International Immunopharmacology 110 (2022) 109063

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Clarithromycin-treated chronic spontaneous urticaria with the negative regulation of FcεRI and MRGPRX2 activation via CD300f

Delu Che [a,b,c], Tao Zhang [b], Tianxiao Zhang [d], Yi Zheng [a], Yajing Hou [b], Songmei Geng [a,c,*], Langchong He [b,*]

[a] Department of Dermatology, Xi'an Jiaotong University Second Affiliated Hospital, Xi'an 710061, China
[b] School of Pharmacy, Xi'an Jiaotong University, Xi'an 710061, China
[c] Center for Dermatology Disease, Precision Medical Institute, Xi'an 710000, China
[d] School of Public Health, Xi'an Jiaotong University, Xi'an 710061, China

ARTICLE INFO

Keywords:
Chronic spontaneous urticarial
Mast cell
Clarithromycin
Leukocyte mono-immunoglobulin-like receptor 3
Negatively regulation

ABSTRACT

Mast cells (MCs) are main effector cells in chronic spontaneous urticaria (CSU). Both Fc epsilon RI (FcεRI)- and MAS-related G coupled receptor-X2 (MRGPRX2)-mediated MC activations affect CSU course. Leukocyte mono-immunoglobulin-like receptor 3 (CD300f) has been shown to regulate FcεRI activation. However, no study has verified CD300f is a target to cure CSU. Therefore this study aimed to verify whether clarithromycin (CLA) regulates FcεRI- and MRGPRX2-mediated MC activations via CD300f and shows therapeutic effect on CSU. The target of CLA was verification. CLA inhibited FcεRI- and MRGPRX2-mediated MC activations were shown *in vivo* and *in vitro*. A single-center, self-comparison study was performed, and CLA-treated CSU was investigated in 28 patients who were not sensitive to the third-generation antihistamines. Serum inflammatory mediators in patients before and after CLA administration were analyzed. CLA effectively inhibited type I anaphylactic reactions and pseudo-allergic reactions in mice. Moreover, CLA inhibited FcεRI- and MRGPRX2-mediated MC signaling pathway activation. Regulatory effects of CLA were decreased significantly after CD300f knockdown. CLA effectively alleviated the symptoms of wheal and itch and reduced serum cytokine levels in patients. CLA negatively regulated FcεRI- and MRGPRX2-mediated MC activation via CD300f and showed significant therapeutic effect on CSU.

1. Introduction

The incidence of chronic spontaneous urticarial (CSU) is from 0.1% to 3% of the general population, and the cardinal symptoms of CSU are wheal and itch. The time course in most of the CSU patients who experience symptoms is more than two years [1]. The etiology and pathogenesis of CSU are complex, especially since the etiology of CSU varies in the different age stages [2]. CSU in children is labeled more frequently as idiopathic than in adult patients, leading to differences in treatment options [3].

Mast cells (MCs) are vital effector cells in CSU, and their activation cause symptoms of chronic urticaria directly. MCs degranulation reaction induces histamine release, and cause wheal and itch in CSU patients. The immunological mechanisms in CSU, which involve the antigen/IgE complex, autoimmune-targeting of IgE receptors and complementary system-mediated pathways. And in this mechanism, Fc epsilon Receptor I (FcεRI) mediates IgE-induced MCs degranulation reaction [4]. However, studies have demonstrated that only 35–40% of CSU patients show high-IgE levels in serum [5], which indicated that there might be other receptors and pathways mediated MCs activation in CSU. Recent studies have confirmed the expression of the MAS-related G protein coupled receptor-X2 (MRGPRX2) at significantly higher levels among the skin MCs of CSU patients [6]. Mouse Mrgprb2 is a homologous receptor of MRGPRX2. This is the key receptor that mediates pseudo-allergic reactions and may also be the key receptor that mediates the process of CSU [7]. Many neurokinins, such as substance P (SP), that widely distributed in fine nerve fibers and released in the central end and peripheral end when the nerve is stimulated, can be which are endogenous agonists of MRGPRX2 can aggravate the skin responses in CSU patients [8], suggesting that MRGPRX2 is closely related to the

* Corresponding authors at: Department of Dermatology, Northwest Hospital, The Second Hospital Affiliated to Xi'an Jiaotong University, 157 Xi Wu Road, Xi'an, Shaanxi, China (S. Geng).
E-mail addresses: gsm312@yahoo.com (S. Geng), helc@mail.xjtu.edu.cn (L. He).

https://doi.org/10.1016/j.intimp.2022.109063
Received 6 May 2022; Received in revised form 7 July 2022; Accepted 12 July 2022
Available online 16 July 2022

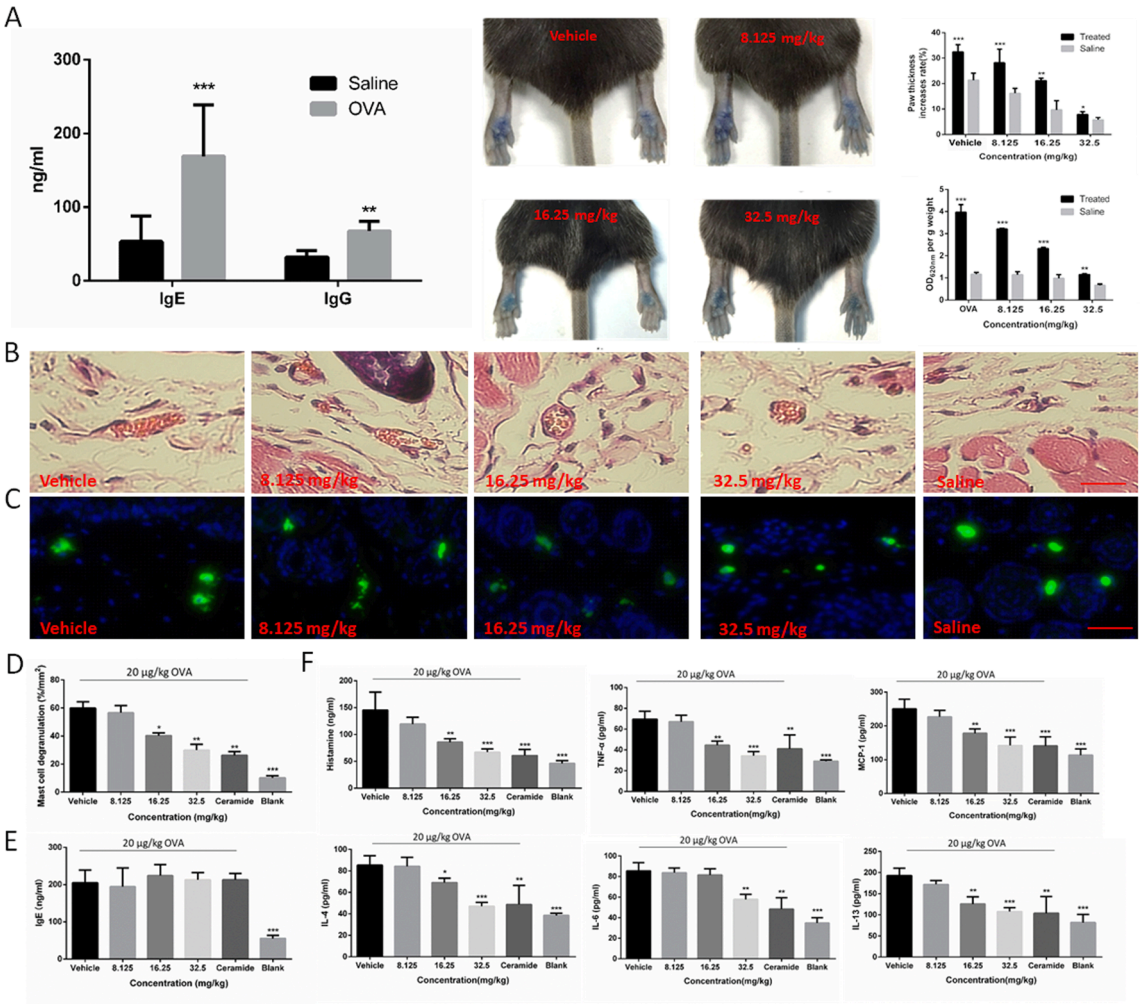

Fig. 1. CLA inhibited Type I anaphylactic reaction induced by IgE in passive anaphylaxis mouse model. A: OVA induced IgE and IgG significantly increase in mouse model analyzed by ELISA and CLA reduced the degree of swelling and Evans blue exudation induced by OVA. B: CLA inhibited skin telangiectasia analyzed by H&E staining. C: CLA inhibited skin MCs degranulation marked by avidin-FITC. D: CLA significantly reduced the percentage of activated MCs induced by IgE. E-F: CLA showed little effect on reduced IgE level, while CLA reduced the levels of histamine, TNF-α, MCP-1, IL-4, IL-6, and IL-13 in the serum analyzed by ELISA. (Vehicle group treated by OVA, experimental groups treated by OVA and different concentrations of CLA, and blank group treated by saline, ceramide was used as a negative control, experiments were repeated 3 times and n = 12. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

occurrence and development of CSU. So inhibition FcεRI- and MRGPRX2-meidated MCs activation might has important value in treating CSU.

Third-generation antihistamines, which are still widely used in clinical practice, act as conventional therapeutic drugs in the treatment of CSU. However, these third-generation antihistamines are not able to achieve satisfactory therapeutic effects in some circumstances [9–10]. Many patients have to use monoclonal antibodies to cure CSU, which are expensive. MCs play an important role in the occurrence of CSU, therefore, the regulation of the activation of MCs' activation provides a new direction for the treatment of CSU.

The leukocyte mono-immunoglobulin-like receptor 3 (LMIR3; CD300f) is a type of inhibitory immune receptor, that expresses on the cytomembranes of neutrophil granulocytes and MCs [11–12], which is regarded as a negatively regulatory factor in inflammatory responses. Ceramide is a family of lipid molecules which consist of sphingoid long-chain base linked to an acyl chain via an amide bond and is the precursor of all complex sphingolipids. Ceramide can inhibit the FcεRI-mediated MCs activation and allergic reactions via CD300f [13]. CD300f, which has been shown to regulate immune diseases in the skin, digestive, and nervous systems, is considered a potential target to treat allergic diseases [14]. However, the regulatory effects of CD300f on MRGPRX2-mediated pseudo-allergic reactions are still unclear, while the exogenous drugs effects on CD300f have not yet been discovered.

