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Quercetin inhibits Mrgprx2-induced pseudo-allergic reaction *via* PLCγ-IP3R related Ca^{2+} fluctuations

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ABSTRACT

An allergic reaction is a potentially fatal hypersensitivity response caused by mast cell activation, particularly histamine and lipid mediators. Histamine release caused by reaction to drugs is considered a pseudo-allergic reaction. Quercetin is known for its anti-allergic immune effect. However, at present, its anti-pseudo-allergic effect and its mechanism are less investigated. Therefore, the purpose of this study was to evaluate the anti-pseudo-allergic effect of Quercetin *in vivo* and to explore the mechanism *in vitro*. The anti-pseudo-allergic activity of Quercetin was evaluated *in vivo* using a mouse model, while Quercetin mechanism of action was examined *in vitro* using HEK293 cells expressing Mrgprx2, a mast cell specific receptor, and LAD2 mast cell line. Our *in vivo* results showed that Quercetin could attenuate Evans blue leakage in the paws and hind paw thickness in C57BL/6 mice in a dose-dependent manner, and could significantly inhibit serum histamine and chemokines release. In addition, it suppressed calcium mobilization and attenuated the release of histamine and MCP-1 in peritoneal mast cells in a dose-dependent manner. Furthermore, it inhibited the vasodilation due to histamine, the release of eosinophils, and the percentage of degranulated mast cells, indicating that Quercetin antagonized mast cell mediators *in vivo*, histamine-induced vasodilation and eosinophil release.

In vitro results showed that Quercetin reduced pseudo-allergic induced calcium influx, suppressed degranulation and chemokines release in a similar way as dexamethasone (100 μM) (mast cell stabilizer) in LAD2 mast cell line. In addition, Quercetin inhibited Mrgprx2-induced both calcium influx and pseudo-allergic reaction in HEK293 cells expressing Mrgprx2. C48/80, a histamine promoter, and Substance P (a neuropeptide) EC_{50} was higher when combined with Quercetin compared to the EC_{50} of these compounds alone, suggesting that Quercetin could inhibit Mrgprx2-induced pseudo-allergic reaction. Furthermore, Quercetin decreased PLCγ-IP3R signaling pathway activation induced by C48/80 in LAD2 mast cell line. In Mrgprx2 knockdown LAD2 cells, the effect of Quercetin (200 μM) reduced C48/80 induced calcium flux and the release of β-hexosaminidase, histamine, MCP-1 and IL-8 compared with non-atopic control (NC) transfected LAD2 human mast cells, suggesting that Quercetin anti-pseudo-allergic effect was related to Mrgprx2. The docking results showed that Quercetin had a good binding affinity with Mrgprx2 similar to the one of Substance P and C48/80. Therefore, Quercetin inhibited Mrgprx2-induced pseudo-allergic reaction *via* PLCγ-IP3R associated Ca^{2+} fluctuations.

Our results validated Quercetin as an effective small molecule inhibiting Mrgprx2-induced pseudo-allergic reaction *via* PLCγ-IP3R associated Ca^{2+} fluctuations, thus highlighting a potential candidate to suppress Mrgprx2 induced pseudo-allergic related diseases.

1. Introduction

In the high-affinity IgE receptor (FcεRI)-independent pathway, mast cells can be activated by many stimuli, including basic secretagogues (such as C48/80 and Substance P), THIQ primitive drugs (such as atracurium), peptidergic drugs (such as icatibant) and fluoroquinolones (such as ciprofloxacin). Recent studies showed that they are all ligands for Mrgprx2, which is an ortholog of the human mas-related G protein-coupled receptor x2 [1,2]. Human skin has rich Substance P binding sites on the surface of Langerhans cells, and when activated, they

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inhibit the antigen-presenting process [3]. Substance P is associated with various skin and systemic pruritus such as atopic dermatitis [4,5] and cholestasis [6]. For chronic urticaria (CU) patients, the pulmonary response to intradermally injected neuropeptides (such as Substance P and vasoactive intestinal peptide) is significantly greater and lasts longer than non-atopic control (NC) subjects. Mrgprx2 induces Ca^{2+} mobilization and mast cells degranulation by activating the phospholipase C (PLC) signaling pathway [7].

So far, no cure for allergic diseases is available. Some medicines such as anti-histamines, immunosuppressants and mast cells stabilizers can only help relieving allergic symptoms and reducing the pain of allergic reactions; furthermore, these medicines have some side effects [8].

Quercetin is a biological brass compound. Dietary foods such as tea, apples, onions, apples and grapes, contain bioflavonoids and Quercetin is the main one in our diet [9,10]. Quercetin has antitumor [11–13], antioxidant [14–17], anti-radiation [18], and antiviral [19,20] effect, and anti-cardiovascular diseases activity. In addition, it improves capillary microcirculation and cerebral blood circulation and inhibits platelet aggregation [21]. Thanks to these properties, it has been used to treat several diseases including cerebral infarction, coronary heart disease and cerebral thrombosis. However, no studies are available on the effect of Quercetin against pseudo-allergic reactions.

Therefore, the present study aimed at determining the anti-pseudo-allergic effect of Quercetin *in vivo*, and the associated mechanism was eventually studied *in vitro*. For our *in vitro* experiments, LAD2 cells and HEK293 expressing Mrgprx2 were used. In our *in vivo* experiments, a mouse model was used to test C48/80 or Substance P-induced pseudo-allergic effect. Our results highlighted the therapeutic potential of Quercetin against mast cells-associated pseudo-allergic diseases and other Mrgprx2 related diseases.

2. Materials and methods

2.1. Drugs and reagents

Quercetin was purchased from Dalianmeilun (Dalian, China) and purified to ≥98%; C48/80 and Substance P were purchased from Sigma-Aldrich Co., LLC. (Shanghai, China); Pluronic F-127, Fluo-3 and AM ester were purchased from Biotium; *p*-nitrophenyl *N*-acetyl-β-D-glucosamide and triton X-100 were purchased from Sigma-Aldrich Co., LLC. (Shanghai, China). mk-459 Millipore Milli-Q Plus ultra-water system was used to obtain ultrapure water used in all water solutions.

2.2. Cell lines and C57BL/6 mice

Human mast cell Laboratory Allergic Disease 2 (LAD2) cell line was provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). LAD2 cells were routinely grown in StemPro-34 medium supplemented with 10 ml/l StemPro nutritional supplements, penicillin (1:100), streptomycin (1:100), 2 mmol/l glutamine and 100 ng/ml human stem cell factor and incubated at 37 °C in an atmosphere containing 5% CO_2. HEK293 expressing Mrgprx2 cells (HEK293-Mrgprx2) were kindly provided by Professor Xinzhong Dong of the Johns Hopkins university, Baltimore, Maryland, USA, and routinely cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U), and streptomycin (100 U) (HyClone, UT). C57BL/6 adult male mice, 7–8 weeks old, were purchased from the experimental animal center of Xi'an Jiaotong University (Xi'an, China). Mice were housed under a 24-hour light/dark cycle, with food and water *ad libitum*.

2.3. Ethical considerations

This study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols for the mouse experiments were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). All animal procedures were performed under chloral hydrate anesthesia.

2.4. Cytotoxicity assay

Cell viability was determined using Cell Counting Kit-8 (CCK-8, Abbkine). LAD2 cells were seeded into 96-well plates (5×10^3 cells/well, 90 μl per well) and treated with 10 μl Quercetin at various concentrations (0, 50, 100, 200 and 400 μM) for 24 h. Next, cells were treated with Cell Counting Kit-8 solution (10 μl) for 2 h. The relative cell viability was measured using a microplate reader (Bio-Rad, Carlsbad, CA, USA) at an absorption of 450 nm. Cell growth inhibition after Quercetin treatment at various concentrations was calculated using the Prism software.

2.5. Intracellular Ca^{2+} mobilization assay

LAD2 or HEK293-Mrgprx2 cells were placed on an EP tube and centrifuged at 1000 rpm for 5 min. The medium was discarded, cells were resuspended in calcium imaging buffer (CIB, prepared in advance) and centrifuged at 1000 rpm and 25 °C for 5 min. The supernatant was discarded and cells were resuspended in incubation buffer (0.5 μl Fluo-3, 2 μl Pluronic F-127 and 997.5 μl CIB) containing Quercetin (0, 50, 100 and 200 μM) and placed in the incubator for 30 min in the dark. Cells were centrifuged at 1000 rpm and 25 °C for 5 min, the supernatant was discarded, cells were re-suspend in CIB, and then placed into a 96-well plate, 100 μl per well. Cells were treated with 100 μl C48/80 (30 μg/ml) or Substance P (4 μg/ml) and, after standing for several minutes, pictures of the cells were taken in blue light under the fluorescence microscope at the following conditions: 1 s exposure time, one shot per second, and a photographing time of 2 min.

2.6. Western blot analysis

LAD2 cells were incubated with medium without any treatment (control) or Quercetin at different concentrations (0, 50, 100 and 200 μM) combined with C48/80 (30 μg/ml) or Quercetin alone at the same mentioned concentrations for 24 h. After incubation, cells were washed twice with cold PBS and lysed in lysis buffer. Proteins were extracted using a Total Protein Extraction Kit. The lysates were centrifuged at 12,000 rpm and 4 °C for 20 min. A BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA) was used to quantify the protein concentration. The protein mix was immunoblotted with the following rabbit anti-human antibodies: anti-PLCγ1 (1:1000, CST), anti-phosphorylated-PLCγ1 (1:1000, CST), anti-IP3R (1:1000, CST), anti-phosphorylated-IP3R (1:1000, CST), anti-ERK1/2 (1:1000, CST), anti-phosphorylated-ERK1/2 (1:1000, CST) or anti-GAPDH (1:1000, CST). Enhanced chemiluminescence was used to visualize proteins and GAPDH was used as a reference for the relative protein level.

