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Imiquimod enhances DNFB mediated contact hypersensitivity in mice

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ABSTRACT

Imiquimod (Imiq) is a synthetic imizoquinoline compound which can act on Toll-like receptor (TLR)7 and transduce signals involved in cell activation. We investigated the role of Imiq on contact hypersensitivity (CHS) and explored the potential mechanisms of mast cells involved in the process. Topical application of Imiq cream augmented DNFB mediated CHS in C57BL/6 mice. Imiq application induced skin inflammation and increased the number of dendritic cells (DCs) in the draining lymph nodes (DLNs). The splenic cell proliferation to DNBS in DNFB and Imiq treated mice was greater than that in mice of DNFB treatment alone. Peritoneal cell-derived mast cells (PCMCs) expressed TLR7 mRNA. The results from toluidine blue staining for mast cells and histamine detection indicated that Imiq alone did not induce mast cell degranulation while Imiq plus DNFB significantly induced mast cell degranulation. Cromolyn, pyrilamine and cimetidine attenuated CHS reaction induced by Imiq. Our findings suggest that Imiq could augment the intensity of CHS reaction. The mechanisms underlying the effect may relate to histamine release by mast cells and induction of DC homing to DLNs. Blocking histamine action in early time of allergen contact is beneficial to the alleviation of CHS.

1. Introduction

Allergic contact dermatitis (ACD) is a common disease that is caused by type IV hypersensitivity responses to allergens such as nickel, dyes, pesticides, cosmetics, etc. [1,2]. The acute skin lesions of ACD include redness, rashes, blisters and even exfoliative dermatitis. If the allergens are not removed, ACD tends to become chronic and manifests papules and scales. The prevalence of contact allergy is rising worldwide. It is estimated that about 20% of the general population suffers from contact allergy to at least one contact allergen, which results in high costs for health care systems as well as in an impairment of the quality of life for the patients [3,4]. Murine contact hypersensitivity (CHS) is widely used to study ACD by using dinitrofluorobenzene (DNFB). CHS consists of two phases, i.e. the sensitization and the challenge. During the sensitization phase, allergens firstly penetrate into the skin and bind to self-protein in the dermis. Skin resident dendritic cells (DCs), the most potent antigen presenting cells, capture the modified proteins and migrate to draining lymph nodes (DLNs), where peptides are presented to T cells and activate the allergen-specific lymphocytes [1,5]. The challenge phase starts from the re-exposure to the same allergen. The specific effector cells are recruited into allergen-exposed skin, in which they release pro-inflammatory cytokines and destroy target cells by cytotoxicity and keratinocyte apoptosis [1,2].

Toll-like receptors (TLRs), expressed in many immunocytes including macrophage, DC, NK and mast cells, are the major innate immune sensors against exogenous foreign invasion [6–8]. Stimulation of specific TLR induces distinct signal transduction and gene expression, which not only leads to the activation of innate immunity but also correlates with the induction of adaptive immunity. It has been appreciated that some TLR engagement by chemical contact allergens elicits an inappropriate immune response that results in allergic contact dermatitis [1,2]. Some allergens directly or indirectly promote the activation of TLR2, 3 or 4, which conduce to the sensitization or stimulation of CHS [9–12]. Mast cells, as highly specialized secretory cells, locate preferentially at the environment interface of the body and play roles in innate and adaptive immune responses [13,14]. Mast cell activation is required for lymphocyte and Langerhans cell migration, which is needed for initiating adaptive immune response [15–17]. The study of mast cell deficient mice confirmed that mast cell activation and histamine released by mast cells play an important role in CHS sensitization stage [18]. Imiquimod (Imiq) is a synthetic imizoquinoline compound, which can act on TLR7 and transduce signals involved in cell activation. After topical application of Imiq, mast cells are crucial for early skin inflammation, Langerhans cell trafficking and CTL production [19].

