

p-Phenylenediamine induces immediate contact allergy and non-histaminergic itch via MRGPRX2

Delu Che^{a,b}, Jiapan Gao^c, Xueshan Du^a, Yi Zheng^a, Yajing Hou^c, Bin Peng^{a,b}, Tao Jia^a, Songmei Geng^{a,b,**}, Langchong He^{c,d,*}

^a Department of Dermatology, Northwest Hospital, The Second Hospital Affiliated to Xi'an Jiaotong University, Xi'an, Shaanxi, China

^b Center for Dermatology Disease, Precision Medical Institute, Xi'an, China

^c School of Pharmacy, Xi'an Jiaotong University, Xi'an, China

^d Pazhou Lab., Guangzhou, China

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ABSTRACT

p-phenylenediamine (PPD) is a common component of hair dye known to induce immediate allergy, even acute dermatitis and contact dermatitis. MAS-related G protein coupled receptor-X2 (MRGPRX2) in mast cells (MCs) mediates small molecular substances-induced pseudo-allergic reactions. However, the role of MRGPRX2 in PPD-induced immediate contact allergy needs further exploration. The aim of this study was to investigate whether PPD activates MCs via MRGPRX2 and induces immediate allergies that contribute to contact dermatitis. Wild-type (WT) and *kit^{w-sh/w-sh}* mice (MUT) were treated with PPD to observe local inflammation and MC degranulation *in vivo*. The release of inflammatory mediators was measured *in vitro*. Histamine 1 receptor (*H₁R*)^{-/-} mice were used to analyze itch type. PPD caused immediate contact allergy in WT mice, induced scratching, and local inflammatory reactions, while exhibiting minimal effects on MUT mice. PPD did not induce histamine release, but induced significant tryptase release *in vivo* and *in vitro*. PPD activated MRGPRX2 to induce MC degranulation *in vitro*. PPD caused immediate contact allergy in WT mice, induced scratching and local inflammatory reactions, while exhibited minimal effect on MUT mice. PPD did not induce histamine release, while induced significant tryptase release *in vivo* and *in vitro*. PPD induced immediate contact allergy by MCs activation via MRGPRX2 and lead to tryptase release. The scratching times showed no significant difference in WT mice or *H₁R*^{-/-} mice, which indicated PPD caused non-histaminergic itch. The results showed that PPD activated MCs via MRGPRX2 and induced immediate contact allergy, leading to the release of tryptase without monoamine release, which might induce non-histaminergic itch.

1. Introduction

Several ingredients of oxidative hair dyes can induce contact dermatitis [1]. *p*-Phenylenediamine (PPD), an aromatic amine with good antioxidant and dyeing properties, is found in paints, rubber, black ink, color developers, clothing, and footwear, and is currently most widely used in hair dyes and henna paint [2]. PPD shows high permeability across the skin barrier as its low molecular weight and strong protein binding ability, which may induce acute dermatitis, facial edema, and contact dermatitis [3]. In many countries, the dosage of PPD is clearly defined though the prevalence of PPD contact allergy is still

high, with no statistically significant association with gender or hair dye usage [4]. In Europe, the prevalence of PPD causing allergic contact dermatitis in the general population is estimated to be 1.5%. In the hairdressing industry, the incidence of allergic contact dermatitis caused by PPD is 19%–35% [5]. Sensitization by black henna tattoos also results in severe elicitation reactions [6]. PPD shows a higher positive rate in patch testing, which is a diagnostic tool for investigating allergic contact dermatitis [7].

PPD-induced allergic reactions are common; however, the mechanism of sensitization is unclear. PPD has been shown to sensitize the most exposed individuals after only two exposures [8]. This would make most hairdressers sensitized to PPD, which does not seem to be the case

* Corresponding author. School of Pharmacy, Xi'an Jiaotong University, Xi'an, China.

** Corresponding author. Department of Dermatology, Northwest Hospital, The Second Hospital Affiliated to Xi'an Jiaotong University, 157 Xi Wu Road, Xi'an, Shaanxi, China.

E-mail addresses: gsm312@yahoo.com (S. Geng), helc@mail.xjtu.edu.cn (L. He).

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Abbreviations

PPD	p-phenylenediamine,
MCs	mast cells
MRGPRX2	MAS-related G protein coupled receptor X2
C 48/80	compound 48/80
WT	Wild-type
MUT	kit ^{w-sh/w-sh} mice,
H ₁ R	Histamine 1 receptor
MPMC	Mouse peritoneal mast cells
LAD2	Laboratory of Allergic Disease 2
MCDM	Mast cell dissociation medium
H&E	Hematoxylin and eosin
TNF- α	Tumor necrosis factor α

MCP-1	Monocyte chemoattractant protein-1
IL-8	Interleutin-8
IL-4	Interleutin-4
IL-6	Interleutin-6
IL-10	Interleutin-10
IL-13	Interleutin-13
CXCL2	C-X-C Motif Chemokine 2
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PLC γ 1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-1
PKC	Protein kinase C
AKT	Protein kinase B
ERK1/2	Extracellular regulated protein kinases
P38	p38 MAP kinase

[9]. The majority of PPD that penetrated is converted into the detoxification products monoacetyl-PPD and diacetyl-PPD by the N-acetyltransferase enzymes. The remaining small amount of PPD without N-acetylation is prone to auto-oxidation reactions, yielding proteinase-active PPD derivatives, which may bind to specific amino acids and might be responsible for sensitization [10]. However, *in vivo* evidence is lacking [11].

Most studies indicate that PPD induces type I and type IV hypersensitivity allergic reactions [12], but it is worth noting that PPD also causes immediate hypersensitivity within minutes, resulting in severe itching and rashes on the scalp [13]. In the patch test, the PPD-induced positive reaction was more associated with the dose per unit area than the excipient volume dispensed [12]. MRGPRX2 on MCs directly mediate MC activation and induce pseudo-allergic reactions [14]. Many small molecular substances such as morphine [15], mivacurium chloride [16], and substance P [17] cause anaphylactoid reactions via MRGPRX2, leading to the degranulation of MCs. MRGPRX2 is a key target that mediates MC activation induced by small molecular substances [14].

This study was conducted to investigate whether PPD induces immediate contact allergy and to clarify whether the mechanism is mediated by MC-related MRGPRX2. In addition, PAMP(9–20) has been shown to induce MC release of tryptase [18]. PAMP(9–20) was used as the positive control to preliminarily analyze the type of pruritus induced by PPD in immediate contact allergy.

2. Materials and methods

2.1. Drugs and reagents

PPD was purchased from Aladdin Industrial Corporation (Shanghai, China). PAMP(9–20) was purchased from NJpeptide (Nanjing, china). Compound 48/80 (C 48/80), p-nitrophenyl N-acetyl- β -D-glucosamide and Pluronic F-127 were purchased from Sigma-Aldrich (St. Louis, USA). Fluo-3, AM ester was obtained from Biotium (California, USA). 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 BSA, pH = 7).

2.2. Animals

C57BL/6 kit^{w-sh/w-sh} and C57BL/6H₁R^{-/-} mice were purchased from Model Animal Research Center of Nanjing University. C57BL/6 mice were purchased from the Experimental Animal Center of Xi'an Jiaotong University. Six mice were used in control and PPD-treated groups for each experiment (n = 6). In order to ensure the accuracy and consistency of the experiment, the experiments *in vivo* were repeated thrice and each group contained totally 18 mice.

Adult male mice ageing 8 weeks were anaesthetized by intraperitoneal injection using pentobarbital sodium (40 mg/kg) and were immobilized by tying the limbs to the board. The hair on the back was depilated by small animal shaver. Then commercial hair removal cream was used to depilate. PPD was dissolved in absolute ethyl alcohol at a concentration of 20 mg/ml. And then it was diluted to the set concentration (5, 10, 20 μ g/ml) and prepared in 50% glycerine for one day assay to show dose relationship between allergy and PPD. While 5 μ g/ml PPD prepared in 50% glycerine was used in three days assay to make the experiment consistent with the patch test. 300 μ g/ml PAMP(9–20) was set as positive control, because it induced less histamine release, which is different with compound 48/80. 50% glycerine was negative control. The drugs were applied as closed-patch to back skin. According to the requirements of the patch experiment, the chemicals were treated on skin for 48 h, and the analysis was finished at 72 h.