2.7. β-Hexosaminidase assay

LAD2 cell suspension was diluted to a final concentration of 5×10^5 cells/ml, and 100 μl per well were seeded in a 96-well plate (the final amount of cells per well was 5×10^4 cells). This plate was placed in a 37 °C saturated humidity cell incubator and incubated overnight. The next day, the plate was centrifuged for 5 min at 25 °C. As regard cells subjected to treatments, the medium was removed from each well, 50 μl TM buffer containing Quercetin at different concentrations (0, 50, 100 and 200 μM) was added and cells were incubated at 37 °C for 30 min. Next, 50 μl TM buffer containing C48/80 (30 μg/ml) or Substance P (4 μg/ml) was added to each well and cells were incubated at 37 °C for additional 30 min. The plate was spun down

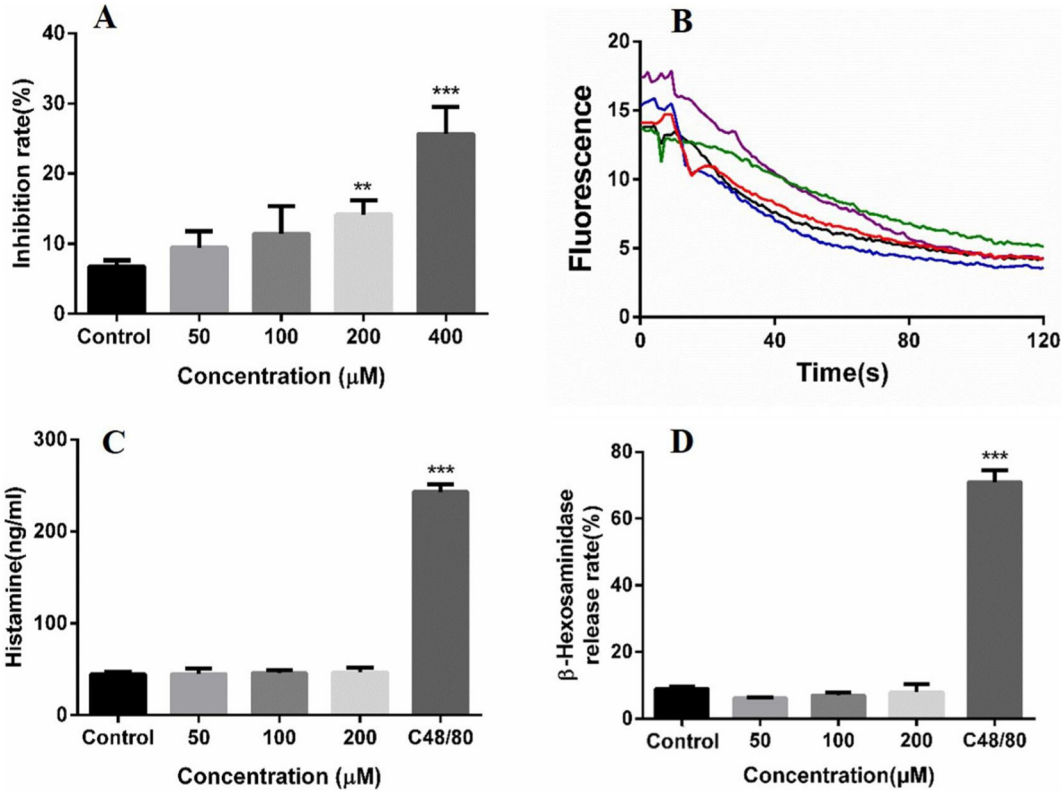

Fig. 1. Effect of Quercetin on human mast cells.
(A) The cytotoxicity in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM, 400 μM Quercetin. (B) Representative imaging traces Ca^{2+} concentrations treated by 200 μM Quercetin in LAD2 cells. (C) The histamine release triggered in LAD2 cells treated with 0, 50 μM, 100 mM, 200 μM Quercetin or 30 μg/ml C48/80 for 30 min. (D) The β-hexosaminidase release triggered in LAD2 cells treated with 0, 50 μM, 100 mM, 200 μM Quercetin or 30 μg/ml C48/80 for 30 min. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

for 5 min, and the supernatant was collected. As regard the blank cells without any treatment, the supernatant was collected and cells were further lysed with 0.1% Triton X-100 for 5 min. The lysate was evenly blown and centrifuged for 5 min at 25 °C to obtain a blank cell lysate. Finally, 50 μl of the supernatant of the treated cells and of the blank cells, and the lysate of the blank cells were added to a new 96-well plate, and 50 μl β-aminohexose (1 μg/l) were added to each well. The 96-well plate was incubated at 37 °C for 90 min. After incubation, 150 μl Na_2CO_3/$NaHCO_3$ (0.1 mol/l) Stop Solution were added to each well to stop the reaction. The 96-well plate was shaken for 2 min at room temperature and absorbance (OD) was measured at 405 nm on a microplate reader.

2.8. Histamine release assay

The cell suspension was diluted to a final concentration of 5×10^5 cells/ml, and 100 μl per well were seeded in a 96-well plate and incubated overnight. The next day the plate was centrifuged for 5 min at 25 °C. As regard the treated cells, the medium was removed from each well and 100 μl TM buffer containing Quercetin at different concentrations (0, 50, 100 and 200 μM) or C48/80 at a final concentration of 30 μg/ml was added. After incubation at 37 °C for 30 min, the plate was centrifuged for 5 min at 25 °C, and the supernatant was collected. Finally, 50 μl of the supernatant of the treated cells and of the blank cells were separately added to two 1.5 ml EP tubes. Hundred μl histamine label were added to each tube and after shaking, the mixture was centrifuged at 12,000 rpm and 4 °C for 10 min before LC-MS detection and analysis.

2.9. Chemokine release assay

LAD2 cells were seeded in 96-well plates (5×10^5 cells/ml per well) and incubated overnight at 37 °C under 5% CO_2. Next, the culture medium was removed, 150 μl Quercetin at different concentrations (0, 50, 100 and 200 μM) and C48/80 (30 μg/ml) or Substance P (4 μg/ml) were added, and cells were incubated at 37 °C and 5% CO_2 at least for 6 h and then 100 μl supernatant was collected. The supernatant was used to measure MCP-1 and IL-8 according to the following kits: Human Chemokine Array Kit, purchased from R&D Systems China Co., Ltd. (Shanghai, China), used for their measurement in the cell supernatant mentioned above, and mouse ELISA Kits, purchased from TIANGEN BIOTECH (BEIJING) CO., LTD (Beijing, China), used for their measurement in the mouse serum. All steps were performed according to the instructions.

2.10. Hind paw swelling and Evans blue extravasation

Mice were randomly divided into 5 groups (n = 5 each group; the experiment was repeated 3 times). Adult male mice were anesthetized using 0.2 ml chloral hydrate 0.35%. After induction of anesthesia for 15 min, 0.2 ml Evans blue saline 0.4% containing Quercetin at different concentrations (0, 1.0, 2.0 and 4.0 mg/ml) were intravenously injected (i.v.) on each mouse front paw. A vernier caliper was used to measure the thickness of the front paw subjected to injection. At 30 min after injection, 5 μl C48/80 (30 μg/ml) or Substance P (4 μg/ml) was administered using a microsyringe in the hind left paw and 5 μl saline was administered as a negative control in the hind right paw. After 15 min, the paw thickness was measured again and recorded. Mice were sacrificed using a dagger and a picture of each paw was taken. The paw

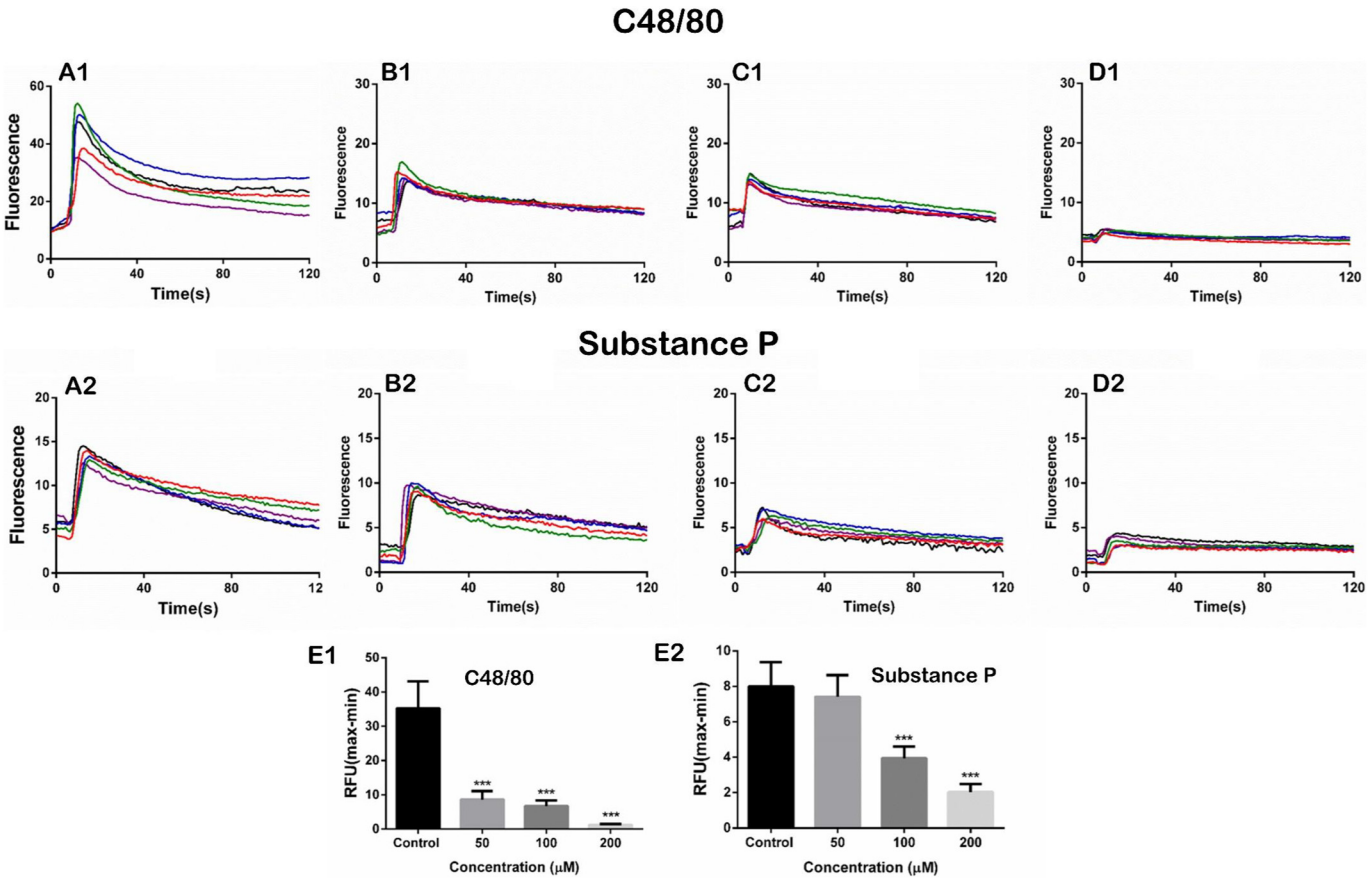

Fig. 2. Quercetin attenuated pseudo-allergic-triggered calcium flux in LAD2 cells.
(A1) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 in LAD2 cells. (B1) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 50 μM Quercetin in LAD2 cells. (C1) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 100 μM Quercetin in LAD2 cells. (D1) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 200 μM Quercetin in LAD2 cells. (E1) Quantification of responding cells of C48/80. Scale bar, 10 mm. (A2) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P in LAD2 cells. (B2) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 50 μM Quercetin in LAD2 cells. (C2) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 100 μM Quercetin in LAD2 cells. (D2) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 200 μM Quercetin in LAD2 cells. (E2) Quantification of responding cells of Substance P. Scale bar, 10 mm. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

tissue was collected, dried at 50 °C for 24 h, and weighed. The tissue was stored in 500 μl acetone:saline (7:3), sectioned in an ultrasonicator for 30 min and centrifuged at 12,000 rpm and 4 °C for 20 min. Supernatants were divided into 200 μl aliquots, placed into 96-well cell culture plates and the OD was read at 620 nm using a spectrophotometer to detect Evans blue content. For studies using Quercetin, 5 μl, C48/80 (30 μg/ml) or Substance P (4 μg/ml) were injected on mice as a positive control.

