

PAPER

Imidazolidinyl urea activates mast cells via MRGPRX2 to induce non-histaminergic allergy

Jiapan Gao,^{1,†} Delu Che,^{2,3,†} Xueshan Du,² Yi Zheng,² Huiling Jing⁴ and Nan Wang^{1,*}

¹Department of Pharmaceutical analysis, School of Pharmacy, Xi'an Jiaotong University, 76, Yanta west road, Xi'an, China, ²Department of Dermatology, Northwest Hospital, The Second Hospital Affiliated to Xi'an Jiaotong University, 157, Xiwu road, Xi'an, Shaanxi, China, ³Center for Dermatology Disease, Precision Medical Institute, East Yinxing Road, Xi'an, China and ⁴Department of Dermatology, Xi'an Hospital of Traditional Chinese Medicine, 69, Fengcheng 8th Road, Xi'an, Shaanxi, China

*Correspondence address. School of Pharmacy, Xi'an Jiaotong University, Yanta Westroad, Xi'an 710061, China. Tel: +86-29-82656788; Fax: +86-29-82655451. E-mail: wangnan2014@xjtu.edu.cn.

†These authors contributed equally to this work.

Abstract

Imidazolidinyl urea (IU) is used as an antimicrobial preservative in cosmetic and pharmaceutical products. IU induces allergic contact dermatitis, however, the mechanism has not yet been elucidated. Mas-related G protein-coupled receptor-X2 (MRGPRX2) triggers drug-induced pseudo-allergic reactions. The aims of this study were to determine whether IU activated mast cells through MRGPRX2 to further trigger contact dermatitis. Wild-type (WT) and Kit^{W-sh}/HNIhrJaeBsmJNju (MUT) mice were treated with IU to observe its effects on local inflammation and mast cells degranulation *in vivo*. Laboratory of allergic disease 2 cells were used to detect calcium mobilization and release of inflammatory mediators *in vitro*. WT mice showed a severe local inflammatory response and contact dermatitis, whereas only slight inflammatory infiltration was observed in MUT mice. Thus, MRGPRX2 mediated the IU-induced activation of mast cells. However, histamine, a typical allergen, was not involved in this process. Tryptase expressed by mast cells was the major non-histaminergic inflammatory mediator of contact dermatitis. IU induced anaphylactic reaction via MRGPRX2 and further triggering non-histaminergic contact dermatitis, which explained why antihistamines are clinically ineffective against some chronic dermatitis.

Key words: imidazolidinyl urea, contact dermatitis, MRGPRX2, mast cell, tryptase

Introduction

Imidazolidinyl urea (IU) is a small-molecule chemical with the molecular formula C₁₁H₁₆N₈O₈. It is also known as imidurea; bis(methylolhydantoinurea)methane; or *N,N'*-methylenebis(*N'*-(1[or3]-hydroxymethyl)-2,5-dioxo-4-imidazolidinyl)urea [1]. IU has broad-spectrum antibacterial activity that mainly inhibits the reproduction of gram-negative and gram-positive bacteria and limits the growth of yeasts and molds to a certain extent. Therefore, it is frequently used as an antimicrobial preservative

in pharmaceutical creams, ointments, cosmetics, and toiletries formulations [2]. However, the decomposition of IU has been reported to release formaldehyde. As a well-known sensitizer, allergic contact dermatitis reactions have been widely reported after exposure to IU [3, 4]. Previously, a survey from a multicenter study in the USA (NACDG) conducted routine testing of IU on patients with suspected contact dermatitis, and the results showed a frequency of sensitization ranged from 1.3 to 3.3%. The lower sensitization frequencies showed in several studies

Received: 30 November 2020; Revised: 16 March 2021; Accepted: 25 March 2021

© The Author(s) 2020. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

in European countries ranging from 0.3 to 1.4%. As one of the most commonly used preservatives, IU is responsible for allergic dermatitis and used clinically as a reagent for patch testing.

Although it has been suggested in many studies that IU can induce allergic reactions, to date, the specific mechanism by which IU activates mast cells and triggers allergic contact dermatitis has not been clarified. Previous studies have shown that anaphylaxis is a systemic hypersensitivity reaction that occurs very rapidly and can be life-threatening. Immunoglobulin E (IgE)-dependent and IgE-independent pathways are two routes of immunologic anaphylaxis [5]. Classically, mast cells membrane surface express high-affinity receptors (FcεRI) of IgE. After being activated, mast cells can generate and release a variety of inflammatory mediators and cytokines involved in allergic reactions, thus causing various allergic symptoms [6]. Anaphylactoids are generally considered to be nonimmune-mediated allergic reactions that the drug component directly acts on mast cells and basophils to cause degranulation [7]. In 2015, McNeil et al. demonstrated that secretagogues and small-molecule drugs can activate LAD2 cells by binding to MRGPRX2, thereby increasing intracellular calcium ion concentrations and inducing degranulation. Mrgprb2 is the mouse ortholog of human MRGPRX2 [8]. Therefore, we speculated that IU, as a small-molecule chemical, was likely to directly bind to MRGPRX2 to activate mast cells, cause their degranulation, and induce an allergic reaction.

In this study, we used wild-type (WT) and Kit^{W^{sh}}/HNhrJaeBsmJNju mice (MUT) to investigate contact dermatitis after exposure of the back skin to IU. PAMP(9-20) was proved as agonist to induced MCs release of tryptase [9]. IU was found to induce non-histaminergic allergic contact dermatitis via the activation of mast cells. Human LAD2 mast cells and HEK293 cells expressing high levels of MRGPRX2 were used to elucidate the mechanism by which calcium mobilization and the release of inflammatory factors in mast cells were involved in triggering contact dermatitis *in vitro*. We found that IU induced non-histaminergic allergic contact dermatitis and mast cell activation through MRGPRX2, which triggered changes in calcium influx-related protein signaling pathways.

Materials and Methods

Drugs and reagents

IU was purchased from Aladdin Industrial Corporation (Shanghai, China). PAMP(9-20) was purchased from NJpeptide (Nanjing, China). Compound 48/80, *p*-nitrophenyl Nacetyl-β-D-glucosamide, Pluronic F-127, and Triton X-100 reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluo-3, AM ester and were obtained from Biotium (California, USA). All the aqueous solutions were prepared at the proper concentrations before use.

Mouse models

C57BL/6 Kit^{W^{sh}}/HNhrJaeBsmJNju mice (MUT) were purchased from the Model Animal Research Center of Nanjing University. And C57BL/6 (WT) mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). The mice were housed at the Experimental Animal Center of Xi'an Jiaotong University. Six mice were used per group.