PPD and PAMP(9–20) were applied to the nape of the neck and back, and scratching behavior was observed for 1 h. A bout of scratching was defined as a hindpaw directed continuous scratching movement at the applied site. Use of both forepaws was classified as grooming behavior and not counted. Scratching behavior was quantified by counting the number of scratching bouts during the 1 h observation period.

For 24 h treated, mice were sacrificed through CO₂ inhalation and skin tissues in the closed-patch location after treated by PPD were weighed and collected into tubes for assay. The skin tissues were treated with chemicals for 48 h and removed at 72 h. 1 ml saline was added to each tube and allowed to stand on ice for 30 min, skin tissues were then cut into pieces, treated supersonically for 30 min, and centrifuged for 10 min at 10000 rpm and 4 °C. Fifty microliters of supernatant from each sample was collected into a clean tube for analysis.

2.3. Peritoneal mast cell purification

Adult male C57BL/6 mice were sacrificed through CO₂ inhalation. A total of 12 ml mast cell dissociation medium (MCDM) (15 ml of MCDM: 1.5 ml of 10 $\times$ Hank's balanced salt solution, 450 μ l of fetal bovine serum, 150 μ l of 1 M HEPES, 12.9 ml of sterile water, pH = 7.2) was used for two to three sequential peritoneal lavages, then centrifuged at 200 g for 10 min at 4 °C. The mouse peritoneal mast cells (MPMC) were isolated and purified by magnetic beads separation. Anti-mouse CD117 and Anti-R-Phycoerythrin (PE) Magnetic (BD Biosciences, New York, USA) was used. MPMC in the pellet were retrieved, and purity was >95% as assayed by morphology. Then MPMC resuspended in DMEM with 100 ng/ml recombinant mouse stem cell factor, and used in 4 h [19].

2.4. Histology

For 24 h treated, mice were sacrificed through CO₂ inhalation and

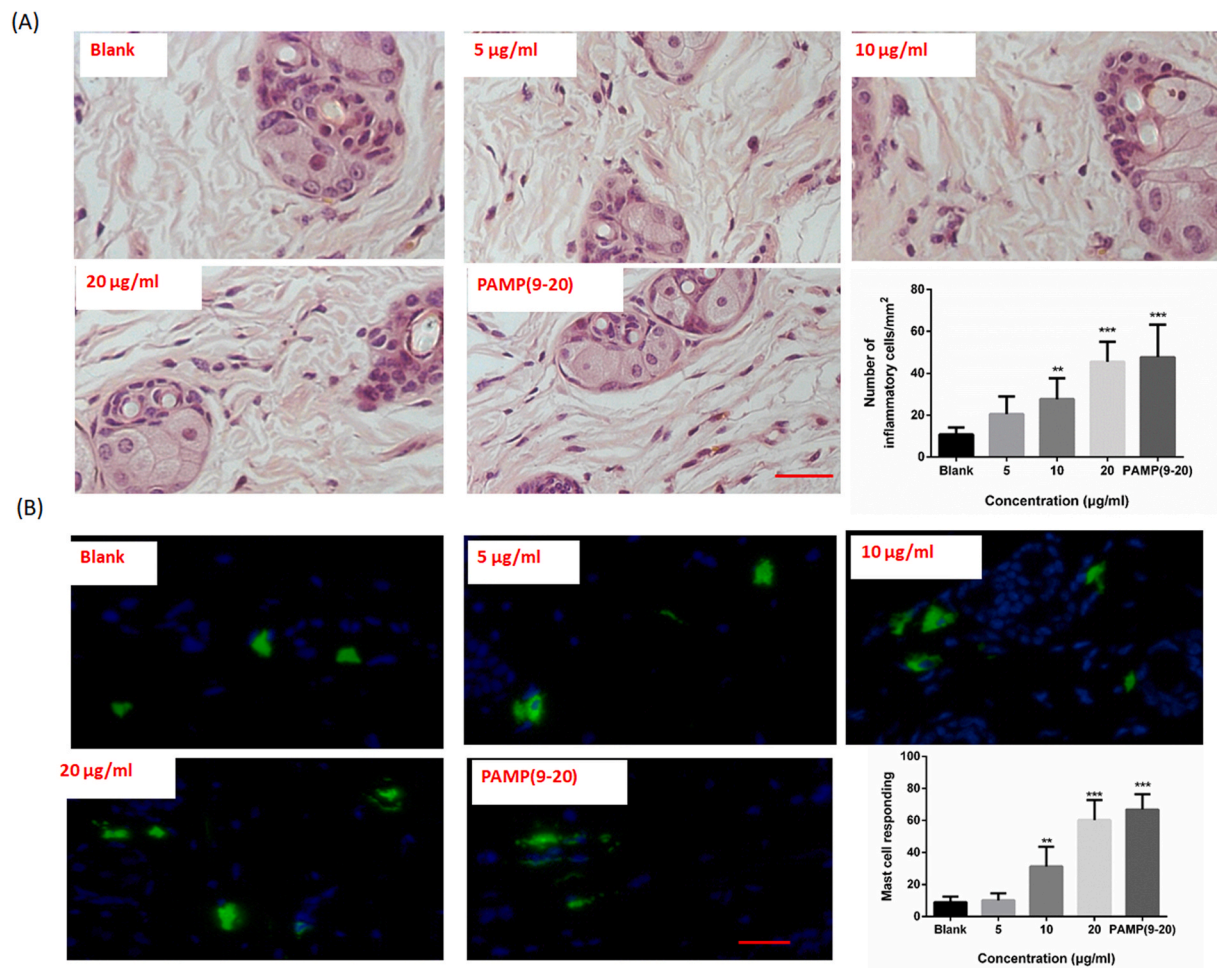


Fig. 1. Topical application of PPD triggers contact allergy. Dorsal skin treated with 50% glycerinum as negative control, PAMP(9–20) as positive control, and 5, 10, 20 µg/ml PPD for one day. (A) PPD induced inflammatory cells infiltration analyzed by hematoxylin and eosin (H&E) staining of skin lesions, scale bars represent 20 µm (B) PPD induced mast cells degranulation reaction significantly at a high dose (the data included 18 data points per group, scale bars represent 20 µm).

skin tissues in the closed-patch location after treated by PPD were weighed and collected into tubes for assay. While the chemicals were treated on skin for 48 h, and the skin tissues was removed at 72 h. Skin samples were fixed in 4% paraformaldehyde and embedded in paraffin. The thickness of the paraffin sections were 5 µm and stained with hematoxylin and eosin (H&E). 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, incubation with 1/500 avidin-FITC (Sigma-Aldrich, St. Louis, USA) for 45 min, sections were washed 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL, U.S.A) was used for marking mast cells and anti-tryptase antibody (Tryptase (E7M2U) Rabbit mAb, Cell Signaling Technology, Inc., Danvers, MA, USA) was used for marking tryptase.

2.5. Ethics statement

Experimental protocols involving mice were approved by Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-711). 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 [20].

2.6. Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells were

kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). The cells were maintained in StemPro-34 medium supplemented with 10 ml/1 StemPro nutrient supplement, 1:100 penicillin-streptomycin, 2 mM L-glutamine and 100 ng/ml human stem cell factor in an atmosphere containing 5% CO₂ at 37 °C and the cells were maintained at a density of 2×10^6 cells/ml [19]. MRGPRX2-expressing HEK293 cells, NC-HEK293 cells and H₁R-expressing HEK293 cells were constructed by HIV-1-based lentiviral vectors, both the cells cultured by DMEM with 10%FBS and 1:100 penicillin-streptomycin [19,21].