Previous studies mainly focused on the effect of TLR on DC

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activation for CHS. Both mast cells and DCs are located in parts of skin and mucosa that are accessible to allergens. They are in close proximity to each other and express multiple TLRs. Does TLR7 ligand Imiq affect CHS by acting on mast cells? Or does Imiq act on mast cells and DCs simultaneously and then participate in CHS? There are few relevant reports on that. In previous studies, we found that mast cell activation by compound 48/80 promoted exogenous DC homing to DLNs and enhanced DC vaccine induced humoral immune response [20]. We also found topical application of Imiq promoted exogenous DC migration to DLNs and enhanced BM-DC efficacy against melanoma [21]. In addition, mast cells treated by Imiq enhanced DC to express co-stimulatory molecules [21]. In this study, we established a murine CHS model to observe the effect of Imiq on CHS and to detect the potential mechanism of mast cells involved in it. We proved that topical application of Imiq cream augmented CHS intensity in C57BL/6 mice. The mechanisms underlying the effect may relate to induction of DC homing to DLNs and histamine release by mast cells.

2. Materials and methods

2.1. Mice and reagents

Female C57BL/6 mice were purchased from Petfuder Animal Center, China. All animals were maintained in animal facility with specific pathogen-free environment and studied at 6–10 weeks of age. All experiments were performed according to the guidelines of local animal experiment ethics committee. Imiq cream was purchased from 3 M health care limited (England, U.K.). Soluble form of Imiq (R837) was purchased from InvivoGen (San Diego, CA, USA). 2,4-dinitrofluorobenzene (DNFB), 2,4-dinitrobenzenesulfonic acid hydrate (DNBS), cromolyn, pyrilamine, cimetidine and compound 48/80 were from Sigma Aldrich (St. Louis, USA). Cell Counting Kit-8 kit was from Multisciences Biotech Co.(Hangzhou, China). PE-conjugated anti-mouse CD11c, CD117 and isotype control antibodies were from eBioscience (San Diego, CA, USA). Recombinant murine interleukin 3 (IL-3) and stem cell factor (SCF) were purchased from Peprotech (Rocky Hill, NJ, USA). Trizol and reverse transcription system were from Takara Bio Co. (Dalian, China). SYBR-PCR Reagent kit was from Vazyme Biotech Co. (Nanjing, China). Red blood cell lysis buffer was from Solarbio (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kit for histamine was from Shanghai Dobio Biotech Co. (Shanghai, China).

2.2. CHS

Mice were sensitized with 100 μl of 0.5% (w/v) DNFB in acetone/olive oil (4:1) on the shaved back and challenged 5 days later with 20 μl of 0.2% DNFB on the right ear (DNFB group). Negative control mice were treated with the solvent only. In order to detect the effect of Imiq on CHS, mice in Imiq + DNFB group received a daily dose of 2.5 mg of 5% Imiq cream on the shaved back skin for successive 3 days before DNFB sensitization. In order to detect the role of mast cells in Imiq + DNFB group, three additional groups were added by intraperitoneal (i.p.) injection with 25 μg/g body weight of cromolyn (cromolyn + Imiq + DNFB group), 50 μg/g of pyrilamine (pyrilamine + Imiq + DNFB group) and 50 μg/g of cimetidine (cimetidine + Imiq + DNFB group) respectively 90 min before DNFB sensitization. Ear thickness was measured before challenge and at 24 h, 48 h, 72 h after challenge with a digital caliper from SMCTW Company (Shanghai, China). The percentage of Ear swelling responses was calculated by the following formula: Ear swelling response (%) = 100 × (post-challenge ear thickness-pre-challenge ear thickness) / pre-challenge ear thickness.

2.3. Histological examination and histamine detection

Imiq treated mice received a daily dose of 2.5 mg of 5% Imiq cream on the shaved back skin for successive 3 days. Control mice received a similar daily dose of Vaseline vehicle cream. Some skin biopsy samples were fixed in 10% formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) or toluidine blue. Some skin samples were snapped frozen in liquid nitrogen and homogenized in normal saline. Peripheral blood of the mice was drawn from orbital vein. The concentration of histamine in the supernatant of homogenized skin and in the blood was determined by ELISA according to the manufacture's instruction. For CHS mouse model, histological examination and histamine detection were carried out one hour after DNFB sensitization similar to that for Imiq treated mice.