Clarithromycin (CLA) is a semisynthetic macrolide antibiotic used widely to treat mild-to-moderate bacterial infections caused by sensitive agents, which is bacteriostatic against many gram positive bacteria including many strains, such as *Helicobacter pylori*, mycobacteriosis, toxoplasmosis, and other infectious diseases. CLA was approved for use in the United States in 1993, and currently more than 3 million prescriptions are filled yearly. Typical indications are for upper respiratory infections, bronchitis, sinusitis, community acquired pneumonia, and skin and tissue infections. In addition, CLA is also used to treat noninfectious inflammatory diseases, such as chronic sinusitis, chronic bronchial asthma [15] and acne rosacea [16]. CLA reduces the degranulated MCs in dermatitis [17]. Moreover, CLA inhibited MCs degranulation *in vivo* and *in vitro* [18–19]. CLA has shown some immune regulatory functions which may act as the key point in the cure of noninfectious inflammatory diseases; the immune regulatory mechanisms of CLA have not yet been elucidated.

In this study, mice models of allergic reactions *in vivo* and degranulation assays of MCs *in vitro* were used to verify the inhibitory effects of

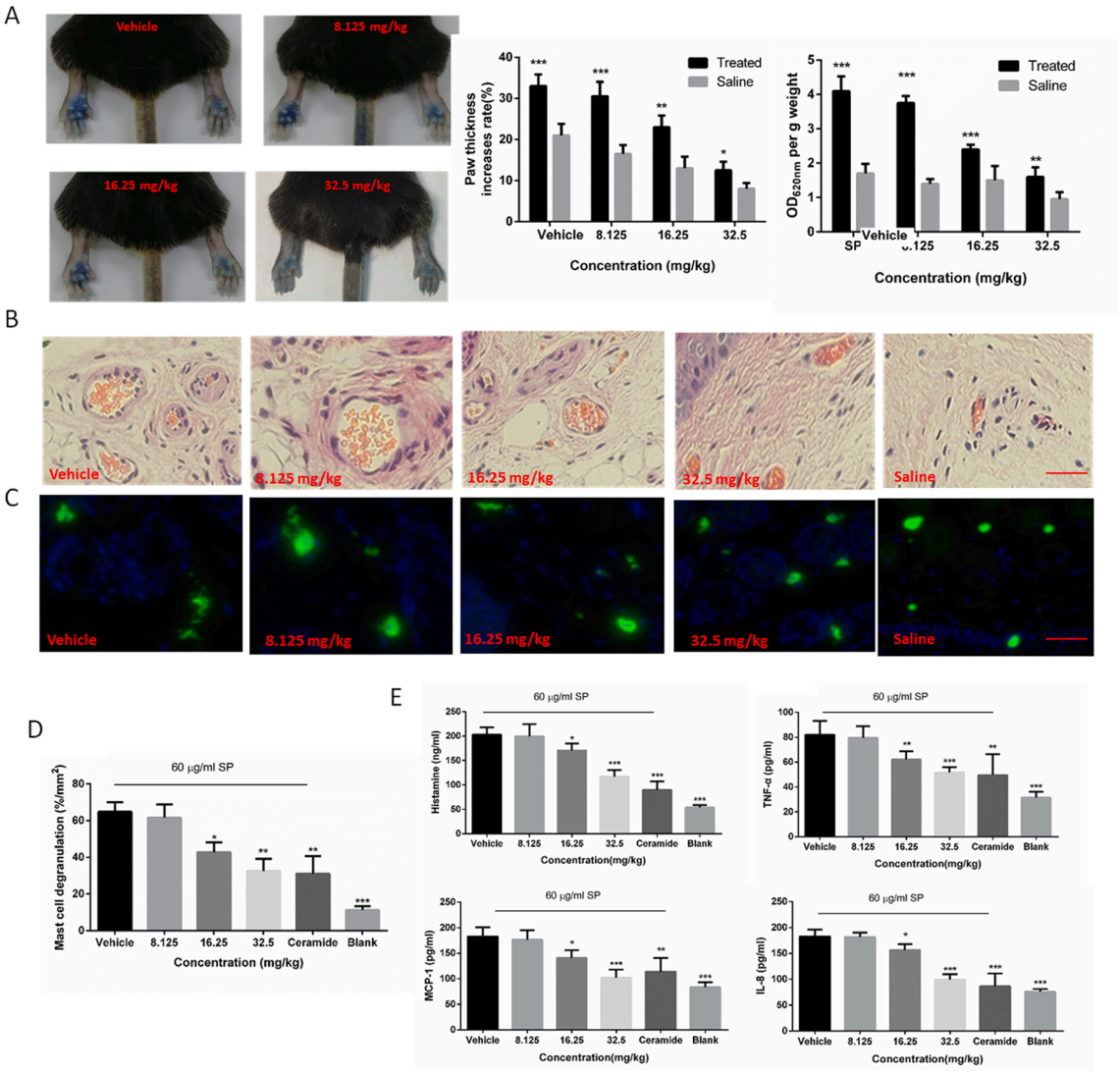

Fig. 2. CLA inhibited Mrgprb2-mediated pseudo-allergic reaction in substance P (SP)-induced passive anaphylaxis mouse model. A: CLA reduced the degree of swelling and Evans blue exudation. B: CLA inhibited SP-indcued skin telangiectasia analyzed by H&E staining. C: CLA inhibited skin mast cells degranulation marked by avidin-FITC. D: CLA significantly reduced the percentage of activated MCs induced by SP. E: CLA obviously reduced SP-indcued the concentration of histamine, TNF-α, MCP-1 and IL-8 in mice serum analyzed by ELISA. (Vehicle group treated by SP, experimental groups treated by SP and different concentrations of CLA, and blank group treated by saline, ceramide was used as a negative control, experiments were repeated 3 times and n = 12. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

CLA on CSU related type I allergic reactions and pseudo-allergic reactions. *si*RNA interference was used to show the target of CLA. In addition, a single-center, a self-comparison study was performed to elucidate the treatment effects of CLA on CSU.

2. Methods

2.1. Reagents

P-nitrophenyl N-acetyl-β-D-glucosamide, ovalbumin (OVA), dinitrophenyl (DNP)-IgE, clarithromycin, substance P (SP), and DNP-BSA were purchased from Sigma-Aldrich (St Louis, MO, USA). TPI-1 (SHP-1 inhibitor) and GS-493 (SHP-2 inhibitor) were purchased from MedChemExpress (Shanghai, China).

2.2. Animals

The 6–8 weeks male C57BL/6 mice used in this study, were purchased from the Experimental Animal Center of the Xi'an Jiaotong University (Xi'an, China). Animals were housed in individual cages in a large colony room, with free access to water, and fed a standard dry chow twice a day. The breeding environment was 20–25 °C, with a relative humidity of 40% on a 12 h light/dark cycle. The mice were divided randomly into the following groups (9 mice per group): blank (treated by saline), control, and the experimental group.

2.3. Ethics statement

The experimental protocols for the mouse model were approved by the Animal Ethics Committee at Xi'an Jiaotong University (Permit Number: XJTU 2019–711). The study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

2.4. Hind paw swelling

For IgE-dependent allergy, 20 μg/kg OVA was injected intraperitoneally into the mice, once a week. After four weeks, CLA (8.125, 16.25,

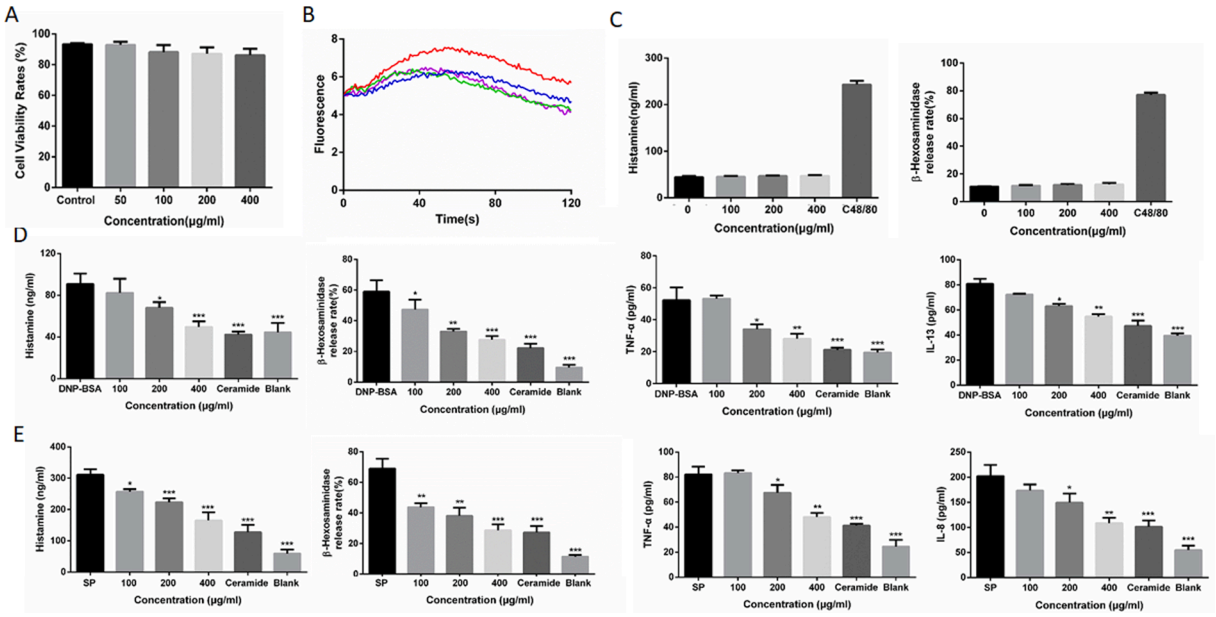

Fig. 3. CLA inhibited FcεRI- and MRGPRX2-mediated MCs activation. A: CLA showed little inhibition effect on LAD2 cells viability for 24 h analyzed by CCK8. B: CLA showed little effect on intracellular Ca^{2+} concentration increase in LAD2 cells. (Cells were identified as responding if the $[Ca^{2+}]i$ rose by at least 50% for at least 10 s, which clearly distinguishes a ligand-induced response from random flickering events and each color line represents an individual cell.). C: CLA did not induce LAD2 cell to release histamine and β-hexosaminidase. D-E: CLA inhibited IgE-induced the release of histamine and β-hexosaminidase, TNF-α and IL-13. CLA reduced SP-caused the release of histamine, β-hexosaminidase, TNF-α and IL-8 analyzed by ELISA (Experiments were repeated 3 times. Only Tyrode's solution buffer was set as blank group, ceramide was used as a negative control. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

and 32.5 mg/kg) was administered intragastrically. The mice were anesthetized using intraperitoneal injections of Pelltobarbitalum Natricum (60 mg/kg) after the CLA treatment for 1 h. The mice were then injected intravenously with 0.4% Evans blue (i.v., 0.2 ml prepared by saline). The paw thickness before any of the treatments was measured using a Vernier caliper.