2.7. Intracellular calcium ion mobilization assay

All drug substances were diluted to the required concentrations in calcium imaging buffer (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, pH = 7.4). The incubation buffer consisted of 0.5 µl of Fluo-3, 2 µl of Pluronic F-127 and 997.5 µl of calcium imaging buffer. MRGPRX2-HEK293 cells, and NC-HEK293 cells were plated at 1×10^4 cells per well in a 96-well plate and incubated overnight. Cells were then washed twice in 100 µl calcium imaging buffer. 100 µl incubation buffer was added, and incubated for 30 min at 37 °C with 5% CO₂. Then, cells were washed twice with calcium imaging buffer and used immediately for imaging. PPD and PAMP(9–20) were added at fifth second after the photos were taken. Cells were identified as responding if the [Ca²⁺]_i rose by at least 50% after the substances were injected. LAD2 cells were

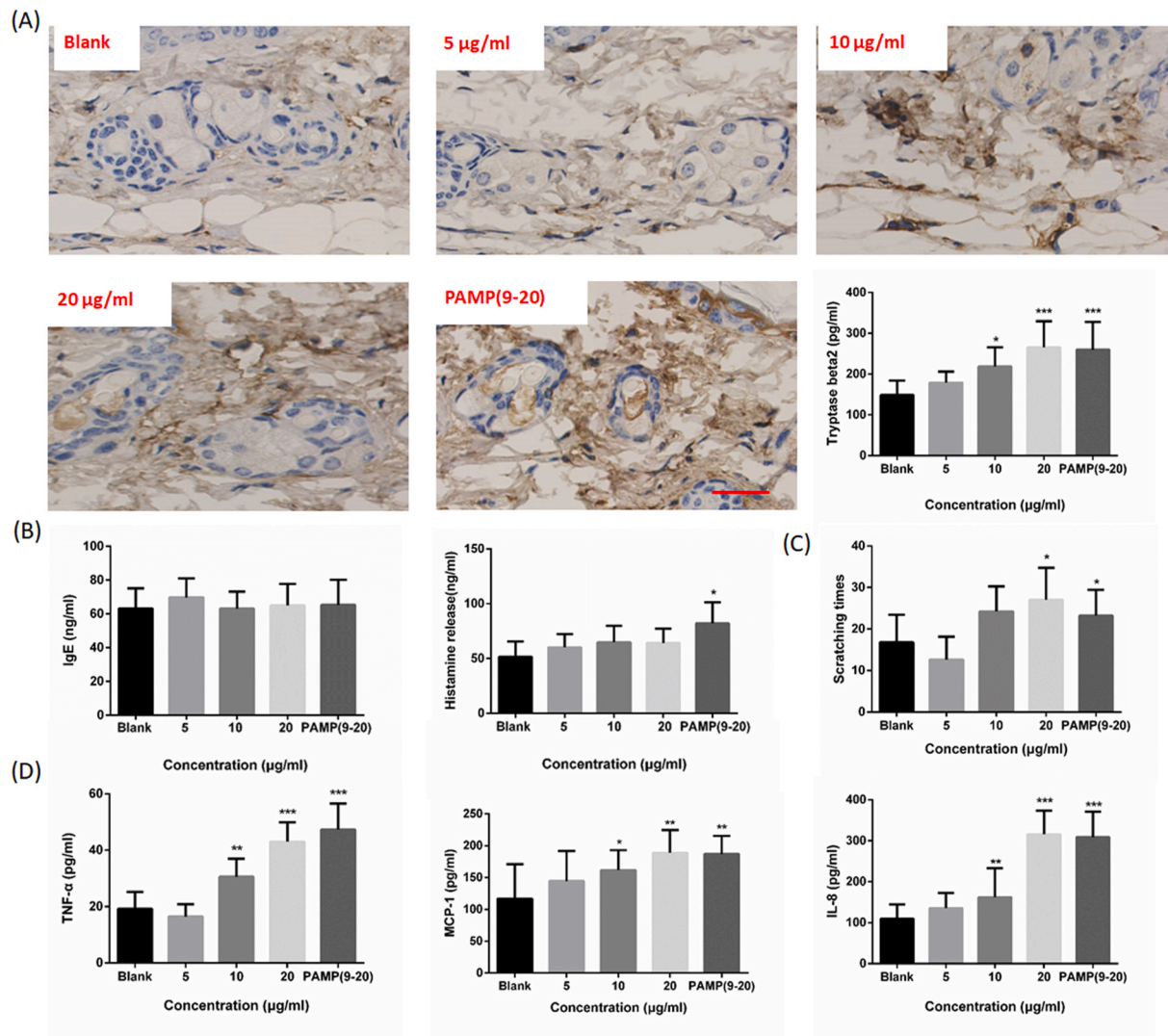


Fig. 2. PPD induced the release of inflammatory mediators with a dose dose-dependent manner. Dorsal skin treated with 50% glycerinum as negative control, PAMP (9–20) as positive control, and 5, 10, 20 µg/ml PPD for one day. (the data included 18 data points per group) (A) PPD induced tryptase beta release in mice skin and serum (B) PPD did not caused IgE release in mice serum or histamine release at the local affected site. (C) Scratching times counted after one day of topical application, PPD induced scratching significantly at a high dose (D) PPD induced TNF-α, MCP-1, IL-8 increase in mice serum. (The data were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.0125$, ** $p < 0.0025$, and *** $p < 0.00125$).

incubated and washed in a 1.5 ml centrifuge tube, then plated at 1×10^4 cells per well in a 96-well plate and following the method described in HEK293 cell calcium influx.

PPD prepared by calcium imaging buffer (50, 100, 200 µg/ml) was added into LAD2 cells, and incubated for 1 h at 37 °C with 5% CO₂. Liquid supernatant was collected. After H₁R-expressing HEK293 cells were incubated by incubation buffer, the liquid supernatant was used to analyze activation of calcium mobilization, histamine was used as positive control. Liquid supernatant and histamine were added at fifth second after the photos were taken. Cells were identified as responding if the [Ca²⁺]_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.

2.8. Small interfering (si)RNA transfection of LAD2 cells

Specific knockdown was achieved using small interfering (si)RNAs targeting MRGPRX2, and non-targeting siRNAs as negative control (NC). The siRNA sequences were as follows: forward, 5'-GUACAACAGU-GAAUGGAAATT-3' and reverse, 5'-UUUCCAUUCACUGUUGUACTT-3'

for MRGPRX2; and forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3' for the control. For transfection, siRNA was delivered at a final concentration of 80 nM using the Lipofectamine® 2000 reagent according to the manufacturer's instructions. Cells were incubated for 48 h to allow knockdown of MRGPRX2.

2.9. Cytokines assay

1×10^6 LAD2 cells and MPMC per well, were incubated in a 96-well plate overnight at 37 °C with 5% CO₂. The culture medium was removed, PPD prepared by Tyrode solution (50, 100, 200 µg/ml) was added at the indicated concentrations, and the cells were incubated for 1 h for histamine, and tryptase assay at 37 °C with 5% CO₂, while 8 h for cytokines assay. Human histamine, 5-HT and tryptase ELISA Kit were purchased from Jianglai Industrial Limited By Share Ltd (Shanghai, China). Human TNF-α, MCP-1, IL-8, IL-4, IL-6, IL-10, and IL-13 ELISA Kit were purchased from Sino Biological Inc (Beijing, China). 30 µg/ml PAMP(9–20) and 10 µg/ml C 48/80 were set as positive control.