2.4. Detection of DCs in DLNs

Imiq was topical applied on the scapular skin of C57BL/6 mice and Vaseline as control for consecutive 3 days. The mice were euthanized 24 h later. The cells from brachial and axillary lymph nodes were pooled and digested with 100 U/ml collagenase A for 30 min at 37 °C. EDTA at a final concentration of 10 mM was added during the last 5 min. Single cell suspensions were stained with PE conjugated anti-mouse CD11c. The cells were determined by FACS and analyzed by NovoExpress software. DCs were identified as $CD11c^+$ cells. The total number of DCs in DLNs equals to the percentage of $CD11c^+$ cells multiplied by the total cellularity in DLNs.

2.5. Lymphocyte proliferation assay

Splenocytes were obtained from each group after 5 days of DNFB sensitization. Single cell suspensions were generated by pressing splenocytes through a 70 μm cell strainer. Splenic erythrocytes were lysed with red blood cell lysis buffer. Duplicate samples of 4×10^5 cells in 200 μl RPMI 1640 supplemented with 10% fetal calf serum (FCS) were seeded in 96-well plates and were stimulated with 50 μg/ml of DNBS (a water-soluble compound with the same antigenicity as DNFB). Three days after co-culture, the ability of lymphocyte proliferation was measured after incubation with Cell Counting Kit-8 reagent for an additional 4 h, and optical density at 450 nm (O.D. 450) was measured for each well.

2.6. Culture of peritoneal cell-derived mast cells (PCMCs)

PCMCs were obtained according to the protocols of Dudeck et al. [22], Mrabet-Dahbi et al. [23] and Malbec et al. [24] with some modifications. In brief, peritoneal cells were collected from C57BL/6 mice after i.p. injection of 5 ml RPMI 1640 medium. Cells at a density of 1×10^6/ml were maintained in RPMI 1640 medium supplemented with 10% FCS, 10 ng/ml IL-3, 30 ng/ml SCF, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). After 48 h, non-adherent cells were removed and fresh complete medium was added to the plate. The cells were refreshed with complete medium every 2 to 3 days. Purity of PCMCs was assessed by expression of CD117 using flow cytometry. PCMCs were used at a purity of > 90%.

2.7. PCMC TLR7 mRNA detection

After overnight co-culture of PCMCs and R837, total RNA was extracted from PCMCs using Trizol reagent according to the manufacturer's instruction. cDNA synthesis was performed with 1 μg of total RNA and reverse transcription system kit. Quantitative RT-PCR was performed to quantify the mRNA levels of TLR7 with the SYBR-PCR Reagent kit. The primers used in this assay were: TLR7 forward: CTG GAGTTCAGAGGCAACCA, TLR7 reverse: CAGGAGGCAAGGAATTC AGG. Mouse GAPDH was used as the endogenous reference. Specificity of resulting PCR products was confirmed by melting curves. The relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method.

The expression of untreated cells was used as a calibrator and set as 1.

2.8. Degranulation assay

The cultured PCMCs were washed with RPMI1640 completely to remove IL-3 and SCF. Duplicate samples of 1×10^5 PCMCs in100μl RPMI 1640 media were seeded into 96-well plate. Compound 48/80 (100 ng/ml), indicated concentrations of R837, DNFB (10 μg/ml) and R837(1 μg/ml) + DNFB(10 μg/ml) were added into specific wells respectively. For total histamine release, PCMCs were lysed in 50 μl of 0.1% Triton X-100. One hour later, the plate was spun at 290 ×*g* for 5 min. The concentration of histamine in the supernatant was determined by ELISA method according to the manufacture's instruction. The percentage of histamine release = 100 × the concentration of histamine of tested cells / the concentration of histamine in Triton X-100 lyzed cells.

