tissues that leads to physiological responses (7). Recently, it became clear that MC regulation is essential for normal functioning of the bowel tissues (8).

The broad range of MC-derived bioactive molecules may explain their involvement in many different pathological conditions including infections, neoplastic diseases as well as many types of allergic disorders of the GI (9). Studies in hypersensitivity and stress models have shown that alterations in mucosal function are attributable to either direct action on epithelial receptors by MC mediators and/or indirect action by neurotransmitters (10). IL-4 is well known as a Th2 cell differentiation promoter although some role in directing Th1 responses has been claimed (11). Cells of several lineages produce IL-4, including CD4 and CD8 T cells, NKT cells, eosinophils, MCs and basophils. IL-4 has been shown not only to elicit the FcεRI expression but also to increase the granule content (12). While several reports found normal/similar numbers of MCs in IL-4-deficient mice other authors demonstrated that the cytokine stimulates a remarkable expansion of intestinal MCs from enhanced signaling through the IL-4Rα chain in rodent model of food allergy (13). Remarkably, differences in the effects of the compound 48/80 have been detected studying humoral immunity in WT mice and MC deficient mice (14).

Annexin A1 (AnxA1) is a 37 KDa glucocorticoid induced monomeric protein with selective distribution in certain tissues. It is synthetized in some immune cells, mainly myeloid cells including macrophages, MCs, eosinophils, and neutrophils, beyond the neuroendocrine system (15–17). Glucocorticoids not only stimulate AnxA1 transcription, but also induce the release into the cytoplasm of its pre-existing forms *via* a receptor-dependent, non-genomic pathway, preceded by phosphorylation at key sites in the N-terminus and other sites (18). The activity of this protein can be reproduced by mimetic peptides of the N-terminal portion (19) including Ac_{2-26}, that have been investigated in many acute (20, 21), chronic (17, 22) and systemic (23) inflammation models. AnxA1 and its active derived peptide Ac_{2-26} share the same receptor FPR-L1 (FPR2/ALX in man) which inhibits cell activation in an autocrine or paracrine pathway (24).

AnxA1 induces the expression of IL-4 and IL-10 in inflammatory conditions (25), two cytokines that play an important role in the regulation of peripheral intestinal tolerance (26, 27). Also, pro-inflammatory cytokines such as IL-6 and TNF-α may induced the AnxA1 protein expression (28). Our research group showed that the induction of peritonitis promotes both reorganization of cytoplasmic granules and *de novo* synthesis of AnxA1, with important effects in the regulation of the inflammatory infiltrate and cytokine production in the mesentery (16).

We have reported the localization of AnxA1 in connective tissue MCs, and that its expression is susceptible not only to glucocorticoid treatment, but also to acute inflammatory response (29). Moreover, previous study demonstrated that AnxA1 inhibits MC degranulation in rodent models (30). However, relatively little is known regarding on the effects of AnxA1 on the biological functions of human MCs and more studies are required to comprehend the effects of exogenous AnxA1/FPR2 system.

While cell lines derived from tumor tissues differ from normal primary MCs, valuable information can be obtained from the HMC-1 cell line derived from a MC leukaemia patient. Although HMC-1 cells are immature, they display many primary characteristics of tissue MCs, such as histamine, heparin, β-hexosaminidase and tryptase expression (31–33). Based on these data, HMC-1 cells are suitable platform for the study of human MC degranulation in acute response (34).

Building upon these observations, we considered the possibility that the effect of Ac_{2-26} peptide could be specific in WT and IL-4 knockout mice. Considering that type 2 responses are implicated in several aspects of gut homeostasis but also in chronic intestinal inflammation, we examined the spontaneous and acute stimulated colonic MC degranulation and cytokine production under Ac_{2-26} treatment in both mouse strains. Moreover, using the HMC-1 cell line we demonstrated that exogenous AnxA1 peptide is capable to interfere with MC degranulation triggered by acute stimulation through formyl peptide receptors (FPRs).

MATERIAL AND METHODS

Animals

All animal experiments were performed in accordance with the Argentina animal welfare legislation and approved by the

Committee on the Ethics of Animal Experiments from Cordoba National University (resolution 1412/2012). Six- to eight-week-old male C57BL/6 wild type (WT) and C57BL/6 interleukin-4-knockout (IL-4 KO) mice maintained under specific-pathogen-free conditions were used for all experiments. The animals were housed with a 12-h light–dark cycle and were allowed food and water ad libitum.

Treatment Protocol

The effects of Ac_{2-26} N-terminal peptide of AnxA1 (Ac-AMVSEFLKQAWFIENEEQEYVQTVK- Invitrogen, USA) were determined by intraperitoneal administration of 1 mg/kg peptide (23). As control, animals received phosphate-buffered saline (PBS). For experiments in **Figures 1–3**, 32 male mice were used, being 16 WT and 16 IL4- KO; experiments were performed twice with 4 mice/group. All mice were euthanized for colon extraction. The organ was weighed and measured and, afterwards, divided into four fragments of 1cm for histological analysis and *ex vivo* assays, respectively.

Histological Analysis

Colonic fragments were opened longitudinally along the entire length and after macro dissection, were fixed in 10% buffered paraformaldehyde for 24 h for histological processing. Fragments were dehydrated in graded ethanol and embedded in paraffin. Serial longitudinal 4µm sections were obtained using microtome (Leica Biosystems, RM2265, Wetzlar, German) and stained with 0.5% Toluidine Blue (Sigma, St. Louis, MO, USA) for MC analysis based on their morphological characteristics. The average number of MC was calculated and recorded. Values are expressed as the mean ± SD of the number of cells per 0.1 mm^2. Sections were analyzed on an Axioskop 2-mot plus Zeiss microscope (Carl Zeiss, Jena, Germany).

Explant Culture

Colon fragments were used for explant culture as follows: tissues were transferred to 48-wells plates containing Roswell Park Memorial Institute medium (RPMI, Gibco/Thermo Fischer, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 1% gentamicin and L-glutamine. Tissues were cultivated with 3µM AnxA1 peptide and 20µg/mL compound 48/80 (Sigma, St. Louis, MO, USA) in a humidified incubator at 5% CO_2 during 24 h according to (35). After incubation supernatants were collected and 20µg/mL compound 48/80 (Sigma, St. Louis, MO, USA) in RPMI medium was again added to cultures. Then, after 24 h the supernatants collected. The control samples were maintained in supplemented RPMI medium alone. All supernatants were stored at -20°C until cytokine quantification and β-hexosaminidase release assays.