After the drugs was applied to back skin, blood was collected and

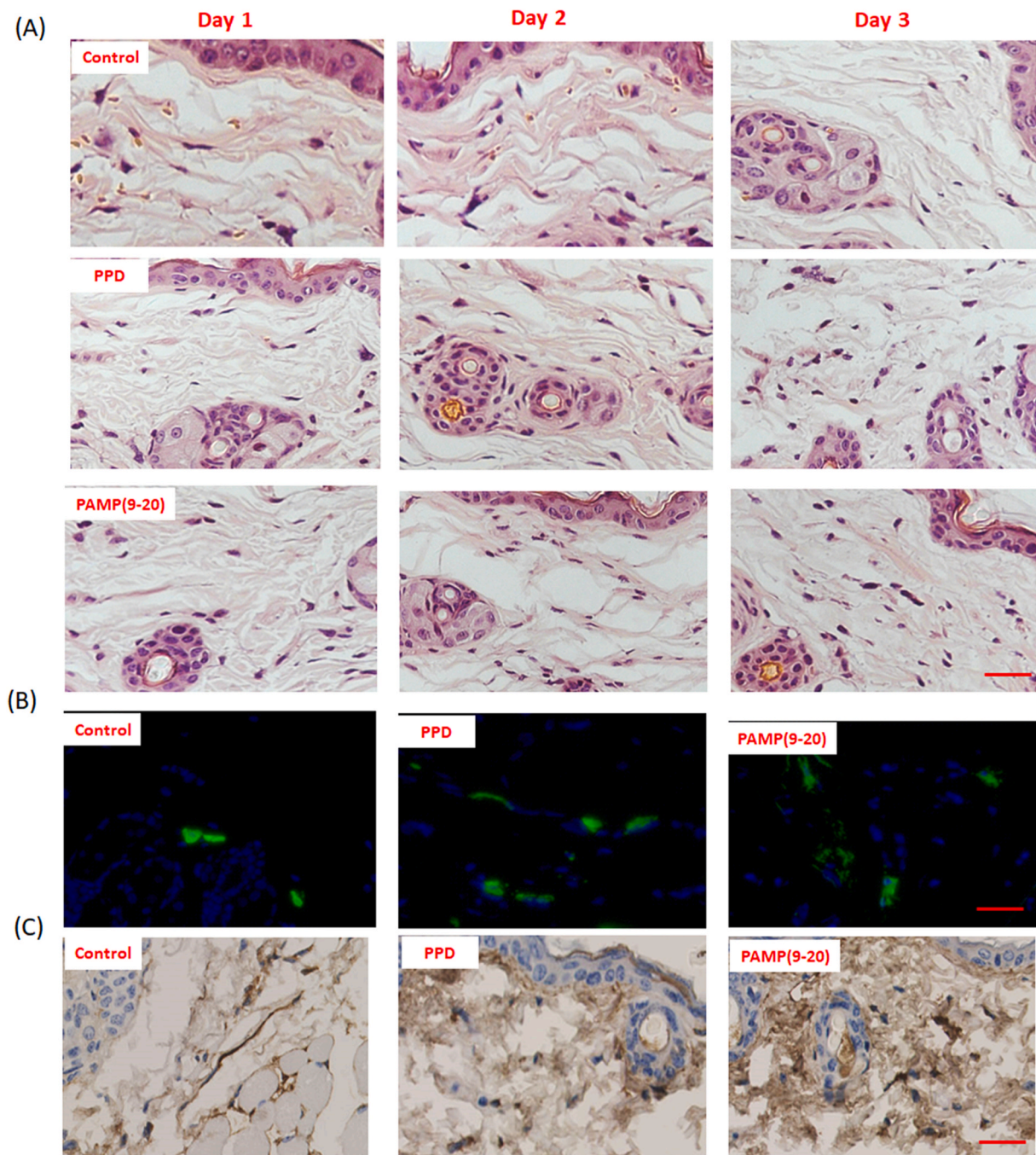


Fig. 3. Topical application of 5 µg/ml PPD triggers contact allergy in three days. Dorsal skin treated with 50% glycerinum as negative control, PAMP(9–20) as positive control, and 5 µg/ml PPD for three days. (A) PPD induced inflammatory cells infiltration analyzed by H&E staining of skin lesions in a time-dependent manner, scale bars represent 20 µm (B–C) PPD induced mast cells degranulation reaction and tryptase beta release in mice skin at the third day (the data included 18 data points per group, scale bars represent 20 µm).

centrifuged to get serum. Mouse IgE ELISA Kit was purchased from BIOTECH CO., LTD. (Beijing, China). Mouse tryptase ELISA Kit were purchased from Jianglai Industrial Limited By Share Ltd (Shanghai, China). Mouse TNF- α , MCP-1, and CXCL-2 ELISA Kit were purchased from Sino Biological Inc (Beijing, China). All steps were performed strictly according to the manufacturers' instructions.

2.10. Western blot analysis

Total proteins from the untreated and PPD-treated LAD2 cells (2 h) were extracted in ice-cold condition using RIPA lysis buffer containing

10% protease inhibitor and a phosphatase inhibitor cocktail (Roche Diagnostics). After 30 min, insoluble protein lysate was removed by centrifuging the samples at 13,500 g for 10 min at 4 °C. The protein concentration was determined using a BCA Protein Quantification kit according to manufacturer's instructions. The protein in the cell lysates was denatured by boiling the samples for 5 min with a 5 × loading sample buffer (Thermo Fisher Scientific, Inc., MA, USA), and equal amounts of protein were separated on a 10% gel using SDS-PAGE (Shaanxi Pioneer Biotech Co., Ltd.). The separated proteins were transferred onto polyvinylidene fluoride membranes and blocked by constant stirring with 5% non-fat milk in Tris-buffered saline (Baihao)

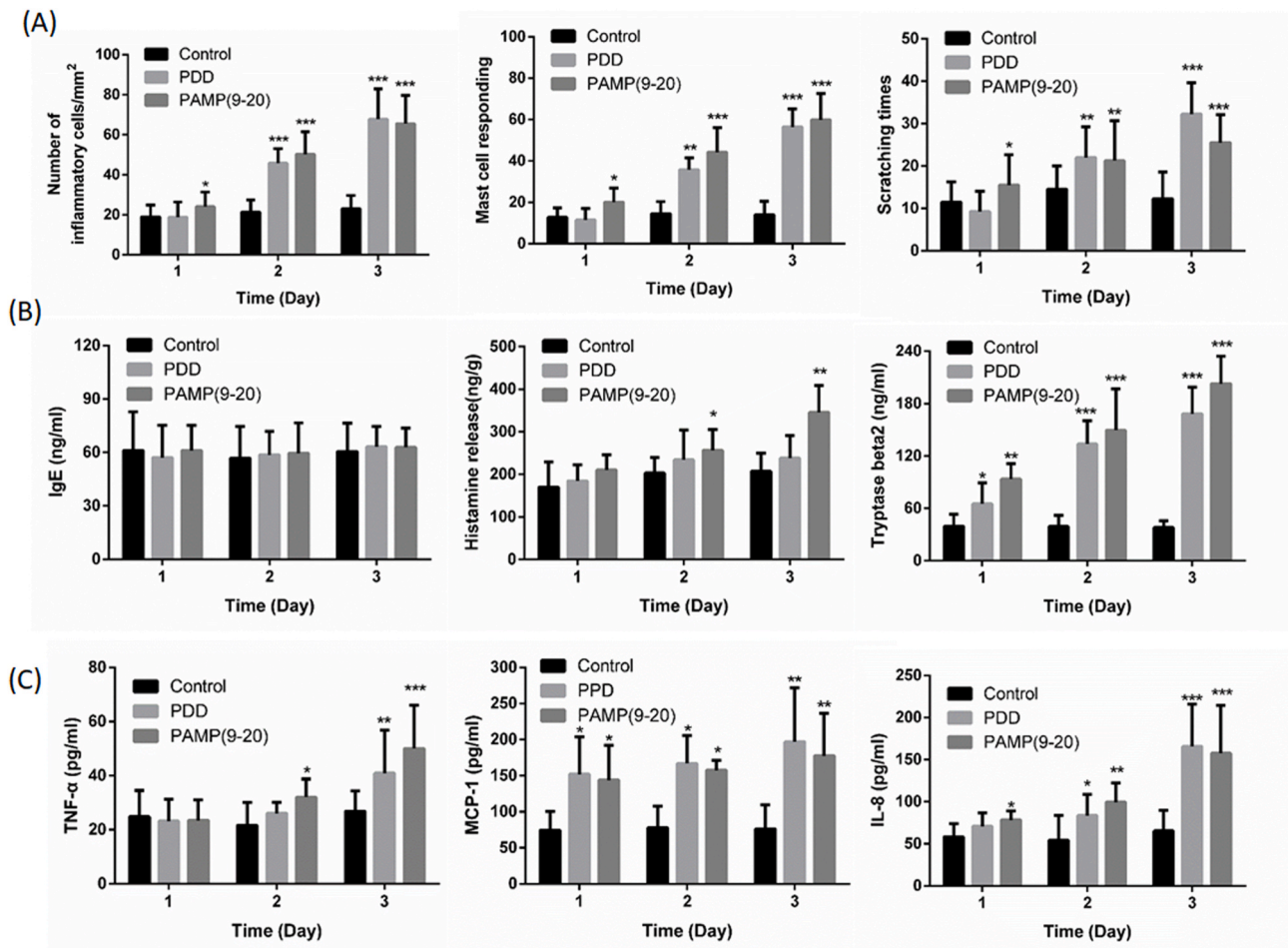


Fig. 4. PPD induced the release of inflammatory mediators with a dose time-dependent manner. (A) PPD induced inflammatory cell infiltration, mast cells activation and scratching significantly (B) PPD did not cause IgE increased in mice serum or histamine increased at the local affected site, while tryptase increased significantly. (C) PPD induced TNF- α , MCP-1, IL-8 increased in mice serum. (the data included 18 data points per group and were calibrated by Bonferroni's test. Differences were considered significant at * $p < 0.016$, ** $p < 0.0033$, and *** $p < 0.0016$).