Histological analysis

Adult male mice aged 6–8 weeks were used in the study and six mice were used per group. The hair on the back was then depilated. For 1-day assay, the concentration of IU was set as 100, 200, and 300 µg/ml and prepared in 50% glycerine. While 100-µg/ml IU prepared in 50% glycerine was used in 3-day assay. The drugs were applied to back skin using the patch test. PAMP (9-20) was set as positive control. About 50% glycerine was negative control. The mice were then sacrificed by CO₂ and the topical application locations were then removed from the skin. The skin was laid flat on filter paper to keep it even and fixed with 4% formaldehyde for 48 h, and then subjected to hematoxylin and eosin (H&E) staining. After dried at 37°C for 30 min and preincubated 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, anti-tryptase antibody was used for marking tryptase and anti-CD3 was used for marking T cells.

Ethics statement

The experimental protocols for the use of mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit number: XJTU 2019-711). This study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals from NIH.

Cell lines

The laboratory of allergic disease 2 (LAD2) cells were maintained in StemPro-34 medium supplemented with 10-ml/l StemPro nutrient supplement, 100 ng/ml human stem cell factor, 2-mM L-glutamine, and 1:100 penicillin-streptomycin in a 37°C incubator with 5% CO₂, which were kindly provided by NIH (USA). Cells were maintained at a density of 2×10^6 cells/ml.

Human MRGPRX2-expressing HEK293 cells (MEGPRX2-HEK293) and NC-HEK293 cells were constructed by human immunodeficiency virus-1-based lentiviral vectors and were cultured in DMEM supplemented with 10% FBS, 100× penicillin-streptomycin.

The β-hexosaminidase release assay

2×10^5 cells per well LAD2 cells were seeded into a 96-well plate and incubated overnight at 37°C with 5% CO₂. The culture medium was removed, and IU (50, 100, and 200 µg/ml) was added which diluted in Tyrode's solution (6.954-g/l NaCl, 0.353-g/l KCl, 0.282-g/l CaCl₂, 0.143-g/l MgSO₄, 0.162-g/l KH₂PO₄, 2.383-g/l HEPES, 0.991-g/l glucose, and 1-g/l BSA, pH 7). Cells treated with 10-µg/ml PAMP (9-20) were used as positive controls and only Tyrode's solution was negative control. Cells were incubated at 37°C for 30 min, then supernatants were collected. Cells were lysed by 0.1% Triton X-100 in Tyrode's solution. The β-hexosaminidase released into the supernatants and cell lysates were quantified by monitoring hydrolysis of *p*-nitrophenyl N-acetyl-β-D-glucosamide in 0.1-M citric acid/sodium citrate buffer (pH 4.5) for 90 min at 37°C. Reactions were terminated by stop buffer (0.1-M sodium carbonate/sodium bicarbonate = 9:1, pH 11.0). The percentage of β-hexosaminidase release was assessed by measuring the absorption of the samples at 405 nm and calculated as follows: absorbance of culture supernatant at 405 nm × 100/absorbance of total cell lysate supernatant at 405 nm.

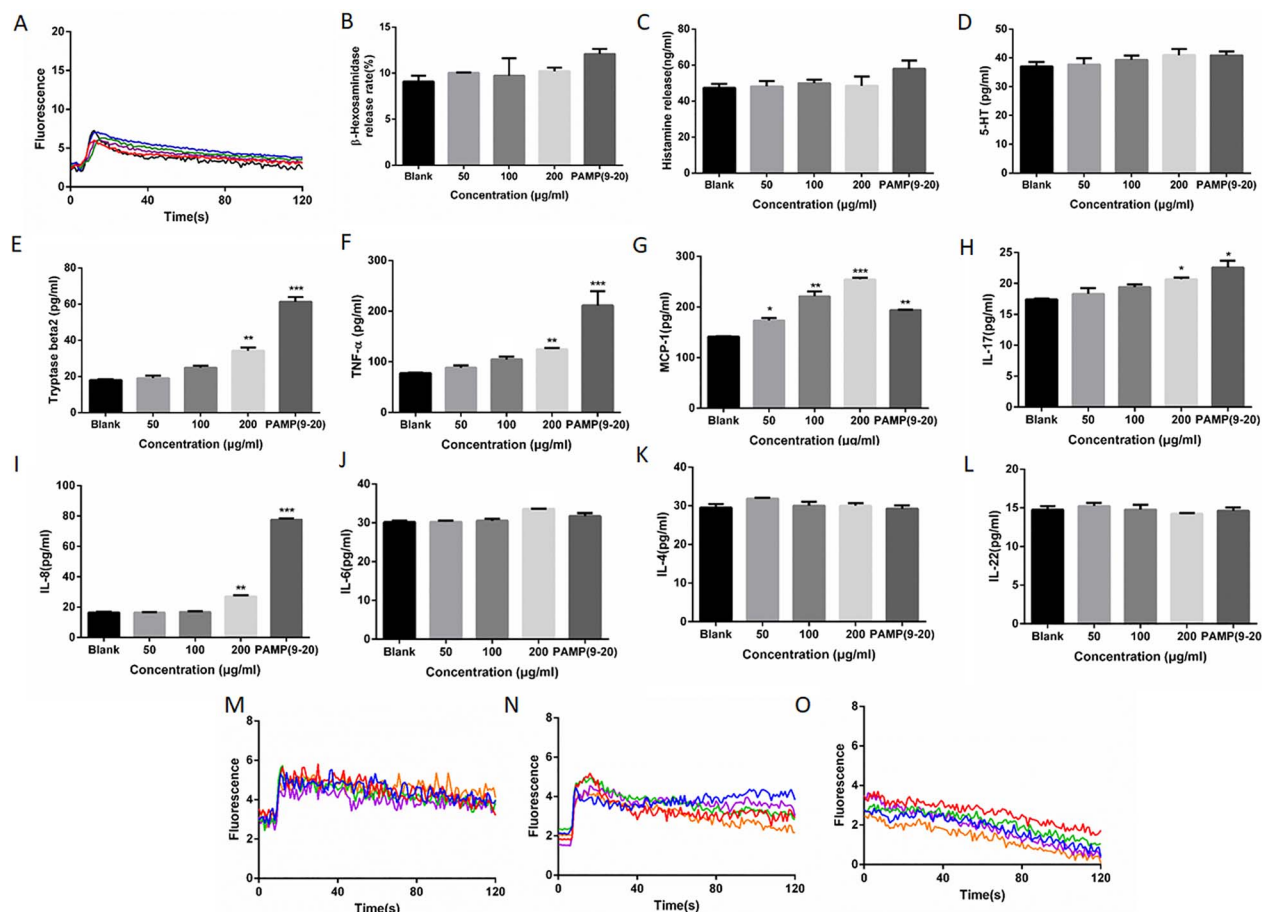


Figure 1: IU activated cell calcium mobilization and induced inflammatory mediator release. (A) IU increased intracellular Ca^{2+} concentration in LAD2 cells. Each colored line represents an individual cell. (B–D) IU did not induce LAD2 cells to release β -hexosaminidase, histamine, or 5-HT. (E–I) IU induced LAD2 cells to release tryptase beta-2, $\text{TNF-}\alpha$, MCP-1, IL-17, and IL-8 in a dose-dependent manner. (J–L) IU did not induce LAD2 cells to release IL-6, IL-4, or IL-22. (M–N) IU and PAMP (9-20) increased the intracellular Ca^{2+} concentration in MRGPRX2-HEK293 cells. (O) IU did not increase the intracellular Ca^{2+} concentration in the negative control HEK293 cells (experiments were repeated three times. The data are presented as the means $\pm$ SEM Analyzed by two-tailed unpaired Student's t-test and were calibrated by Bonferroni's test. Differences were considered significant at * $P < 0.0125$, ** $P < 0.0025$, and *** $P < 0.00125$).