Quantification of TNF-α, IL-4, IL-10 and IL-12 Cytokines

Cytokines were evaluated using commercial immunoassay kits (Mouse Set OptEIA™ for TNF-α: Code 555268; IL-4: Code 555232; IL-10: Code 555252, BD Biosciences. IL-12: Code 14-7122, BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. For the tests, 25µl of undiluted explant supernatants were used.

Human MC Line HMC-1

The human MC line HMC-1 was kindly provided by Dr. Joseph H. Butterfield (Mayo Clinic, Rochester, MN, USA) and cultured in Iscove's Modified Dulbecco's Medium- (IMDM, Gibco-Thermo Fisher) supplemented with 10% FBS (Cultilab, Campinas, SP, Brazil), 40U/ml penicillin/streptomycin (Sigma) and 1.2 mM α-thioglycerol (Sigma, St. Louis, MO, USA). HMC-1 cells (5×10^5 cells/well in 24-well plates) were pre-treated with

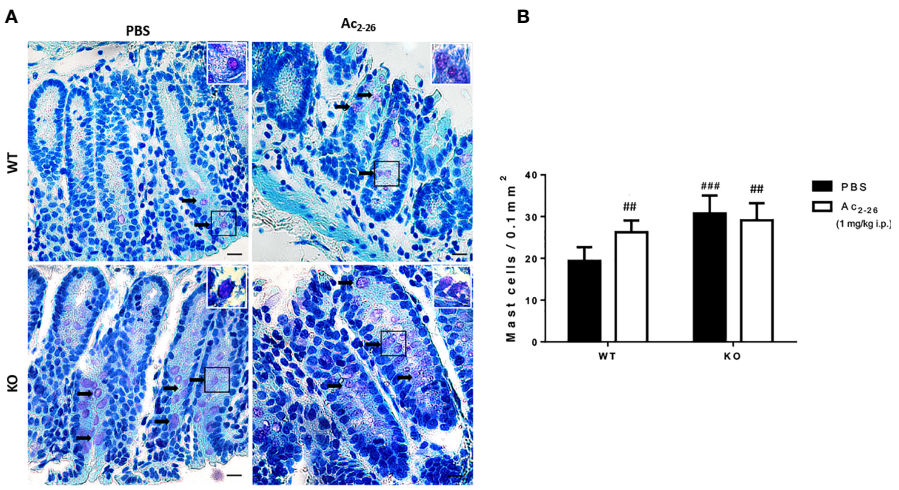

FIGURE 1 | Histological analysis of the distal colon of C57BL/6 WT and C57BL/6 IL-4 KO mice. **(A)** Representative colon sections after PBS or peptide Ac2-26 treatment (1 mg/kg i.p.). Arrows indicate examples of MCs in the tissue. Insets show higher magnification. Staining: Toluidine Blue. Sections: 4µm. Scale bars: 20 µm. **(B)** Quantitative analysis of MCs in the colon fragments. Experiments were performed twice with n = 4 animals/group. Results of cell numbers/mm^2 are expressed as the mean ± S.D, n = 8. Data were analyzed using one-way ANOVA. $^{\#\#}p < 0.01$ compared to WT PBS group. $^{\#\#\#}p < 0.001$ compared to WT PBS group.

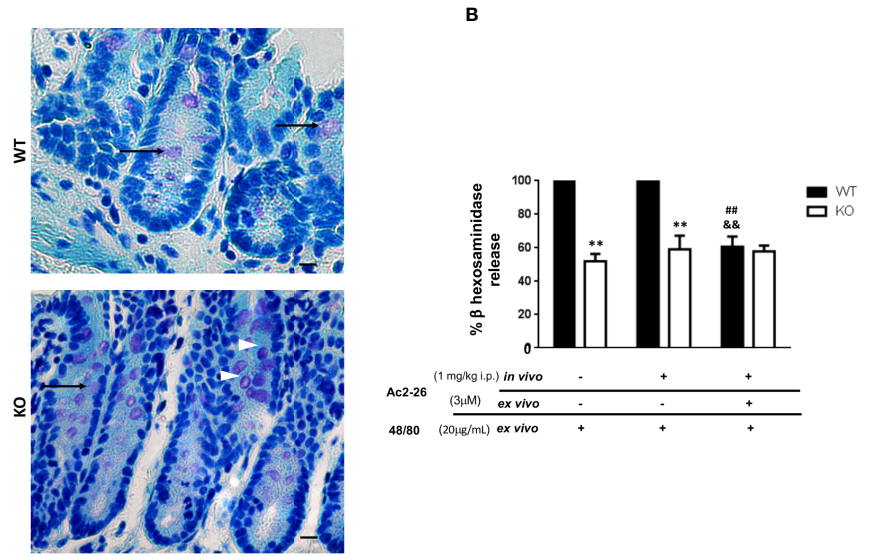

FIGURE 2 | Effect of peptide Ac2-26 on MC degranulation in colon explants. C57BL/6 WT and C57BL/6 interleukin-4- KO mice were pre-treated with PBS (-) or 3μM Ac2 -26 (+) and stimulated with 20μg/mL compound 48/80 *ex vivo* for 24 (h) **(A)** Representative colon sections with examples of intact (arrowheads) and degranulated (arrows) MCs. Staining: Toluidine Blue. Sections: 4μm. Scale bars: 20 μm. **(B)** Degranulation of MCs was assessed by an enzymatic assay. Experiments were performed twice with n = 4 animals/group. Values of the percentage of β hexosaminidase release are expressed as the mean ± S.D., n = 8. Data were analyzed using one-way ANOVA (post Tukey multiple-comparison test). **p <0.01 compared to respective WT group. $^{\#\#}$p <0.01 compared to untreated *in vivo* and *ex vivo* (-/-) and $^{\&\&}$p <0.01 compared to treated and not treated *ex vivo* (-/+).