containing Tween-20 at room temperature for 2 h. The membranes were then incubated overnight at 4 °C with the following primary antibodies: anti-PLC γ 1 (1:1000, #5690, Cell Signaling Technology [CST]), anti-phosphorylated-PLC γ 1 (P-PLC γ 1, Ser1248) (1:1000, #8713, CST), anti-phosphorylated-P38 (P-P38, Thr180/Tyr182) (1:1000, #4511, CST), anti-Akt (1:1000, #4691, CST), anti-phosphorylated-Akt (P-Akt, Ser473), (1:1000, #4060, CST), anti-Erk1/2 (1:1000, p44/42 MAPK (Erk1/2), #9102, CST), anti-phosphorylated-Erk1/2 (P-Erk1/2, P-p44/42 MAPK (Erk1/2) [Thr202/Tyr204], 1:1000, #9101, CST), and anti-GAPDH (1:2000, #2118, CST). The membranes were then washed thrice with TBST at every 10 min interval and then incubated with secondary antibodies (at a dilution of 1:20,000 in TBST) for 1 h at 37 °C. The membranes were again washed three times with TBST for 10 min and developed using an enhanced chemiluminescence (ECL) kit. A Lane 1DTM transilluminator was used to capture the images of the developed blots, and Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the protein levels.

2.11. Statistical analysis

The summary of the division of mice and cell-lines in the experiments showed in [Supplementary Tables 1 and 2](#) Experiments were repeated 3 times. Data are expressed as mean $\pm$ S.E.M. (S.E.M. was calculated by 18 mice *in vivo*) 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. For the

paired comparison samples, the treated groups were compared with the negative control group. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$. For the multiple doses samples, the treated groups compared respectively with the negative control group were calibrated by Bonferroni's test.

3. Results

3.1. Topical application of PPD triggered contact allergy

One day after treatment, histopathological samples and mouse sera were analyzed. Histopathology of the lesion showed PPD-induced inflammatory infiltration in the dermis in a dose-dependent manner. A high concentration of PPD caused a greater inflammatory cell infiltration response ([Fig. 1A](#)). Moreover, PPD significantly induced MCs activation and degranulation reactions at a high dose of avidin-FITC ([Fig. 1B](#)).

Tryptase release in the skin and sera of mice was significantly increased ([Fig. 2A](#)). The total IgE levels in both the positive and treated groups were similar to those in the negative group. Unexpectedly, PPD didn't cause an increase in histamine levels at the affected site ([Fig. 2B](#)). Scratching times were counted one day after treatment, and the results showed that the degree of itch was significant at a high dose of PPD ([Fig. 2C](#)). PPD also induced the release of TNF- α , MCP-1, and IL-8 in a dose-dependent manner ([Fig. 2D](#)).

The PPD in the patch test was in contact with the skin of patients for

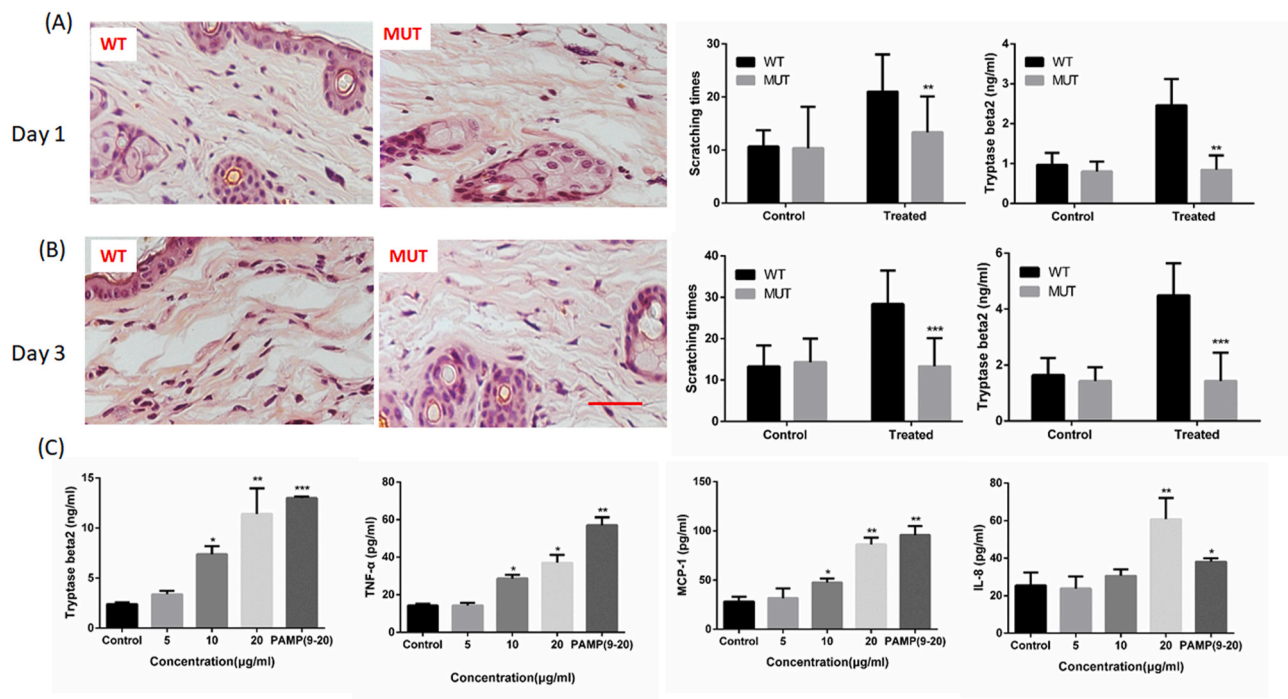


Fig. 5. *Kit^{w-sh/w-sh}* (MUT) deficient mice show resistance to PPD triggered allergy. WT and MUT mice treated with 20 $\mu\text{g/ml}$ PPD for one day and 5 $\mu\text{g/ml}$ PPD for three days. 50% glycerin was used as negative control and PAMP(9–20) was used as positive control (the data included 18 data points per group). (A) PPD induced inflammatory cells infiltration in WT mice, while showed little effect on MUT mice at a high dose, scratching times and tryptase beta in serum after treatment for one day (B) PPD induced inflammatory cells infiltration in WT mice, while showed little effect on MUT mice at a low dose (scale bars represent 20 μm), scratching times and tryptase beta in serum after treatment for 3 days (C) PPD induced mouse primary mast cells (MPMC) release tryptase, TNF- α and MCP-1 in a dose-dependent manner, while induced IL-8 release at a high dose.

two days, and the result was analyzed at the third day. To be consistent with the patch test, an assay of a mouse model treated with a small concentration of PPD (5 $\mu\text{g/mL}$) for three days was used to verify the sensitization role of PPD. On the first day after treatment, there was no visible inflammatory infiltration in the dermis or scratching. However, after treatment for two to three days, symptoms of contact allergy appeared, presenting with inflammatory cell infiltration and scratching. The severity level increased over time. In contrast, the negative control group did not present any lesions (Figs. 3A and 4A). In addition, PPD induced MCs activation and degranulation on day 3 (Figs. 3B and 4A). As the number of days of treatment increased, the level of tryptase in mouse skin and serum increased significantly (Figs. 3C and 4B). Total IgE concentration was not significantly different between the treated and negative groups (Fig. 4B). There were few significant differences in the increase in histamine levels during the three days (Fig. 4B). As the number of treatment days increased, the levels of TNF- α , MCP-1, and IL-8 in mouse serum increased significantly in a time-dependent manner (Fig. 4C).