2.11. Peritoneal mast cell purification

Adult male and female mice 7–8 weeks of age were sacrificed through CO_2 inhalation. A total of 12 ml of ice-cold mast cell dissociation media (MCDM; HBSS with 3% fetal bovine serum and 1 mM HEPES, pH 7.2) were used to make two sequential peritoneal lavages and cells were spun down for 5 min at 200*g* and 4 °C. The pellet from each mouse was resuspended in 2 ml MCDM, layered over 4 ml of an isotonic 70% Percoll suspension (2.8 ml Percoll, 310 μl 10× HBSS, 40 μl 1 M HEPES, 850 μl MCDM), and spun down for 20 min at 500*g* and 4 °C. Mast cells were recovered in the pellet. According to our experience, 800 μl CIB solution or TM solution was added to the precipitated mouse cells for calcium flow and release.

2.12. Histological analysis

Mice were randomly divided into 5 groups (n = 5 each group; the experiment was repeated 3 times). Anesthetized mice were treated with 5 μl test drug or 30 μg/ml C48/80 in the left paw and 5 μl saline in the right paw; saline and C48/80 were used as blank and negative control, respectively. Fifteen minutes later, the paw skin injection site was excised, washed with PBS, fixed with 4% formaldehyde for 48 h and subjected to toluidine blue staining and HE staining. Images were immediately captured using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.13. siRNA transfection of LAD2 cells

Specific knockdown was achieved using either small interfering RNAs (siRNAs) targeting Mrgprx2 or a negative control siRNA. A smart pool of double-stranded siRNAs targeting Mrgprx2 and non-specific siRNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: Negative Control siRNA, forward, 50-UUCUCCGAACGUGUCACGUTT-30, and reverse, 50-ACG UGACACGUUCGGAGAATT-30; MRGPRX2 knockdown siRNA, forward, 50-GUACAACAGUGAAUGGAAATT-30, and reverse, 50-UUUCCAUUC ACUGUUGUACTT-30. siRNA was transfected at a final concentration of

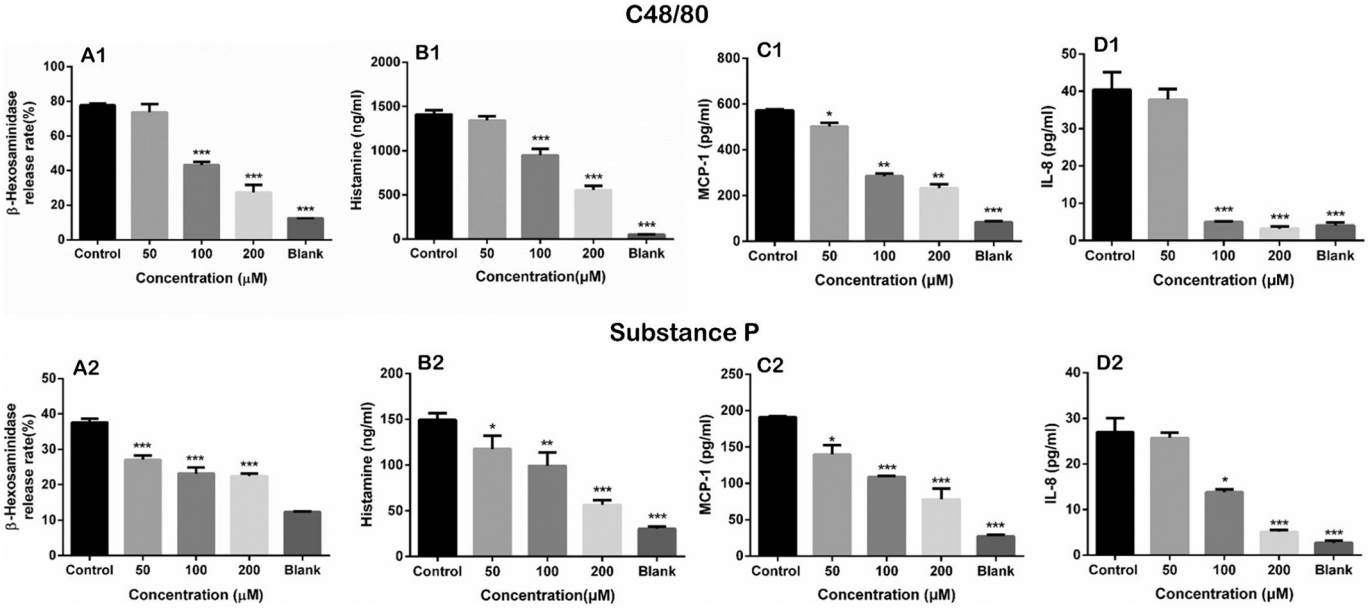

Fig. 3. Quercetin decreased pseudo-allergic-triggered degranulation and chemokines released in LAD2 cells.
(A1) The β-hexosaminidase release triggered in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (B1) The histamine release triggered in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (C1) The MCP-1 release in LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. (D1) The IL-8 release in LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. (A2) The β-hexosaminidase release triggered in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P for 30 min. (B2) The histamine release triggered in LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P for 30 min. (C2) The MCP-1 release in LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P. (D2) The IL-8 release in LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P. Chemokine secretion decreased in LAD2 cells were detected with an ELISA array. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

1 μM using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Cells were incubated for 48 h to allow Mrgprx2 knockdown.

2.14. EC_{50} assay

A 96-well plate with a black rim and transparent bottom was used and 2×10^4 HEK293-Mrgprx2 cells in 100 μl were seeded in each well and incubated overnight. A total amount of 100 μl dye (Calcium 5 Assay Kit, Molecular Devices, USA) was added into each well and cells were incubated at 37 °C in the dark for 60 min. Two solutions, such asC48/80 (30 μg/ml)/Substance P (4 μg/ml) diluted in 1 × HBSS + 20 mm HEPES buffer and Quercetin (200 μM) combined with C48/80 (30 μg/ml)/ Substance P (4 μg/ml), were placed into a clear 96 orifice plate. FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, USA) was used to measure fluorescence intensity. EC_{50} was calculated using Prism 7 software.

2.15. Molecular docking assay

Surflex-DockMode of the SYBYL-X 2.0 program package (Tripos, St. Louis, MO, USA) was used to evaluate molecular docking. Mrgprx2 structure was predicted using I-TASSER, a server to predict protein structure and function. The predicted Mrgprx2 structure was used to dock with Quercetin, Substance P and C48/80.

3. Results

3.1. Cytotoxicity of Quercetin on human mast cells

The effect of Quercetin on the viability of LAD2 cells was assessed using the CCK-8 assay. Quercetin did not significantly affect cell viability even at the highest concentration used of 400 μM (Fig. 1A). Therefore, the concentrations of Quercetin below 400 μM were used for subsequent experiments. In addition, Quercetin did not have any effect on calcium flux (Fig. 1B), histamine (Fig. 1C) and β-hexosidase release (Fig. 1D). Thus, Quercetin did not have any activating effect on LAD2 cells.

3.2. Quercetin attenuated pseudo-allergic-mediated calcium flux in LAD2 cells

Ca^{2+} influx has a prominent influence on histamine release and mast cell degranulation. C48/80 can mobilize calcium stores in mast cells. Substance P can activate mast cells, causing intracellular Ca^{2+} mobilization and cell degranulation, releasing histamine [22]. In our study, Quercetin reduced C48/80 (Fig. 2A1–E1) or Substance P (Fig. 2A2–E2) induced calcium flux in LAD2 human mast cells in a dose-dependent manner (0, 50, 100 and 200 μM). In addition, the effect of Quercetin (100 μM) that reduced C48/80 and induced calcium flux was similar to the effect obtained with dexamethasone (100 μM) (mast cell stabilizer) in the same mast cells (Fig. 13A, B). Therefore, Quercetin could reduce pseudo-allergic-triggered Ca^{2+} fluctuations.

3.3. Quercetin effect on pseudo-allergic-mediated degranulation and chemokines released in LAD2 cells

Mast cells activation can lead to the rapid secretion of pre-formed inflammatory mediators such as β-hexosaminidase, histamine and synthetic cytokines (including MCP-1 and IL-8), which are essential for allergic inflammation [23]. To verify whether Quercetin can suppress C48/80 or Substance P-induced mast cells activation by promoting degranulation, LAD2 cells were used to detect changes in β-hexosaminidase and histamine, MCP-1 and IL-8. Quercetin could inhibit the secretion of β-hexosaminidase (Fig. 3A1, A2) and histamine (Fig. 3B1, B2) due to C48/80 or Substance P treatment, respectively.

