

All-trans-retinoic acid activated mast cells via Mas-related G-protein-coupled receptor-X2 in retinoid dermatitis

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Abstract

Background: Retinoic acid (RA)-induced dermatitis is the most frequent side-effect limiting its widespread use. However, the exact mechanisms triggering dermatitis are not fully understood, including the role of skin mast cells. The newly discovered Mas-related G-protein-coupled receptor-X2 (MRGPRX2) in mast cells mediates pseudoallergic drug reactions in several types of dermatitis. A possible contribution of MRGPRX2 to contact dermatitis induced by RA has hitherto not been examined.

Objectives: To investigate whether all-trans-RA (ATRA) activates mast cells via MRGPRX2/MrgprB2 (the mouse orthologue), contributing to the pathogenesis of retinoid-induced dermatitis.

Methods: Wild-type (WT) and MrgprB2^{-/-} mice were treated with topical ATRA to observe local inflammation and mast cell degranulation in vivo by the use of haematoxylin and eosin and immunofluorescence staining. Release of histamine and release of β -hexosaminidase were measured and calcium influx was detected in Laboratory of Allergic Disease 2 (LAD2) cells with specific knockdown targeting MRGPRX2 by small interfering RNA (siRNA) and in primary cells from MrgprB2^{-/-} mice.

Results: As compared with WT mice, MrgprB2^{-/-} mice showed resistance to ATRA-triggered contact dermatitis and local inflammatory reactions in the paws. ATRA activated mast cells via the MrgprB2 pathway in murine cells, and via the MRGPRX2 pathway in human mast cells.

Conclusions: ATRA-induced dermatitis could be achieved by activating mast cells via MRGPRX2/MrgprB2, which may provide a potential therapy target to reduce the side-effect.

KEYWORDS

all-trans-retinoic acid (ATRA), contact dermatitis, mast cell, MRGPRX2

1 | INTRODUCTION

Retinoic acid (RA) is one of the most commonly used drugs for the treatment of dermatological diseases, such as psoriasis, acne,

keratoacanthoma, and squamous cell carcinoma. It mainly regulates the proliferation, differentiation and apoptosis of keratinocytes, and the activity of sebaceous glands, through RA receptors (RARs) and retinoid X receptors. RA also has anti-inflammatory and immune regulatory functions. All-trans-RA (ATRA) is one of the most frequently

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used retinoids in clinical treatment. As early as 1975, it was reported that ATRA triggered cutaneous reactions by patch testing, which manifested as erythema, scaling and other morphologies on normal skin, known as "retinoid dermatitis."^{1,2} Retinoid dermatitis has become the most frequent side-effect of topical retinoids, from which 85% to 95% of patients might suffer. Therefore, many researchers have studied this to obtain a better understanding of the mechanism of retinoids and to identify ways to avoid this side-effect. It has been found that the concentration and the dosage of retinoids were positively correlated with the severity of symptoms. As compared with retinol and retinaldehyde, topical tretinoin and tazarotene can be highly irritant.^{2,3} Some researchers believe that retinoid dermatitis is an inflammatory reaction, with changes in the skin barrier being closely related to skin mast cells, probably mediated by the RA-degrading enzyme Cyp26b1 affecting ATP receptors of mast cell.⁴ Babina et al indicated that ATRA could act on RAR α in mast cells, leading to the release of cytokines such as interleukin (IL)-1 β , tumour necrosis factor- α and IL-8 from mast cells.^{5,6} However, its mechanism of action is still not fully understood, and further research on retinoid-induced dermatitis will possibly help to avoid side-effects in clinical applications.

Mas-related G-protein-coupled receptor-X2 (MRGPRX2) is highly expressed on the membranes of skin mast cells (MC_{TCs}).⁷⁻⁹ It has also been reported that MRGPRX2 can be expressed in human keratinocytes.¹⁰ MRGPRX2 can be activated by compound 48/80 (C48/80), substance P, and host defence peptides, and this causes degranulation in a dose-dependent manner with no IgE increase.¹¹⁻¹³ MRGPRX2 has been proven to be related to many skin diseases, such as chronic urticaria, atopic dermatitis, red man syndrome, and rosacea.¹⁴ Fujisawa et al⁷ found that the expression of MRGPRX2 on mast cells was upregulated in the skin of patients with severe chronic urticaria. Azimi et al¹⁵ considered that *Staphylococcus* δ -toxin and vancomycin, which lead to atopic dermatitis and red man syndrome, respectively, could activate MRGPRX2. Also, many US Food and Drug Administration-approved peptidergic drugs, such as atracurium, mivacurium, rocuronium, terbufine hydrochloride, sisomicin sulfate, and sulfamethoxazole, cause pseudoallergic drug reactions in humans, manifesting as skin flushing and vascular permeability.¹⁶⁻¹⁸ In order to investigate the function of MRGPRX2 in vivo, MrgprB2, the mouse orthologue of human MRGPRX2, is often used.¹¹

More and more agonists of MRGPRX2 are being discovered. As retinoid dermatitis is similar to a pseudoallergic reaction, we hypothesized whether ATRA, as a small-molecule compound without antigenicity, could activate MRGPRX2/MrgprB2 and induce mast cell degranulation, and trigger retinoid dermatitis as a result. We designed experiments to investigate the effect of ATRA in activating MrgprB2 in vivo and in vitro, as well as ATRA-activated MRGPRX2, by testing the degranulation and calcium influx of human mast cells.

2 | MATERIALS AND METHODS

2.1 | Drugs and reagents

ATRA, C48/80 and histamine were from Sigma-Aldrich (St. Louis, Missouri). Histamine.2HCl (A, A, B, B-D4, 98%) was from Cambridge Isotope Laboratories (Massachusetts), and high-performance liquid chromatography-grade methanol and acetonitrile were from Thermo Fisher Scientific (Pittsburgh, Pennsylvania). Mass spectrometry (MS)-grade formic acid was from Sigma-Aldrich. Fluo-3, AM ester and Pluronic F-127 were from Biotium (Fremont, California). Tyrode's solution buffer was prepared fresh on the day of use (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 bovine serum albumin [BSA], pH 7). The *p*-nitrophenyl-N-acetyl- β -D-glucosamide and Triton X-100 reagents were from Sigma-Aldrich, and were prepared in the proper concentrations prior to use. All aqueous solutions were prepared with ultrapure water produced with an MK-459 Millipore Milli-Q Plus ultra-pure water system (Billerica, Massachusetts).