3.2. Mast cells mediated PPD-induced contact allergy in mice

MCs are key effector cells in mice and mediate pseudo-allergic reactions [14]. *Kit^{w-sh/w-sh}* mice (MUT) have been used to verify the target of PPD-induced contact allergy [15]. Further studies confirmed that MUT mice show a slight dermatitis reaction and scratching compared to WT mice, indicating that MCs are key effector cells in PPD-induced pathological changes. PPD (20 $\mu\text{g/mL}$) was used in this study because this concentration could cause obvious dermatitis and cytokine release of mice. However, the concentration of tryptase in the sera of WT mice was significantly higher than that in the sera of MUT mice (Fig. 5A and B). Moreover, PPD induced mouse peritoneal mast cells (MPMC) to release tryptase, TNF- α , MCP-1 and IL-8 in a dose-dependent manner (Fig. 5C).

3.3. MRGPRX2 mediated PPD-induced human mast cells activation

The relationship between PPD and MRGPRX2 required further verification. Cells were identified as responding if $[\text{Ca}^{2+}]$ increased 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 [14]. PPD directly increased the intracellular Ca^{2+} concentration of the LAD2 cells *in vitro* (Fig. 6A). In addition, PPD increased the intracellular Ca^{2+} concentration in MRGPRX2-HEK293 cells (Fig. 6B) but it did not alter the intracellular Ca^{2+} concentration in NC-HEK293 cells (Fig. 6C). PPD did not cause LAD2 cells to release histamine and 5-HT, but induced a dose-dependent release of tryptase *in vitro* (Fig. 6D). PPD induced TNF- α , MCP-1, and IL-8 release in LAD2 cells (Fig. 6E), but did not induce the release of IL-4, IL-10, and IL-13 (Fig. 6F).

After knockdown of the expression of MRGPRX2 in LAD2 cells treated with the same concentration of PPD, the release of tryptase, TNF- α , MCP-1, and IL-8 by MRGPRX2-knockdown LAD2 cells was lower than that of NC-LAD2 cells (Fig. 7A), which demonstrated the possible partial involvement of MRGPRX2 in the PPD-induced activation of MCs.

The phosphorylation levels of ERK and P38, which are key proteins in the pathways downstream of MRGPRX2, were increased in a dose-dependent manner (Fig. 7B). The phosphorylation levels of PLC γ 1 and AKT showed little change after PPD treatment (Fig. 7C). However, PKC phosphorylation was downregulated (Fig. 7D).

3.4. PPD caused non-histaminergic itch

PPD induced scratching and tryptase increase in the serum of WT and *H1R^{-/-}* mice. In turn, the level of serum histamine showed no significant difference compared to the blank group. In addition, the scratching times and serum tryptase levels were similar in WT and *H1R^{-/-}* mice (Fig. 8A and B). The liquid supernatant of PPD-treated LAD2 cells did

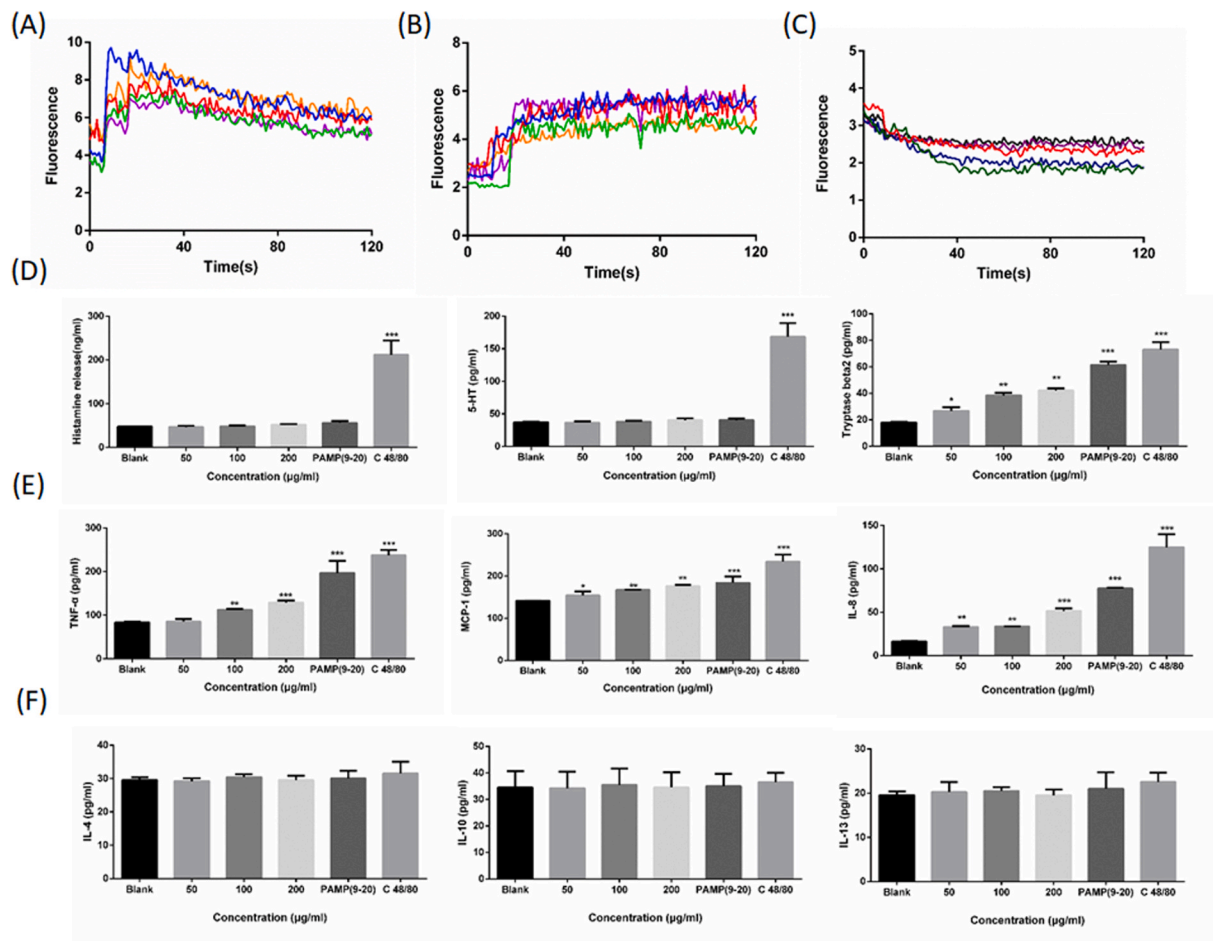


Fig. 6. PPD induced LAD2 cells activation and media release *in vitro*. (A–C) PPD increased the intracellular Ca^{2+} concentration in LAD2 cells and MRGPRX2-HEK293 cells, while did not increase the intracellular Ca^{2+} concentration in NC-HEK293 cells. (PPD and PAMP(9–20) were added at tenth second after the photos were taken. Cells were identified as responding if the $[\text{Ca}^{2+}]$ 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.) (D) PPD did not induce LAD2 cells to release histamine, or 5-HT, while induced tryptase beta2 release in a dose-dependent manner. (E) PPD induced LAD2 cells to release TNF- α , MCP-1 and IL-8 in a dose-dependent manner. (F) PPD did not induce LAD2 cells to release IL-4, IL-10, or IL-13.

not increase the intracellular Ca^{2+} concentration of H_1R -expression HEK293 cells, indicating that PPD-induced itch was histamine-independent (Fig. 8C).

4. Discussion

PPD is the most well-known ingredient of oxidative hair dye products, and the only one that is regularly patch tested in the European baseline series [22]. Higher numbers of strong and extremely strong reactions have been registered in patch tests. The clinical relevance of PPD showed high sensitization rate [23]. Patch test positivity for PPD is associated with changes in the phenotype of peripheral blood T cells [24]. PPD increased in naive cells, decreased regulatory T cell frequency and the $\text{CD4}^+/\text{CD8}^+$ ratio, and increased the expression of the skin-homing marker cutaneous lymphocyte antigen [24]. PPD-allergic patients showed an increased expression of T cell markers and Th2/Th9-associated cytokines in both skin and stimulated peripheral blood mononuclear cells; however, the preclinical presence of CD8^+ T cells has not been confirmed [25]. 4-Nitroaniline and 4,4'-azodianiline formed during the oxidation of PPD are potent sensitizers. PPD-sensitized patients react to 4-nitroaniline and 4,4'-azodianiline at concentrations equimolar to PPD [26]. Our study showed that PPD induced immediate contact allergic reactions via MRGPRB2 and caused inflammatory infiltration and scratches (Figs. 1–5).