2.9. Statistical analysis

All experimental groups consisted of three to five mice, and all experiments were performed at least twice. The results were represented as the means ± standard deviations. All statistical analyses were performed using GraphPad Prism software. Comparisons between two groups were determined using Student's *t*-test, and multiple group comparisons were assessed with one way ANOVA followed by Bonferroni's post hoc tests. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Imiq augmented CHS reaction

To examine the effect of Imiq on CHS, one group of C57BL/6 mice (Imiq + DNFB group) firstly received a daily dose of Imiq cream on the shaved back skin for successive 3 days, at the same time the mice in the control group (DNFB group) received a similar dose of Vaseline cream. Then the two groups of mice were sensitized and challenged with DNFB. As shown in Fig. 1, only acetone/olive oil solvent treatment (negative control group) did not induce obvious ear edema. When DNFB was applied on the ear of DNFB sensitized mice (DNFB group), obvious ear swelling could be seen. When DNFB was applied on the ear of Imiq pretreated DNFB- sensitized mice (Imiq + DNFB group), the mice mounted further intense CHS than that of DNFB group. The kinetics of CHS response was similar in DNFB and in Imiq + DNFB group with a maximal ear swelling at 48 h. Therefore, topical application of Imiq significantly intensified the ability of DNFB induced CHS in C57BL/6 mice.

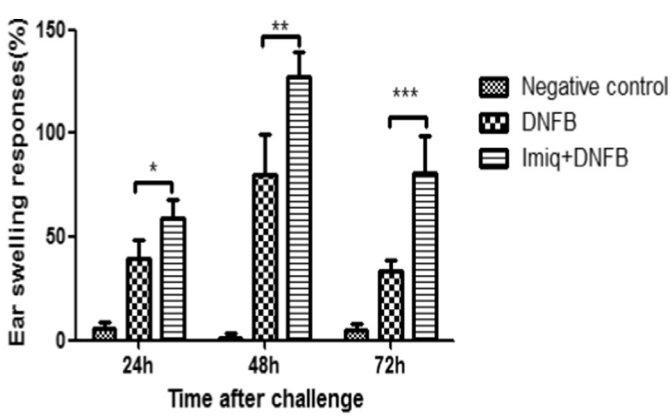

Fig. 1. Effect of Imiq on DNFB mediated CHS.
C57BL/6 mice were treated with Imiq (Imiq + DNFB group) or Vaseline cream (DNFB group) respectively for successive 3 days. On day 3, 100 μl of 0.5%DNFB was applied on the shaved back. Five days later of DNFB treatment, the ears of these mice were challenged with 20 μl of 0.2%DNFB. The ear thickness was measured at various time points. Ear swelling response (%) was calculated by the following formula: 100 × (ear thickness after challenge-ear thickness before challenge) / ear thickness before challenge. n = 4, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.2. Imiq mediated inflammation and mast cell changes in the skin

In order to examine the effect of Imiq on the local site of application, the treated skins were fixed in 10% formalin and embedded in paraffin. Sections were cut and stained with H&E or toluidine blue. As shown in Fig. 2A, compared with negative control group, skin samples treated with Imiq demonstrated obvious epidermal thickening, inflammatory cell infiltration and capillary hyperemia in H.E. staining. Mast cells contain many metachromatic granules which can be specifically stained with toluidine blue. As shown in Fig. 2B, some mast cells were identified in toluidine blue staining skin samples. No obvious difference was found in the number of mast cells between Imiq treated skin samples and those in negative control group. We then investigated the effect of Imiq on histamine levels in skin and in blood. As shown in Fig. 2C, there was no significant difference in the amount of histamine in skin between Imiq treated mice and control mice (Left column of Fig. 2C). The blood sample displayed a similar result to the skin (right column of Fig. 2C).