2.2 | Mouse model

Male C57BL/6 mice aged 8 weeks were purchased from the Experimental Animal Centre of Xi'an Jiaotong University. C57BL/6 STOCK Kit^{W-sh/HNhrJaeBsmJNJu} (Kit^{W-sh/W-sh}) mice were purchased from the Model Animal Research Centre of Nanjing University. C57BL/6 MrgprB2-knockout mice were provided as a kind gift from the laboratory of Xinzhong Dong at Johns Hopkins University. The mice were housed in individual cages in a large colony room with free access to water, and were fed standard dry food twice daily. The breeding environment had a temperature of 20°C to 25°C, with a relative humidity of 40% and a day/night cycle of 12/12 hours. To set up a mouse model of retinoid dermatitis, the mice were randomly divided into two groups (six mice per group), and the dorsal skin was shaved with an electric shaver. The experimental group was treated with topical 0.1% ATRA cream (0.2 mg of ATRA in 200 mg of vaseline) daily, and vaseline as solvent was given to the control group. All animal experiments involving equal treatments were conducted by experimenters who were blinded to the conditions.

2.3 | Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols involving mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animals were under pentobarbital sodium anaesthesia when undergoing any operation.

2.4 | Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, Bethesda, Maryland). The cells were maintained in StemPro-34 medium supplemented with 10 mL/L StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mM L-glutamine and 100 ng/mL human stem cell factor (SCF) in an atmosphere containing 5% CO₂ at 37°C, and maintained at a density of 2×10^6 cells/mL. Human MRGPRX2-expressing HEK293 cells were constructed by the use of human immunodeficiency virus-1-based lentiviral vectors. MrgprB2-expressing HEK293 cells were provided by the laboratory of Xinzhong Dong at Johns Hopkins University. Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM).

2.5 | Histology

Skin samples were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 mm) were stained with haematoxylin and eosin. After being dried at 37°C for 30 minutes and preincubated in blocking solution (10% normal goat serum [vol/vol] and 0.2% Triton X-100 [vol/vol] in phosphate-buffered saline [PBS], pH 7.4) for 2 hours at 25°C, and incubated with 1:500 fluorescein isothiocyanate-avidin for 45 minutes, sections were washed three times with PBS, and a drop of Fluoro-mount G (Southern Biotech, Birmingham, Alabama) was added. Images were taken immediately with a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.6 | Peritoneal mast cell purification

Adult male C57BL/6 mice aged 8 weeks were killed by CO₂ inhalation. A total of 12 mL of ice-cold mast cell dissociation medium (MCDM) (15 mL of MCDM, 1.5 mL of $10 \times$ HBSS, 450 μ L of fetal bovine serum, 150 μ L of 1 M HEPES, and 12.9 mL of sterile water, pH 7.2) was used for two to three sequential peritoneal lavages, in which the MCDM and cells were mixed and then centrifuged at 200g for 10 minutes at 4°C. The cells from each mouse were isolated by magnetic bead separation. Anti-mouse CD117 and anti-R-phycoerythrin (PE) magnetic particles (BD Biosciences, New York, New York) were used for isolation and purification of mouse peritoneal mast cells (MPMCs). The mast cells in the pellet were retrieved, and their purity was >95% as assayed by morphology. The mast cells were then resuspended in DMEM with 100 ng/mL recombinant mouse SCF, seeded into 96-well plates, and used within 4 hours.

2.7 | Hindpaw swelling and extravasation

Male C57BL/6 mice, Kit^{W-sh/W-sh} mice and C57BL/6 MrgprB2-knockout mice were anaesthetized with an intraperitoneal injection of 80 mg/kg 1% pelltobarbitalum natricum. Fifteen minutes after the induction of anaesthesia, each mouse was injected intravenously with 0.2 mL of 0.4% Evans blue dye (EBD) in saline. A Vernier caliper was used to measure the thickness of the paw before any

injection. Five minutes later, 5 μ L of ATRA (0.1, 0.2 or 0.4 μ g/mL) was administered with a microinjector into the left paw, and saline was administered only into the right paw as a blank control. Fifteen minutes later, the paw thickness was measured again and recorded. The mice were then killed with CO₂, and a photograph of each paw was obtained. The paw tissues were collected, dried for 24 hours at 50°C, and weighed. EBD was extracted by a 12-hour incubation in 500 μ L of acetone-saline (7:3) at 37°C. Tissues were cut into pieces, placed for 10 minutes in an ultrasonic machine, and centrifuged for 20 minutes at 1000 g. The supernatant was equally distributed in 200- μ L aliquots into 96-well cell culture plates, and the optical density (OD) was read at 620 nm with a spectrophotometer. For studies using drug substances, the mice were injected with 5 μ L of a 30 μ g/mL solution of C48/80 as a positive control.

2.8 | β -Hexosaminidase assay

LAD2 cells were incubated overnight at 37°C with 5% CO₂, whereas MPMC's were used within 4 hours after isolation. The culture medium was removed, the drug substances were added at the indicated concentrations, and the cells were incubated for 30 minutes at 37°C with 5% CO₂. The 96-well plate was then centrifuged at 200g for 5 minutes at 4°C. To determine the total β -hexosaminidase content, the cells were lysed with 0.1% Triton X-100 in Tyrode's solution buffer. The β -hexosaminidase released into the supernatants and in the cell lysates was quantified by the hydrolysis of *p*-nitrophenyl-*N*-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The reaction was stopped by the addition of stop buffer (0.1 M sodium carbonate/sodium bicarbonate, pH 11). The percentage of β -hexosaminidase release was assessed by measuring the absorption of the samples at 405 nm with a microplate spectrophotometer, and was calculated as follows: absorbance of culture supernatant at 405 nm $\times$ 100/absorbance of total cell lysate supernatant at 405 nm. C48/80 (30 μ g/mL) was used as a positive control.

2.9 | Histamine release assay

In 96-well plates, 1×10^6 LAD2 cells were incubated overnight at 37°C with 5% CO₂, whereas MPMC's were used within 4 hours. Following incubation, the culture medium was removed, the drug substances were added at the indicated concentrations, and the cells were incubated for 30 minutes at 37°C with 5% CO₂. The 96-well plate was centrifuged at 200g for 5 minutes at 4°C, and the supernatant was selected. Histamine-2HCl was used as an interior label. An LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used to apply the liquid chromatography electrospray ionization tandem MS (LC-ESI-MS/MS) method. Histamine was evaluated with the system by employing a HILIC column (Venusil HILIC, 2.1×150 mm, 3 μ m; Agela Technologies, Tianjin, China) and isocratic elution with acetonitrile-water containing 0.1% formic acid and 20 mM ammonium formate (77:23, vol/vol) at a flow rate of 0.3 mL/min.