MRGPRX2 and $\text{Fc}\epsilon\text{RI}$ are the key receptors that mediate MCs activation and induce allergic reactions. However, $\text{Fc}\epsilon\text{RI}$ - and MRGPRX2-triggered MCs activation routes are independent of each other. $\text{Fc}\epsilon\text{RI}$ -triggered degranulation is delayed and shows a compound exocytosis pattern, while MRGPRX2-mediated degranulation is rapid and associated with a quick and transient peak of intracellular calcium followed by the secretion of individual granules [27]. Moreover, the type of cytokines released was significantly different in $\text{Fc}\epsilon\text{RI}$ - and MRGPRX2-mediated allergic reactions [28]. Nevertheless, the different types of small molecule compounds that induce pseudo-allergic reactions via MRGPRX2 could also release different sensitizing media. Compound 48/80 and substance P cause MCs to release a substantial amount of histamine in a short period of time via MrgprB2 , while the PAMP(9–20)-induced MrgprB2 activation releases more tryptase and fewer monoamines [18]. Recent studies have confirmed that the MrgprB2 activation of MCs excites non-histaminergic itch sensory neurons and mediates non-histaminergic itch [18]. The results of our study indicated that PPD induced MC activation and released a mass of tryptase, which suggested that PPD-induced scratching might be non-histaminergic dependent (Figs. 1–4). MC activation can release many different cytokines. MRGPRX2 and $\text{Fc}\epsilon\text{RI}$ are the key receptors that mediate MCs activation and induce different patterns of MCs degranulation that mainly manifest with a difference in the release with respect to the sensitizing medium. TNF- α , MCP-1, and IL-8 are typical

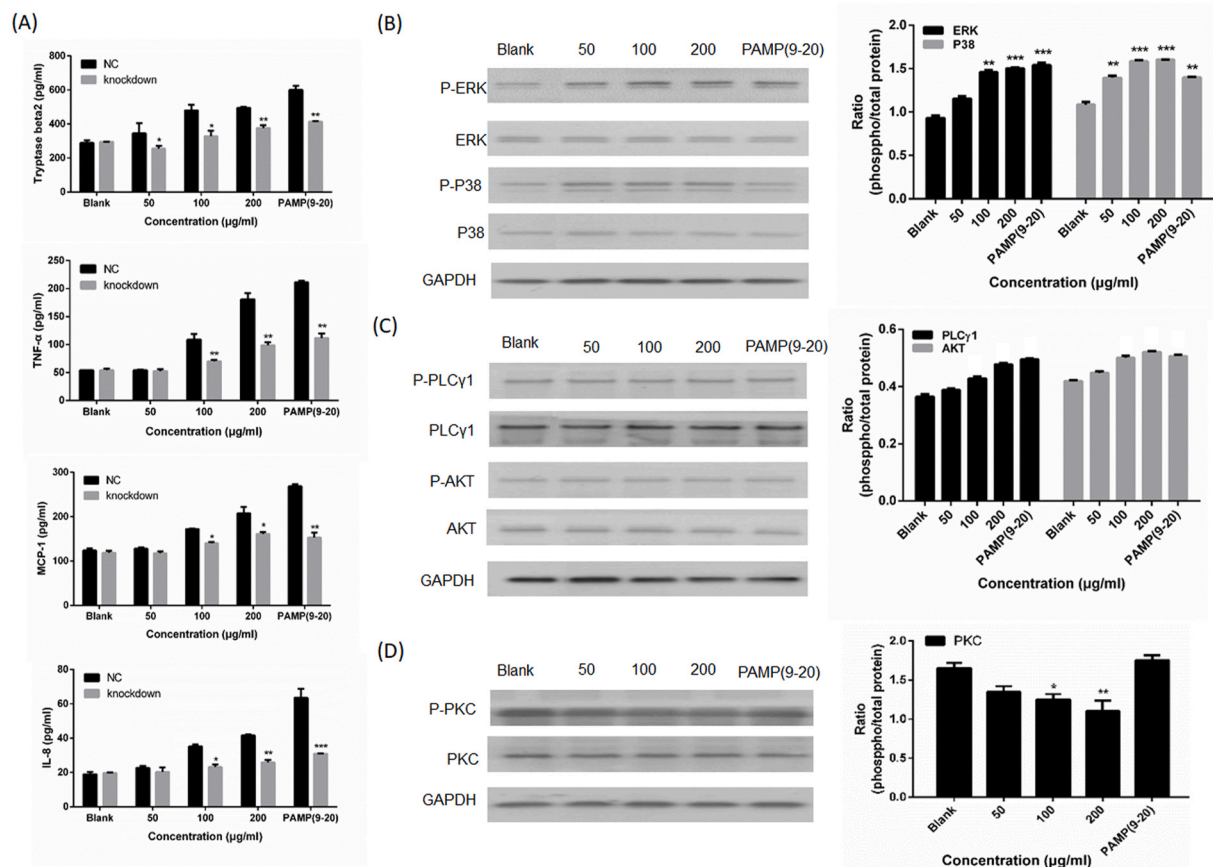


Fig. 7. PPD activated LAD2 cells via MRGPRX2 signal pathway. (A) After knockdown the expression of MRGPRX2 in LAD2 cells by siRNA, the release of tryptase, TNF- α , MCP-1 and IL-8 were lower than those of NC-LAD2 cells treated with the same concentration of PPD (B) PPD up-regulated the phosphorylation level of ERK and P38, (C) PPD showed little effect on the phosphorylation level of PLC γ 1 and AKT, (D) PPD down-regulated the phosphorylation level of PKC.

cytokines released after MRGPRX2 activation [15], while Fc ϵ R1-mediated MC activation, which has been more deeply studied, induces the release of TNF- α , MCP-1, IL-4, IL-10, IL-13, etc. We analyzed some typical cytokines released by MCs to show the different effects of PPD on MRGPRX2. However, PPD only induced TNF- α , MCP-1, and IL-8 release, but did not induce IL-4, IL-10, and IL-13 release (Fig. 6), suggesting that PPD-induced inflammation might be lower than type I anaphylaxis.

Our data showed that the phosphorylation levels of ERK and P38 were increased in a dose-dependent manner after treatment with PPD. However, PPD downregulated PKC phosphorylation. PKC plays an important role in controlling MC degranulation [29]. MCs degranulate while PKC activates and phosphorylates its substrate of serine and threonine. Activation of PKC phosphorylates membrane and cytoskeleton proteins to induce MC degranulation. Inhibition of PKC phosphorylation can effectively inhibit inflammatory responses [30]. Inactivated PKC cannot induce MCs to release histamine and leukotriene [31], which may be a reason that PPD could activate MRGPRX2 while not causing the release of histamine (Fig. 7).

Tryptases cleave proteinase-activated receptors (PARs) present on itch-sensory neurons [32]. PAR2 is preferentially expressed in 5ht1f + neurons [33], while afferent neurons mediate tryptase-induced itch. Tryptase activates PAR2 in the nerve centers of the skin, intestines, and other regions, and may be a mechanism by which mast cells promote neurogenic inflammation. Tryptase can activate sensory neurons and induce non-histaminergic itching.

MRGPRX2-mediated pseudo-allergic reactions vary from person to person, and not all individuals develop contact allergic reactions after contact with PPD. One reason is that the compounds involved in the activation of MRGPRX2 are positively correlated with the concentration

and dose rate [34]. However, pseudo-allergic reactions might vary within the body; for example, human stem cell factor can affect pseudo-allergic reactions by inhibiting degranulation of MCs [35]. However, it has been reported that naturally occurring single nucleotide polymorphisms (SNPs) in the *MRGPRX2* gene can influence the responsiveness to MRGPRX2 ligands [36]. In addition, the expression of MRGPRX2 in the skin MCs of different people showed different degrees of response to C48/80 or substance P, which has been proven to be an agonist of MRGPRX2. This could be due to differences in the expression quantities of MRGPRX2. Its content could be a key factor that can reduce the pseudo-allergic reactions induced by PPD. Inhibition of MRGPRX2-mediated MCs degranulation and cytokine release could play a significant role in preventing PPD-induced immediate contact allergy.