Histamine release assay

1×10^6 cells per well LAD2 cells were treated by IU (50, 100, and 200 $\mu\text{g/ml}$) for 30 min at 37°C with 5% CO_2 , and then the supernatant was collected. Histamine was evaluated with the system by employing an HILIC column (Venusil HILIC, 2.1×150 mm, 3 μm , Agela Technologies, Tianjin, China) and isocratic elution was performed with acetonitrile-water containing 0.1% formic acid and 20-mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min. An LCMS-8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used in the applied LC-ESI-MS/MS method.

Chemokine release assay

Human tryptase ELISA Kit were purchased from Jianglai Industrial Limited By Share Ltd (Shanghai, China). Human $\text{TNF-}\alpha$, MCP-1, IL-8, IL-17, and IL-22 ELISA Kit were purchased from Sino Biological Inc. (Beijing, China). 1×10^6 cells per well LAD2 cells were treated by IU (50, 100, and 200 $\mu\text{g/ml}$) for 30 min at 37°C with 5% CO_2 , and then the supernatant was used for assay.

For mice serum assay, blood was collected after the drugs was applied to back skin, and centrifuged to get serum. Mouse tryptase ELISA Kit were purchased from Jianglai Industrial Limited By Share Ltd (Shanghai, China). Mouse $\text{TNF-}\alpha$, MCP-1, and IL-8 ELISA Kit were purchased from Sino Biological Inc. (Beijing, China). Mouse IgE ELISA Kit was purchased from BIOTECH CO., Ltd (Beijing, China). All steps were performed strictly according to the manufacturers' instructions.

For histamine release assay, skin tissues after treated by IU were weighed and collected into tubes. Skin tissues were then cut into pieces and add 1-ml saline, treated supersonically for 30 min. After centrifuged, 50 μl of supernatant from each sample was collected for analysis.

Intracellular Ca^{2+} mobilization assay

For imaging, the incubation buffer was diluted to the required concentration by calcium imaging buffer (CIB; 125-mM NaCl, 3-mM KCl, 2.5-mM CaCl_2 , 0.6-mM MgCl_2 , 10-mM HEPES, 20-mM glucose, 1.2-mM NaHCO_3 , 20-mM sucrose, and pH 7.4) which contained 4- μM Fluo-3 AM and 0.1% Pluronic F-127. Cells were washed twice with CIB and imaged at 488-nm excitation. Drugs

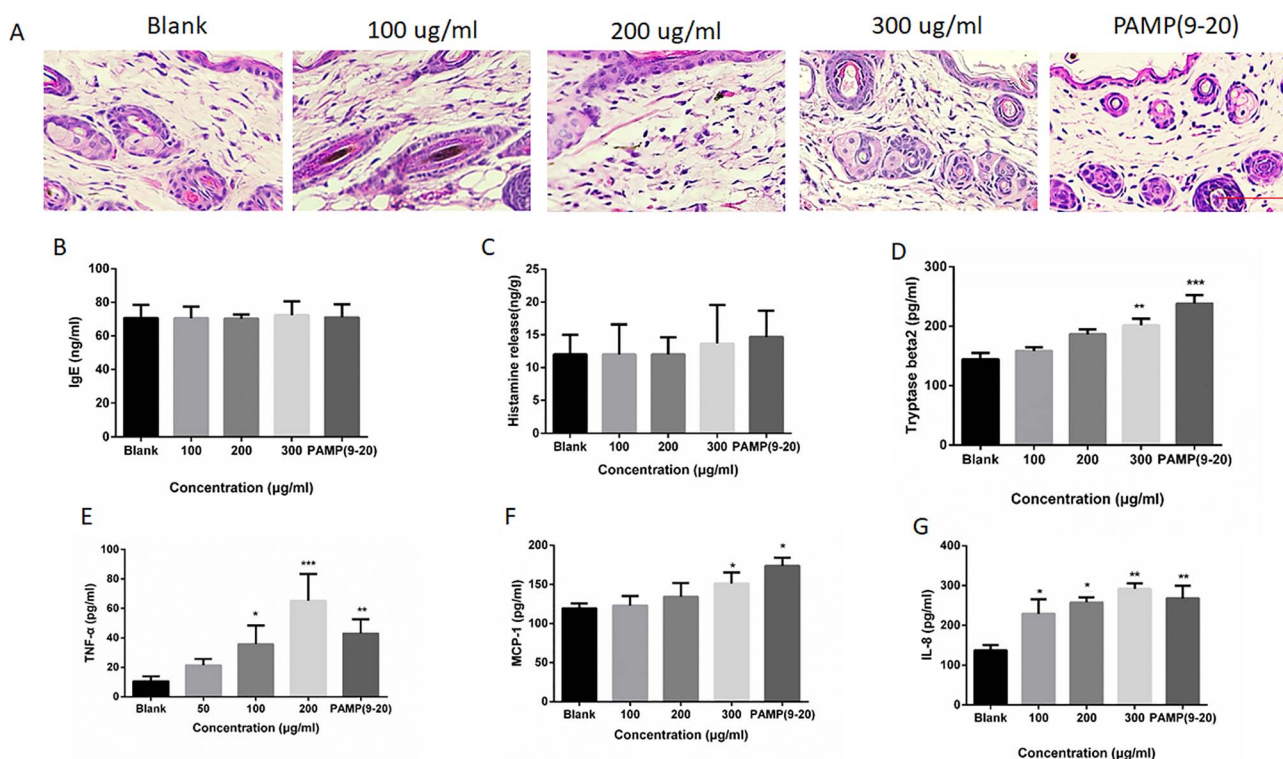


Figure 2: Topical application of IU induced contact allergy. Dorsal skin treated with 50% glycerol as the negative control; PAMP (9-20) as the positive control; or 100, 200, or 300-µg/ml IU for 1 day. (A) H&E staining of skin lesions. (B–H) Serum IgE, histamine, tryptase beta-2, TNF- α , MCP-1, and IL-8 levels ($n = 6$ per group; experiments were repeated three times. The data are presented as the means $\pm$ SEM. Analyzed by two-tailed unpaired Student's t -test and were calibrated by Bonferroni's test. Differences were considered significant at * $P < 0.0125$, ** $P < 0.0025$, and *** $P < 0.00125$).