OVA (20 μg/ml) was injected into the left paw. For an IgE-independent allergy, after anesthesia, the mice were injected with 5 μl SP (30 μg/ml) for 15 min directly into the left paw. Saline, as a negative control, was injected into the right paw. The thickness of each paw was measured and the paw size was compared with photographs that were taken before the treatment. The tissues were collected and weighed after being dried for 12 h at 50 °C. The tissues were extracted in acetone-saline (500 μl, 7:3). They were cut into pieces, super sonicated, and centrifuged to extract the Evans Bule. The OD of the supernatant was measured at 620 nm.

2.5. Skin avidin and H&E (hematoxylin-eosin staining) staining

After treated like the hind paw swelling experiments. The paw skin washed with PBS and fixed with 4% formaldehyde for 48 h and subjected to stain. After dried at 37 °C for 30 min and pre-incubated in blocking solution (10% normal goat serum (v/v), 0.2% Triton X-100 (v/v) in PBS, pH 7.4) for 2 h at 25 °C, followed by incubation with 1/500 FITC-avidin for 45 min. Sections were washed 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL. U.S.A) was added. Images were taken immediately using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.6. Analysis of the mouse serum

The mice received 8.125, 16.25, and 32.5 mg/kg CLA intragastrically. For an IgE-dependent allergy, 20 μg/kg OVA was injected intraperitoneally into the mice once a week for four weeks. Blank group was treated by saline. Then mice were given intravenous injections with OVA (100 μg /kg) into the tail. For an IgE-independent allergy, 60 μg/ml SP was injection intraperitoneally in the treated and control groups. The serum was collected 8 h later, and the release of histamine was analyzed using an ELISA kit (Jianglai Biotechnology Co., LTD, Shanghai, China). The mouse interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), and interleukin-13 (IL-13) ELISA Kits were purchased from Sino Bio. CO., LTD. (Beijing, China) according to provided instructions.

2.7. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) cells available for study of human mast cell biology in lieu of primary mast cell cultures derived from bone marrow; or peripheral or cord blood. The LAD2 mast cell line most closely resembles primary human mast cell cultures. LAD2 human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA), and these were maintained in a StemPro-34 medium containing StemPro nutrient supplement (10 mg/l), human SCF (100 ng/ml), and L-glutamine (2 mmol/l). Cells were maintained at a density of 2×10^6 cells/ml.

2.8. Cytotoxicity assays

The LAD2 cells were seeded into a 96-well plate then centrifuged to remove the medium. The cells treated with CLA (100 μl) and maintained in the medium for 24 h in an incubator. Cell viability was determined using Abbkine-Cell Counting Kit assays (California, USA). Next, 10 μl of Cell Counting Kit solution was added to each well followed by incubation for 2 h. Further, the relative cell viability was assessed by detection of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA). Survival rate of LAD2 cells was calculated as:

$$[(OD_{Treated} - OD_{Blank})/(OD_{Control} - OD_{Blank})] \times 100\%.$$

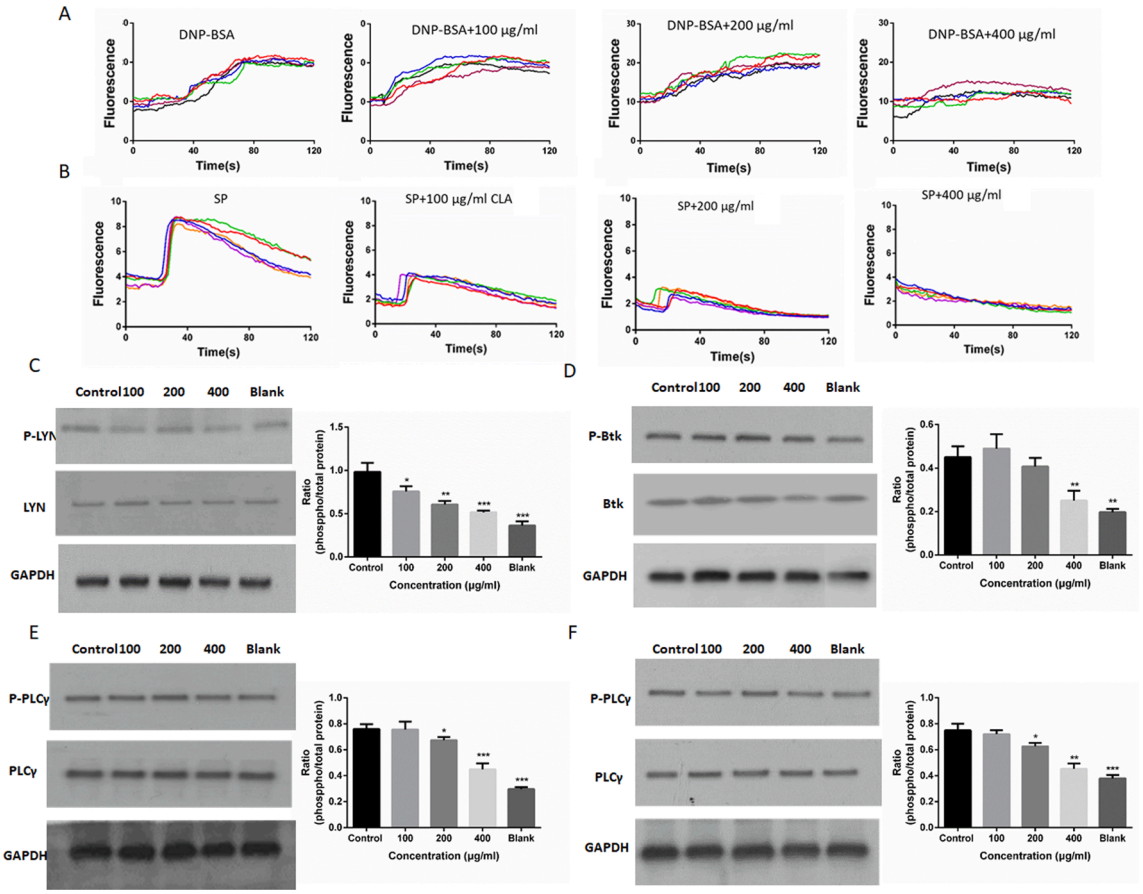

Fig. 4. CLA inhibited calcium signaling pathway activation. A-B: CLA inhibited intracellular Ca^{2+} concentration increase mediated by FcεRI and MRGPRX2 analyzed by calcium imaging. C-E: CLA down-regulated the level of phosphorylated LYN, Btk and PLC-γ increased by IgE. F: CLA decreased the level of phosphorylated PLC-γ caused by SP (Experiments were repeated 3 times. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

2.9. β-hexosaminidase release assay

The LAD2 cells were incubated for 24 h with DNP-IgE (2 μg/ml) as a medium. The LAD2 were incubated in CLA, prepared using a Tyrode's solution buffer for 20 min. Then cells were incubated with DNP-BSA (100 μl, 2 μg/ml) for 40 min. Only Tyrode's solution buffer was set as blank group, and only DNP-BSA was set as control group.

For inhibition of SP-induced β-hexosaminidase release, the LAD2 cells were incubated with CLA for 30 min, then stimulated with SP (16 μg/ml). A 0.1% Triton X-100 was used to lyse the LAD2 cells. The supernatants and cell lysates were hydrolyzed with p-nitrophenyl N-acetyl-β-D-glucosamide at 37 °C for 90 min. The samples were measured at 405 nm after addition of a stop buffer. Only Tyrode's solution buffer was set as blank group, only SP was set as control group. For the experiments on antagonism against CD300f, the CD300f antibody (2 ug/ml) was added in LAD2 cells for 1 h, then CLA and SP were added. For the experiments on antagonism against SHPs, 60 nM TPI-1 and 90 nM GS-493 were added separately in LAD2 cells for 24 h, CLA and SP were added.

2.10. Chemokine release assay

The LAD2 cells were incubated for 24 h with DNP-IgE (2 μg/ml) as a medium. The medium was then removed and 100 μl CLA containing DNP-BSA (2 μg/ml) or SP (16 μg/ml), prepared in Tyrode's solution was added; this was incubated for 8 h in the incubator. Human ELISA Kits were purchased from Sino Bio CO., LTD. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group.

2.11. Intracellular Ca^{2+} mobilization assay

Calcium imaging buffer (CIB) was used to prepare the incubation buffer. Fluo-3 final work concertation at 2.5 μM. CLA was diluted with the incubation buffer. 1×10^5 LAD2 cells were incubated with 2 μg/ml DNP-IgE for 24 h before treatment. Untreated LAD2 cells were used for the SP-induced MC activation. The cells were washed twice using CIB and added to the incubation buffer. Thirty minutes later, the cells were plated in a 96-well plate. The cells were identified as responsive if the $[Ca^{2+}]$i rose by at least 50% after treatment with 100 μl DNP-BSA (4 μg/ml) or SP (16 μg/ml) under blue light.

2.12. siRNA transfection

The siRNA targeting CD300f (forward: 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse: 5′-ACGUGACACGUUCGGAGAATT-3′) and non-targeting siRNA as negative control (NC) (forward: 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse: 5′-ACGUGACACGUUCGGAGAATT-3′) were obtained from GenePharma Co., Ltd. (Shanghai, China). The LAD2 cells were incubated for 48 h to knockdown CD300f expression with using a concentration of 80 nM Lipofectamine® 3000 reagent. The efficiency of the siRNA to knockdown CD300f expression was established using the western blot technique, RT-PCR and immunofluorescent staining. The CD300f Polyclonal antibody was obtained from Invitrogen (Shanghai, China). Sequences of the primers were, forward: 5′CACCAGTCACCCAAGAAGAA3′, reverse: 5′ CAGCAGCAATATGGTGAAGATG3′.