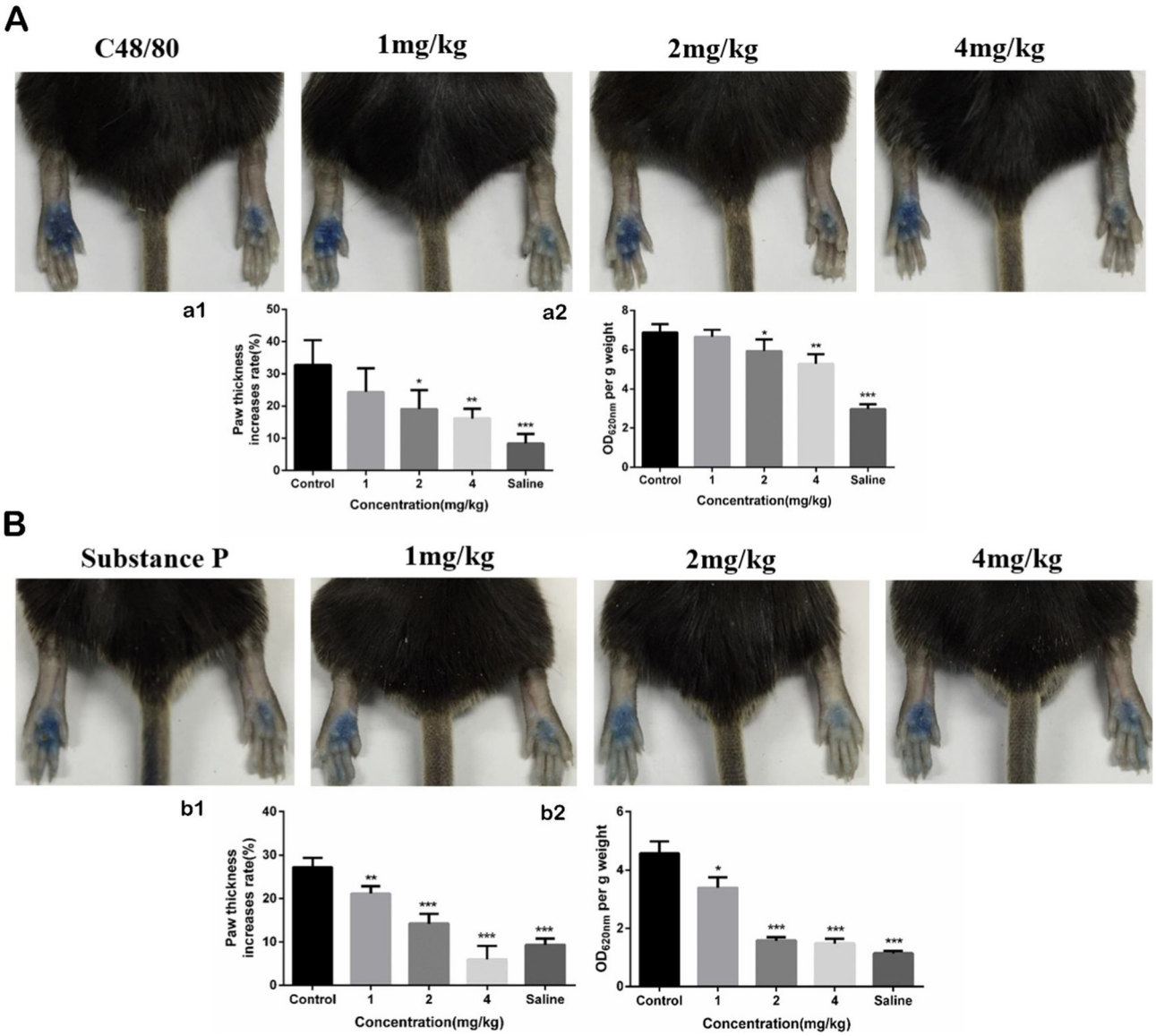

Fig. 4. *In vivo* assessment of the anti-anaphylactoid effect of Quercetin induced cutaneous flare in mice.
(A) Cutaneous flare reactions of mice treated with different concentrations of Quercetin. Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 1 mg/ml, 2 mg/ml, 4 mg/ml Quercetin combined with 30 μg/ml C48/80 in the left paw, or saline in the right paw. (a1) Quantification of paw thickness increase after 15 min. (a2) Quantification of Evans blue leakage into the paw after 15 min. (B) Cutaneous flare reactions of mice treated with different concentrations of Quercetin. Representative images of Evans blue stained extravasation 15 min after intraplantar injection of 1 mg/ml, 2 mg/ml, 4 mg/ml Quercetin combined with 4 μg/ml Substance P in the left paw, or saline in the right paw. (b1) Quantification of paw thickness increase after 15 min. (b2) Quantification of Evans blue leakage into the paw after 15 min. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

Compared to C48/80 or Substance P alone, Quercetin inhibited MCP-1 (Fig. 3C1, C2) and IL-8 (Fig. 3D1, D2) secretion, respectively. In addition, the effect of Quercetin (100 μM) that reduced the release of β-hexosaminidase, histamine, MCP-1 and IL-8 was similar to the effect obtained with dexamethasone (100 μM) (mast cell stabilizer) in in the same mast cells (Fig. 12C, D). Therefore, Quercetin attenuated the release of β-hexosaminidase, histamine, MCP-1 and IL-8 in LAD2 cells treated with C48/80 or Substance P, indicating that Quercetin could inhibit pseudo-allergic-induced mast cell degranulation and release of chemokines.

3.4. *In vivo* assessment of the anti-pseudo-allergic effect of Quercetin

To validate whether Quercetin could inhibit pseudo-allergic reactions *in vivo*, C57BL/6 mice were used to evaluate the effect of Quercetin on paw edema. Administration of Quercetin at 0, 1.0, 2.0 and 4.0 mg/ml resulted in a dose-dependent decrease in the thickness of the hind paw (Fig. 4a1, b1) induced by C48/80 or Substance P. Moreover, Quercetin could attenuate the Evans blue extravasation (Fig. 4a2, b2) in a dose-dependent manner. Furthermore, mice serum histamine (Fig. 5A1, A2), MCP-1 (Fig. 5B1, B2), and IL-8 (Fig. 5C1, C2) release showed that Quercetin could significantly inhibit the degranulation induced by C48/80 or Substance P. HE staining of the paw skin showed that Quercetin inhibited the vasodilatation caused by histamine and the release of eosinophils was reduced. Next, the relevant paw skin sections were stained with toluidine blue and the results showed a significant decrease in the percentage of degranulated mast cells after Quercetin treatment, indicating that Quercetin antagonized mast cell mediators *in vivo* (Fig. 14). Furthermore, Quercetin reduced C48/80 induced calcium flux in peritoneal mast cells in a dose-dependent manner (0, 50, 100

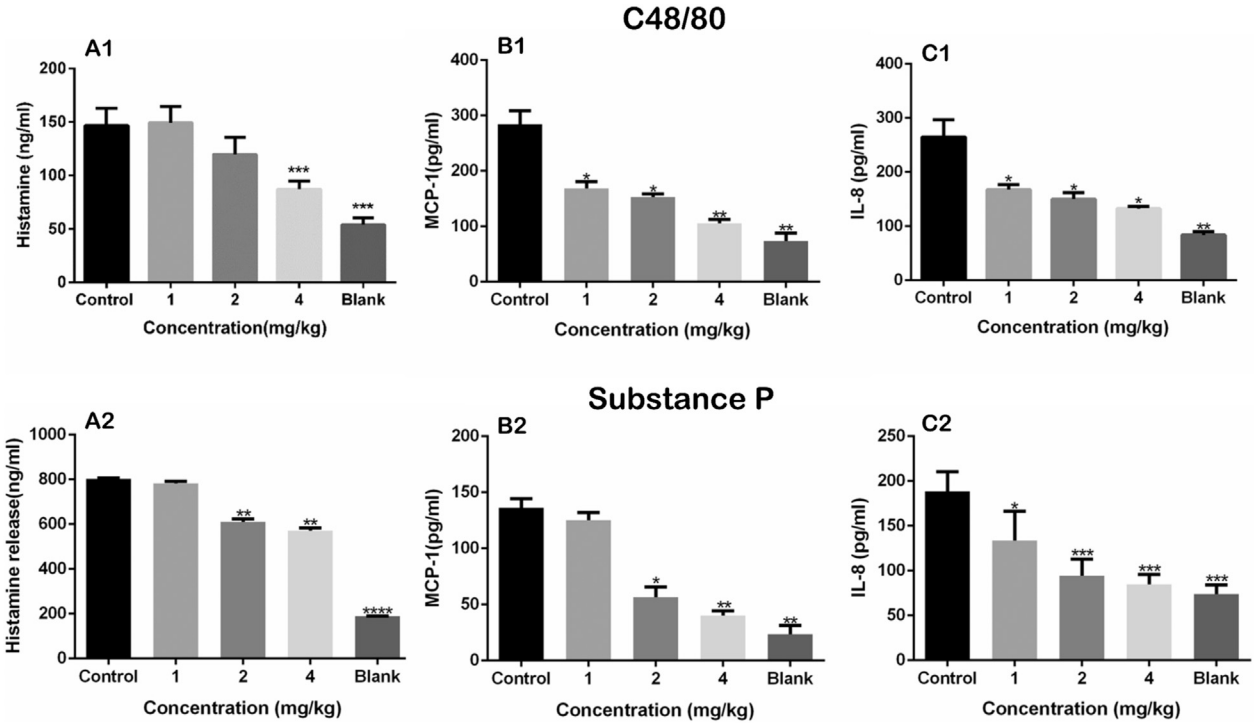

Fig. 5. Quercetin decreased C48/80 or Substance P-triggered degranulation and chemokines released in C57 mice.
(A1) The histamine release triggered in mice treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (B1) The MCP-1 release in mice were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. (C1) The IL-8 release in mice were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. (A2) The histamine release triggered in mice treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P for 30 min. (B2) The MCP-1 release in mice were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P. (C2)The IL-8 release in mice were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 4 μg/ml Substance P. Chemokine secretion decreased in mice were detected with an ELISA array. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