2.10 | Analysis of mouse serum

ATRA cream was made from 0.1% ATRA added to vaseline matrix, and vaseline alone was used as a negative control. After treated by ATRA cream or vaseline, blood of mice was collected and centrifuged to obtain serum. Histamine release was analysed with the LC-ESI-MS/MS method. The mouse IgE enzyme-linked immunosorbent assay kit was from Biotech (Beijing, China), and was used to measure IgE levels in supernatants according to the instructions provided.

2.11 | Western blot analysis

Total proteins from the MRGPRX2-knockdown LAD2 cells and NC-LAD2 cells were extracted in ice-cold conditions into RIPA lysis buffer containing 10% protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). After 30 minutes, insoluble protein lysate was removed by centrifuging the samples at 13 500g for 10 minutes at 4°C. The protein concentration was determined with a BCA Protein Quantification kit according to the manufacturer's instructions. The protein in the cell lysates was denatured by boiling the samples for 5 minutes with a 5 × loading sample buffer from Thermo Fisher Scientific (Waltham, Massachusetts), and equal amounts of protein were separated on a 10% gel by the use of sodium dodecylsulfate polyacrylamide gel electrophoresis (Shaanxi Pioneer Biotech, Xi'an, China). The separated proteins were transferred onto polyvinylidene fluoride membranes from Hangzhou Microna Membrane Technology (Hangzhou, China), and blocked by constant stirring with 5% non-fat milk in Tris-buffered saline (Baihao, Xi'an, China) containing Tween-20 (TBST) (Shaanxi Pioneer Biotech, Xi'an, China) at room temperature for 2 hours. The membranes were then incubated overnight at 4°C with the following primary antibodies: anti-MRGPRX2 (1:250, #ab129548; Abcam, Cambridge, UK) and anti-glyceraldehyde-3-phosphate dehydrogenase (1:2000, #2118; CST, Danvers, Massachusetts). The membranes were then washed three times with TBST with 10-minute intervals, and then incubated with secondary antibodies (at a dilution of 1:20 000 in TBST) for 1 hour at 37°C. The membranes were again washed three times with TBST for 10 minutes, and developed with an enhanced chemiluminescence kit. A Lane 1DTM transilluminator (Beijing Creation Science, Beijing, China) was used to capture the images of the developed blots, and IMAGE-PRO PLUS 5.1 software (Media Cybernetics, Rockville, Maryland) was used to quantify the protein levels.

2.12 | Intracellular calcium ion mobilization assay

All drug substances were diluted to the required concentrations in calcium imaging buffer (CIB) (125 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 20 mM glucose, 1.2 mM NaHCO₃, and 20 mM sucrose, with the pH adjusted to 7.4 with NaOH). The incubation buffer consisted of 0.5 µL of Fluo-3, 2 µL of Pluronic F-127, and 997.5 µL of CIB. MRGPRX2-HEK293 cells, MrgprB2-HEK293 cells and normal control (NC)-HEK293 cells were plated at 1 × 10⁴ cells per well in a 96-well plate and incubated

overnight at 37°C with 5% CO₂, whereas mouse peritoneal mast cells were plated at 1 × 10⁴ cells in 96-well plates in DMEM with 0.05% recombinant mouse SCF, and seeded for 2 hours. The cells were then washed twice in 100 µL of CIB. Then, 100 µL of incubation buffer was added, and the plate was incubated for 30 minutes at 37°C with 5% CO₂. Then, the cells were again washed twice with CIB. The cells were used immediately for imaging. For the calcium imaging, the cells were magnified 200 times, and one photograph per second was obtained under blue light. Cells were identified as responding if the intracellular calcium ion concentration ([Ca²⁺]_i) rose by at least 50% after the substances were injected. LAD2 cells were incubated and washed in a 1.5-mL eppendorf (EP) tube, and then placed at 1 × 10⁴ cells per well in a 96-well plate. The cell fluorescence changes were read with a fluorescence microscope same as HEK293 cell.

2.13 | Small interfering RNA transfection of LAD2 cells

Specific knockdown was achieved with small interfering RNAs (siRNAs) targeting MRGPRX2, and non-targeting siRNAs as NCs. A SMART pool of double-stranded siRNAs targeting MRGPRX2 and non-specific siRNAs were obtained from Shanghai GenePharma (Shanghai, China). The siRNA sequences were as follows: MRGPRX2 forward, 5'-GUACAACAGUGAAUGGAAATT-3'; and MRGPRX2 reverse, 5'-UUUCCAUUCACUGUUGUACTT-3'; control forward, 5'-UUCUCCGAACGUGUCACGUTT-3'; and control reverse, 5'-ACGUGACACGUUCGGAGAATT-3'. For transfection, siRNA was delivered at a final concentration of 80 nM by use of the Lipofectamine 2000 reagent according to the manufacturer's instructions. Expression of MRGPRX2 mRNA and expression of MRGPRX2 protein were tested after knockdown. The cells were incubated for 48 hours to allow knockdown of MRGPRX2. These cells were then used for the β-hexosaminidase assay and histamine release assay.

2.14 | Statistical analysis

Experiments were repeated three times. Data are expressed as mean ± standard error of the mean, and were analysed with two-tailed unpaired Student's *t* test. An independent-samples analysis of variance was used to determine statistical significance in comparisons of the data by the use of SPSS. Differences were considered significant at *P* < 0.05, *P* < 0.01, and *P* < 0.005.

3 | RESULTS

3.1 | Topical application of ATRA triggered contact dermatitis

It has been reported that clinical symptoms of ATRA-triggered dermatitis appear 4 days after application.² In order to investigate the early stage of "retinoid dermatitis," we set up a mouse model to mimic ATRA-triggered dermatitis by topically applying 0.1% ATRA cream daily on the dorsal skin after shaving; vaseline was given as a solvent

control. At the first day after treatment, there was no visible erythema and scaling. After 2 to 3 days, symptoms of contact dermatitis appeared, with erythema, dryness, and scaling. In contrast, mice treated with vaseline as control did not show any lesions (Figure 1A). Histopathology of the lesion showed epidermal thickening and inflammatory infiltration in the dermis. The longer the treatment, the thicker the epidermis became, and the more massive the inflammatory cell infiltration (Figure 1B). After treatment with ATRA, especially after 2 to 3 days, immunofluorescence staining of avidin showed a degranulated status of mast cells in the dermis, as compared with non-degranulated mast cells in the control group (Figure 1C). Scratches in 20 minutes were counted 24 hours after treatment. Mice treated with ATRA scratched more than the those in the control group, with an increase in the histamine level in serum from the first

day, suggesting that retinoid dermatitis could induce pruritus and is closely related to skin mast cells (Figure 1D,E). However, IgE concentrations showed no significant differences between the treated and vaseline groups (Figure 1F), indicating that the release of histamine was not related to the IgE pathway.