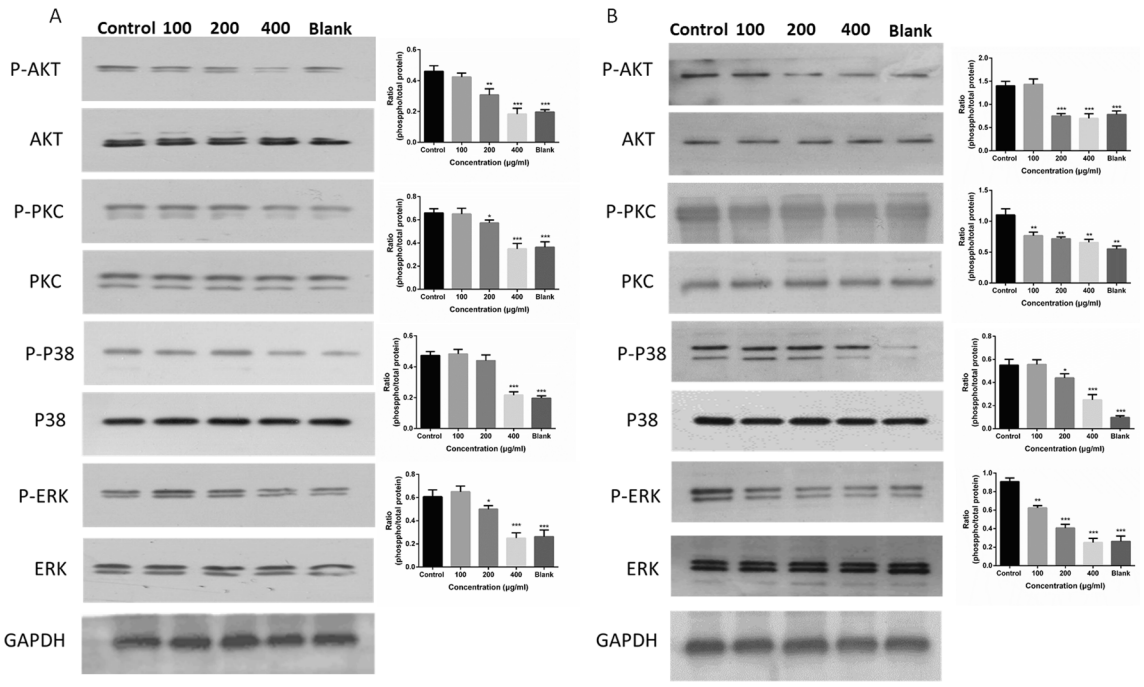

Fig. 5. CLA inhibited cytokine signaling pathways activation. A: CLA down-regulated the phosphorylation level increase of AKT, PKC, P38 and ERK mediated by FcεRI. B: CLA down-regulated the phosphorylation level increase of AKT, PKC, P38 and ERK mediated by MRGPRX2 (Experiments were repeated 3 times. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

2.13. Molecular docking analysis and molecular dynamics simulation

The 3D structures of Clarithromycin were obtained from Pubchem (https://pubchem.ncbi.nlm.nih.gov/). The Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (https://www.pdbus.org/) was used to obtain target gene crystal structures. Discovery Studio v. 2016 (Dassault Systèmes, Vélizy-Villacoublay, France) was used to optimize the crystal structures by removing redundant ligands, adding charge, adding hydrogens, and removing water before running docking experiments. Autodock Vina v. 1.1.2 (Scripps Research Institute, San Diego, CA, USA) and Smina was used to perform a molecular docking analysis. The results obtained were verified by the consistency of the above two programs. Finally, the results that both scoring and conformational fit were selected. Discovery Studio v. 2016 and Pymol were employed to visualize the docking model.

The GROMACS 2019 software was used for molecular dynamics simulations. The docking complex was used as the initial conformation for all-atomic molecular dynamics simulation. Amber 99sB-ILDN field parameters were used for both protein and EB molecules with the aid of ACPYPE Server (https://www.bio2byte.be/acpype/) Server generates UNK molecular topology file, select dodecahedron solvation box, set the system boundary and compounds in recent distance of 1.0 nm, use TIP3P water model and based on method of VERLET cut random Na^+ and Cl^- added to the complex system to counteract the charge of the protein. Then, the system energy was minimized, the temperature was controlled by NVT and the pressure was controlled by NPT. The system temperature was 300 K and the pressure was constant at 101.325 kPa. Based on the above equilibrium, the free dynamics of the system was simulated for 100 ns.

2.14. Western blotting

The untreated LAD2 cells were used for the activation of SP-induced MCs. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group. The LAD2 cells were incubated with DNP-IgE (2 μg/ml) and used to activate the IgE-dependent MCs. Total proteins from the untreated, SP or DNP-BSA treated LAD2 cells (2 h) were extracted using a RIPA lysis buffer containing 10% protease inhibitor and phosphatase inhibitor. This was denatured by boiling with the sample buffer. The proteins were separated using SDS-PAGE (10% gel), then transferred onto polyvinylidene fluoride membranes, and blocked for 2 h at room temperature by constant stirring in 5% non-fat milk in TBST. The membranes were incubated overnight at 4 °C with the following primary antibodies (Cell Signaling Technology): anti-GAPDH (#2118,1:2000), anti-P-Fyn (#6943, 1:1000), anti-Fyn (#4023, 1:1000), anti-P-Lyn (#2731, 1:1000), anti-Lyn (#2796, 1:1000), anti-P-Btk (#87141, 1:1000), anti-Btk (#8547, 1:1000), anti-P-P38 (#4511, 1:1000), anti-P-PLCγ1 (#8713, 1:1000,), anti-PLCγ1 (#5690, 1:1000,), anti-P-Erk1/2 (#9101, 1:1000), anti-Erk1/2 (#9102, 1:1000), anti-P-Akt (#4060, 1:1000), anti-Akt (#4691, 1:1000). The membranes were incubated with secondary antibodies (1:20,000 in TBST) for 1 h at 37 °C after washed thrice with TBST, after which they were developed using an enhanced chemiluminescence kit. The results were analyzed using a Lane 1DTM transilluminator with Image-Pro Plus 5.1 software.

2.15. Clinical efficacy evaluation of clarithromycin in the treatment of CSU

A single-center, self-control study was used. Samples were obtained from the Department of Dermatology, Second Affiliated Hospital of Xi'an Jiaotong University, where CLA used to treat CSU. The CSU patients included in our study did not respond to treatment with third-generation antihistamines. In our study, the CSU patients were allowed to take the same antihistamines that they were using before the implementation of the CLA treatment. The CSU patients continued taking the CLA sustained-release tablets (250 mg/day) for two weeks in accordance with the doctor's instructions. These patients returned to the doctor after two weeks. The peripheral blood samples of the patients newly diagnosed with CSU were first obtained during the study period. After two weeks of clarithromycin treatment, the peripheral blood samples were obtained again and clinical and laboratory data were collected, and the quality was managed by repeating the tests more than

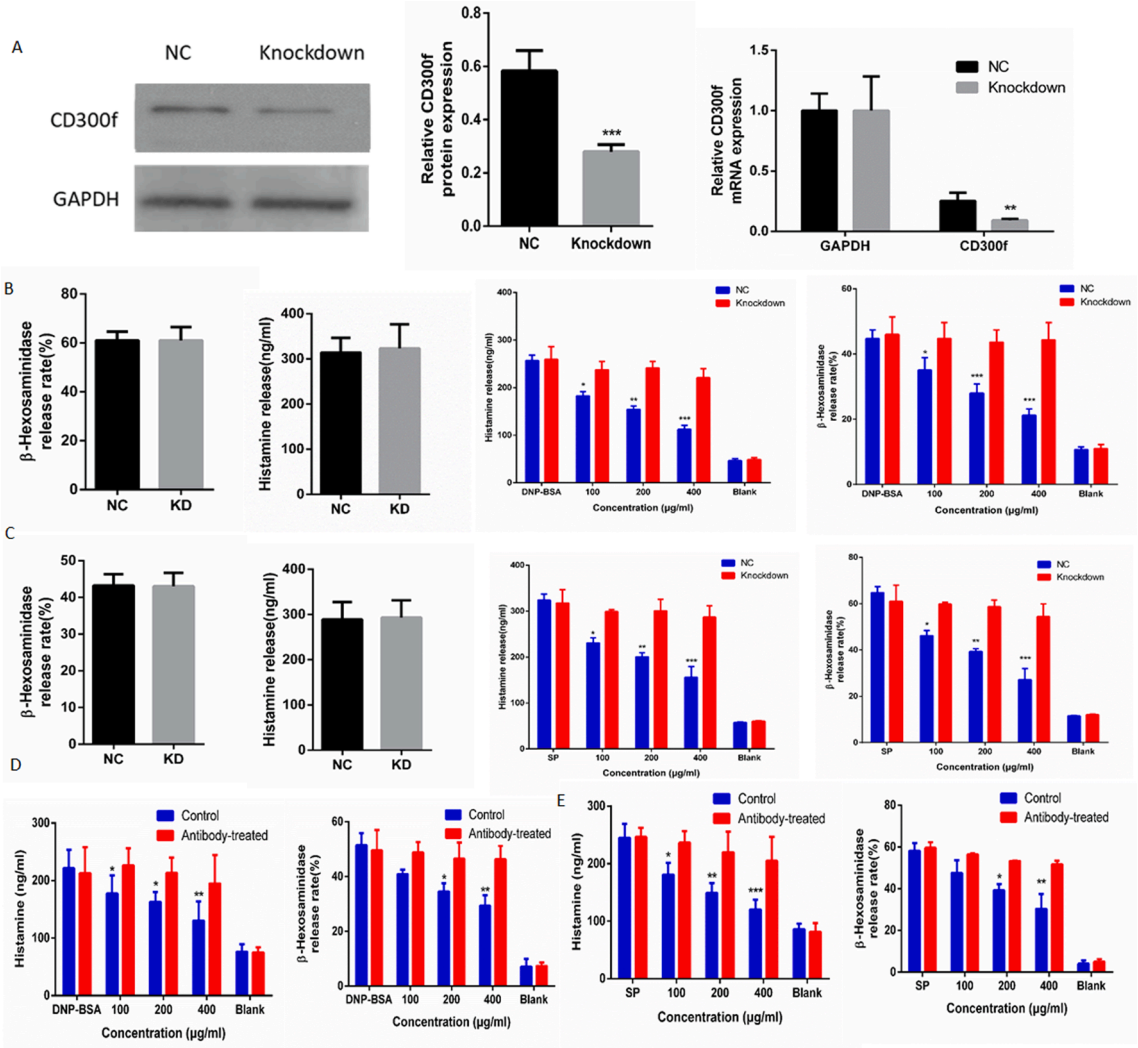

Fig. 6. CLA negative regulated LAD2 cells activization via CD300f. A: Efficiency of *si*RNA transfection in LAD2 cells analyzed by western blot and RT-PCR. B-C: After knockdown CD300f expression, the release of histamine and β-hexosaminidase caused by IgE and SP were not affected in knockdown-LAD2 cells. While CLA showed little effect on FcεRI- and MRGPRX2-mediated the release of histamine and β-hexosaminidase in knockdown-LAD2 cells compared with NC-LAD2 cells. D-E: After CD300f antibody treated, the inhibition effect of CLA on FcεRI and MRGPRX2-mediated the release of histamine and β-hexosaminidase was significantly decrease. (Experiments were repeated 3 times. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

three times. ELISA was used to analyze concentrations of the serum TNF-α, IL-13, IL-4, IL-6, and tryptase. The effectiveness of treatment was assessed using the urticaria activity score (UAS), which includes the scores of the wheal and itch. The CSU patients understood the purpose and content of the experiment. They understood that CLA was not a drug used commonly for the treatment CSU; however they were willing to accept CLA for treatment.