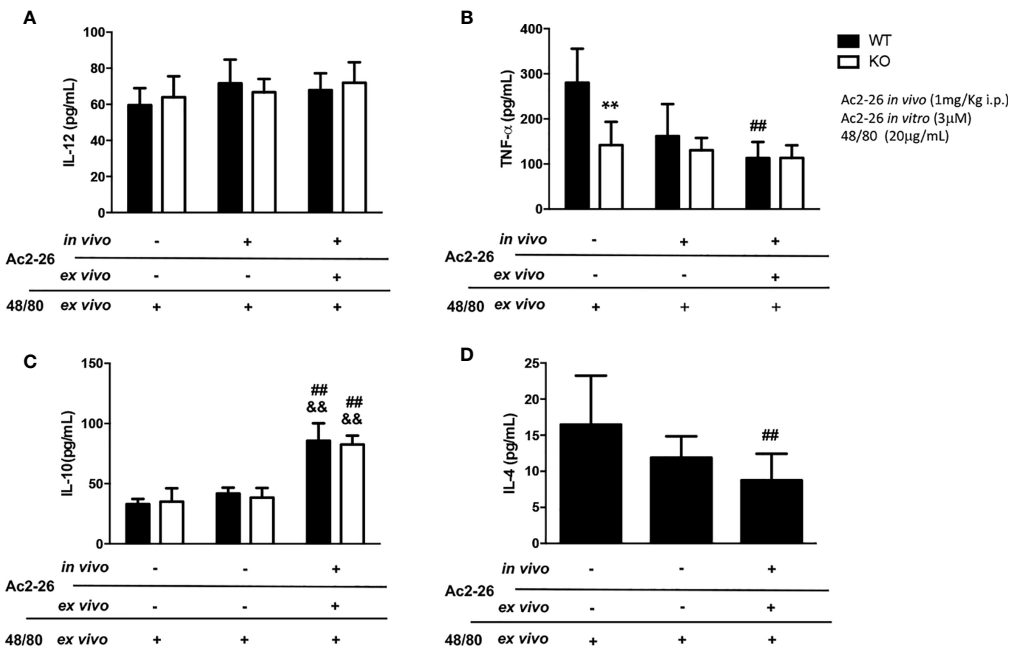

FIGURE 3 | Cytokine production by colon explants from C57BL/6 WT and C57BL/6 IL-4 KO mice treated *in vivo* with 1 mg/kg i.p. $Ac_{2\text{-}26}$ (+) or PBS (-). ELISA determined the concentration of IL-12 **(A)**, TNF-α **(B)**, IL-10 **(C)** and IL-4 **(D)** in explants stimulated for 24 h, with 20μg/mL compound 48/80 and with or without 3μM Ac2-26 treatment. Experiments were performed twice with n = 4 animals/group. Values of concentration in pg/mL are expressed as the mean ± S.D, n = 8. Data were analyzed using one-way ANOVA (post Tukey multiple-comparison test). **p <0.01 *vs*. respective WT group. $^{\#\#}$p <0.01 *vs*. untreated *in vivo* and *ex vivo* (-/-) and $^{\&\&}$p <0.01 *vs* treated *in vivo* and not treated *ex vivo* (-/+).

Ac_{2-26} peptide (3, 5 and 10uM) in addition to 5μg/mL of the antagonist of FPRs, BOC-2/Boc-FLFLFL (Sigma, St. Louis, MO, USA) or PBS at 37°C in a humidified incubator at 5% CO_2. Then, cells were stimulated with 20 μg/mL compound 48/80 (Sigma, St. Louis, MO, USA) at the same conditions. Culture supernatants were collected, and total cell lysates were obtained by adding 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS to the pellets. The number of viable cells was determined by trypan blue staining.

β-hexosaminidase Release Assay

The degranulation of MCs was determined by measuring β-hexosaminidase release in the extracellular medium (36). In a 96-well ELISA plate, samples were incubated with 60 μL of the substrate 1mM p-nitrophenyl-N-acetyl-b-D-glycosaminidase in 0.05M citrate, pH 4.5 (Sigma, St. Louis, MO, USA). The plate was incubated at 37°C for 1 h. The reaction was stopped by adding 0.1M Na2CO3-NaHCO3 buffer pH 10. The absorbance was measured in a microplate reader at 415nm. The percentage of β hexosaminidase release was calculated as follows: β-hexosaminidase release rate (%) = 100 × {(supernatant - blank supernatant)/[(supernatant - blank supernatant) + (total cell lysate]}.

Statistical Analysis

Statistical comparisons were performed with GraphPad Prism 6 (San Diego, CA, USA) using one-way ANOVA followed by Bonferroni multiple comparison post-test or unpaired t-test. All data are the mean ± SD. P values < 0.05 were considered statistically significant.

RESULTS

Histological Analysis

To characterize MCs in WT and KO mice, we analyzed and quantified distal colon sections of PBS or peptide Ac_{2-26}-treated animals. In both strains we found singly dispersed MCs with no aggregates located predominantly in the lamina propria (**Figure 1A**). The MC density in WT-PBS group was significantly lower compared to peptide Ac_{2-26} treated mice; in the colon mucosa of IL-4 KO mice the frequency of MCs was significantly higher compared with WT PBS group, although for PBS and AnxA1 peptide Ac_{2-26} treated hosts, MC numbers were similar (**Figure 1B**).

Ac_{2-26} Modulates MC Acute Reaction in Colonic Explants of WT but Not IL-4 KO Mice

Initially, in order to examine the effect of AnxA1 in colonic MCs, C57BL/6 WT and IL-4 KO mice received intraperitoneal administration of peptide Ac_{2-26} or PBS. After 24 h the colon was obtained as described in Materials & Methods for explant cultures under different conditions and the degranulation of MCs was determined by assessing the activity of β-hexosaminidase (**Figures 2A, B**). Resident MCs from the colon of IL-4 KO mice displayed a significantly reduced degranulation induced by compound 48/80 when compared to WT group ($p<0.01$); on the other hand, the treatment with AnxA1 peptide Ac_{2-26} did not modify the degranulation pattern of MCs in both WT and IL-4 KO mice. However, when explant cultures were re-exposed *ex vivo* to the peptide $Ac_{2-26,}$ the degranulation triggered by compound 48/80 was selectively abolished in WT, but not in IL-4 KO samples ($p<0.01$).