3.2 | MrgprB2^{-/-} mice showed resistance to ATRA-triggered dermatitis

ATRA-induced dermatitis was ameliorated in MC-deficient Kit^{W-sh/W-sh} mice, indicating that retinoid dermatitis was mast cell-dependent dermatitis.⁴ As ATRA triggered contact dermatitis without IgE increase, we suspected that it was related to a pseudoallergic reaction via MrgprB2 in mice. To confirm this, MrgprB2^{-/-} mice were treated with

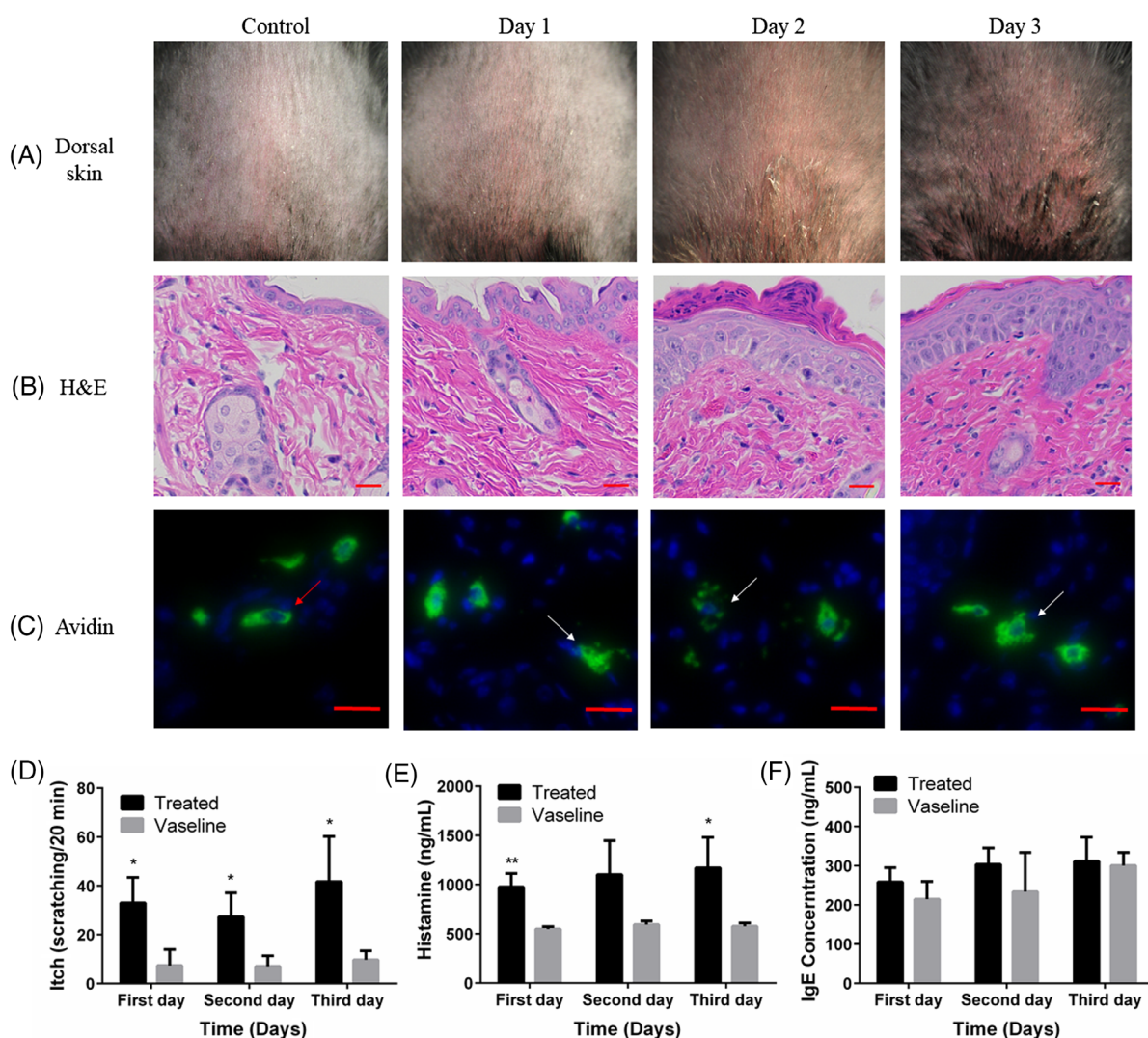


FIGURE 1 Topical application of all-trans-retinoic acid (ATRA) triggers contact dermatitis. Photographs of dorsal skin treated with vaseline (Control) or 0.1% ATRA cream for 1 day, 2 days, and 3 days, respectively, are presented from left to right. (A), Clinical photographs. (B), Haematoxylin and eosin (H&E) staining of skin lesions. (C), Immunofluorescence staining of avidin. Scale bars: 20 μ m. The red arrows point to one of the undegranulated mast cells; the white arrows point to degranulated mast cells. (D), Numbers of scratches in 20 minutes were counted 24 hours after topical application of drugs. (E), Serum histamine concentration. (F), IgE concentration in serum. Six mice were used per group, and experiments were repeated three times. Data are presented as mean $\pm$ standard error of the mean, and were analysed with a two-tailed unpaired Student's *t* test. Differences were considered to be significant at **P* < 0.05, ***P* < 0.001, and ****P* < 0.005

0.1% ATRA cream for 3 days, like wild-type (WT) C57 mice, and vaseline was given as a control. Mutant mice showed similar erythema and scaling as WT mice (Figure 2A), as well as epidermal thickening, whereas inflammatory infiltration was not as severe as in WT mice (Figure 2B). However, the skin mast cells did not release granules, according to the avidin staining of lesions (Figure 2C). The scratching intensity of mutant mice was not as marked as in the WT and control groups (Figure 2D). The histamine concentration in serum increased after treatment with ATRA (Figure 2E). There were no significant differences in IgE concentrations between control, WT and mutant groups (Figure 2F).