were added to the well at 10 s after initial imaging, and responses were monitored for an additional 120 of 1-s intervals. All the drugs were diluted to the required concentration by CIB.

siRNA transfection of LAD2 cells

The siRNA sequences were as follows: Negative Control siRNA, forward, 50-UUCUCCGACGUGACAGGTT-30, and reverse, 50-ACGUGACAGUUCGAGAAATT-30; MRGPRX2 knockdown siRNA, forward, 50-GUACAACAGUGAAUG GAAATT-30, and reverse, 50-UUCCAUCAGUGUUGUACTT-30 (Shanghai GenePharma Co., Ltd, Shanghai, China). For transfection, the siRNAs were delivered at a final concentration of 1 μ M by Lipofectamine[®] 3000 transfection reagent in accordance with the instructions. Cells were incubated for 48 h to allow for MRGPRX2 knockdown. The efficiency of siRNA to knockdown MRGPRX2 expression was proved by Human Mas-related G-protein coupled receptor member X2, MRGPRX2 ELISA Kit (MyBioSource, LLC, San Diego, CA).

Western blot analysis

RIPA lysis buffer containing 10% protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics) were used for extracting total protein. And the protein concentration was determined with a BCA Protein Quantification kit. After the protein was denatured by boiling with 5 $\times$ loading sample buffer (Thermo Fisher Scientific, Inc. MA, USA). Equal amounts of protein were separated on a 10% SDS-PAGE gel (Shaanxi Pioneer

Biotech Co., Ltd, Shanghai, China). Following electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride membranes (Hangzhou Microna Membrane Technology Co., Ltd, Hangzhou, China). Membranes were blocked with 5% nonfat milk in Tris-buffered saline (Baihao) containing Tween-20 (TBST; Shaanxi Pioneer Biotech Co., Ltd) for 2 h at room temperature with continuous agitation. The membranes were then washed three times with TBST every 10 min followed by incubation with secondary antibodies at a dilution of 1:20 000 in TBST for 1 h at 37°C, after which the membranes were washed three times with TBST for 10 min and developed using an enhanced chemiluminescence (ECL) kit.

The primary antibodies were as following: anti-GAPDH (1:2000, #2118, CST), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (1:1000, #8713, CST), anti-PLC γ 1 (1:1000, #5690, Cell Signaling Technology CST), anti-phosphorylated-P38 (P-P38, Thr180/Tyr182) (1:1000, #4511, CST), anti-phosphorylated-Akt (P-Akt, Ser473) (1:1000, #4060, CST), anti-Akt (1:1000, #4691, CST), anti-phosphorylated-Erk1/2 (P-Erk1/2, Thr202/Tyr204, 1:1000, #9101, CST), and anti-Erk1/2 (1:1000, #9102, CST).

Statistical analysis

Experiments were repeated three times. Data are expressed as mean $\pm$ SEM and analyzed one-tail paired Student's t -test. An independent samples analysis of variance was used to determine statistical significance in comparisons of the data using the SPSS software. Differences were considered significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$. Multiple doses were calibrated

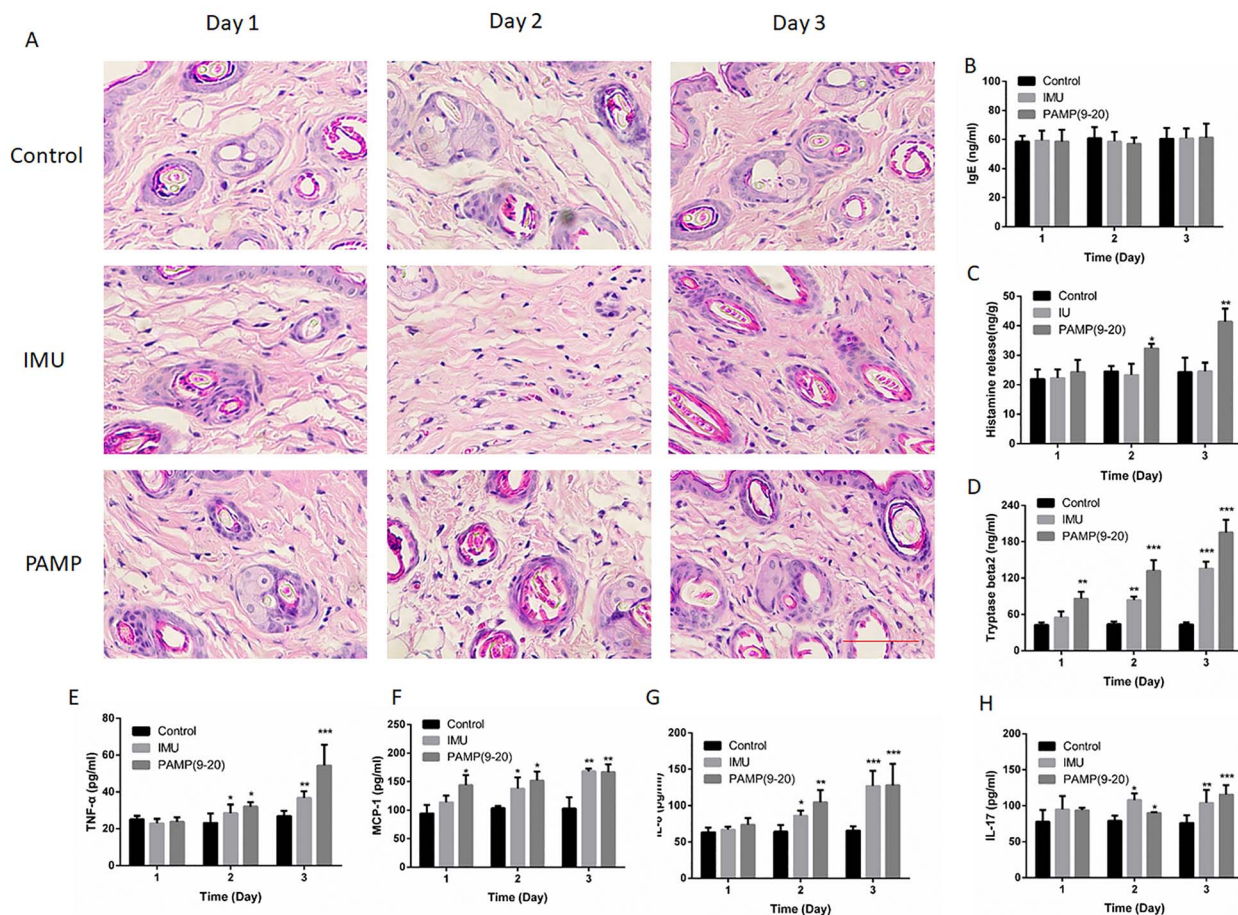


Figure 3: Topical application of 100-µg/ml IU induces contact allergy in 3 days. Dorsal skin treated with 50% glycerol as the negative control, PAMP (9-20) as the positive control, or 100-µg/ml IU for 3 days. (A) H&E staining of skin lesions. (B–H) Serum IgE, histamine, tryptase beta-2, TNF-α, MCP-1, IL-8, and IL-17 levels ($n = 6$ per group; experiments were repeated three times. Data are expressed as mean $\pm$ SEM and analyzed one-tail paired Student's *t*-test. Differences were considered significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$).