Effects of Ac_{2-26} Treatment on the Cytokine Release of Colonic Explants Stimulated by Compound 48/80

In supernatants of colonic explants of WT and IL-4 KO mice stimulated as above we also assessed IL-12, IL-10, TNF- α and IL-4 levels (**Figure 3**). While no differences in IL-12 were observed, after peptide Ac_{2-26} and compound 48/80 stimulation *ex vivo*, we found a significant increment in IL-10 levels, a pivotal regulatory cytokine, in both mouse strains ($p<0.01$). In contrast to untreated WT group, IL-4 KO mice showed reduced release of the pro-inflammatory cytokine TNF-α, that was not modified by compound 48/80 stimulation *ex vivo* with or without Ac_{2-26} treatment. Interestingly, the treatment with Ac_{2-26} *ex vivo* yielded a significant decrease in TNF-α and IL-4 release upon compound 48/80 stimulation in colonic explants from WT mice ($p < 0.05$).

In Vitro Ac_{2-26} Peptide Treatment Inhibits Degranulation of HMC-1 Cells

As studies in animals, especially those related to pharmaceutical products, do not always reproduce the complexity of human allergic diseases (37), next we tested the effects of exogenous AnxA1 peptide on acute reaction of human MCs. For that purpose, we took advantage of the well-established cell line HMC-1 to confirm the effect Ac_{2-26}. The HMC-1 cells were pre-treated with 3, 5, or 10 peptide Ac_{2-26} (μM), for 30 min prior to stimulation with 20 μg/mL compound 48/80 and the degranulation was assessed by the release of β-hexosaminidase. As shown in **Figure 4**, pretreatment with the optimal dose (10 μM) of peptide Ac_{2-26} significantly inhibited human MC degranulation and this reduction was selectively blocked when FPRs antagonist BOC-2 was added before stimulation. The pre-treatment with both peptide Ac_{2-26} and the antagonist BOC-2 at the doses evaluated did not affect cell viability and survival of HMC-1 cells (**Figure 5**). Collectively, these *in vitro* studies indicate that AnxA1 peptide can inhibit degranulation of HMC-1 cells in a direct pathway, suggesting that the exogenous AnxA1 peptide/FPR axis may alter human MC function.

DISCUSSION

The MCs are widely distributed throughout most tissues, especially at the mucosal interface where they can respond to a variety of stimuli, with notable differences based on their tissue residency. Previous report using microarray assays indicated that

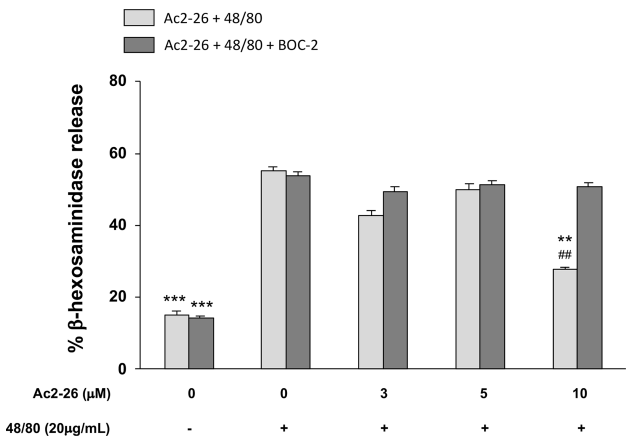

FIGURE 4 | Exogenous AnxA1 (peptide Ac2-26) inhibits HMC-1 cell line degranulation induced by compound 48/80. HMC-1 cells were pre-treated with 3, 5, or 10μM peptide Ac2-26 for 30 min in the absence or presence of FPRs antagonist BOC-2 (5μg/mL). Degranulation was assessed measuring the activity of β-hexosaminidase in culture supernatants. Results are expressed as percentage of enzyme release. All experiments were conducted in triplicate. Data are expressed as the mean ± S.D from four different experiments and analyzed using one-way ANOVA. ***p <0.001 compared to cells treated with 48/80 alone or 48/80 + Ac2-26 (3 or 5 μM); **p <0.01 compared to cells treated with 48/80 + Ac2-26 (10 μM) + BOC-2; ##p <0.01 compared to cells treated with 48/80 alone.

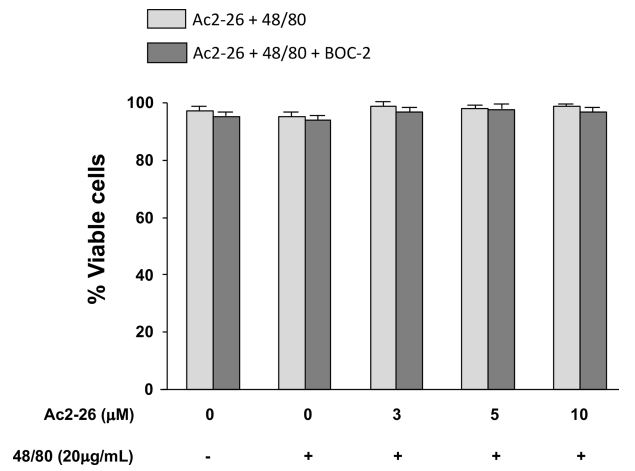

FIGURE 5 | Exogenous AnxA1 (peptide Ac2-26) did not alter viability of HMC-1 cell line. Cells were pre-treated with 3, 5, or 10μM peptide Ac2-26 for 30 min in the absence or presence of FPRs antagonist BOC-2 (5μg/mL). The cells were then stimulated with 20 μg/mL compound 48/80 for 30 min. Cell viability was assessed by trypan blue exclusion assay and results are expressed as percentage. All experiments were performed in triplicate. Data are expressed as the mean ± S.D from four different experiments.

the expression of genes coding for MC proteases Cpa3, Mcpt4, Mcpt5, Mcpt6, and Mcpt10 is restricted to MCs in the peritoneal cavity, which are connective tissue cells. However, the lack of expression of Mcpt1 and Mcpt2 in peritoneal MCs is to be expected, since the expression Mcpt1 and Mcpt2 is found in intestinal MCs (38). Specifically, in the intestine, mucosal MCs express cysteinyl leukotrienes and have high TLR expression, suggesting their commitment to inflammatory responses (39). Due to their location, they secrete a wide range of mediators many of which may affect the intestinal epithelial barrier directly (40).