2.16. Inclusion criteria

The inclusion criteria were: age between 6 and 80 years, patients diagnosed with CSU (duration greater than or equal to six weeks), the symptoms of wheal and itch which did not improve significantly after antihistamine therapy according to UAS, the blood parameters being normal and suitable for the patients taking clarithromycin, and volunteering to participate in the program and be able to perform as required. The volunteers continued to take the CLA sustained-release tablets (250 mg/day) for two weeks according to the doctor's instructions, and they returned to the doctor after two weeks.

2.17. Exclusion criteria

The exclusion criteria were: CSU patients with atopic dermatitis, contact dermatitis, asthma, and other immune diseases; patients with definite indications of *H. pylori* infection and other microbial disturbances; patients with histories of chronic bacterial or fungal infections; patients diagnosed with physical urticaria (including urticaria factitia), cholinergic urticaria, or urticaria vasculitis; patients unable to cooperate or adhere to treatment and with poor predictive compliance; pregnant and nursing women; and patients who were also participating in other clinical trials.

2.18. Serum assay

A sample of 1 ml fresh blood was centrifuged and the serum collected for ELISA. Inflammatory mediators were detected using ELISA kits.

2.19. Ethical approval.

This study was registered at Chinese Clinical Trail Registry, and the

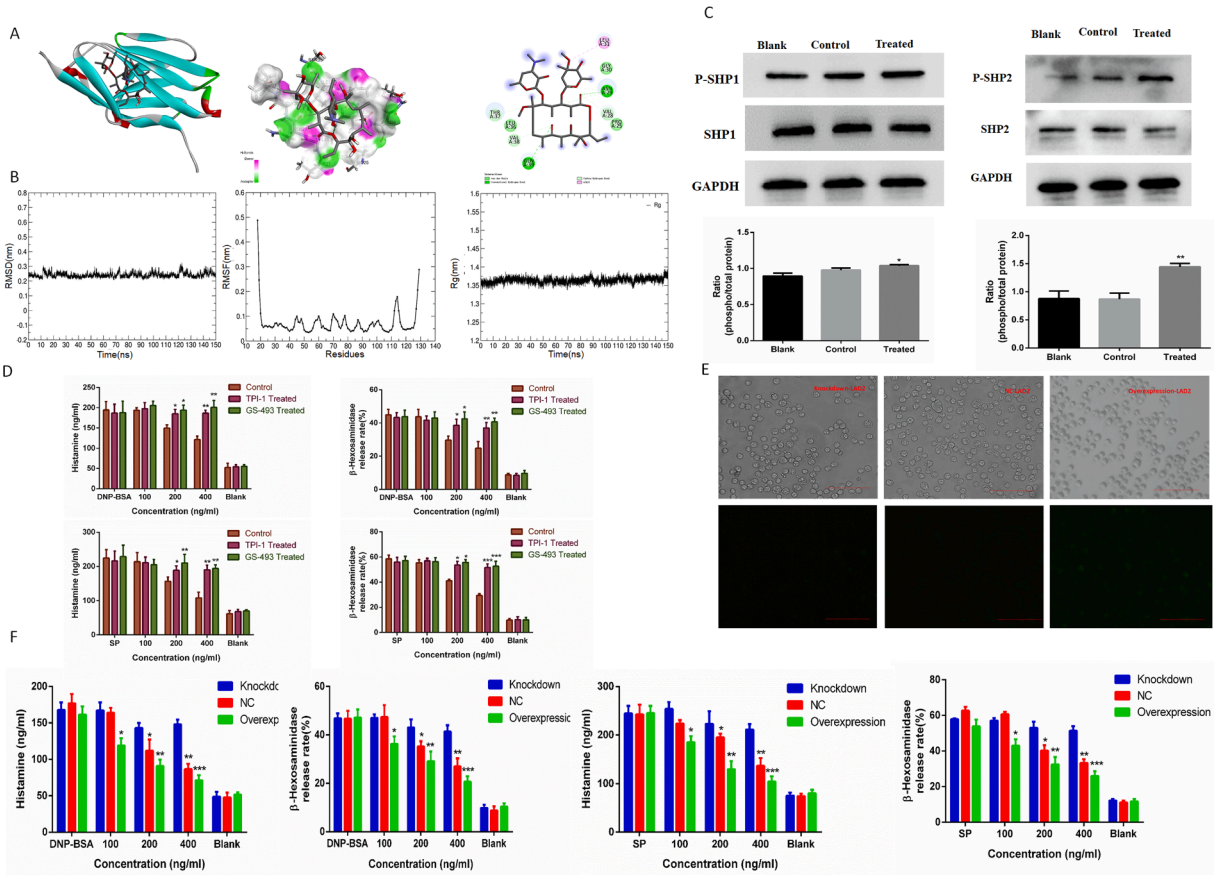

Fig. 7. CLA affect CD300f downstream signaling pathways. A: The docking mode of 2NMS-Clarithromycin. The hydrogen bonds surface of docking pocket. Creates a surface colored by hydrogen bond type, with receptor donors colored in green and receptor acceptors in cyan. 2D diagram of Clarithromycin and the binding site residues of 2NMS. B: Plot showing the RMSD values of 2NMS-Clarithromycin through 150 ns time period of Molecular dynamics simulation. RMSF of residues the 2NMS-Clarithromycin complex. Rg of 2NMS-Clarithromycin complex. C: CLA up regulated both the phosphorylation level of SHP-1 and SHP-2. D: After treated with TPI-1 and GS-493, the inhibition of CLA on FcεRI- and MRGPRX2-meidated LAD2 cells degranulation reaction was significantly decrease and showed little on the release of histamine and β- hexosaminidase. E: The efficiency of knockdown CD300f expression and overexpression CD300f were measured by immunofluorescence. F: Compared with the knockdown-LAD2, CLA could inhibite NC-LAD2 on FcεRI and MRGPRX2-mediated the release of histamine and β-hexosaminidase, and over expression CD300f, the inhibition was significantly enhanced (Experiments were repeated 3 times. Only Tyrode's solution buffer was set as blank group, only DNP-BSA or SP was set as control group. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

registration number is ChiCTR1900021411. The ethical approval was approved by Ethics Committee at Xi'an Jiaotong University and conformed to the ethical standard (2019-027).

2.20. Statistical analysis

Data are presented as the mean ± SEM. The data were analyzed using analysis of variance and one-tailed paired Student's t-tests with the SPSS software. The differences were considered significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Statistical analysis for clinical trials was performed using R (https://www.r-project.org/). The continuous variables are presented as means ± SDs. They were analyzed using the Student's *t* test or Fishers test. The categorical data are presented using numbers and percentages. Their associations were tested using the Chi-square test or Fisher's exact test. $P < 0.05$ was considered to be significant.

3. Results

3.1. CLA inhibits FcεRI- and MRGPRX2-mediated anaphylactic reactions in mice

In our study, ceramide was set as a positive control which has been proved work on CD300f to negative regulate of FcεRI mediated MC activation [13]. FcεRI-mediated passive anaphylaxis mouse model was used. IgE and IgG were significantly increase after treated by OVA, which showed OVA-induced type I anaphylactic reactions mouse model could be used in next experiments (Fig. 1A). CLA showed an inhibitory effect on OVA-induced type I anaphylactic reactions in mice in a dose-dependent manner. CLA reduced the exudation of Evans blue and the degree of swelling in the paw skin of the mice (Fig. 1A). Pathological examinations of H&E confirmed that CLA reduced IgE-induced angiotelectasis in mice (Fig. 1B). The reseason was CLA inhibiting MCs degranulation of the skin marked by avidin-FITC (Fig. 1C). CLA Significantly reduced the percentage of activated MCs (Fig. 1D). However, CLA showed little effect on reduced IgE level, which release by B cells (Fig. 1E), while CLA reduced the levels of histamine, TNF-α, MCP-1, IL-4, IL-6, and IL-13 in the serum of the mice, inhibiting the inflammatory reactions induced by IgE (Fig. 1F).

Moreover, MRGPRX2-mediated passive anaphylaxis mouse model was used to study the inhibition effect of CLA on Mrgprb2-mediated MCs activation, which is a homologous receptor of MRGPRX2 in mouse (Fig. 2). CLA also inhibited Mrgprb2-mediated pseudo-allergic reactions in mice. H&E staining and immunofluorescent staining showed that CLA inhibited the SP-induced MCs degranulation reaction of the skin to red, indicating the degree of telangiectasia (Fig. 2A-C). CLA significantly reduced the percentage of activated MCs induced by SP (Fig. 2D). CLA reduced the levels of histamine, TNF-α, MCP-1, and IL-8 in the serum