by Bonferroni's test. Differences were considered significant at * $P < 0.0125$, ** $P < 0.0025$, and *** $P < 0.00125$.

Results

IU activated calcium mobilization in LAD2 cells and induced inflammatory mediator release

The activation of calcium mobilization and the release of inflammatory mediators are characteristics of mast cells activation. Calcium imaging analysis revealed that IU directly activated LAD2 cells and increased the intracellular Ca^{2+} concentration to 200 µg/ml (Fig. 1A). However, IU did not cause LAD2 cells to release β -hexosaminidase, histamine, or 5-HT (Fig. 1B–D). After treatment with 0, 50, 100, or 200-µg/ml IU, the amount of tryptase beta-2, TNF-α, MCP-1, IL-17, and IL-8 increased in a dose-dependent manner (Fig. 1E–I). Moreover, IU did not induce the release of IL-6, IL-4, or IL-22 (Fig. 1J–L). These results demonstrated that IU directly activated human mast cells and induced the release of inflammatory mediators that were different from those released during typical allergic reactions. Furthermore, to investigate whether MRGPRX2 could be activated by IU, HEK293 cells expressing MRGPRX2 were used to test calcium influx. IU and PAMP (9-20) activated Ca^{2+} mobilization in MRGPRX2-HEK293 cells (Fig. 1M and N), but IU

had little influence on Ca^{2+} mobilization in negative control HEK293 cells (Fig. 1O), indicating that MRGPRX2 may be a key receptor in IU-induced mast cell activation.

Topical application of IU triggered contact dermatitis

To investigate IU-induced contact dermatitis in mice, we established a mouse model. After shaving the mice, 100, 200, or 300-µg/ml IU, prepared with 50% glycerol, was applied to the back skin of the mice for 1 day, and 100-µg/ml IU was applied to the back skin of the mice for 3 consecutive days. In this experiment, 50% glycerol was used as the negative control and PAMP (9-20) was used as the positive control. We analyzed the release of various inflammatory mediators in mouse serum and used the back skin for H&E staining.

One day after treatment with different concentrations of IU, H&E staining revealed that, with increasing treatment concentrations, dermal inflammation was aggravated and vasodilation was significantly enhanced, compared with the glycerol-treated controls (Fig. 2A). However, serum total IgE concentrations were similar in the treated groups and the control group, indicating that IU-induced dermatitis was not related to the IgE pathway (Fig. 2B). In addition, the local histamine concentrations showed no change after IU treatment (Fig. 2C). However, the levels of