3.3. Imiq induced DLN hypertrophy and DC accumulation

Skin resident DC activation and migration to DLNs is a prerequisite for CHS [1,25]. We determined whether pretreatment with Imiq altered the migration of DC to local DLNs. Imiq cream was topically applied onto the scapular skin for consecutive 3 days. Twenty-four hours after the last Imiq application, DLN cells were collected and analyzed by flow cytometry. DCs were identified as $CD11c^+$cells. Compared with mice in control group, DLNs of Imiq treated mice were macroscopically enlarged. The number of DLN cells was about three times that of the control group. As shown in Fig. 3, the proportions of $CD11c^+$DCs in DLN cells are 4.36% (control) and 5.56% (Imiq treated) respectively. The total number of DCs in DLNs of Imiq treated mice was significantly higher than that of the control mice. Thus, treatment with Imiq had enhancing effect on DC homing to DLNs.

3.4. Imiq augmented the specific proliferation of splenocytes to DNBS

Mice pretreated with Imiq increased total cellularity and DC accumulation in DLNs (Fig. 3). The net effect of Imiq in adaptive immune response was further investigated by in vitro specific lymphocyte proliferation assay. Splenocytes obtained from mice treated with DNFB (DNFB and Imiq + DNFB group) showed greater lymphocyte proliferation significantly, compared with those from vehicle treated mice (Negative control group). The strongest lymphocyte proliferation was observed in the mice treated with Imiq and DNFB (Imiq + DNFB group), which was significantly greater than that of only DNFB treatment (Fig. 4).

3.5. PCMC identification and TLR7 mRNA detection

Peritoneal cells from C57BL/6 mice were cultured in SCF and IL-3 containing medium as described in materials and methods 2.6. After one month of culture, approximately 5×10^7 cells were derived from one mouse peritoneal cells. We found out that most of the propagated cells contained many granules that could be stained by toluidine blue, which was one of the characteristics of mast cells. We also detected CD117 (a characteristic marker of mast cells) expression on these cells by flow cytometry. As shown in Fig. 5A, under the same PMT gain, the fluorescence intensity of CD117-stained cells (PCMC-CD117) was