3.3 | ATRA triggered local inflammatory reactions via MrgprB2

Hindpaw swelling and extravasation have been used to evaluate drug-induced pseudoallergic reactions.^{11,19} In vivo analysis showed that ATRA triggered local inflammatory reactions in WT C57 mice. The degrees of rate of increase in paw thickness and EBD exudation were higher than those in mice injected with normal saline solution, and

were proportional to drug concentrations (Figure 3A). However, mast cell-deficient Kit^{W-sh/W-sh} mice showed resistance to this reaction, in that there was no visible swelling or EBD extravasation in either ATRA-injected or normal saline-injected mice, according to the rate of increase in paw thickness and OD_{620 nm} per gram of weight (Figure 3B). Furthermore, in contrast to WT mice, MrgprB2^{-/-} mice did not present with inflammatory reactions triggered by ATRA; that is, there was no difference between the two paws injected with ATRA and normal saline (Figure 3C). C48/80 was used as a positive control in the experiment.

3.4 | ATRA activated murine mast cells via MrgprB2

In order to confirm whether mast cells could be activated by ATRA in vitro, we purified MPMCs, and tested calcium influx and degranulation, including release of histamine and β -hexosaminidase, after treatment with ATRA. The [Ca²⁺]_i values in MPMCs from C57 WT mice were increased by ATRA (Figure 4A,B). Moreover, MPMCs from WT mice released β -hexosaminidase and histamine in a dose-dependent manner after being treated with ATRA for 30 minutes (Figure 4C,D).

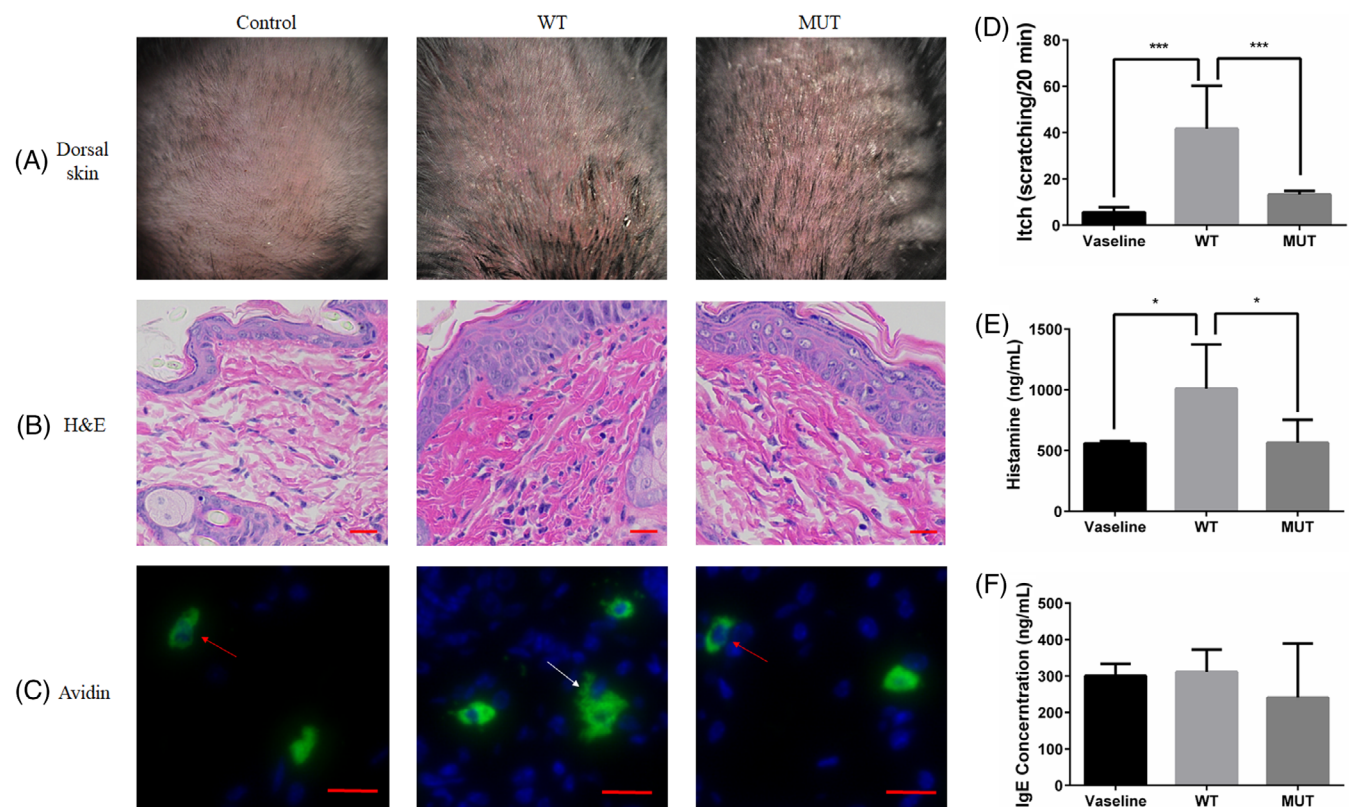


FIGURE 2 MrgprB2^{-/-} mice show resistance to all-*trans*-retinoic acid (ATRA)-triggered dermatitis. Wild-type (WT) mice treated with vaseline (Control) or 0.1% ATRA cream for 3 days and ATRA-treated mutant mice (MUT) are presented, respectively, from left to right. (A), Clinical photographs. (B), Haematoxylin and eosin (H&E) staining. (C), Immunofluorescence staining of avidin in skin lesions. Scale bars: 20 μ m. The red arrows point to non-degranulated mast cells; the white arrow points to one of the degranulated mast cells. (D), Numbers of scratches in 20 minutes were counted 24 hours after topical application of drugs. (E), Serum histamine concentration. (F), IgE concentration in serum. Six mice were used per group, and experiments were repeated three times. Data are presented as mean $\pm$ standard error of the mean, and were analysed with a two-tailed unpaired Student's *t* test. Differences were considered to be significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. MrgprB2, mouse Mas-related G-protein-coupled receptor-X2