Depending on the intrinsic secretory phenotype, MCs exhibit different activation profiles from resting, low activation with small secretion of mediators to highly activated (4). In the GI, the MCs exert their biological functions mainly by humoral functions, releasing a range of mediators with some of them involved in monocyte and macrophage activation (TNF-α and IL-6) and others exhibiting regulatory functions (TGFβ1 and IL-10) (41). Remarkably, the perception on MC function has been expanded and now it is well accepted that they perform additional and unexpected activities in strict collaboration with immune and non-immune cells (5). However, little is known about the way MCs interact with or affect the biologic activities of other tissue-resident cells during an inflammatory response.

In this work we found a significantly higher frequency of intact MCs in the colon mucosa of IL-4 KO mice. Consistent with this, IL-4 deficient mice have a higher number of peritoneal MCs (42) and IL-4-deficient bone marrow cell cultures result in a constant increase in the number of MCs and the expression of FcεRI (43), supporting the role of IL-4 as an endogenous regulator of MC development. On the other hand, after the administration of peptide Ac_{2-26} *in vivo*, while the MC count remained unchanged in the IL-4 KO mice, it increased notably in the WT samples, suggesting an improvement in the identification in the tissue. In agreement, using an intraocular inflammation model, our group demonstrated that the administration of Ac_{2-26} significantly increases the number of the number of intact mast cells and decreased the proportion of degranulated cells (25), confirming that in mice treated with mimetic peptide, its ability to prevent cellular degranulation results in higher numbers of MCs in tissue sections. Furthermore, we found that without any stimulation, the frequency of degranulated MCs in the colon mucosa of WT and IL-4 KO mice was similar, which is in agreement with previous evidence. MC maturation, phenotype and function can vary according to anatomical location, strain background, *in vivo* or *in vitro* studies or cytokine milieu. For instance, IL-4 regulates positively the proliferation and expression of surface receptors, although its deficiency does not influence the number, size, granularity or histamine content, suggesting the compensatory activity of other factors. In the gastrointestinal tract, the largest MC population is found in the mucosa contributing to the regulation of epithelial barrier and participating in both innate and adaptive immune responses. We provide evidence for a negative modulation for basal MC function in the GI tract of IL-4 deficient mice. In agreement, mouse MCs derived from bone marrow cells cultured with IL-3, released higher levels of β-hexosaminidase upon substance P challenge only after 6 days of treatment with SCF plus IL-4 (44). Other groups instead

reported that despite a lack of degranulation in response to anti-IgE, peritoneal MCs from IL-4/IL-13- or IL-4R-deficient mice do not have a general degranulation defect, as they responded normally to IgE-independent stimuli such as compound 48/80, or ionomycin (45). One possible explanation for these differences is that the sensitivity of MCs to activation by non-immunological stimuli is dependent on the MC population examined (44). The inability of IL-4 KO mice to orchestrate strong MC activation was not altered by the treatment *in vivo* and *ex vivo* with exogenous peptide $Ac_{2\text{-}26}$. However, the IL-4 production in WT mice was affected using the same treatment protocol. Accordingly, in naive T cells from mouse, overexpression of AnxA1 significantly increased IFNγ and reduced IL-4 production, while AnxA1-silenced T cells exhibited decreased IFNγ and increased IL-4 production (46).

It is well accepted that AnxA1 is an important endogenous regulator of MCs, inhibiting their activation when cells are in the resting state and limiting the extent of degranulation and activation response (47). We hypothesize that differences in MC population in normal conditions could determine differential release of MC mediators and the pattern of anaphylactic or acute response after specific stimuli. We uncovered that, after *in vivo* and *ex vivo* administration of $Ac_{2\text{-}26}$, WT and KO mice colon explants released higher levels of IL-10 when compared to untreated animals. These results suggest that some of the anti-inflammatory effects of AnxA1 may result from a subsequent release of IL-10 (48). Still, MCs can affect various immune cells by releasing cytokines and chemical mediators (49), therefore the production of IL-10 could be an indirect effect. On the other hand, high expression of TNF-α in activated MCs plays an instrumental role is the pathogenesis of colitis in mice (50). Our data showed an overall reduced production of the pro-inflammatory cytokine TNF-α levels in colon explant cultures from animals lacking IL-4 gene, while administration *ex vivo* of exogenous peptide $Ac_{2\text{-}26}$ attenuated TNF-α levels in WT mice. The data highlights that depending on the stimulus, MCs calibrate their pattern of mediator release, modulate the amplification of inflammation, or are involved in the resolution of the immune responses (5). Also, it is difficult to determine the biological relevance of MCs as sources of cytokines in settings where multiple different immune cells represent alternative potential sources of the same products (51). Our findings in the cytokine release experiments illustrate the overall response of the colonic tissue upon compound 48/80 stimulation, which represents a suitable approach to understand how activation or mimetic peptide inhibition of MCs, produces certain outcomes.

Many studies have shown the role of MCs in visceral hypersensitivity reaction mechanisms based on *in vitro* assays using human HMC-1 cell line (52–54). For that reason, the human HMC-1 cell line is considered a good model to evaluate the role of AnxA1 on cell function. Previous reports demonstrated that the release of histamine and prostaglandin D2 by cord-derived human MCs (CBDMCs) activated by IgE-FcRε1 crosslinking (55) or treatment with the compound 48/80 (56) was inhibited by pre-treatment with cromoglycate, nedocromil, dexamethasone, and human recombinant AnxA1. Remarkably, our study demonstrated that the AnxA1-derived peptide $Ac_{2\text{-}26}$ inhibited *in vitro* the degranulation of HMC-1 cells stimulated with compound 48/80 in FPR-dependent manner, as assessed by blocking the degranulation system using the antagonist BOC-2. Former results with mouse model revealed that AnxA1 is able to downregulate MCs function in allergic disorders (56). Moreover, using human cord-blood derived MCs it has been shown that AnxA1 is an important regulator of MC reactivity to compound 48/80 exerting a negative feedback effect through a mechanism that depends at least partly on the FPR receptor (47).