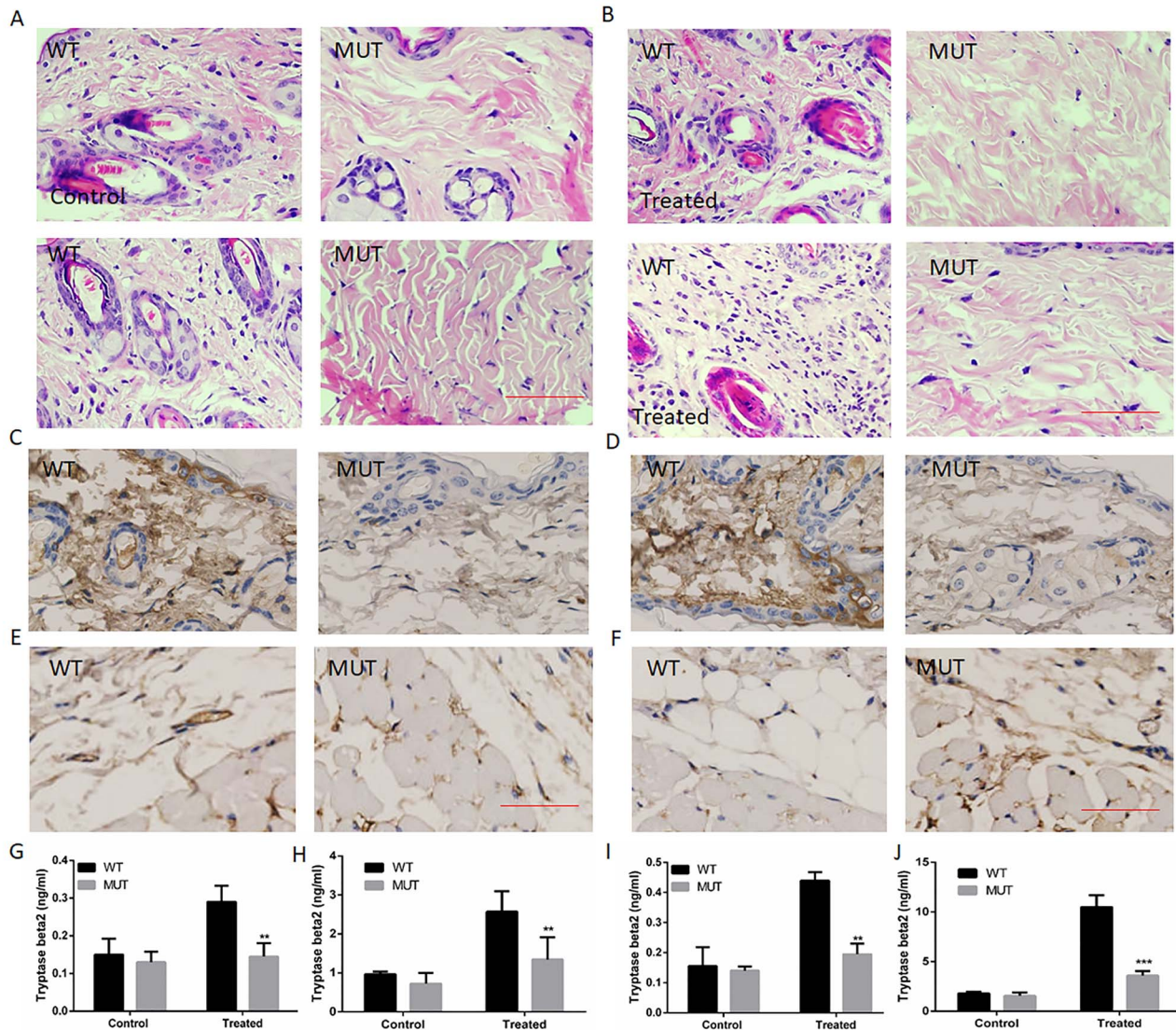


Figure 4: Mast cells mediate IU-induced contact allergy. *Kit^{W-sh}/HNIhrJaeBsmJNju* mice (MUT) show resistance to IU-induced allergy. WT and MUT mice were treated with 300- μ g/ml IU for 1 day or 100- μ g/ml IU for 3 days. (A and B) H&E staining of skin lesions for 1 and 3 days. (C and D) Tryptase beta-2 in mice skin increased after treated with IU in WT mice for 1 and 3 days. (E and F) IU did not induce T cells activation and infiltration in mice skin for 1 and 3 days. (G and H) Tryptase release in mice skin and serum was significantly increased for 1 day. (I and J) Tryptase release in mice skin and serum was significantly increased for 3 days ($n = 6$ per group; experiments were repeated three times. Data are expressed as mean $\pm$ SEM and analyzed one-tail paired Student's t-test. Differences were considered significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$).

other inflammatory mediators, such as tryptase beta-2, TNF- α , MCP-1, and IL-8, increased in a dose-dependent manner after IU treatment (Fig. 2D-G).

Mast cells mediated IU-induced contact dermatitis.

When used in patch tests, IU remains in contact with the patients' skin for 3 days. For consistency with the patch test, mice were treated with IU (100 μ g/ml) for 3 days. On the first day after treatment, there was no visible inflammatory infiltration in the dermis (Fig. 3A). After 2-3 days of treatment, inflammatory infiltration was observed, whereas the negative control group did not show any lesions (Fig. 3A). Total IgE concentrations showed no significant differences between the IU-treated and negative control groups (Fig. 3B). Few significant differences were observed in local histamine levels during the 3 days of treatment (Fig. 3C). As the number of days of treatment increased, the

serum levels of tryptase beta-2, TNF- α , MCP-1, IL-8, and IL-17 increased significantly (Fig. 3D-H).

As IU induced contact dermatitis without an increase in total IgE levels, we hypothesized that mast cells directly activating mediated anaphylaxis. To test this, WT and *Kit^{W-sh}/HNIhrJaeBsmJNju* mice (MUT) were administered 300- μ g/ml IU for 1 day or 100- μ g/ml IU for 3 days. Glycerol (50%) was used as the negative control. MUT mice showed slight inflammatory infiltration after 1 and 3 days of treatment (Fig. 4A and B). In addition, tryptase beta-2 in mice skin increased after treated with IU in WT mice for 1 and 3 days (Fig. 4C and D). However, CD3 was used to mark T cells and the results showed that IU did not induce T cells activation and infiltration in mice skin (Fig. 4E and F). Moreover, tryptase release in mice skin and serum was significantly increased (Fig. 4G-J).

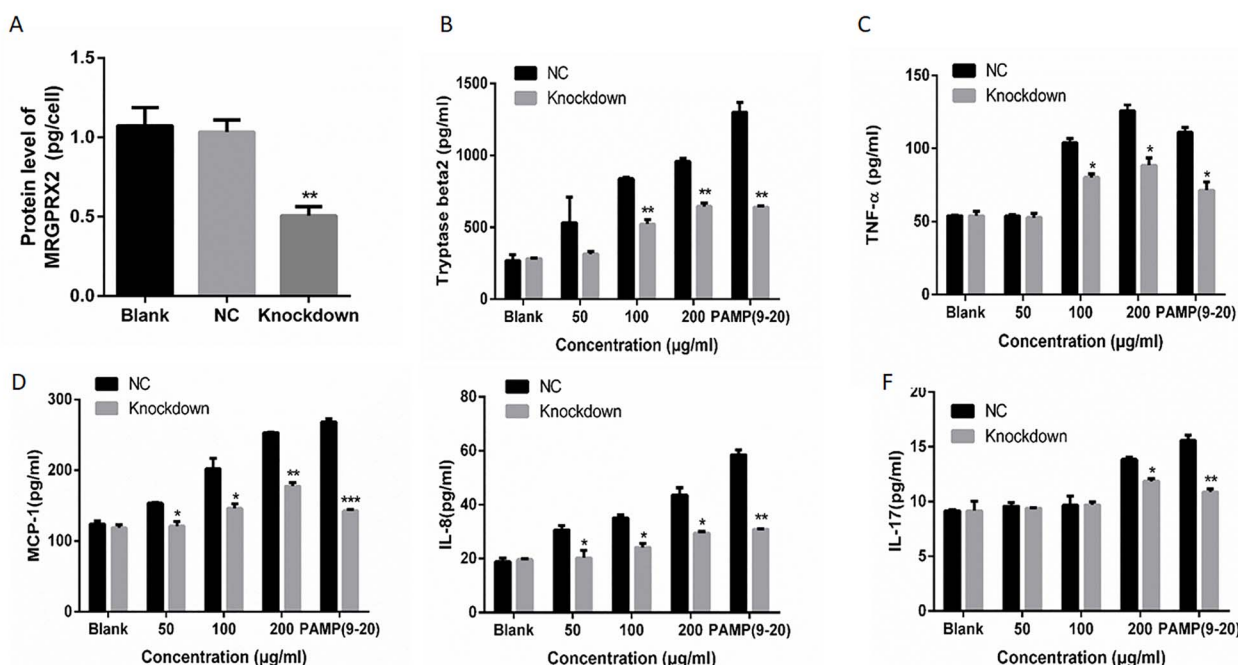


Figure 5: IU activated LAD2 cells via MRGPRX2. (A) protein level of MRGPRX2 analysis after siRNA transfection. (B–F) The release of tryptase beta-2, TNF- α , MCP-1, IL-8, and IL-17 in MRGPRX2-knockdown LAD2 cells treated with IU at concentrations of 50, 100, 200 $\mu\text{g/ml}$, or 10 $\mu\text{g/ml}$ PAMP (9-20) for 30 min, compared with negative control LAD2 cells ($n = 6$ per group; experiments were repeated three times). Data are expressed as mean $\pm$ SEM and analyzed one-tail paired Student's t-test. Differences were considered significant at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

MRGPRX2 mediated the IU-induced activation of human mast cells

MRGPRX2 knockdown was performed in LAD2 cells to verify the interaction between MRGPRX2 and IU. After siRNA transfection, the protein level of MRGPRX2 was downregulated expression (Fig. 5A). Treatment with 0, 50, 100, and 200- $\mu\text{g/ml}$ IU or 10- $\mu\text{g/ml}$ PAMP (9-20), inflammatory mediators were released at lower levels from MRGPRX2-knockdown LAD2 cells than from negative control LAD2 cells, indicating that MRGPRX2 played an important role in the IU-induced activation of mast cells (Fig. 5B–F).