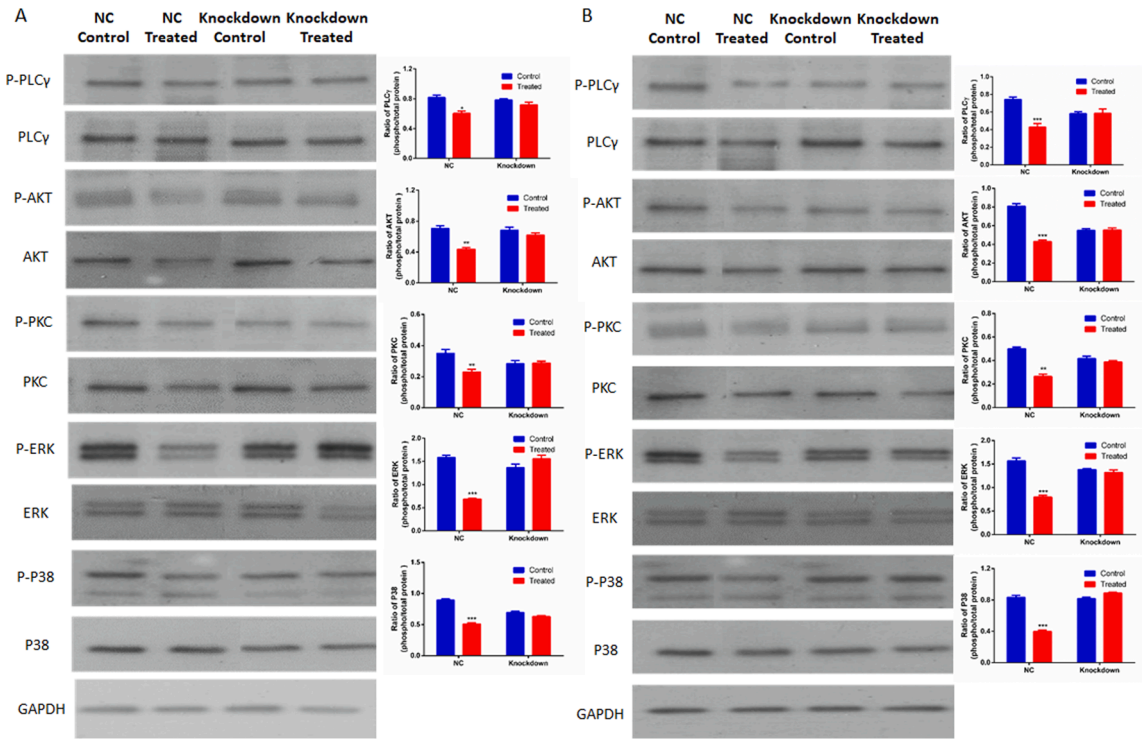

Fig. 8. Inhibition effect of CLA on signaling pathways decrease after knockdown CD300f. A: There were small reduction in the FcεRI downregulations of the phosphorylation of AKT, PKC, and ERK in the knockdown control group and the down-regulation effect of CLA on the phosphorylation level increase of PLC-γ, AKT, PKC, P38 and ERK mediated by FcεRI was significantly decrease. B: After knockdown the expression of CD300f, there were some impact in the phosphorylation of PLC-γ, AKT, PKC, ERK, and p38 in the MRGPRX2 downregulations, while the down-regulation effect of CLA on the phosphorylation level increase of PLC-γ, AKT, PKC, P38 and ERK mediated by MRGPRX2 was significantly decrease. (Experiments were repeated 3 times. Only DNP-BSA or SP was set as control group. Data are presented as the mean ± SEM. The differences were considered significant at *P < 0.05, **P < 0.01, ***P < 0.001.).

Table 1
CLA decreased the Symptoms in CU patients.

Variable		Before treatment	After treatment	*P* value
	Score			
UAS	1	0	4	
	2	0	15	1.24×10^{-8}
	3	1	4	
	4	8	1	
	5	6	1	
	6	13	3	
Wheal score				
	0	0	2	
	1	1	18	2.11×10^{-7}
	2	10	5	
	3	17	3	
Itch score				
	0	0	2	
	1	0	20	6.26×10^{-10}
	2	13	2	
	3	15	4	

Table 2
CLA decreased the level of inflammatory medium in serum of CU patients.

Variable	Before treatment (pg/ml)	After treatment (pg/ml)	t-statistics	*P* value
TNF-α	1259.88 ± 257.60	1120.95 ± 234.84	4.44	1.39×10^{-4}
IL-13	248.18 ± 45.77	208.47 ± 41.33	4.22	2.45×10^{-4}
IL-4	104.08 ± 18.85	81.22 ± 12.48	7.63	3.34×10^{-8}
IL-6	52.95 ± 6.56	47.39 ± 6.75	4.68	7.26×10^{-5}
Tryptase	4143.68 ± 3379.15	2598.05 ± 2512.38	6.43	6.90×10^{-7}

that were induced by SP (Fig. 2E).

3.2. CLA inhibits FcεRI- and MRGPRX2-mediated MC activation

CLA showed weak effects on LAD2 cell proliferation (Fig. 3A). CLA showed weak effects on the concentration of intracellular Ca^{2+} without obvious fluorescent changes (Fig. 3B). LAD2 cells were identified as responding if the $[Ca^{2+}]i$ rose by at least 50% for at least 10 s, which clearly distinguishes a ligand-induced response from random flickering events and each color line represents an individual cell. CLA did not induce the release of histamine and β-hexosaminidase in the LAD2 cells (Fig. 3C). However, CLA inhibited FcεRI- and MRGPRX2-mediated mast cell activations *in vitro* in a dose-dependent manner. CLA inhibited the release of β-hexosaminidase and histamine induced by IgE and reduced the release of TNF-α and IL-13 in the LAD2 cells (Fig. 3D). In addition, CLA reduced the release of histamine, β-hexosaminidase, TNF-α, and IL-8 induced by SP (Fig. 3E).

Both FcεRI- and MRGPRX2-mediated MC activations presented with an increased Ca^{2+} flux. CLA inhibited the increase in the concentration of intracellular Ca^{2+} mediated by FcεRI and MRGPRX2 (Fig. 4A-B). The western blotting results showed that with the use of CLA in LAD2 cells, the levels of phosphorylated Lyn, Btk and PLC-γ increased by IgE, were downregulated. These are key proteins in the calcium signaling pathways of FcεRI and MRGPRX2 (Fig. 4C-E). CLA also decreased the levels of phosphorylated PLC-γ that were increased by SP (Fig. 4F).

In addition, the results showed that CLA inhibited the

phosphorylation levels of proteins in the release of mast cell cytokines among the signaling pathways, which are the downstream of FcεRI and MRGPRX2. CLA downregulated the phosphorylation of AKT, PKC, P38, and ERK induced by IgE and SP (Fig. 5).

3.3. CLA regulates mast cell activation via CD300f negatively

CD300f in LAD2 cells was downregulated by *si*RNA transfections, as per the results of the western blotting and RT-PCR (Fig. 6A). The degranulation reactions of LAD2 cells were not affected after the knockdown of the CD300f expression. The release of histamine and β-hexosaminidase caused by IgE (Fig. 6B) and SP in knockdown-LAD2 cells did not show a significant difference from that in the NC-LAD2 cells (Fig. 6C). Moreover, after CD300f antibody treated, the inhibition effect of CLA on FcεRI and MRGPRX2-mediated the release of histamine and β-hexosaminidase was significantly decrease (Fig. 6D-E).

We performed molecular docking between 2NMS and Clarithromycin. The binding energies was −7.2 kcal/mol. Discovery Studio were employed to visualize the molecular docking conformations (Fig. 7A). The docking complexes were selected for molecular dynamics simulation experiments. The Root Mean Square Deviation (RMSD) was used to indicate the degree of molecular structure change and to measure the stability of the complex system. All compounds were stable during operation. RMSD fluctuation of 2NMS-Clarithromycin system is less than 0.3 nm. Root Mean Square Fluctuation (RMSF) showed the Fluctuation and structural flexibility of amino acid residues of receptor protein. RMSF analysis showed that the RMSF value of the amino acid residues binding Clarithromycin in receptor protein was less than 0.5 nm, reflecting that Clarithromycin binding can make the structure of the binding region more stable to a certain extent. Radius of gyration (Rg) measures for compactness of protein. The Rg values of 2NMS-Clarithromycin system was both less than 1.4 nm. The molecular dynamics simulation results indicated that the complexes remained stable with favorable conformations throughout 150 ns. Above all, it suggested that the Clarithromycin were stable at the binding site of 2NMS during the interactions. The foregoing results suggest that Clarithromycin bound 2NMS proteins (Fig. 7B). Moreover, both the phosphorylation levels of SHP-1 and SHP-2 were upregulated by CLA, which led to inhibitory effects via CD300f (Fig. 7C). Moreover, after treated with TPI-1 and GS-493, the inhibition of CLA on FcεRI- and MRGPRX2-meidated LAD2 cells degranulation reaction was significantly decrease and showed little on the release of histamine and β- hexosaminidase (Fig. 7D). In further research, we knockdown CD300f expression and overexpression CD300f used shRNA plasmid and the CD300f expression were measured by immunofluorescence (Fig. 7E). The MC degranulation experiment showed that compared with the knockdown-LAD2, CLA could inhibite WT-LAD2 on FcεRI and MRGPRX2-mediated the release of histamine and β-hexosaminidase, and after overexpression CD300f, the inhibition was significantly enhanced (Fig. 7F).

After knockdown CD300f, there were small reduction in the FcεRI downregulations of the phosphorylation of AKT, PKC, and ERK in the knockdown control group (Fig. 8A). And in the MRGPRX2 downregulations, there were some impact in the phosphorylation of PLC-γ, AKT, PKC, ERK, and p38 (Fig. 8B). However, the inhibitory effects of CLA on the signaling pathways of FcεRI and MRGPRX2, showed a significant decrease. The downregulations of the phosphorylation of PLC-γ, AKT, PKC, P38, and ERK caused by CLA and mediated by FcεRI and MRGPRX2 also showed a significant decrease (Fig. 8).

3.4. CLA shows therapeutic effects on CSU

The present study used peripheral blood samples from 43 patients who were not sensitive to the third-generation antihistamines and used CLA to treat CSU. After the follow-up visit, 28 subjects were finally included in our study. Only five patients did not respond well to the CLA treatment. In comparison to patients without CLA treatment, the wheal and itch was relieved significantly in 23 patients after the use of CLA. The effective rate was 82.14%. CLA was found to decrease the UAS, wheal scores and itch scores in patients with CSU (Table 1).

The serum indices of the patients were further evaluated and CLA was found to significantly decrease the levels of TNF-α, IL-13, IL-4, IL-6 and tryptase in CSU patients (Table 2).

4. Discussion

MCs are key effectors in regulating inflammatory responses [20–21]. IgE-induced mast cell degranulation via FcεRI plays an important role in immune diseases. However, a growing number of reports suggest that MRGPRX2 is involved in the development of many allergic diseases and it may be as a potential therapeutic target [22–23]. The quantity of MRGPRX2 is higher under the skin lesions that occur in atopic dermatitis in CSU patients. It also plays a key role in the erythroid syndromes caused by ciprofloxacin hydrochloride and vancomycin [24]. In addition, MRGPRX2 can be activated by hemoglobin and it induces the activation of MCs in the lung, which are involved in the occurrence and development of allergic asthma [25]. Another study showed that MRGPRX2 also participated in some IgE-induced diseases, such as allergic asthma and that FcεRI and MRGPRX2 exerted a coordinating effect on the development of the disease [26]. MCs are vital effector cells in CSU, however, there is no mouse model for CSU. MCs activation might cause the release of inflammatory mediators other than histamine, such as tryptase, IL-4, IL-6 and IL-13, which also showed effect on CSU. Both FcεRI- and MRGPRX2-mediated MCs activation showed important effect on CSU occurrence and development. Inhibition of FcεRI- and MRGPRX2-mediated MCs activation has potential therapeutic value for CSU. So in our study, FcεRI- and MRGPRX2-mediated the passive anaphylaxis model was applied to our study.