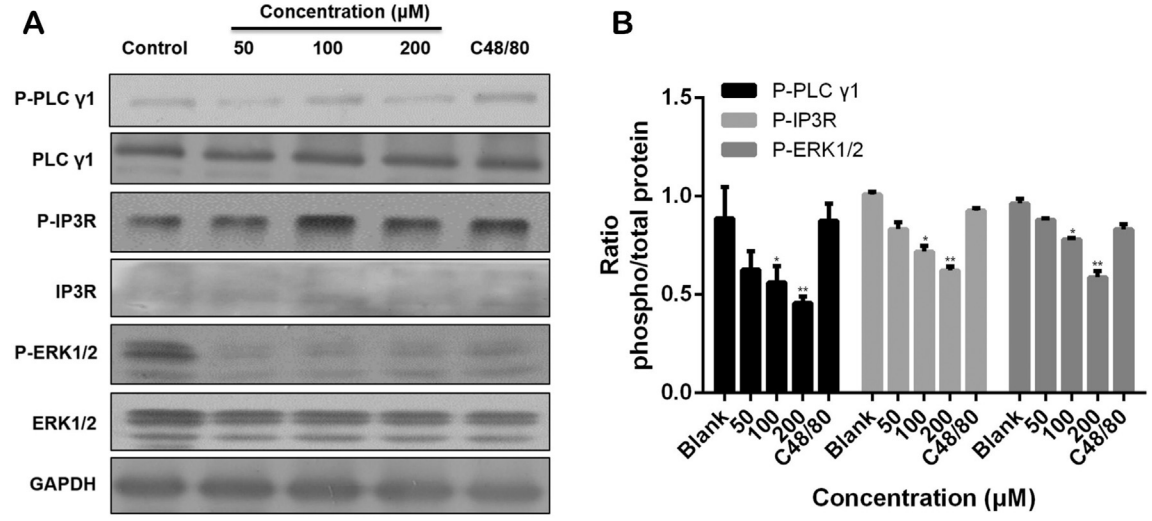

Fig. 6. Quercetin decreased the activation of the PLCγ-IP3 signaling pathway regulating calcium fluctuations in mast cells.
Effect of Quercetin on the protein expression of PLCγ, IP3R in LAD2 cells. (A) Western blot analysis of the expression levels of PLCγ, IP3R, ERK1/2, Phosphorylation-PLCγ, Phosphorylation-IP3R and Phosphorylation-ERK1/2 in LAD2 cells treated with Quercetin and C48/80. (B) Quantification of PLCγ, IP3R, ERK1/2, Phosphorylation-PLCγ, Phosphorylation-IP3R and Phosphorylation-ERK1/2 protein expression in (A) by densitometric analysis. The data are presented as mean ± S.D. (n = 3).Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

and 200 μM) and Quercetin (0, 100, 200 μM) attenuated the release of histamine and MCP-1 in peritoneal mast cells (Fig. 13). Therefore, Quercetin inhibited Evans blue extravasation in mice skin, reduced C48/80 induced calcium flux and attenuated the release of histamine and MCP-1 in peritoneal mast cells as the concentration increased, suggesting the anti-pseudo-allergic effect of Quercetin.

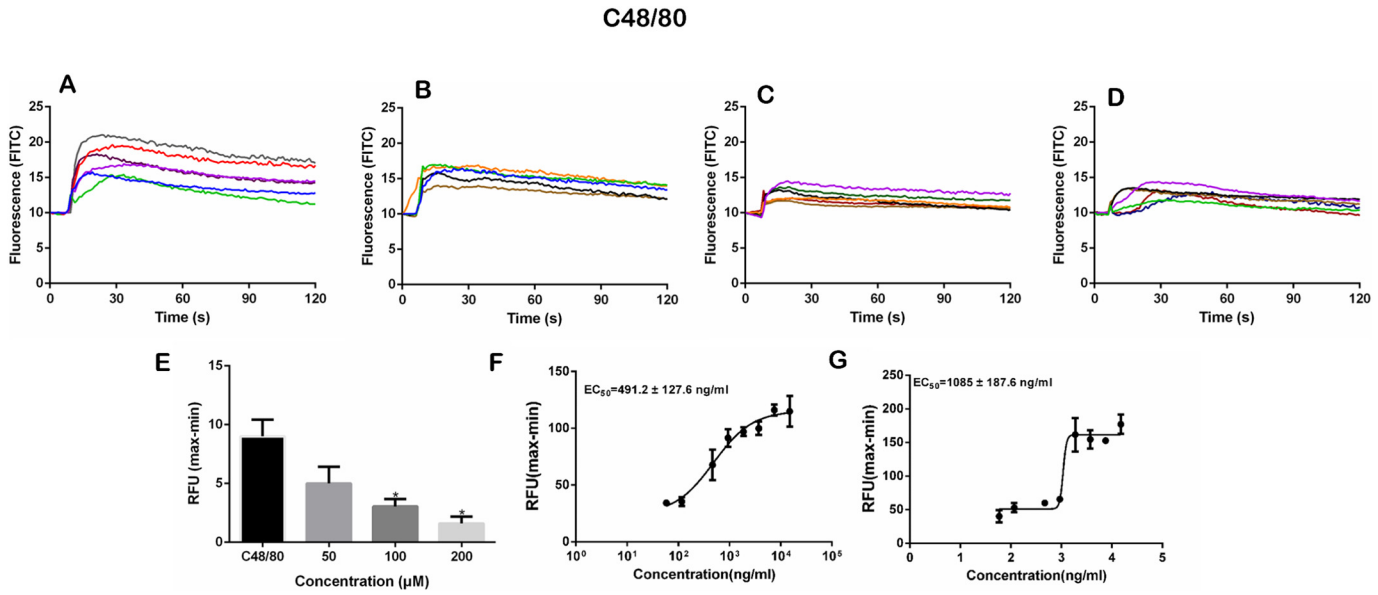

Fig. 7. Quercetin attenuated C48/80-triggered calcium flux in Mrgprx2-expressing HEK293 cells.
(A) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 in Mrgprx2-expressing HEK293 cells. (B) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 50 μM Quercetin in Mrgprx2-expressing HEK293 cells. (C) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 100 μM Quercetin in Mrgprx2-expressing HEK293 cells. (D) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 200 μM Quercetin in Mrgprx2-expressing HEK293 cells. (E) Quantification of responding cells of C48/80. Scale bar, 10 mm. (F) FlexStation 3 determine the EC50 value of C48/80 acting on the MRGPRX2 receptor. (G) FlexStation 3 determine the EC50 value of C48/80 acting on the MRGPRX2 receptor after administration of quercetin. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

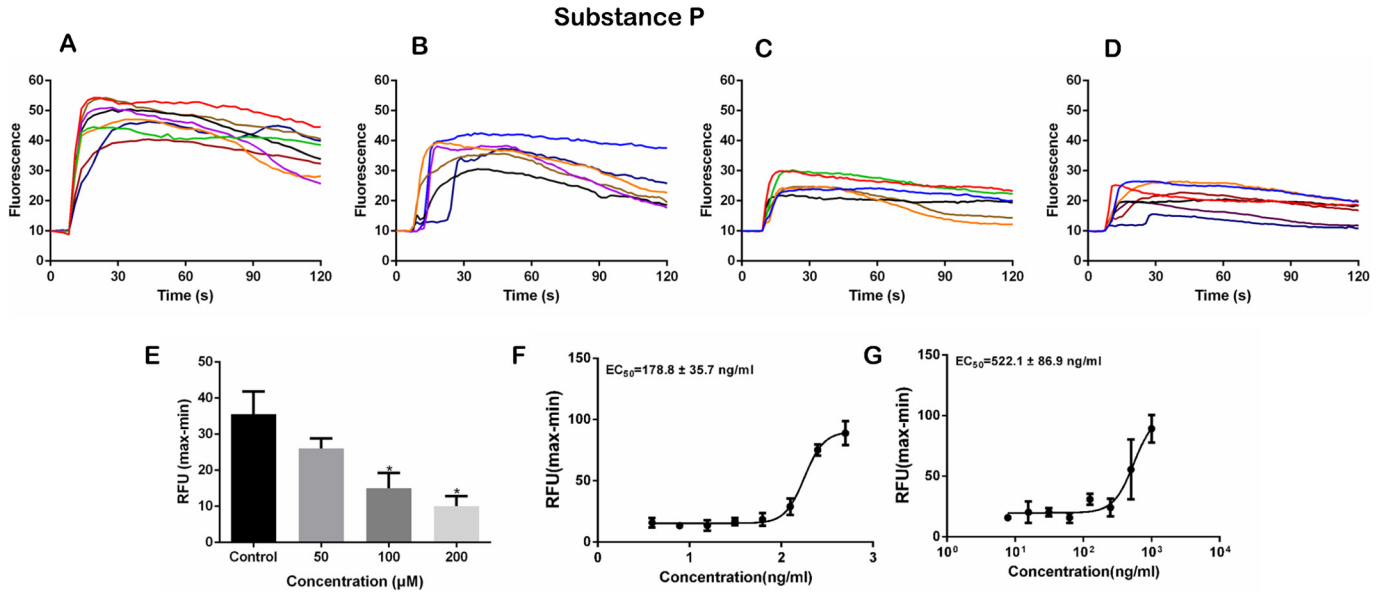

Fig. 8. Quercetin attenuated Substance P-triggered calcium flux in Mrgprx2-expressing HEK293 cells.
(A) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P in Mrgprx2-expressing HEK293 cells. (B) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 50 μM Quercetin in Mrgprx2-expressing HEK293 cells. (C) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 100 μM Quercetin in Mrgprx2-expressing HEK293 cells. (D) Representative imaging traces Ca^{2+} concentrations treated by 4 μg/ml Substance P and 200 μM Quercetin in Mrgprx2-expressing HEK293 cells. (E) Quantification of responding cells of Substance P. Scale bar, 10 mm. (F) FlexStation 3 determine the EC50 value of Substance P acting on the MRGPRX2 receptor. (G) FlexStation 3 determine the EC50 value of Substance P acting on the MRGPRX2 receptor after administration of quercetin. The data are presented as mean ± S.D. (n = 3). Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

3.5. Effect of Quercetin on the activation of the PLCγ-IP3 signaling pathway regulating calcium fluctuations in mast cells

Phosphorylated PLCγ1, IP3R and ERK1/2 (Fig. 6A) expression decreased in the LAD2 cells treated with Quercetin at the concentration of 50, 100 or 200 μM and 30 μg/ml C48/80 as the Quercetin concentration increased, as compared to the cells treated with C48/80 alone, indicating that Quercetin inhibited pseudo-allergic-induced PLCγ-IP3-Ca^{2+} signaling pathway activation and mast cell activation.