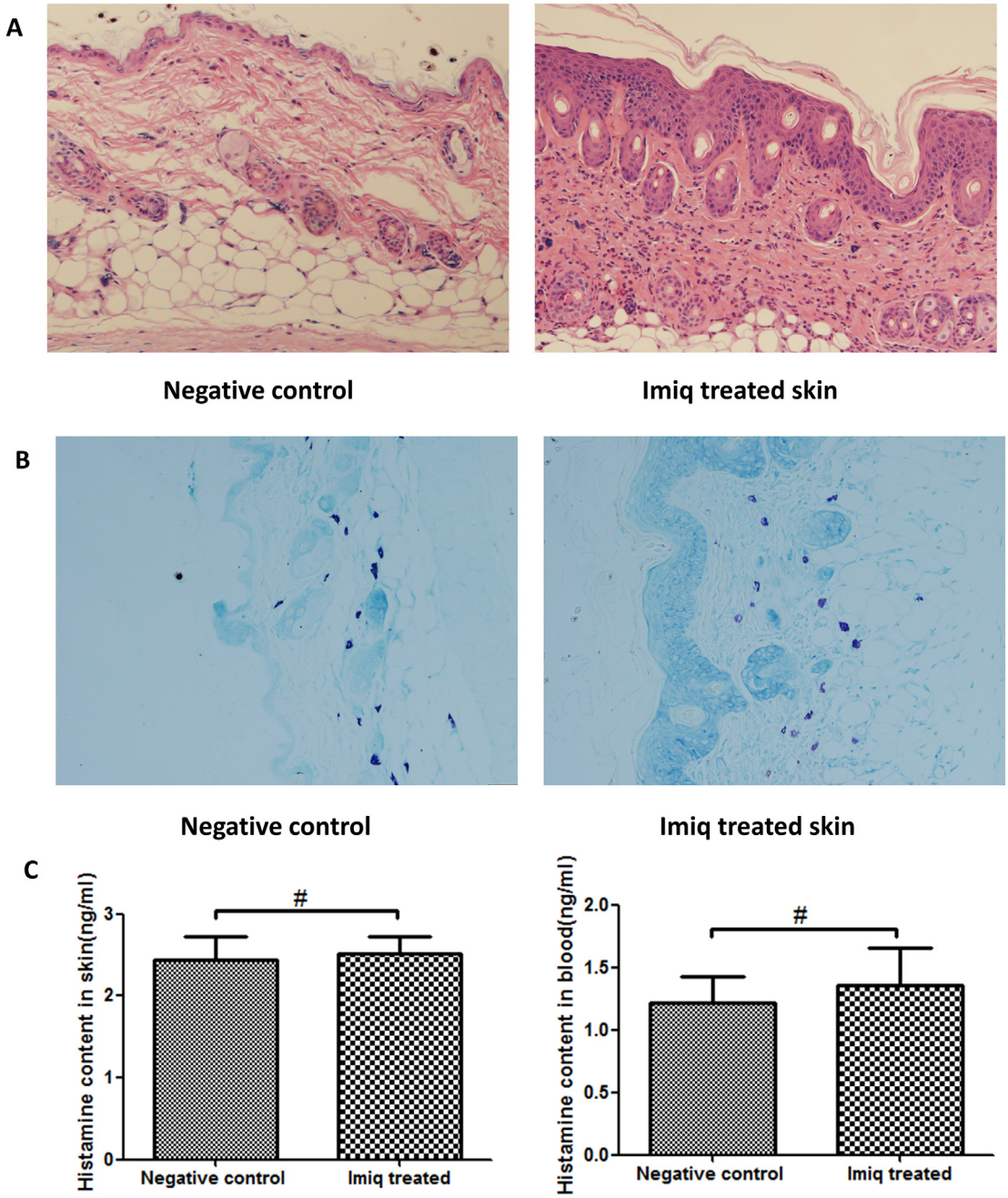

Fig. 2. Histological analysis and histamine detection of Imiq or vehicle cream treated mice.
Mice were treated with Imiq or vehicle cream once daily for successive 3 days. Paraffin sections were prepared and stained with hematoxylin and eosin (H&E) or toluidine blue. The histamine levels in the supernatant of homogenized skin and in blood were detected by ELISA. (A) Typical H&E stained skin samples. (B) Typical toluidine blue stained skin samples. (C) Histamine levels in skin (left) and in blood (right) for mice treated with Imiq or vehicle cream. n = 5, $^{\#}P > 0.05$.

significantly higher than that of isotype control stained cells (PCMC-negative control), which indicated that CD117 was expressed on most of the cells. Therefore, PCMCs were propagated successfully. Then we analyzed the expression of TLR7 mRNA in PCMCs with no stimulation or stimulated with R837 for 24 h, the expression of untreated cells was used as a calibrator and set as 1. As shown in Fig. 5B, TLR7 mRNA expression in low dosage of R837-treated PCMCs was at the similar level to that in untreated PCMCs, while the expression in high dosage of R837-treated PCMCs was significantly increased compared to that in untreated cells.

3.6. Imiq enhanced DNFB mediated mast cell degranulation

As shown in Fig. 2B and C, Imiq treatment alone did not induce obvious mast cell degranulation. We further examined the effect of Imiq on degranulation of mast cells in DNFB-mediated CHS. Fig. 6A displayed the typical results of toluidine blue staining samples from vehicle treated control mice (Negative control group), DNFB sensitized mice (DNFB group) and DNFB sensitized mice pretreated with Imiq (Imiq + DNFB group). The skin samples from DNFB group and Imiq + DNFB group demonstrated fewer mast cells compared with that from negative control. As shown in Fig. 6B, histamine content in skin treated with DNFB and Imiq + DNFB was lower than that from negative control mice, whereas histamine in blood increased significantly compared