In summary, $Ac_{2\text{-}26}$ showed a strong inhibitory activity in WT colon, reducing the production of TNF-α and IL-4 as well as the MC degranulation. In IL-4 KO mice, the constitutively expanded MCs population seemed less reactive to acute stimulation, although upon $Ac_{2\text{-}26}$ treatment, the release of IL-10 the hallmark anti-inflammatory cytokine of the intestinal mucosa-was similar to WT hosts. Herein, we have uncovered properties that include HMC-1 cells with focus particularly on allergic or acute responses. AnxA1 may contribute to biological regulation of human cell line HMC-1, *via* paracrine mechanisms mediated by FPRs. Our study sheds additional light on the function of intestinal MCs and HMC-1 after AnxA1 treatment, delineating new strategies to reduce the release of inflammatory mediators.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Ethics of Animal 145 Experiments from Cordoba National University (resolution 1412/2012).

AUTHOR CONTRIBUTIONS

MO designed and performed *in vitro* experiments, helped with the analysis of data and wrote the manuscript. JP performed *in vivo* and *in vitro* experiments. AG performed histological processing and data analysis. SG evaluated the results and contributed to revising the manuscript. SO conceived and coordinated the design of the study, obtained funding for the project, evaluated the results, contributed to drafting the manuscript and supervised the process. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Galli SJ, Grimbaldeston M, Tsai M. Immunomodulatory Mast Cells: Negative, as Well as Positive, Regulators of Immunity. *Nat Rev Immunol* (2008) 8:478–86. doi: 10.1038/nri2327
2. Kalesnikoff J, Galli SJ. New Developments in Mast Cell Biology. *Nat Immunol* (2008) 9:1215–23. doi: 10.1038/ni.f.216
3. Moon TC, Befus AD, Kulka M. Mast Cell Mediators: Their Differential Release and the Secretory Pathways Involved. *Front Immunol* (2014) 5:569. doi: 10.3389/fimmu.2014.00569
4. Galli SJ, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast Cells as "Tunable" Effector and Immunoregulatory Cells: Recent Advances. *Annu Rev Immunol* (2005) 23:749–86. doi: 10.1146/annurev.immunol.21.120601
5. Komi EA,D, Wöhrl S, Bielory L. Mast Cell Biology at Molecular Level: A Comprehensive Review. *Clin Rev Allergy Immunol* (2020) 58:342–65. doi: 10.1007/s12016-019-08769-2
6. Albert-Bayo M, Paracuellos I, González-Castro AM, Rodríguez-Urrutia A, Rodríguez-Lagunas MJ, Alonso-Cotoner C. Intestinal Mucosal Mast Cells: Key Modulators of Barrier Function and Homeostasis. *Cells* (2019) 8:135. doi: 10.3390/cells8020135
7. Bingham CO3rd, Austen KF. Mast-Cell Responses in the Development of Asthma. *J Allergy Clin Immunol* (2000) 105:S527–34. doi: 10.1016/s0091-6749(00)90056-3
8. Wouters MM, Vicario M, Santos J. The Role of Mast Cells in Functional GI Disorders. *Gut* (2016) 65:155–68. doi: 10.1136/gutjnl-2015-309151
9. Bischoff SC. Physiological and Pathophysiological Functions of Intestinal Mast Cells. *Semin Immunopathol* (2009) 31:185–205. doi: 10.1007/s00281-009-0165-4
10. Yu LC, Perdue MH. Role of Mast Cells in Intestinal Mucosal Function: Studies in Models of Hypersensitivity and Stress. *Immunol Rev* (2001) 179:61–73. doi: 10.1034/j.1600-065x.2001.790107.x
11. Biedermann T, Zimmermann S, Himmelrich H, Gumy A, Egeter O, Sakrauski AK, et al. IL-4 Instructs TH1 Responses and Resistance to *Leishmania Major* in Susceptible BALB/C Mice. *Nat Immunol* (2001) 2:1054–60. doi: 10.1038/ni725
12. Lorentz A, Wilke M, Sellge G, Worthmann H, Klempnauer J, Manns MP, et al. IL-4-Induced Priming of Human Intestinal Mast Cells for Enhanced Survival and Th2 Cytokine Generation Is Reversible and Associated With Increased Activity of ERK1/2 and C-Fos. *J Immunol* (2005) 174:6751–6. doi: 10.4049/jimmunol.174.11.6751
13. Burton OT, Darling AR, Zhou JS, Noval-Rivas M, Jones TG, Gurish MF, et al. Direct Effects of IL-4 on Mast Cells Drive Their Intestinal Expansion and Increase Susceptibility to Anaphylaxis in a Murine Model of Food Allergy. *Mucosal Immunol* (2013) 6:740–50. doi: 10.1038/mi.2012.112
14. McLachlan JB, Shelburne CP, Hart JP, Pizzo SV, Goyal R, Brooking-Dixon R, et al. Mast Cell Activators: A New Class of Highly Effective Vaccine Adjuvants. *Nat Med* (2008) 14:536–41. doi: 10.1038/nm1757
15. Perretti M, Flower RJ. Measurement of Lipocortin 1 Levels in Murine Peripheral Blood Leukocytes by Flow Cytometry: Modulation by Glucocorticoids and Inflammation. *Br J Pharmacol* (1996) 118:605–10. doi: 10.1111/j.1476-5381.1996.tb15444.x
16. Oliani SM, Paul-Clark MJ, Christian HC, Flower RJ, Perretti M. Neutrophil Interaction With Inflamed Postcapillary Venule Endothelium Alters Annexin 1 Expression. *Am J Pathol* (2001) 158:603–15. doi: 10.1016/S0002-9440(10)64002-3
17. Oliani SM, Ciocca GA, Pimentel TA, Damazo AS, Gibbs L, Perretti M. Fluctuation of Annexin-A1 Positive Mast Cells in Chronic Granulomatous Inflammation. *Inflamm Res* (2008) 5:450–6. doi: 10.1007/s00011-008-7222-7