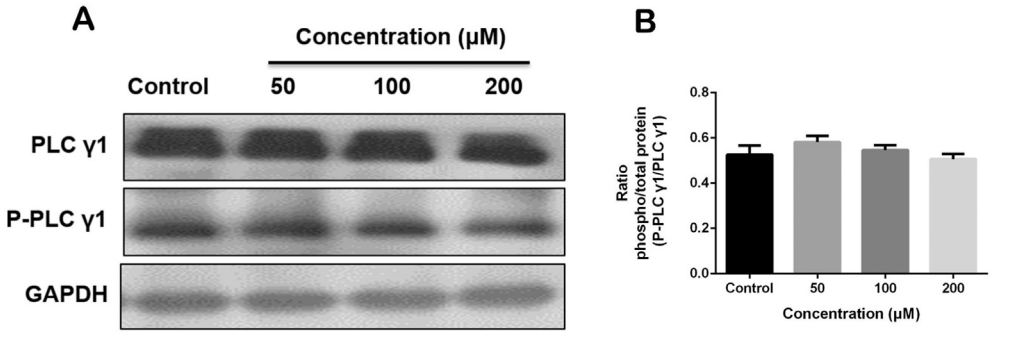

Fig. 9. Effect of Quercetin on PLCγ signaling pathway.
Effect of Quercetin on the protein expression of PLCγ in LAD2 cells. (A) Western blot analysis of the expression levels of PLCγ and Phosphorylation-PLCγ in LAD2 cells treated with Quercetin. (B) Quantification of PLCγ and Phosphorylation-PLCγ protein expression in (A) by densitometric analysis. The data are presented as mean ± S.D. (n = 3).Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

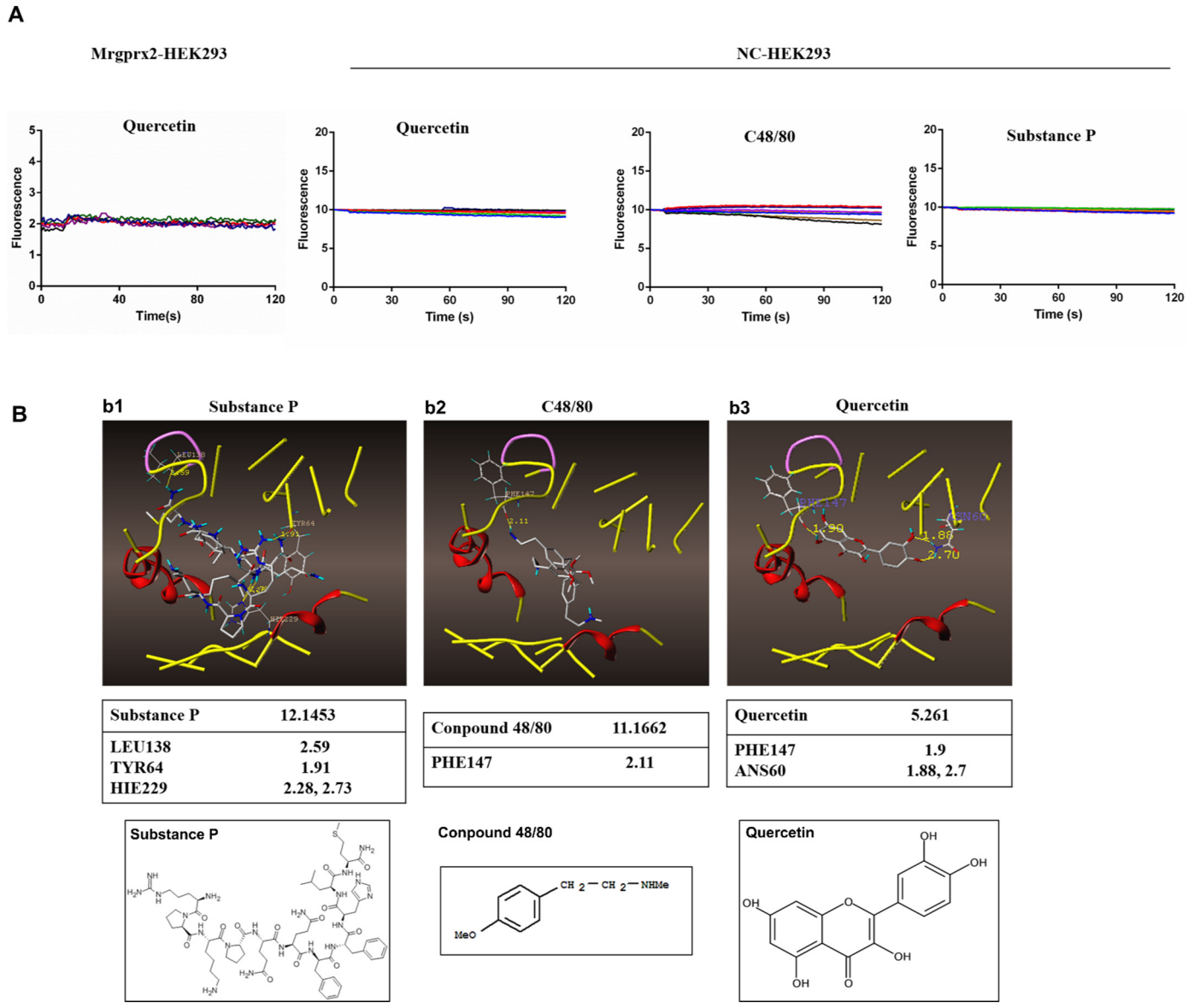

Fig. 10. Molecular docking modeling of Quercetin with Mrgprx2.
(A) Representative imaging traces Ca^{2+} concentrations treated by 200 μM Quercetin in Mrgprx2-expressing HEK293 cells; Representative imaging traces Ca^{2+} concentrations treated by 200 μM Quercetin, 30 μg/ml C48/80 and 4 μg/ml Substance P in NC-HEK293 cells. (B) Ribbon model of small molecule and Mrgprx2. (b1) Ribbon model of Substance P and Mrgprx2; (b2) Ribbon model of Conpound 48/80 and Mrgprx2; (b3) Ribbon model of Quercetin and Mrgprx2;

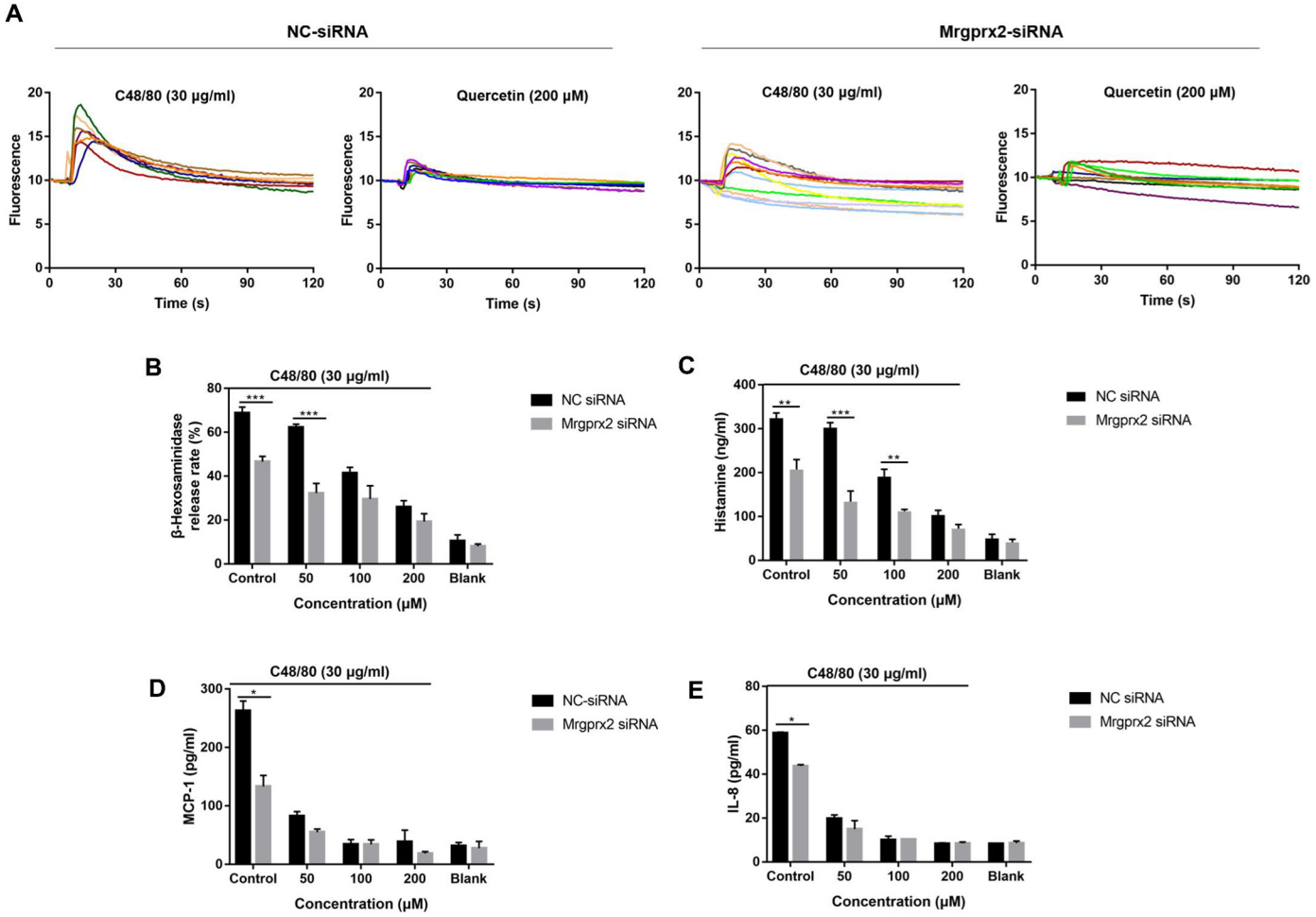

Fig. 11. Quercetin attenuated C48/80-triggered calcium flux and chemokines released in Mrgprx2-siRNA LAD2 cells.
(A) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 200 μM Quercetin in NC-siRNA and Mrgprx2-siRNA LAD2 cells. (B) The β-hexosaminidase release triggered in NC-siRNA and Mrgprx2-siRNA LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (C) The histamine release triggered in NC-siRNA and Mrgprx2-siRNA LAD2 cells treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (D) The MCP-1 release in NC-siRNA and Mrgprx2-siRNA LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. (E) The IL-8 release in NC-siRNA and Mrgprx2-siRNA LAD2 cells were treated with 0, 50 μM, 100 μM, 200 μM Quercetin combined with 30 μg/ml C48/80. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

3.6. Mechanism of the anti-pseudo-allergic effect of Quercetin

C48/80 and Substance P can induce mast cells activation through the Mrgprx2 receptor [24]. Therefore, HEK293-Mrgprx2 cells were pretreated with Quercetin (0, 50, 100 and 200 μM) before activation by C48/80 (Fig. 7A–E) or Substance P (Fig. 8A–E). Quercetin inhibited C48/80 or Substance P-induced Ca^{2+} fluctuations in HEK293-Mrgprx2 cells. In addition, Quercetin did not have any effect on calcium flux at a dose of 200 μM (Fig. 10A) in HEK293-Mrgprx2 cells. Moreover, Quercetin (only at a dose of 200 μM), C48/80 and Substance P did not have any effect on calcium flux (Fig. 10A) in NC-Mrgprx2 cells.