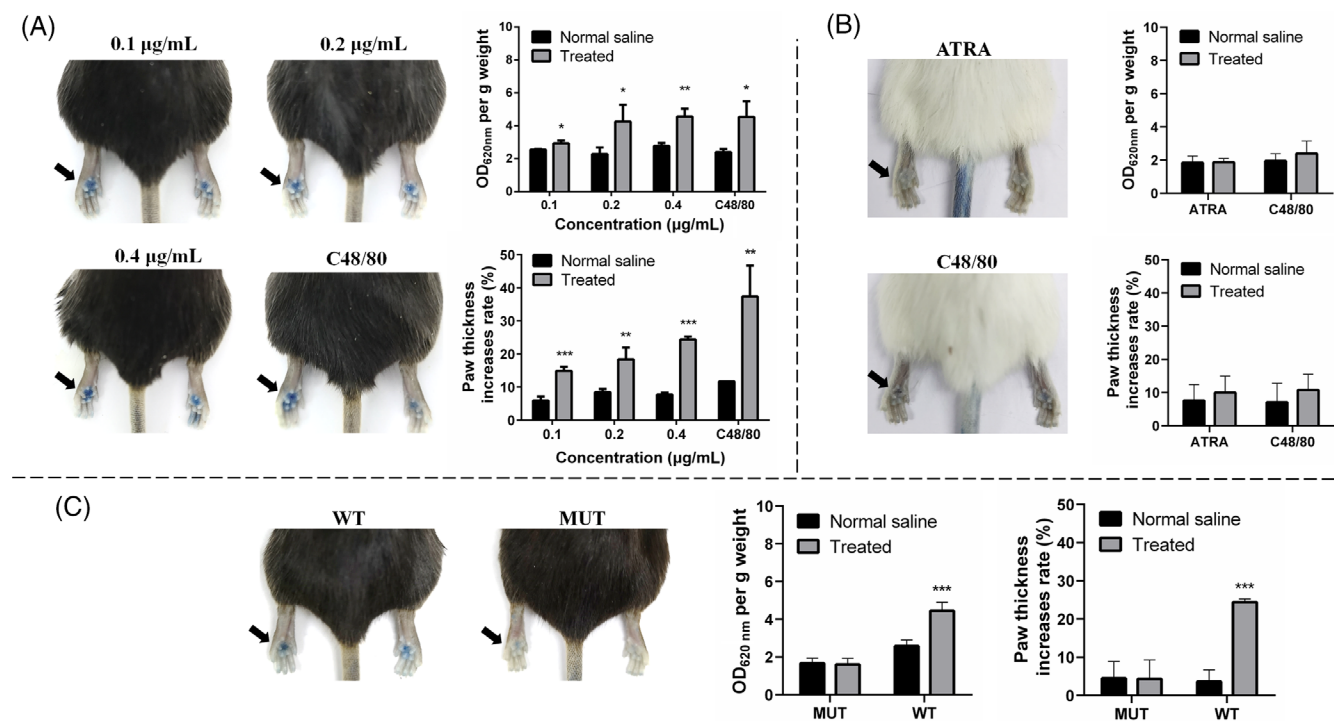


FIGURE 3 Retinoic acid (RA) induced local inflammatory reactions. (A), Wild-type (WT) C57 mice were injected with all-*trans*-RA (ATRA) at concentrations of 0.1, 0.2 or 0.4 µg/mL, or with 30 µg/mL C48/80, into the left paw (black arrows), and with normal saline solution into the right paw as a blank control. OD_{620 nm} values per gram of weight were measured to illustrate Evans blue dye exudation on both paws; the rate of increase in paw thickness represents the degree of swelling. (B), Paws of C57BL/6 STOCK Kit^{W-sh}/HnhrJaeBsmJNju (Kit^{W-sh/W-sh}) mice were treated with 0.4 µg/mL ATRA or 30 µg/mL compound 48/80 (C48/80) on the left (black arrows) and with normal saline solution on the right. (C), The left paws of MrgprB2^{-/-} mice (MUT) and WT C57 mice were treated with 0.4 µg/mL ATRA (black arrows); normal saline solution was injected into the right paw. Six mice were used per group, and experiments were repeated three times. Data are presented as mean ± standard error of the mean, and were analysed with a two-tailed unpaired Student's *t* test. Differences were considered to be significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. MrgprB2, mouse Mas-related G-protein-coupled receptor-X2; OD, optical density

In contrast, MPMCs purified from MrgprB2^{-/-} mice showed no alteration in [Ca²⁺]_i (Figure 4E,F) or the release of β-hexosaminidase and histamine after treatment with ATRA (Figure 4G,H), indicating that ATRA could activate murine mast cells through MrgprB2. Furthermore, we treated MrgprB2-HEK293 cells with ATRA to identify the activating effect on MrgprB2, which resulted in an increase in [Ca²⁺]_i (Figure 4I) as compared with NC-HEK293 cells (Figure 4J).

3.5 | ATRA activated human mast cells via MRGPRX2

To investigate whether MRGPRX2 could be activated by ATRA, calcium influx was tested. Fluorescence imaging traces showed that, at a concentration of 80 µg/mL, ATRA altered the [Ca²⁺]_i of MRGPRX2-HEK293 cells (Figure 5A), as compared with NC-HEK293 cells (Figure 5B). In addition, calcium influx and degranulation of human mast cells induced by ATRA were observed in LAD2 cells. Calcium influx of LAD2 cells was altered at a concentration of 200 µg/mL (Figure 5C), and β-hexosaminidase and histamine release were increased in a dose-dependent manner after treatment with 200 µg/mL ATRA for 30 minutes (Figure 5D,E). Also, after treatment with the same concentration of ATRA, the rate of β-hexosaminidase

release and the amount of histamine released after MRGPRX2 knock-down in LAD2 cells were obviously lower than those in NC-LAD2 cells (Figure 5F,G), showing that there was MRGPRX2-mediated ATRA-induced activation of mast cells. Specific knockdown of MRGPRX2 in LAD2 cells was achieved with siRNA, which led to obvious decreases in the expression of MRGPRX2 protein and MRGPRX2 mRNA as compared with NC-LAD2 cells (Figure 5H-J).

4 | DISCUSSION

The pathogenesis of retinoid dermatitis is complex, including changes in keratinocytes and inflammatory cells in the dermis. It is commonly believed that contact dermatitis comprises 80% irritant contact dermatitis and 20% allergic contact dermatitis. Retinoid dermatitis had been thought to be allergic contact dermatitis,²⁰ that is, a type IV delayed hypersensitivity reaction that occurs only in a sensitized individual.^{21,22} A strong relationship between ATRA and skin mast cells has been confirmed, as mast cell-deficient Kit^{W-sh/W-sh} mice did not show as serious dermatitis as WT mice.⁴ Mast cell-mediated drug hypersensitivity mainly consists of allergic/immune reaction, pharmacological interaction with immune receptors (p-i reactions), and pseudo-allergy.²³ The most frequent form is the IgE-mediated type I allergic