18. Oliani SM, Christian HC, Manston J, Flower RJ, Perretti M. An Immunocytochemical and in Situ Hybridization Analysis of Annexin 1 Expression in Rat Mast Cells: Modulation by Inflammation and Dexamethasone. *Lab Invest* (2000) 80:1429–38. doi: 10.1038/labinvest.3780150
19. Perretti M, Getting SJ, Solito E, Murphy PM, Gao JL. Involvement of the Receptor for Formylated Peptides in the *In Vivo* Anti-Migratory Actions of Annexin 1 and Its Mimetics. *Am J Pathol* (2001) 158:1969–73. doi: 10.1016/S0002-9440(10)64667-6
20. Gavins FN, Dalli J, Flower RJ, Granger DN, Perretti M. Activation of the Annexin 1 Counter-Regulatory Circuit Affords Protection in the Mouse Brain Microcirculation. *FASEB J* (2007) 21:1751–8. doi: 10.1096/fj.06-7842com
21. Gastardelo TS, Damazo AS, Dalli J, Flower RJ, Perretti M, Oliani SM. Functional and Ultrastructural Analysis of Annexin A1 and Its Receptor in Extravasating Neutrophils During Acute Inflammation. *Am J Pathol* (2009) 174:177–83. doi: 10.2353/ajpath.2009.080342
22. Gibbs L, Carollo MG, Damazo AS, Oliani SM, Perretti M. Time-Dependent Expression of Annexin 1 in a Model of Chronic Granulomatous Inflammation. *Inflamm Res* (2002) 51:300–6. doi: 10.1007/pl00000308
23. Stuqui B, de Paula-Silva M, Carlos CP, Ullah A, Arni RK, Gil CD, et al. Ac2-26 Mimetic Peptide of Annexin A1 Inhibits Local and Systemic Inflammatory Processes Induced by *Bothrops Moojeni* Venom and the Lys-49 Phospholipase A2 in a Rat Model. *PloS One* (2015) 10(7):e0130803. doi: 10.1371/journal.pone.0130803
24. Bena S, Brancaleone V, Wang JM, Perretti M, Flower RJ. Annexin A1 Interaction With the FPR2/ALX Receptor: Identification of Distinct Domains and Downstream Associated Signaling. *J Biol Chem* (2012) 287:24690–7. doi: 10.1074/jbc.M112.377101
25. Gimenes AD, Andrade TR, Mello CB, Ramos L, Gil CD, Oliani SM. Beneficial Effect of Annexin A1 in a Model of Experimental Allergic Conjunctivitis. *Exp Eye Res* (2015) 134:24–32. doi: 10.1016/j.exer.2015.03.013
26. Connelly TM, Koltun WA, Sangster W, Berg AS, Hegarty JP, Harris L 3rd, et al. An Interleukin-4 Polymorphism Is Associated With Susceptibility to *Clostridium Difficile* Infection in Patients With Inflammatory Bowel Disease: Results of a Retrospective Cohort Study. *Surgery* (2014) 156:769–74. doi: 10.1016/j.surg.2014.06.067
27. Toptygina AP, Semikina EL, Bobyleva GV, Miroshkina LV, Petrichuk SV. Cytokine Profile in Children With Inflammatory Bowel Disease. *Biochem (Mosc).* (2014) 79:1371–5. doi: 10.1134/S0006297914120116
28. Yang YH, Aeberli D, Dacumos A, Xue JR, Morand EF. Annexin-1 Regulates Macrophage IL-6 and TNF *via* Glucocorticoid-Induced Leucine Zipper. *J Immunol* (2009) 183:1435–45. doi: 10.4049/jimmunol.0804000
29. Oliani SM, Perretti M. Cell Localization of the Anti-Inflammatory Protein Annexin 1 During Experimental Inflammatory Response. *Ital J Anat Embryol* (2001) 106:69–77.
30. Bandeira-Melo C, Bonavita AG, Diaz BL, E Silva PM, Carvalho VF, Jose PJ, et al. A Novel Effect for Annexin 1-Derived Peptide Ac2-26: Reduction of Allergic Inflammation. *J Pharmacol Exp Ther* (2005) 313:1416–22. doi: 10.1124/jpet.104.080473
31. Nilsson G, Blom T, Kusche-Gullberg M, Kjellén L, Butterfield JH, Sundström C, et al. Phenotypic Characterization of the Human Mast-Cell Line HMC-1. *Scand J Immunol* (1994) 39:489–98. doi: 10.1111/j.1365-3083.1994.tb03404.x
32. Butterfield JH, Weiler D, Dewald G, Gleich GJ. Establishment of an Immature Mast Cell Line From a Patient With Mast Cell Leukemia. *Leuk Res* (1988) 12:345–55. doi: 10.1016/0145-2126(88)90050-1
33. Sundström M, Vliagoftis H, Karlberg P, Butterfield JH, Nilsson K, Metcalfe DD, et al. Functional and Phenotypic Studies of Two Variants of a Human Mast Cell Line With a Distinct Set of Mutations in the C-Kit Proto-Oncogene. *Immunology* (2003) 108:89–97. doi: 10.1046/j.1365-2567.2003.01559.x
34. Guhl S, Babina M, Neou A, Zuberbier T, Artuc M. Mast Cell Lines HMC-1 and LAD2 in Comparison With Mature Human Skin Mast Cells–Drastically Reduced Levels of Tryptase and Chymase in Mast Cell Lines. *Exp Dermatol* (2010) 19:845–7. doi: 10.1111/j.1600-0625.2010.01103.x
35. Prates J, Franco-Salla GB, Dinarte Dos Santos AR, da Silva WA Jr, da Cunha BR, Tajara EH, et al. ANXA1Ac_{2-26}; Peptide Reduces ID1 Expression in Cervical Carcinoma Cultures. *Gene* (2015) 570:248–54. doi: 10.1016/j.gene.2015.06.021
36. Balletta A, Lorenz D, Rummel A, Gerhard R, Bigalke H, Wegner F. Human Mast Cell Line-1 (HMC-1) Cells Exhibit a Membrane Capacitance Increase When Dialysed With High Free-Ca2+ and Gtpγs Containing Intracellular Solution. *Eur J Pharmacol* (2013) 720:227–36. doi: 10.1016/j.ejphar.2013.10.022
37. Bracken MB. Why Animal Studies Are Often Poor Predictors of Human Reactions to Exposure. *J R Soc Med* (2009) 102:120–2. doi: 10.1258/jrsm.2008.08k033
38. Dahlin JS, Ding Z, Hallgren J. Distinguishing Mast Cell Progenitors From Mature Mast Cells in Mice. *Stem Cells Dev* (2015) 24:1703–11. doi: 10.1089/scd.2014.0553