IU triggered calcium mobilization during contact dermatitis by activating PLC- γ and p38

The calcium signaling pathway is important for mast cell degranulation, and an increase in intracellular Ca^{2+} concentration is necessary for both degranulation and cytokine release. However, increased levels of phosphorylated PLC γ 1, PKC, ERK, and p38 are important for mast cell degranulation and the synthesis and release of cytokines in the MRGPRX2 signaling pathway. After treatment with IU, the levels of phosphorylated PLC γ 1 and P38, which are key proteins in pathways downstream of MRGPRX2, increased in a dose-dependent manner (Fig. 6A). However, the levels of phosphorylated PKC and ERK, in the cytokine synthesis signaling pathway, were not significantly increased (Fig. 6B).

Discussion

MRGPRX2 has specific functions in mediating mast cell degranulation and regulating inflammatory responses [10]. Additionally,

several studies have shown that MRGPRX2 specifically triggers drug-induced pseudo-allergic reactions and induces mast cells to release histamine and various inflammatory and immunomodulatory substances. However, different agonists can activate MRGPRX2, and they may induce the release of different factors and different pathological changes. Dong et al. demonstrated that PAMP (9-20) increases mast cell activation via Mrgrprb2-induced non-histaminergic dermatitis in mice by releasing tryptase instead of histamine and exciting non-histaminergic itch sensory neurons [11]. Tryptase and its receptor, PAR-2, expressed by mast cells, are the major non-histaminergic inflammatory mediators of atopic dermatitis [12].

Our study showed that the specific mechanism by which IU induced contact dermatitis in mice was not related to the IgE pathway and that histamine, which is considered a classical allergen, was not involved in this process. Significant differences in the tissue at the application site and the inflammatory mediators induced by the topical application of IU in WT and MUT mice revealed that mast cells directly activating played an important role in contact dermatitis in mice. The activation of mast cells by IU increased the levels of phosphorylated PLC γ 1 and p38 but showed no effect on the levels of phosphorylated PKC and ERK1/2, members of the mitogen-activated protein kinase family, as important cell signaling pathways, they can couple diverse cell surface proteins to inflammatory gene expression for inflammatory response [13, 14]. It has been reported that ERK plays an important role in mast cell degranulation and that the MEK-ERK pathway is required for cytokine production [15, 16]. However, whether the downregulation of phosphorylated ERK directly leads to the lack of a significant change in histamine concentrations is unknown; thus, further research is needed to

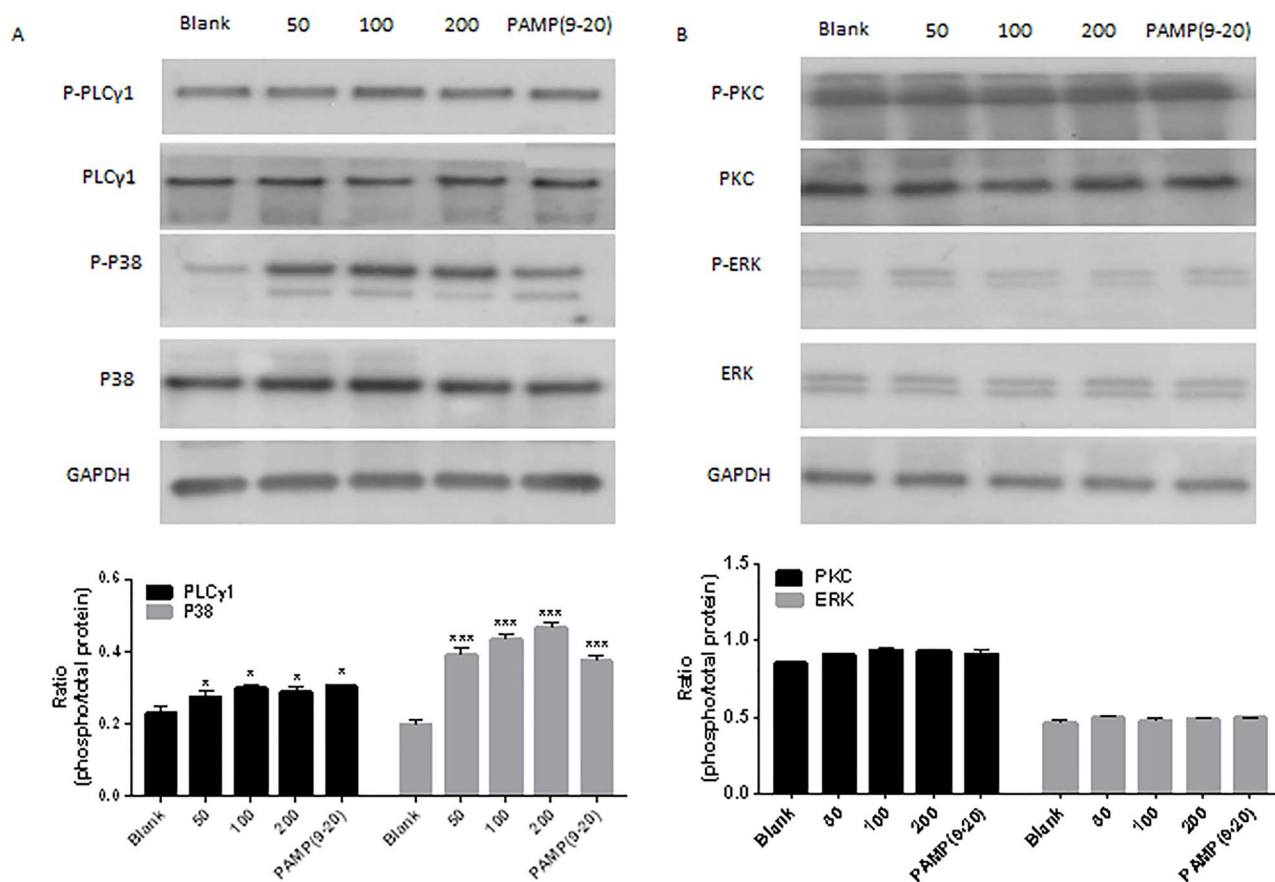


Figure 6: IU upregulation of the phosphorylation of downstream members of the MRGPRX2 signaling pathway. (A) IU upregulation of the levels of phosphorylated PLC γ 1 and p38. (B) IU showed no effect on the levels of phosphorylated PKC or ERK (experiments were repeated three times. The data are presented as the means $\pm$ SEM and analyzed by two-tailed unpaired Student's t-test and were calibrated by Bonferroni's test. Differences were considered significant at * $P < 0.0125$, ** $P < 0.0025$, and *** $P < 0.00125$).

clarify this. In addition, PPD downregulated the phosphorylation level of PKC. PKC is involved in controlling the process of mast cell degranulation [17]. After mast cell activation, multiple signal transduction pathways in the cells are activated to induce degranulation. The activated PKC catalyzes the phosphorylation of specific target protein serine or threonine residues, and can phosphorylate membrane proteins and cytoskeleton proteins, triggering mast cells degranulation. Inhibition of PKC phosphorylation can effectively reduce the occurrence of inflammatory responses [18]. Moreover, inactivated PKC cannot induce mast cell release histamine and leukotriene [19], which may be a reason why PPD could activate MRGPRX2 without causing histamine release.