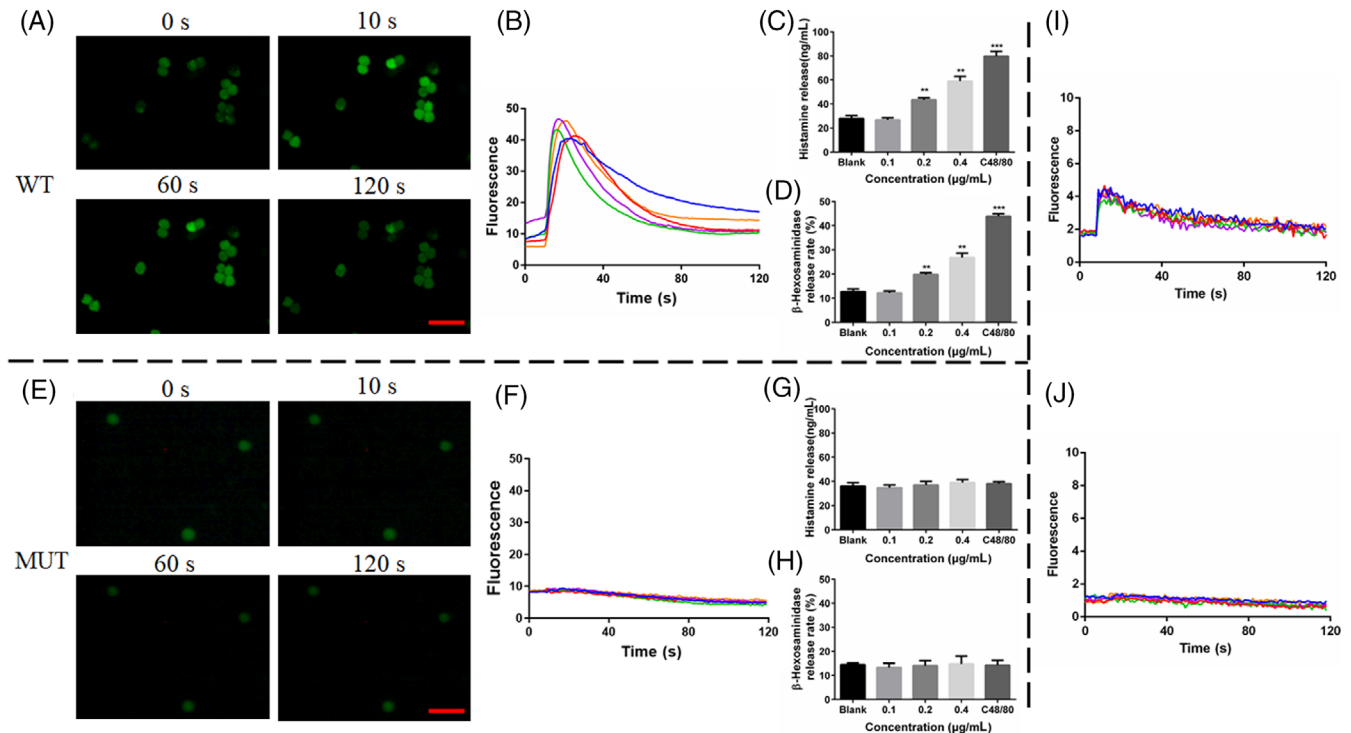


FIGURE 4 Degranulation and calcium influx of mouse peritoneal mast cells (MPMC) and MrgprB2-HEK293 cells. (A), Calcium images of wild-type (WT) MPMCs. Fluorescence intensity represents the intracellular Ca^{2+} concentration. All-*trans*-retinoic acid (ATRA) (0.4 μ g/mL) was added at 10 seconds. Scale bars: 100 μ m. (B), Representative imaging traces; each coloured line represents an individual cell. (C,D), Histamine concentration (C) and β -hexosaminidase release rate (D) of MPMC supernatant after treatment with ATRA at concentrations of 0.1, 0.2 and 0.4 μ g/mL, and with 30 μ g/mL compound 48/80 (C48/80), for 30 minutes. (E), Calcium images of MrgprB2^{-/-} mouse (MUT) MPMCs treated with 0.4 μ g/mL ATRA at 10 seconds. Scale bars: 100 μ m. (F), Representative imaging traces of mutant MPMCs; each coloured line represents an individual cell. (G,H), Histamine concentration (G) and β -hexosaminidase release rate (H) of mutant MPMC supernatant after treatment with 0.1, 0.2, 0.4 μ g/mL ATRA and 30 μ g/mL C48/80 for 30 minutes. (I,J), Representative calcium imaging traces of MrgprB2-HEK293 cells (I) and normal control (NC)-HEK293 cells (J); each coloured line represents an individual cell. Experiments were repeated three times. Data are presented as mean $\pm$ standard error of the mean, and were analysed with a two-tailed unpaired Student's *t* test. Differences were considered to be significant at **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. MrgprB2, mouse Mas-related G-protein-coupled receptor-X2

reaction, which occurs rapidly and is induced by antigens via prior exposure. However, the irritation and itch after the first contact with ATRA in many patients are hard to explain by such a mechanism.

Pseudoallergy could occur in the respiratory tract and the skin, mostly within minutes or hours, without prior exposure. Non-steroidal anti-inflammatory drugs, quinolones, muscle relaxants, radiocontrast media and other drugs have been found to trigger pseudoallergic reactions, via MRGPRX2. This receptor recognizes a certain chemical motif that was found to be crucial for IgE-independent, direct mast cell stimulation.²³ Could ATRA induce pseudoallergic reactions via MRGPRX2 in contact dermatitis?

In our research, we focused on the early stage of RA-induced dermatitis, in order to investigate the role of MRGPRX2 in mediating pseudoallergic reactions. Injection with ATRA caused paw swelling and extravasation in WT mice, but MrgprB2^{-/-} mice were resistant to this reaction. In vitro, ATRA could induce MPMC degranulation in a dose-dependent manner and altered calcium influx in WT mice, whereas MPMCs from MrgprB2^{-/-} mice showed no change. Calcium influx of MrgprB2-HEK293 cells could also be altered by ATRA,

indicating the activating effect of ATRA on mast cells. However, MrgprB2^{-/-} mice also showed epidermal thickening and inflammatory infiltration in the dermis, which may, partly, be a result of ATRA's effect on tight junctions and the function of the epidermal barrier.²⁴ Moreover, RAR α and Cyp26b1 in mast cells also participated in the ATRA-induced activation, as previously reported.