The CD300 receptors are type of I transmembrane proteins expressed in both the myeloid and lymphoid lineages, modulating immune responses by their stimulatory and inhibitory capabilities. These molecules have an IgV-like extracellular and transmembrane domains, and a cytoplasmic tail that could be short or long depending on their functionality. CD300f exhibits an inhibitory capacity due to its long cytoplasmic tail that contains the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) [27–28]. The binding of CD300f to its physiological ligands, ceramide and sphingomyelin, inhibits IgE-mediated mast cell activation. CD300f also suppresses human mast cell-dependent allergic responses [29].

The activation of CD300f showed positive effects on the treatment of immune and inflammatory diseases. Some studies have indicated that CD300f expressed on microglial cells, and activations of CD300f showed resistance to cerebral malaria by suppressing the neuroinflammatory responses [30]. CD300f is an important target in the regulation of MC activation, but whether it can negatively regulate MRGPRX2-mediated pseudo-allergic reactions, has not been clearly reported. The results of clinical trials confirmed that CLA decreased the wheal scores, itch scores, and serum inflammatory mediators in CSU patients. CLA showed a good curative effect in treating CSU. The results showed that CD300f regulated MRGPRX2-mediated mast cell activations, negatively. CLA could negatively regulate both FcεRI- and MRGPRX2-mediated mast cell activations via CD300f by inhibiting both type I allergic reactions and pseudo-allergic reactions. Our study indicated a partial elucidation of the mechanisms of CLA in immune regulation.

CD300f mediates inhibitory signals by activating Src homology 2 (SH2) 4 domain-containing phosphatases and CD300f activation induced interactions with phosphatase- 1 (SHP-1) [31]. CD300f reduced the induction of pro-inflammatory mediator expressions via its association with SHP-1 to block TLR Signaling [32]. However, both CD300a and CD300f which belong to CD300 family, showed negative regulatory effects. These were the differential effects of SHP-1 and SHP-2 in the inhibitory effects of CD300a and CD300f. The triggering of CD300a showed weak inhibitory activities against TRIF-mediated cellular

activations, because CD300a only affected SHP-1. The triggering of CD300f induced phosphorylation of both SHP-1 and SHP-2 to showed inhibitory effects on MyD88- and TRIF-mediated cellular activations by combining the actions of SHP-1 and SHP-2, which may be marks of CD300f activation [33]. Our results showed that CLA presented the inhibitory effects on FcεRI- and MRGPRX2-mediated MC activations by up regulating both the phosphorylation levels of SHP-1 and SHP-2.

CSU is characterized by both autoimmune and allergic diseases, the pathogenesis of which is not fully understood. Skin MCs are recognized as the main effector cells, which can be activated and are able to release histamine and other pro-inflammatory agents that induce the dilations of blood vessels in CSU patients [34]. However, while the root cause of the activation of MCs are largely unknown, the autoimmune antibodies IgE and IgG are both factors affecting CSU. FcεRI activation induced MC degranulation is a key step in both the IgE-induced type I and IgG induced type II hypersensitivity reactions. Thus, FcεRI activation is a key step in the occurrence and development of CSU [35]. IgG activates MCs requiring complement participation which is IgE-independent. Pathogenic IgG directly cross-link FcεRI to cause histamine release, and the activation of cells is enhanced by complement participation [36]. However, IgG and IgE antibodies are presented in approximately only 40% of CSU patients [37].

The role of MRGPRX2 in skin diseases has attracted increasing attentions recently. The activation of MCs via MRGPRX2 may contribute to neurogenic inflammations, itch, and pruritic skin diseases, such as CSU and atopic dermatitis (AD) [38]. Increased numbers of MRGPRX2-positive cells have been reported in the skin of patients with CSU. In complementary studies, patients with CSU responded to intradermal applications of the MRGPRX2 agonists, such as endogenous polypeptides. SP, vasoactive intestinal peptides and eosinophil cationic proteins, which have been proven to activate MRGPRX2 (found elevated in CSU patients), with augmented wheal reactions. MRGPRX2 activation caused increased skin reactivities in CSU patients [39].

A single conventional dose of second generation H_1 antihistamines (sgAH) is recommended as a first-line treatment, in each guideline, to treat CSU. Each guideline recommends the regular application rather than an on-demand use of sgAH to achieve maximum clinical benefits [40–41]. In addition, all the guidelines recommend short-term oral corticosteroids to control the progression of CSU. Guidelines of urticaria for second-line treatments show significant differences. International guidelines suggest that the dose of sgAH can be increased up to four times if urticaria symptoms cannot be controlled after two to four weeks of first-line treatments. The United States guidelines offer a variety of options for second-line therapies, including increasing the dose of sgAH, combinations with other types of sgAHs, combinations with H_2 receptor antagonist, combinations with leukotriene receptor blockers, and increasing the doses of first-generation H_1 antihistamines (fgAH) before sleep [41]. However, the Chinese guidelines suggest that the dose of sgAHs can be increased, and that fgAH be combined with sgAH before bedtime, if the urticaria symptoms were controlled after one to two weeks of treatment. However, according to international guidelines, there is not sufficient evidence to recommend the use of the H_2 receptor antagonists, LTRA or fgAH before bedtime. Omagzumab is used as a third-line therapy according to international guidelines, while the use of doxepin or hydroxazine is recommended in the United States guidelines [40–41]. In the Chinese guidelines, cyclosporine or biological agents are used as third-line therapies. However, there are no drugs that target the degranulation of MCs in the current urticaria treatment guidelines. Thus, developing drug candidates that inhibit FcεRI and MRGPRX2 activation, simultaneously, will be advantageous of in the treatment of CSU.

CLA displayed significant effects in the treatment of CSU patients who are not sensitive to the antihistamine drugs. CLA showed a negative regulation on FcεRI-mediated type I allergic reactions, and MRGPRX2-mediated pseudo-allergic reactions as an exogenous stimulant of CD300f by up regulating both the phosphorylation levels of SHP-1 and SHP-2. Our study confirmed that CLA, which has immunomodulatory effects, maybe a potential drug for the treatment of allergic diseases.

5. Funding sources

This work was supported by the National Natural Science Foundation of China (Grant number: 81930096).

CRediT authorship contribution statement

Delu Che: Conceptualization, Methodology, Writing – original draft. **Tao Zhang:** Methodology, Formal analysis, Writing – original draft. **Tianxiao Zhang:** Methodology, Formal analysis, Data curation. **Yi Zheng:** Methodology, Formal analysis, Writing – original draft. **Yajing Hou:** Methodology, Formal analysis. **Songmei Geng:** Supervision, Resources, Conceptualization, Methodology. **Langchong He:** Supervision, Resources, Conceptualization, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgments

We thank the patients and their families for their participation in these studies. This work was supported by the National Natural Science Foundation of China (Grant number: 81930096).

References

[1] L. Webster, N.L. Rider, M.E. Archambault, Evaluating and managing chronic idiopathic urticaria in adults, JAAPA 31 (7) (2018) 22–26, https://doi.org/10.1097/01.JAA.0000534976.46365.11.

[2] I. Cherrez Ojeda, E. Vanegas, M. Felix, V. Mata, S. Cherrez, D. Simancas-Racines, L. Greiding, J. Cano, A. Cherrez, J.C. Calderon. Etiology of chronic urticaria: the Ecuadorian experience. World Allergy Organ. J., 11(1) (2018) 1, 0.1186/s40413-017-0181-0.

[3] D. Poddighe, M. De Amici, G.L. Marseglia, Spontaneous (autoimmune) chronic urticaria in children: current evidences, diagnostic pitfalls and therapeutic management, Recent. Pat. Inflamm. Allergy Drug Discov. 10 (1) (2016) 34–39, https://doi.org/10.2174/1872213x10666160219163502.

[4] S. Altrichter, J.S. Fok, Q. Jiao, P. Kolkhir, P. Pyatilova, S.M. Romero, J. Scheffel, F. Siebenhaar, C. Steinert, D. Terhorst-Molawi, Y.K. Xiang, M.K. Church, M. Maurer, Total IgE as a marker for chronic spontaneous urticarial, Allergy Asthma Immunol. Res. 13 (2) (2021) 206–218, https://doi.org/10.4168/aair.2021.13.2.206.

[5] M. Cugno, A. Tedeschi, B. Frossi, F. Bossi, A.V. Marzano, R. Asero, Detection of low-molecular-weight mast cell-activating factors in serum from patients with chronic spontaneous urticarial, J. Investig. Allergol Clin. Immunol. 26 (5) (2016) 310–313, https://doi.org/10.18176/jiaci.0051.

[6] D. Fujisawa, J. Kashiwakura, H. Kita, Y. Kikukawa, Y. Fujitani, T. Sasaki-Sakamoto, K. Kuroda, S. Nunomura, K. Hayama, T. Terui, C. Ra, Y. Okayama, Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticarial, J. Allergy Clin. Immunol. 134 (3) (2014) 622–633, https://doi.org/10.1016/j.jaci.2014.05.004.

[7] H. Kühn, P. Kolkhir, M. Babina, M. Düll, S. Frischbutter, J.S. Fok, Q. Jiao, M. Metz, J. Scheffel, K. Wolf, A.E. Kremer, M. Maurer, Mas-related G protein-coupled receptor X2 and its activators in dermatologic allergies, J. Allergy Clin. Immunol. 147 (2) (2021) 456–469, https://doi.org/10.1016/j.jaci.2020.08.027.

[8] S. Roy, C. Chompunud Na Ayudhya, M. Thapaliya, V. Deepak, H. Ali, Multifaceted MRGPRX2: New insight into the role of mast cells in health and disease, J. Allergy Clin. Immunol. 148 (2) (2021) 293–308, https://doi.org/10.1016/j.jaci.2021.03.049.

[9] Y. Shi, S. Zhou, Q. Zheng, Y. Huang, P. Hao, M. Xu, L. Zhang, X. Xiao, H. Zheng, Y. Li, Systematic reviews of pharmacological and nonpharmacological treatments for patients with chronic urticarial, Medicine (Baltimore) 98 (20) (2019) e15711.