siRNA interference was used to knockdown Mrgprx2 gene expression in LAD2 mast cells. In our study, Quercetin (200 μM) reduced C48/80 (Fig. 11A) and induced calcium flux in NC transfected LAD2 human mast cells, but in Mrgprx2 knockdown LAD2 cells, Quercetin (200 μM) reduced C48/80 (Fig. 11A), but induced a weak calcium flux. Furthermore, Quercetin (0, 50, 100 and 200 μM) attenuated the release of β-hexosaminidase, histamine, MCP-1 and IL-8 in NC transfected LAD2 cells treated with C48/80 (Fig. 11B, C, D, E), but in Mrgprx2 knockdown LAD2 cells, the effect of Quercetin (0, 50, 100 and 200 μM) on attenuating the release of β-hexosaminidase, histamine, MCP-1 and IL-8 was weak (Fig. 11B, C, D, E). The interaction between small molecules and receptors can be characterized by EC_{50} values. C48/80 EC_{50}, when combined with Quercetin (200 μM) was 1085 ± 187.6 ng/ml (Fig. 7G) compared with EC_{50} of C48/80 alone that was 491.2 ± 127.6 ng/ml (Fig. 7F). Substance P EC_{50}, when combined with Quercetin (200 μM) was 522.1 ± 86.9 ng/ml (Fig. 8G) compared with EC_{50} of Substance P alone that was 178.8 ± 35.7 ng/ml (Fig. 8F), indicating that Quercetin could inhibit Mrgprx2-induced pseudo-allergic reaction. In addition, LAD2 cells were incubated with Quercetin alone at 0, 50, 100, 200 μM for 24 h, resulting in no change of the levels of phosphorylated PLCγ1 (Fig. 9). Therefore, Quercetin action was not exerted in the downstream pathway (PLCγ1 signaling pathway) of the anaphylactoid reaction. Quercetin dose-dependent inhibition of HEK293-Mrgprx2 cell activation indicated that Quercetin anti-pseudo-allergic effect might be related to Mrgprx2 mediated signaling pathway.

3.7. Interaction between Quercetin and Mrgprx2 by molecular docking

A detailed analysis of the interaction between Quercetin, C48/80 or Substance P with Mrgprx2 is shown in Fig. 10B. Based on the docking results, Substance P formed a hydrogen bond with LEU138 (2.59 Å long), TYR64 (1.91 Å long) and HIE229 (2.28 Å and 2.73 Å long) (Fig. 10b1); C48/80 formed a hydrogen bond with PHE147 (2.11 Å

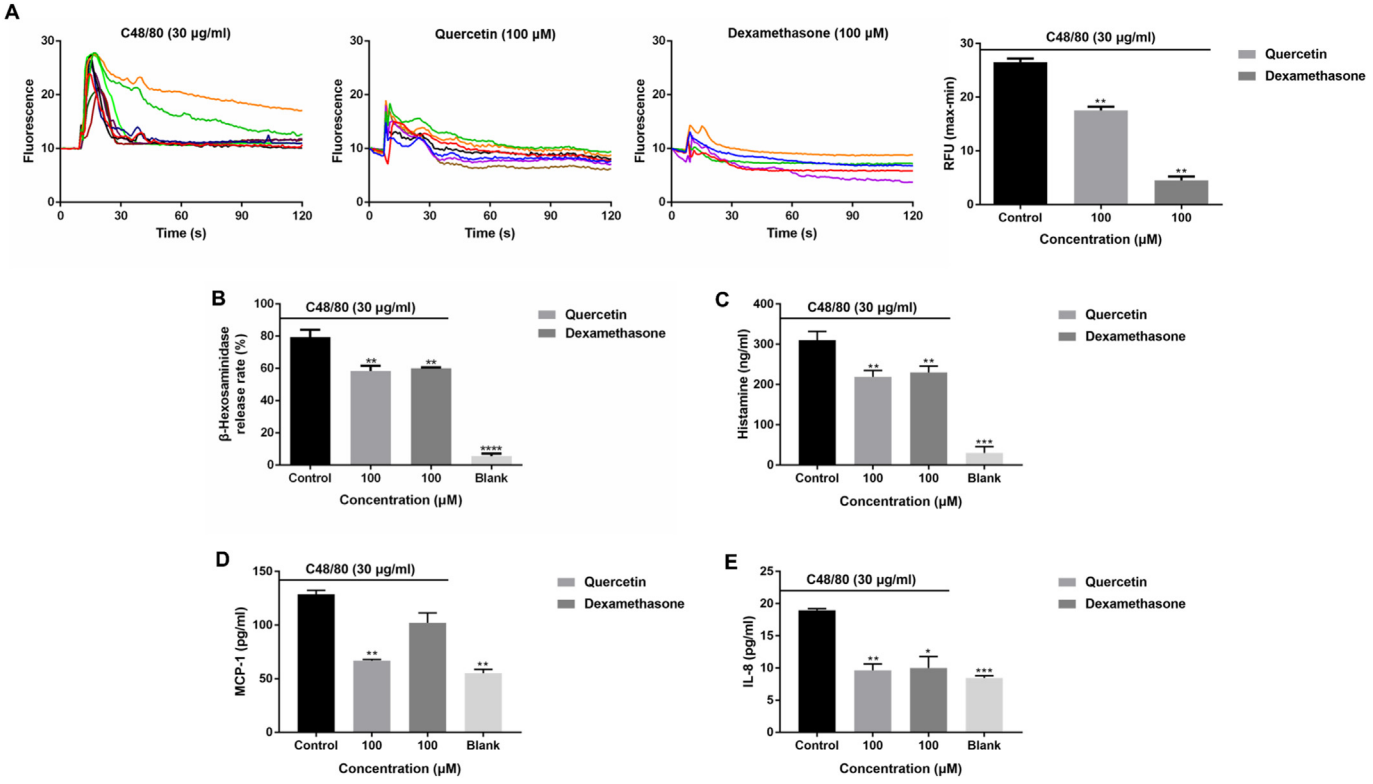

Fig. 12. Dexamethasone attenuated C48/80-triggered calcium flux and chemokines released in LAD2 cells.
(A) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80, Quercetin and dexamethasone in LAD2 cells; Quantification of responding cells of C48/80. Scale bar, 10 mm. (B) The β-hexosaminidase release triggered in LAD2 cells treated with 100 μM Quercetin and 100 μM dexamethasone combined with 30 μg/ml C48/80 for 30 min. (C) The histamine release triggered in LAD2 cells treated with 100 μM Quercetin and 100 μM dexamethasone combined with 30 μg/ml C48/80 for 30 min. (D) The MCP-1 release in LAD2 cells were treated with 100 μM Quercetin and 100 μM dexamethasone combined with 30 μg/ml C48/80. (E) The IL-8 release in LAD2 cells were treated with 100 μM Quercetin and 100 μM dexamethasone combined with 30 μg/ml C48/80. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

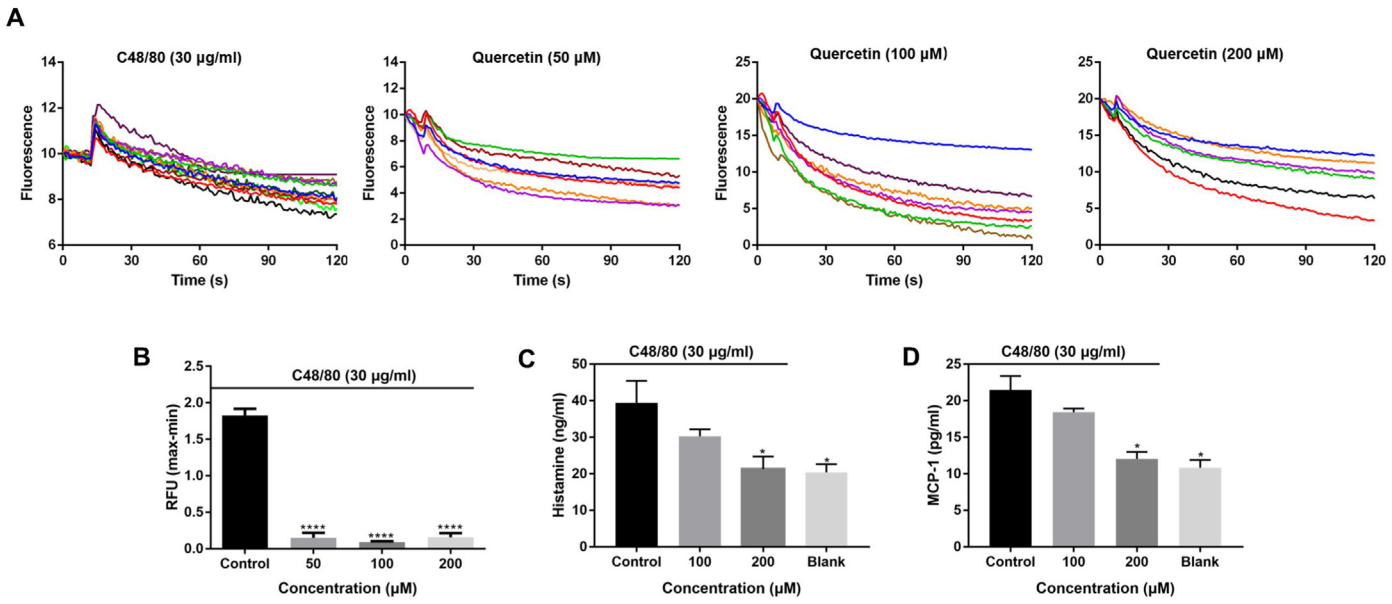

Fig. 13. Quercetin attenuated C48/80-triggered calcium flux and chemokines released in mouse peritoneal mast cells.
(A) Representative imaging traces Ca^{2+} concentrations treated by 30 μg/ml C48/80 and 50, 100, 200 μM Quercetin in mouse peritoneal mast cells. (B) Quantification of responding cells of C48/80. Scale bar, 10 mm. (C) The histamine release triggered in mouse peritoneal mast cells treated with 100, 200 μM Quercetin combined with 30 μg/ml C48/80 for 30 min. (D) The MCP-1 release in mouse peritoneal mast cells were treated with 100, 200 μM Quercetin combined with 30 μg/ml C48/80. Two-tailed unpaired Student's *t*-test was used to determine significance in statistical comparisons, and statistical significance was accepted at $p < .05$ (*$p < .05$, **$p < .01$, ***$p < .001$).