Although MrgprB2 is the mouse homologue of human MRGPRX2, it shares only 52% sequence identity with MRGPRX2.¹² Many drugs or basic activators are able to activate these at different concentrations, as shown by the results of a previous study testing the half-maximum effective concentration (EC_{50}) values.¹¹ In our study, we found that MRGPRX2 could be activated by ATRA at a much higher concentration than MrgprB2, suggesting that pseudoallergic reactions induced by ATRA occur more easily in the mouse. In humans, clinical doses of ATRA may not always trigger pseudoallergic reactions, but incorrect topical use yielding high concentrations could still be one of the reasons for the side-effect. On the other hand, whether individual susceptibility is affected by the expression of MRGPRX2 has not been studied. We expect this to

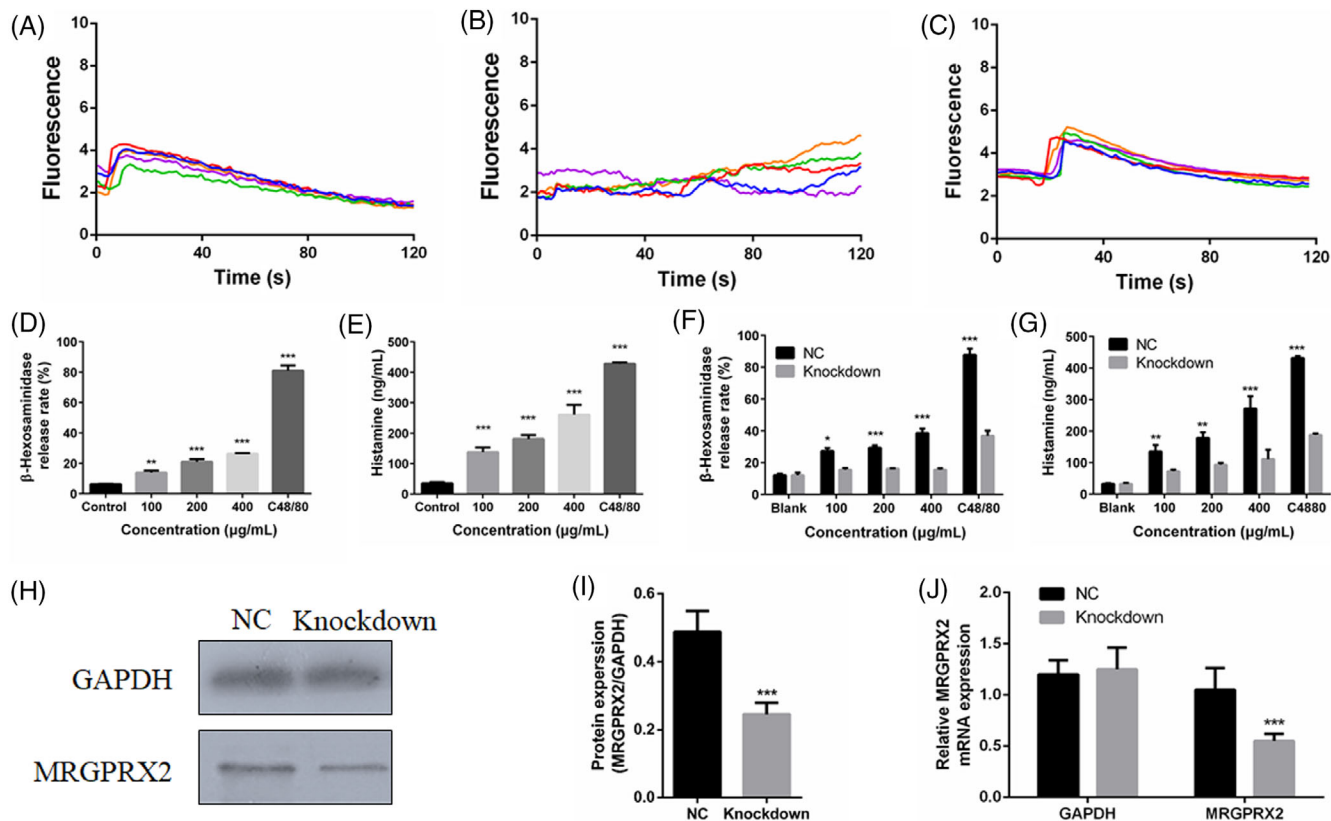


FIGURE 5 All-*trans*-retinoic acid (ATRA) activated human mast cells via Mas-related G-protein-coupled receptor-X2 (MRGPRX2). (A,B), MRGPRX2-HEK293 cells (A) and normal control (NC)-HEK293 (B) cells were treated with 80 μg/mL ATRA. Calcium imaging traces are shown. Each coloured line represents an individual cell. (C), Intracellular Ca^{2+} concentrations of Laboratory of Allergic Disease 2 (LAD2) cells were altered by ATRA (200 μg/mL); each coloured line represents an individual cell. (D,E), β-Hexosaminidase release rate (D) and histamine concentration (E) of LAD2 supernatant after treatment with 100, 200 or 400 μg/mL ATRA, or with 30 μg/mL compound 48/80 (C48/80), for 30 minutes. (F,G), β-Hexosaminidase (F) and histamine (G) release in transfected MRGPRX2-knockdown LAD2 cells treated with ATRA at concentrations of 100, 200 or 400 μg/mL, or with 30 μg/mL C48/80, for 30 minutes, as compared with NC-LAD2 cells. (H), MRGPRX2 protein expression after knockdown by small interfering RNA transfection. (I), Quantification of MRGPRX2 protein expression by densitometric analysis. (J), Expression of MRGPRX2 mRNA after knockdown. GAPDH, glyceraldehyde-3-phosphate dehydrogenase

be so, as the expression of MRGPRX2 on mast cells has been found to be upregulated in the skin of patients with severe chronic urticaria.⁷

Our results indicate that ATRA could lead to the activation of mast cells via MrgprB2/MRGPRX2, which is consistent with the symptoms observed in clinical practice. Activation of skin mast cells induced by ATRA could contribute to the pathogenesis of retinoid dermatitis. The histamine level in serum increased, and immunofluorescence staining of avidin showed a degranulated status of mast cells in the dermis after treatment with ATRA for 1 day. We consider that retinoid dermatitis is similar to a pseudoallergic reaction, manifested as vasodilatation accompanied by itching or pain, without an increase in IgE.¹⁷

On the basis of the above results, we conclude that a pseudoallergic reaction via MrgprB2/MRGPRX2 on skin mast cells may contribute to retinoid dermatitis. Apart from contact dermatitis, mast cells play a key role in several skin diseases, such as atopic dermatitis and psoriasis, as well as wound healing.^{25,26} Whether MRGPRX2-mediated mast cell releasing inflammatory factors are involved in the pathogenesis of other cutaneous conditions is worthy

of more research. Saikosaponin A has been proved to inhibit C48/80-induced pseudoallergy via the MRGPRX2 pathway.²⁷ To avoid severe side-effects, stopping the application of ATRA and some antihistamine drugs could be helpful, not to mention the traditional treatment with topical corticosteroids and calcineurin inhibitors. We are looking forward to more research on the inhibition of the pseudoallergic reaction.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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