We found that contact dermatitis triggered by IU induced the specific release of IL-8 and IL-17. IL-8 (CXCL2) is a chemokine that stimulates neutrophils to participate in the inflammatory process by upregulating the expression of CD11b on the neutrophil surface and inducing the shedding of L-selectin [20, 21]. There is evidence that IL-17, released by Th17 cells, plays an important role in the regulation of innate and adaptive immune responses, particularly in Th1- and/or Th2-mediated autoimmune diseases represented by rheumatoid arthritis and allergic asthma [22]. It is also involved in the production of proinflammatory cytokines and matrix metalloproteinases and recruitment of neutrophils and eosinophils, which are important pathogenic factors in contact dermatitis [23].

Conclusion

IU, a water-soluble fungicide and formaldehyde releaser introduced in 1970, is the second most widely used preservative in cosmetics after parabens in the USA and Europe. It is present in 8–10% of cosmetic products. Although there have been many reports of contact dermatitis caused by cosmetic additives or preservatives, this is the first study to explore the specific mechanism of contact dermatitis induced by IU. The results of our study explain why antihistamines are clinically ineffective against most types of chronic dermatitis and offer the possibility of discovering new drugs for the treatment of non-histaminergic dermatitis.

Authors' contributions

Jiapan Gao wrote the manuscript. Delu Che and Nan Wang contributed to experimental design. Jiapan Gao and Delu Che performed experiments and collected the data. Xueshan Du, Yi Zheng, and Huiling Jing contributed to the data analysis. All authors read and approved the manuscript.

Funding

The present study was supported by the National Science Foundation for Post-doctoral Scientists of China (no. 2019M653672)

and National Natural Science Foundation of China (grant nos. 81930096 and 81704088).

Conflict of Interest

The authors have no conflicts of interest to report.

References

- de Groot AC, White IR, Flyvholm MA et al. Formaldehyde-releasers in cosmetics: relationship to formaldehyde contact allergy. Part 1. Characterization, frequency and relevance of sensitization, and frequency of use in cosmetics. *Contact Dermatitis* 2010;**62**:2–17.
- de Groot AC, Flyvholm MA, Lensen G et al. Formaldehyde-releasers: relationship to formaldehyde contact allergy. Contact allergy to formaldehyde and inventory of formaldehyde-releasers. *Contact Dermatitis* 2009;**61**:63–85.
- Marks JG, Belsito DV, DeLeo VA et al. North American contact dermatitis group patch-test results, 1996–1998. *Arch Dermatol* 2000;**136**:272–3.
- Williams KW, Sharma HP. Anaphylaxis and urticarial. *Immunol Allergy Clin North Am* 2015;**35**:199–219.
- Wang N, Che DL, Zhang T et al. Saikosaponin A inhibits compound 48/80-induced pseudo-allergy via the MRGPRX2 pathway in vitro and in vivo. *Biochem Pharmacol* 2017;**148**:147–54.
- Lieberman P, Garvey LH. Mast cells and anaphylaxis. *Curr Allergy Asthma Rep* 2016;**16**:20.
- Subramanian H, Gupta K, Ali H. 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* 2016;**138**:700–10.
- McNeil BD, Pundir P, Meeker S et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature* 2015;**519**:237–41.
- Kamohara M, Matsuo A, Takasaki J et al. Identification of MrgX2 as a human G-protein-coupled receptor for proadrenomedullin N-terminal peptides. *Biochem Biophys Res Commun* 2005;**330**:1146–52.
- Ali H. Mas-related G protein coupled receptor-X2: a potential new target for modulating mast cell-mediated allergic and inflammatory diseases. *J Immunobiol* 2016;**1**:115.
- Meixiong J, Anderson M, Limjunyawong N et al. Activation of mast cell-expressed mas-related G-protein-coupled receptors drives non-histaminergic itch. *Immunity* 2019;**50**:1163–71.
- Sanders KM, Nattkemper LA, Rosen JD et al. Non-histaminergic itch mediators elevated in the skin of a porcine model of scabies and of human scabies patients. *J Invest Dermatol* 2019;**139**:971–3.
- Thalhamer T, McGrath MA, Harnett MM. MAPKs and their relevance to arthritis and inflammation. *Rheumatology (Oxford)* 2008;**47**:409–14.
- Schett G, Zwerina J, Firestein G. The p38 mitogen-activated protein kinase (MAPK) pathway in rheumatoid arthritis. *Ann Rheum Dis* 2008;**67**:909–16.
- Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol* 2006;**6**:218–30.
- Tanifuji S, Aizu-Yokota E, Funakoshi-Tago M et al. Licochalcones suppress degranulation by decreasing the intracellular Ca^{2+} level and tyrosine phosphorylation of ERK in RBL-2H3 cells. *Int Immunopharmacol* 2010;**10**:769–76.
- Nechushtan H, Leitges M, Cohen C et al. Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase C beta. *Blood* 2000;**95**:1752–7.
- Song T, Zheng YM, Vincent PA et al. Canonical transient receptor potential 3 channels activate NF- κ B to mediate allergic airway disease via PKC- α /I κ B- α and calcineurin/I κ B- β pathways. *FASEB J* 2016;**30**:214–29.
- Kimata M, Shichijo M, Miura T et al. Ca^{2+} and protein kinase C signaling for histamine and sulfide leukotrienes released from human cultured mast cells. *Biochem Biophys Res Commun* 1999;**257**:895–900.
- Hoch R, Schraufstatter IU, Cochrane CG. In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptor. *J Lab Clin Med* 1996;**128**:134–45.
- Mukaida N, Harada A, Matsushima K. Interleukin-8(IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev* 1998;**9**:9–23.
- Ghoreschi K, Laurence A, Yang X et al. T helper 17 cell heterogeneity and pathogenicity in autoimmune disease. *Trends Immunol* 2011;**32**:395–401.
- Harrington LE, Hatton RD, Mangan PR et al. Interleukin 17-producing CD4⁺ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 2005;**6**:1123–32.