[10] M. Maurer, U. Raap, P. Staubach, G. Richter-Huhn, A. Bauer, E.M. Oppel, U. Hillen, D. Baeumer, M. Reinhardt, N. Chapman-Rothe, Antihistamine-resistant chronic

spontaneous urticaria: 1-year data from the AWARE study, Clin. Exp. Allergy 49 (5) (2019) 655–662.
[11] A. Maehara, A. Kaitani, K. Izawa, E. Shiba, M. Nagamine, A. Takamori, M. Isobe, S. Uchida, K. Uchida, T. Ando, K. Maeda, N. Nakano, D. Voehringer, A. Roers, T. Shimizu, H. Ogawa, K. Okumura, T. Kitamura, J. Kitaura, Role of the ceramide-CD300f interaction in gram-negative bacterial skin infections, J, Invest. Dermatol. 138 (5) (2018) 1221–1224, https://doi.org/10.1016/j.jid.2017.11.025.
[12] E. Abadir, P.A. Silveira, R.E. Gasiorowski, M. Ramesh, A. Romano, A.H. Mekkawy, Targeting CD300f to enhance hematopoietic stem cell transplantation in acute myeloid leukemia, Blood Adv. 4 (7) (2020) 1206–1216, https://doi.org/10.1182/bloodadvances.2019001289.
[13] K. Izawa, Y. Yamanishi, A. Maehara, M. Takahashi, M. Isobe, S. Ito, A. Kaitani, T. Matsukawa, T. Matsuoka, F. Nakahara, T. Oki, H. Kiyonari, T. Abe, K. Okumura, T. Kitamura, J. Kitaura, The receptor LMIR3 negatively regulates mast cell activation and allergic responses by binding to extracellular ceramide, Immunity 37 (5) (2012) 827–839, https://doi.org/10.1016/j.immuni.2012.08.018.
[14] S. Uchida, K. Izawa, T. Ando, H. Yamada, K. Uchida, N. Negishi, A. Kaitani, A. Maehara, M. Nagamine, A. Kamei, A. Takamori, K. Maeda, N. Nakano, T. Shimizu, H. Ogawa, K. Okumura, A. Nagahara, S. Watanabe, J. Kitaura, CD300f is a potential therapeutic target for the treatment of food allergy, Allergy 75 (2) (2020) 471–474, https://doi.org/10.1111/all.14034.
[15] T.A. Seemungal, T.M. Wilkinson, J.R. Hurst, W.R. Perera, R.J. Sapsford, J. A. Wedzicha, Long-term erythromycin therapy is associated with decreased chronic obstructive pulmonary disease exacerbations, Am. J. Respir. Crit. Care Med. 178 (2008) 1139–1147, https://doi.org/10.1164/rccm.200801-145OC.
[16] A.M. D'Erme, A. Boca, M. Sabau, N. Milanesi, F. Simonacci, M. Gola, Successful treatment of rosacea fulminans in a 59-year-old woman with macrolide antibiotics and prednisone, Int. J. Dermatol. 55 (8) (2016) e470–e472, https://doi.org/10.1111/ijd.13277.
[17] Y. Hashimoto, Y. Kaneda, N. Takahashi, S. Akashi, I. Arai, S. Nakaike, Clarithromycin inhibits the development of dermatitis in NC/Nga mice, Chemotherapy 49 (5) (2003) 222–228, https://doi.org/10.1159/000072445.
[18] V. Ivetić Tkalčević, S. Cužić, M.D. Kramarić, M.J. Parnham, V. Eraković Haber, Topical azithromycin and clarithromycin inhibit acute and chronic skin inflammation in sensitized mice, with apparent selectivity for Th2-mediated processes in delayed-type hypersensitivity, Inflammation. 35 (1) (2012) 192–205, https://doi.org/10.1007/s10753-011-9305-9.
[19] I. Kazama, K. Saito, A. Baba, T. Mori, N. Abe, Y. Endo, H. Toyama, Y. Ejima, M. Matsubara, M. Yamauchi, Clarithromycin dose-dependently stabilizes rat peritoneal mast cells, Chemotherapy 61 (6) (2016) 295–303, https://doi.org/10.1159/000445023.
[20] H. Bonnekoh, J. Scheffel, N. Kambe, K. Krause, The role of mast cells in autoinflammation, Immunol. Rev. 282 (1) (2018) 265–275, https://doi.org/10.1111/imr.12633.
[21] C. Akin, Mast cell activation syndromes, J. Allergy Clin. Immunol. 140 (2) (2017) 349–355, https://doi.org/10.1016/j.jaci.2017.06.007.
[22] H. Ali, Mas-related G protein coupled receptor-X2: A potential new target for modulating mast cell-mediated allergic and inflammatory diseases, J. Immunobiol. 1 (4) (2016) 115.
[23] A. Takamori, K. Izawa, A. Kaitani, T. Ando, Y. Okamoto, A. Maehara, A. Tanabe, M. Nagamine, H. Yamada, S. Uchida, K. Uchida, M. Isobe, T. Hatayama, D. Watanabe, T. Ando, T. Ide, M. Matsuzawa, K. Maeda, N. Nakano, N. Tamura, K. Ikeda, N. Ebihara, T. Shimizu, H. Ogawa, K. Okumura, J. Kitaura, Identification of inhibitory mechanisms in pseudo-allergy involving Mrgprb2/MRGPRX2-mediated mast cell activation, J. Allergy Clin. Immunol. 143 (3) (2019) 1231–1235, https://doi.org/10.1016/j.jaci.2018.10.034.
[24] E. Azimi, V.B. Reddy, E.A. Lerner, Brief communication: MRGPRX2, atopic dermatitis and red man syndrome, Itch (Phila) 2 (1) (2017) e5.
[25] W. Manorak, C. Idahosa, K. Gupta, S. Roy, R. Panettieri Jr, H. Ali, Upregulation of Mas-related G Protein coupled receptor X2 in asthmatic lung mast cells and its activation by the novel neuropeptide hemokinin-1, Respir. Res. 19 (1) (2018) 1, https://doi.org/10.1186/s12931-017-0698-3.
[26] J. An, J.H. Lee, H.K. Won, Y. Kang, W.J. Song, H.S. Kwon, Y.S. Cho, H.B. Moon, T. B. Kim, Clinical significance of serum MRGPRX2 as a new biomarker in allergic asthma, Allergy 75 (4) (2020) 959–962, https://doi.org/10.1111/all.14084.
[27] J. Vitallé, I. Terrén, A. Orrantia, O. Zenarruzabeitia, F. Borrego, CD300 receptor family in viral infections, Eur. J. Immunol. 49 (3) (2019) 364–374, https://doi.org/10.1002/eji.201847951.
[28] J. Vitallé, I. Terrén, A. Orrantia, A. Bilbao, P.M. Gamboa, F. Borrego, O. Zenarruzabeitia, The expression and function of CD300 molecules in the main players of allergic responses: Mast Cells, basophils and eosinophils, Int. J. Mol. Sci. 21 (9) (2020) 3173, https://doi.org/10.3390/ijms21093173.
[29] K. Izawa, M. Isobe, T. Matsukawa, S. Ito, A. Maehara, M. Takahashi, Y. Yamanishi, A. Kaitani, T. Oki, K. Okumura, T. Kitamura, J. Kitaura, Sphingomyelin and ceramide are physiological ligands for human LMIR3/CD300f, inhibiting FcεRI-mediated mast cell activation, J. Allergy Clin. Immunol. 133 (270–273) (2014) e1–e7, https://doi.org/10.1016/j.jaci.2013.08.008.
[30] R.J. Davidson, In vitro activity and pharmacodynamic/pharmacokinetic parameters of clarithromycin and azithromycin: why they matter in the treatment of respiratory tract infections, Infect. Drug Resist. 12 (2019) 585–596, https://doi.org/10.2147/IDR.S187226.
[31] D. Alvarez-Errico, J. Sayós, M. López-Botet, The IREM-1 (CD300f) inhibitory receptor associates with the p85 subunit of phosphoinositide 3-kinase, J. Immunol. 178 (2) (2007) 808–816, https://doi.org/10.4049/jimmunol.178.2.808.
[32] S.M. Lee, E.J. Kim, K. Suk, W.H. Lee, CD300F blocks both MyD88 and TRIF-mediated TLR signaling through activation of Src Homology Region 2 domain-containing phosphatase 1, J. Immunol. 186 (11) (2011) 6296–6303, https://doi.org/10.4049/jimmunol.1002184.
[33] E.J. Kim, S.M. Lee, K. Suk, W.H. Lee, CD300a and CD300f differentially regulate the MyD88 and TRIF-mediated TLR signalling pathways through activation of SHP-1 and/or SHP-2 in human monocytic cell lines, Immunology 135 (3) (2012) 226–235, https://doi.org/10.1111/j.1365-2567.2011.03528.x.
[34] M.K. Church, P. Kolkhir, M. Metz, M. Maurer, The role and relevance of mast cells in urticaria, Immunol. Rev. 282 (1) (2018) 232–247, https://doi.org/10.1111/imr.12632.
[35] P. Kolkhir, M.K. Church, K. Weller, M. Metz, O. Schmetzer, M. Maurer, Autoimmune chronic spontaneous urticaria: what we know and what we do not know, J. Allergy Clin. Immunol. 139 (6) (2017) 1772–1781, https://doi.org/10.1016/j.jaci.2016.08.050.
[36] E.W. Langley, J. Gigante, Anaphylaxis, urticarial, and angioedema, Pediatr. Rev. 34 (2013) 247–257, https://doi.org/10.1542/pir.2019-0056.
[37] K. Vasagar, B.M. Vonakis, L.M. Gober, A. Viksman, S.P. Gibbons Jr, S.S. Saini, Evidence of in vivo basophil activation in chronic idiopathic urticarial, Clin. Exp. Allergy 36 (2006) 770–776, https://doi.org/10.1111/j.1365-2222.2006.02494.x.
[38] H. Subramanian, K. Gupta, H. Ali, Roles of Mas-related G protein-coupled receptor X2 on mast cell-mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases, J. Allergy Clin. Immunol. 138 (2016) 700–710, https://doi.org/10.1016/j.jaci.2016.04.051.
[39] M. Shtessel, N. Limjunyawong, E.T. Oliver, K. Chichester, L. Gao, X. Dong, S. S. Saini, MRGPRX2 activation causes increased skin reactivity in patients with chronic spontaneous urticarial, J, Invest. Dermatol. 141 (3) (2021) 678–681, https://doi.org/10.1016/j.jid.2020.06.030.
[40] T. Zuberbier, W. Aberer, R. Asero, A.H. Abdul Latiff, D. Baker, B. Ballmer-Weber, et al., The EAACI/GA2LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticarial, Allergy 73 (7) (2018) 1393–1414, https://doi.org/10.1111/all.13397.
[41] J.A. Bernstein, D.M. Lang, D.A. Khan, T. Craig, D. Dreyfus, F. Hsieh, J. Sheikh, D. Weldon, B. Zuraw, D.I. Bernstein, J. Blessing-Moore, L. Cox, R.A. Nicklas, J. Oppenheimer, J.M. Portnoy, C.R. Randolph, D.E. Schuller, S.L. Spector, S. A. Tilles, D. Wallace, The diagnosis andmanagement of acute and chronic urticarial: 2014 update, J. Allergy Clin. Immunol. 133 (5) (2014) 1270–1277, https://doi.org/10.1016/j.jaci.2014.02.036.