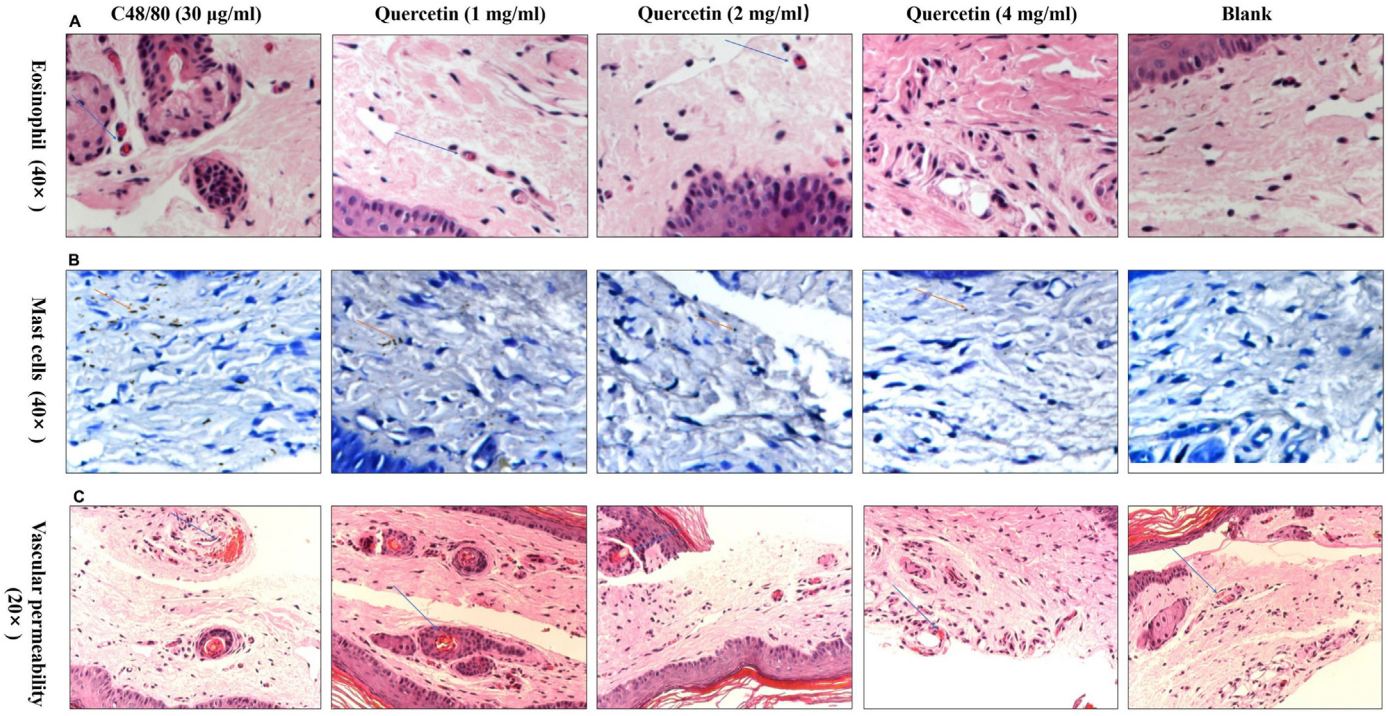

Fig. 14. Quercetin inhibited C48/80-induced local anaphylaxis by inhibiting mast cells degranulation.
Treat group indicates that 30 μg/ml C48/80 with different Quercetin concentrations (0, 1, 2, 4 mg/ml) were injected into the left paw. Saline was simultaneously injected into the right paw as the blank control. (A) Quercetin inhibited C48/80-induced eosinophil release as demonstrated by H&E staining of skin tissue sections. (B) Quercetin inhibited C48/80-induced mast cells degranulation (toluidine blue staining). (C) Quercetin inhibited C48/80-induced hemangiectasis as demonstrated by H&E staining of skin tissue sections. All experiments were repeated at least three times (n = 5). Data are expressed as mean ± SD and analyzed using two-tailed unpaired Student's *t*-test.

long) (Fig. 10b2) and Quercetin formed a hydrogen bond with PHE147 (1.90 Å long) and ASN60 (1.88 Å and 2.70 Å long) (Fig. 10b3). The results showed that the affinity of Quercetin for hydrogen bonds might be correlated with the affinity of Mrgprx2 for Substance P or C48/80 indicating that Quercetin and Mrgprx2 showed good binding ability. This binding hypothesis might provide valuable information for the design of structure-based Quercetin derivatives, which might act as effective small molecular inhibitors of Mrgprx2 mediated signaling pathway.

4. Discussion

CU is a systemic daily wheal lasting for at least 6 weeks [25]. Mast cells play an important role in inducing a rubella response by releasing various vasoactive steroids and functionally diverse proteases, chemokines and cytokines [26]. It is known that 30% to 50% of CU patients have an autoimmune basis because these patients show autoantibodies against the high-affinity IgE receptor FcεRI or unusual IgE. About 50% of the remaining CU patients have a true “idiopathic” condition [25,27]. Recently, Mrgprx2 was found to be a receptor for basic peptides in human umbilical cord blood-derived mast cells, including Substance P, C48/80, VIP, cortistatin, and somatostatin [28]. One study showed that Mrgprx2 expression in skin mast cells of severe CU patients was significantly higher than that of NC patients. Human skin mast cells are capable of expressing functional Mrgprx2, which induces rapid degranulation not only in response to Substance P but also to major basic protein and eosinophil peroxidase. Therefore, blocking human skin mast cells by Mrgprx2 might provide a new method for the prevention and treatment of severe CU. Mrgprx2 may be a new target for treating rubella in severe CU patients [29]. However, effective treatments for urticaria need to be further studied; thus, it is of utmost importance to perform a relevant basic research. In this study, our results showed that Quercetin anti-pseudo-allergic effect was mediated by Mrgprx2, giving the basis for subsequent investigations to find anti-CU drugs.

In our *in vitro* study, LAD2 cells were used to evaluate the anti-pseudo-allergic effect of Quercetin. In addition, C48/80 and Substance P were used as positive mast cell activators. Quercetin dose-dependently suppressed calcium mobilization in LAD2 cells and attenuated the release of β-hexosaminidase, histamine, MCP-1 and IL-8 in a similar way as compared to the effect of dexamethasone (100 μM) (mast cell stabilizer), indicating that Quercetin could inhibit mast cell degranulation and chemokines release. In our *in vivo* study, Quercetin suppressed the anti-pseudo-allergic effect induced by C48/80 or Substance P in C57BL/6 mice and the mice histamine, MCP-1, and IL-8 serum content demonstrated that Quercetin could significantly inhibit the degranulation induced by pseudo-allergic reaction. Quercetin could inhibit the hind paw swelling and Evans blue extravasation in mouse skin in a dose-dependent manner. Quercetin dose-dependently suppressed calcium mobilization in peritoneal mast cells and attenuated the release of histamine and MCP-1, suggesting an anti-pseudo-allergic effect. In addition, it inhibited the vasodilatation caused by histamine, reduced the release of eosinophils and the percentage of degranulated mast cells, indicating that Quercetin antagonizes mast cells to its threshing *in vivo* and histamine-induced vasodilation and eosinophils release.

Drugs that directly trigger mast cell activation produce diacylglycerol and IP3 through the PKC pathway, leading to the release of calcium ions from the endoplasmic reticulum [30,31]. The inhibition of PLCγ antigen-stimulating Rac and Cdc42 in RBL-2H3 cells leads to the formation of filamentous structures [32]. As a result of PLCγ phosphorylation, more downstream molecules, such as IP3R, PKC and MAPK, are activated, leading to the desquamation of mast cells and the production of proinflammatory cytokines [33]. In our study, Quercetin inhibited the phosphorylation of PLCγ1, IP3R and ERK1/2, indicating that Quercetin inhibited C48/80-induced PLCγ-IP3-Ca^{2+} signaling

pathway activation and mast cell activation.

Finally, our results showed that the anti-pseudo-allergic effect of Quercetin was activated by the inhibition of mast cells. Additionally, Quercetin inhibited pseudo-allergic-induced Ca^{2+} fluctuations in HEK293-Mrgprx2 cells. A previous study reported that C48/80 and Substance P EC_{50} were 470.1 ± 86.9 ng/ml and 152.3 ± 48.0 ng/ml, respectively, which were associated with the activation of pseudo-allergic reactions in these cells [1]. In our study, the EC_{50} values of C48/80 and Substance P were similar to these results [1]. C48/80 and Substance P EC_{50} were both higher when combined with Quercetin compared to the EC_{50} of these compounds alone, suggesting that Quercetin could inhibit Mrgprx2-induced pseudo-allergic reaction, and indicating that Quercetin exerted an antagonist effect through the Mrgprx2 receptor mediated signaling pathway. Furthermore, Quercetin action was not exerted in the downstream pathway of anaphylactoid reaction. Moreover, siRNA interference was used to knockdown Mrgprx2 gene expression in LAD2 mast cells and the results showed that in Mrgprx2 knockdown LAD2 cells, the effect of Quercetin (200 μM) reduced C48/80, induced calcium flux and the release of β-hexosaminidase, histamine, MCP-1 and IL-8 in a weaker way compared with NC transfected LAD2 human mast cells. Our results demonstrated that Quercetin anti-pseudo-allergic effect was related to Mrgprx2 mediated signaling pathway. The docking results showed that the affinity of Quercetin for hydrogen bonds might be correlated with the affinity of Mrgprx2 for Substance P or C48/80, indicating that Quercetin and Mrgprx2 showed good binding ability. Related literature indicates that C48/80 and Substance P can induce mast cells activation through the Mrgprx2 receptor [24]. This hypothesis might provide valuable information for the design of structure-based Quercetin derivatives that might act as effective Mrgprx2 inhibitors. It also indicated that the inhibitory effect of Quercetin on mast cells was associated to the Mrgprx2 mediated signaling pathway.

Fewtrell and Gomperts are the first demonstrating that Quercetin can suppress calcium influx in peritoneum fat cells [34]. Quercetin also inhibits ion-vector induced histamine release from rat peritoneal mast cells, indicating that it has a role other than receptor mediated calcium intramolecular flow [35]. However, they did not explore the anti-pseudo-allergic effects of Quercetin. Therefore, we are the first using an *in vivo* mouse model and HEK293-Mrgprx2 cells to explore the anti-pseudo-allergic effects of Quercetin, and we are thus the first demonstrating that Quercetin could inhibit Mrgprx2-induced pseudo-allergic effect *via* PLCγ-IP3R related Ca^{2+} fluctuations.

In conclusion, our findings demonstrated that Quercetin might be a potential candidate for the development of anti-CU therapies.

4.1. Statistical analysis

The group data is denoted as the average value ± SD. Independent sample variance analysis was used to determine that significant statistical comparison using SPSS. Differences were considered significant at $p < .05$ (*$p < .05$, **$p < .01$, and ***$p < .001$).

Conflict of interest

We wish to confirm that there is no known conflict of interest with this publication and that there is no significant financial support for the work that may affect its results.

Acknowledgments

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