39. Frossi B, Mion F, Sibilano R, Danelli L, Pucillo CEM. Is it Time for a New Classification of Mast Cells? What Do We Know About Mast Cell Heterogeneity? *Immunol Rev* (2018) 282:35–46. doi: 10.1111/imr.12636
40. McLeod JJ, Baker B, Ryan JJ. Mast Cell Production and Response to IL-4 and IL-13. *Cytokine* (2015) 75:57–61. doi: 10.1016/j.cyto.2015.05.019
41. Bischoff SC. Mast Cells in Gastrointestinal Disorders. *Eur J Pharmacol* (2016) 778:139–45. doi: 10.1016/j.ejphar.2016.02.018
42. Chen XJ, Lycke N, Enerbäck L. Surface and Gene Expression of Immunoglobulin E Receptors on Mast Cells and Mast-Cell Numbers in Interleukin-4-Gene Knockout Mice. *Immunology* (1999) 96:544–50. doi: 10.1046/j.1365-2567.1999.00727.x
43. Speiran K, Bailey DP, Fernando J, Macey M, Barnstein B, Kolawole M, et al. Endogenous Suppression of Mast Cell Development and Survival by IL-4 and IL-10. *J Leukoc Biol* (2009) 85:826–36. doi: 10.1189/jlb.0708448
44. Karimi K, Redegeld FA, Blom R, Nijkamp FP. Stem Cell Factor and Interleukin-4 Increase Responsiveness of Mast Cells to Substance P. *Exp Hematol* (2000) 28:626–34. doi: 10.1016/s0301-472x(00)00161-2
45. Fish SC, Donaldson DD, Goldman SJ, Williams CM, Kasaian MT. Ige Generation and Mast Cell Effector Function in Mice Deficient in IL-4 and IL-13. *J Immunol* (2005) 174:7716–24. doi: 10.4049/jimmunol.174.12.7716
46. Huang P, Zhou Y, Liu Z, Zhang P. Interaction Between ANXA1 and GATA-3 in Immunosuppression of CD4+ T Cells. *Mediators Inflamm* (2016) 2016:1701059. doi: 10.1155/2016/1701059
47. Sinniah A, Yazid S, Perretti M, Solito E, Flower RJ. The Role of the Annexin-A1/FPR2 System in the Regulation of Mast Cell Degranulation Provoked by Compound 48/80 and in the Inhibitory Action of Nedocromil. *Int Immunopharmacol* (2016) 32:87–95. doi: 10.1016/j.intimp.2016.01.003
48. Ferlazzo V, D'Agostino P, Milano S, Caruso R, Feo S, Cillari E, et al. Anti-Inflammatory Effects of Annexin-1: Stimulation of IL-10 Release and Inhibition of Nitric Oxide Synthesis. *Int Immunopharmacol* (2003) 3:1363–9. doi: 10.1016/S1567-5769(03)00133-4
49. Yoshino N, Takeshita R, Kawamura H, Sasaki Y, Kagabu M, Sugiyama T, et al. Mast Cells Partially Contribute to Mucosal Adjuvanticity of Surfactin in Mice. *Immun Inflamm Dis* (2018) 6:117–27. doi: 10.1002/iid3.204
50. Rijnierse A, Koster AS, Nijkamp FP, Kraneveld AD. TNF-Alpha Is Crucial for the Development of Mast Cell-Dependent Colitis in Mice. *Am J Physiol Gastrointest Liver Physiol* (2006) 29:G969–976. doi: 10.1152/ajpgi.00146.2006
51. Mukai K, Tsai M, Saito H, Galli SJ. Mast Cells as Sources of Cytokines, Chemokines, and Growth Factors. *Immunol Rev* (2018) 282:121–50. doi: 10.1111/imr.12634
52. Botschuijver S, Roeselers G, Levin E, Jonkers DM, Welting O, Heinsbroek SEM, et al. Intestinal Fungal Dysbiosis Is Associated With Visceral Hypersensitivity in Patients With Irritable Bowel Syndrome and Rats. *Gastroenterol* (2017) 153:1026–39. doi: 10.1053/j.gastro.2017.06.004
53. Gong L, Li J, Tang Y, Han T, Wei C, Yu X. The Antinociception of Oxytocin on Colonic Hypersensitivity in Rats Was Mediated by Inhibition of Mast Cell Degranulation *via* Ca(2+)-NOS Pathway. *Sci Rep* (2016) 6:31452. doi: 10.1038/srep31452
54. van Diest SA, van den Elsen LW, Klok AJ, Welting O, Hilbers FW, van de Heijning BJ, et al. Dietary Marine N-3 Pufas Do Not Affect Stress-Induced Visceral Hypersensitivity in a Rat Maternal Separation Model. *J Nutr* (2015) 145:915–22. doi: 10.3945/jn.114.201731
55. Yazid S, Sinniah A, Solito E, Calder V, Flower RJ. Anti-Allergic Cromones Inhibit Histamine and Eicosanoid Release From Activated Human and Murine Mast Cells by Releasing Annexin A1. *PloS One* (2013) 8:e58963. doi: 10.1371/journal.pone.0058963
56. Sinniah A, Yazid S, Bena S, Oliani SM, Perretti M, Flower RJ. Endogenous Annexin-A1 Negatively Regulates Mast Cell-Mediated Allergic Reactions. *Front Pharmacol* (2019) 10:1313. doi: 10.3389/fphar.2019.01313

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