Moreover, tryptase can activate afferent neurons via PAR2, which directly triggers neuronal activation, thereby promoting the itch effect. Interactions between tryptase and nerve cells PAR2 in skin can trigger neuro-inflammatory responses and promote the release of inflammatory factors by nerve cells, which may be a mechanism by which tryptase promotes neurogenic inflammation. In clinical practice, analysis for monoamine and tryptase would provide a better treatment for itching caused by allergic reactions.

5. Conclusion

PPD induced immediate contact allergy by activating MCs via MRGPRX2 and led to tryptase release, which may induce non-histaminergic itch. Inhibition of MRGPRX2-mediated MCs degranulation and cytokine release could play a significant role in preventing PPD-induced immediate contact allergy.

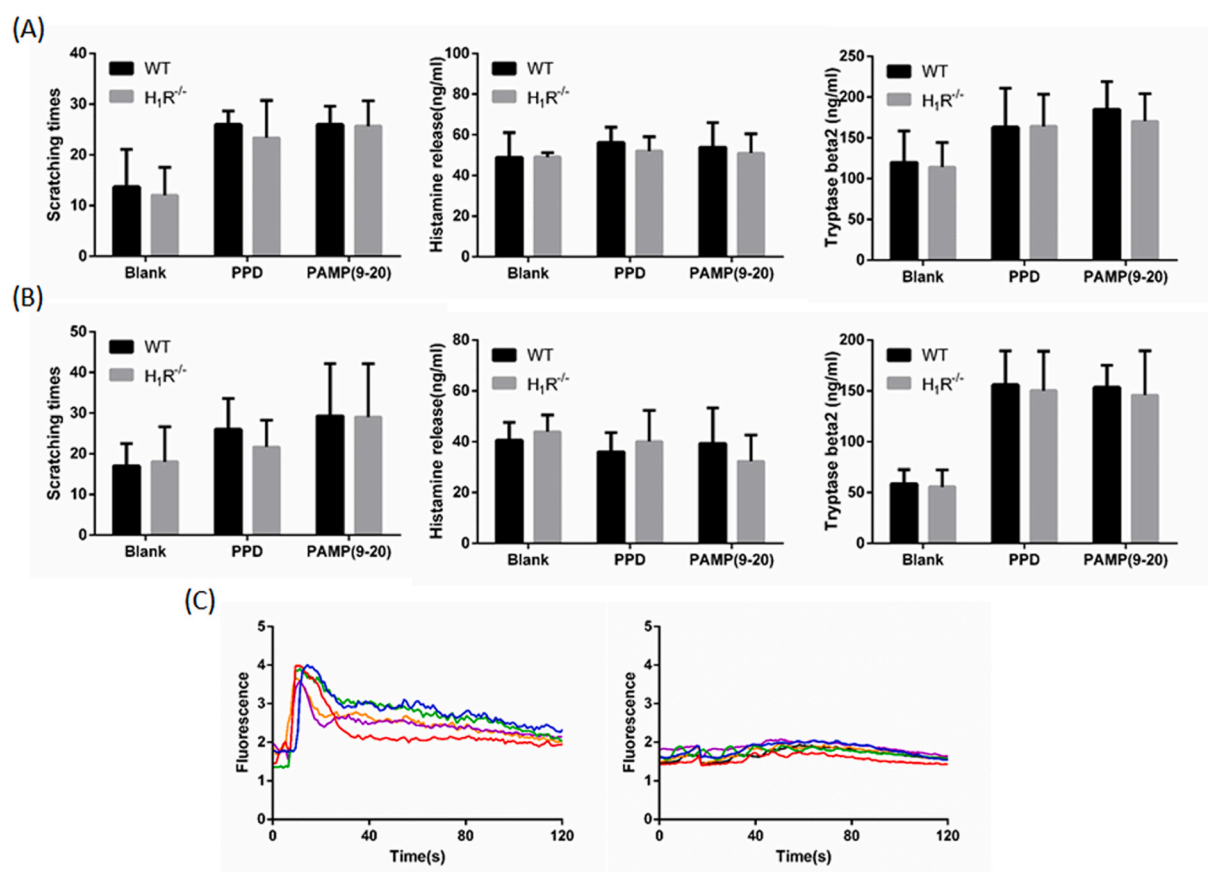


Fig. 8. Release content of PPD-treated LAD2 cells did not activate H_1R . WT and $H_1R^{-/-}$ mice treated with 20 μ g/ml PPD for one day and 5 μ g/ml PPD for three days. 50% glycerinum was used as negative control and PAMP(9–20) was used as positive control. (A–B) There was no significant difference in scratching times, histamine and tryptase release after PPD treated in WT and $H_1R^{-/-}$ mice, either for 1 day or 3 days (the data included 18 data points per group, scale bars represent 20 μ m), scratching times and tryptase beta in serum after treatment for one day (C) The liquid supernatant of PPD-treated LAD2 cells did not increased the intracellular Ca^{2+} concentration of H_1R -expression HEK293 cells, 4 μ g/ml histamine was used as positive control (left).

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Author contributions

Delu Che: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - Original Draft, Visualization. Jiapan Gao: Methodology, Resources, Writing - Review & Editing. Xueshan Du: Software, Formal analysis, Data Curation. Yi Zheng: Methodology, Investigation, Data Curation. Yajing Hou: Software, Formal analysis. Bin Peng: Software, Formal analysis. Tao Jia: Software, Formal analysis. Songmei Geng: Conceptualization, Resources, Writing - Review & Editing. Langchong He: Conceptualization, Validation, Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

The authors have no conflicts of interest to report.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbi.2021.109751>.

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Supplementary Tables

Supplementary Table 1: The summary of the division of mice [16, 19, 21]

experimental objective	Experiment content	Strain	Repetition in an experiment
Topical application of PPD triggers contact allergy for 24 h	1. H&E staining 2. Immunofluorescent staining for avidin-FITC 3. immunohistochemical staining for tryptase 4. Serum index analysis by ELISA	C57 BL/6 mice	Six mice per group, totally 90 mice
Topical application of PPD triggers contact allergy for 72 h	1. H&E staining 2. Immunofluorescent staining for avidin-FITC 3. immunohistochemical staining for tryptase 4. Serum index analysis by ELISA	C57 BL/6 mice	Six mice per group, totally 108 mice
Analysis of mast cell function in PPD-induced allergy	1. H&E staining 2. Serum index analysis by ELISA	1. C57 BL/6 mice 2. Kit ^{w-sh/w-sh} mice (C57 BL/6 genetic background)	Six mice per group, totally 72 mice
Peritoneal mast cell purification (MPMC)	Media release analysis by ELISA	C57 BL/6 mice	10 mice for one time, totally 30 mice
PPD-induced non-histamine itch	Serum index analysis by ELISA	1. C57 BL/6 mice 2. H ₁ ^{-/-} mice (C57 BL/6 genetic background)	Six mice per group, totally 108 mice

Supplementary Table 2: The summary of the division of cell-lines [16, 19, 21]

experimental objective	Experiment content	Strain	Repetition in an experiment
PPD induced media release in vitro	Media release analysis by ELISA	Laboratory of Allergic Disease 2 (LAD2)	Five well per group in 96-well plates
PPD induced cells activation in vitro	Intracellular calcium ion mobilization assay	1. Laboratory of Allergic Disease 2 (LAD2), 2. MRGPRX2-HEK293 cells 3. NC-HEK293 cells	Five well per group in 96-well plates
PPD activated MCs via MRGPRX2	1. siRNA transfection 2. Media release analysis by ELISA	Laboratory of Allergic Disease 2 (LAD2)	3 well per group in 6-well plates for siRNA transfection, and Five well per group in 96-well plates
PPD activated MRGPRX2 signaling pathway	Western Blotting	Laboratory of Allergic Disease 2 (LAD2),	3 well per group in 6-well plates
PPD-treated LAD2 cells did not activate H1R	Intracellular calcium ion mobilization assay	1. H1R -HEK293 cells 2. NC-HEK293 cells	Five well per group in 96-well plates