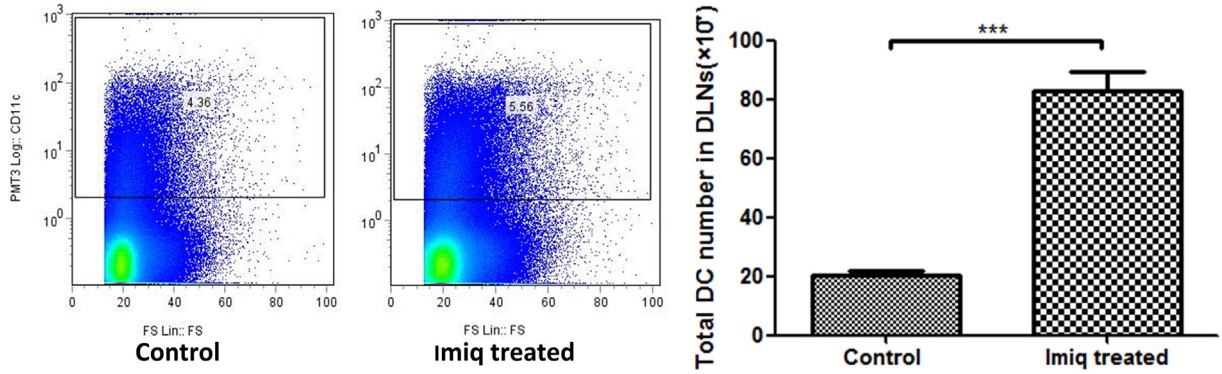

Fig. 3. Analysis of DCs in DLNs by flow cytometry.
Imiq cream was topically applied on the scapular skin of C57BL/6 mice for consecutive 3 days. Twenty-four hour after the last Imiq application, the cells from brachial and axillary lymph nodes were collected and analyzed by flow cytometry. Total DC number in DLNs equals to the percentage of $CD11c^{+}$ cells multiplied by the total number of DLN cells. n = 4, ***P < 0.001.

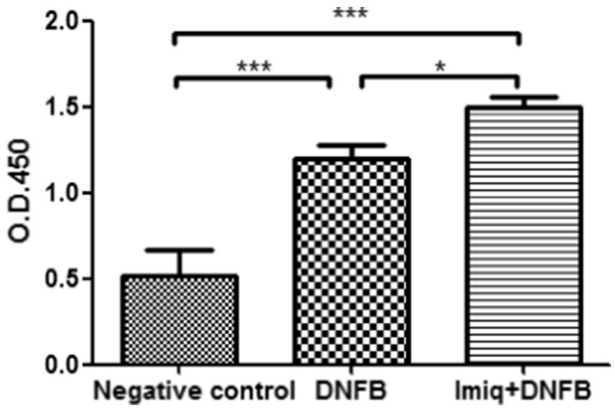

Fig. 4. Imiq augmented the specific proliferation of splenocytes to DNBS.
Splenocytes from each group were harvested and stimulated with DNBS in vitro for 72 h. Cell proliferation was measured by Cell Counting Kit-8. Data are expressed as the mean optical density at 450 nm (O.D. 450) ± SD. n = 3,*P < 0.05, ***P < 0.001.

with that from negative control. Imiq pretreatment further decreased histamine concentration in skin and increased histamine in blood. Fig. 6C showed the results of in vitro experiments on PCMCs. R837 (soluble form of Imiq) at three concentrations could not induce histamine release from PCMCs significantly. Compound 48/80 (a typical mast cell activator) and DNFB led to obvious histamine release from PCMCs. R837 supplement to DNFB-containing medium enhanced histamine release by PCMCs in that DNFB inducing 32% of mast cell degranulation and supplement of R837 increasing the percentage to 51%.

3.7. Blocking histamine alleviated the effect of Imiq on CHS

Both in vitro and in vivo assays confirmed that Imiq combined with DNFB treatment resulted in larger amount of histamine release from mast cells. We further examined the roles of histamine on Imiq enhanced DNFB-induced CHS in mice. First, three groups of mice were treated with cromolyn, pyrilamine and cimetidine 90 min before DNFB sensitization respectively. Then CHS reactions were measured 48 h after DNFB challenge. Pretreatment of the animals with cromolyn, pyrilamine and cimetdine caused a significant decline of ear thickness, as shown in Fig. 7, the ear thickness levels in the above three groups were similar to that in single DNFB treatment.

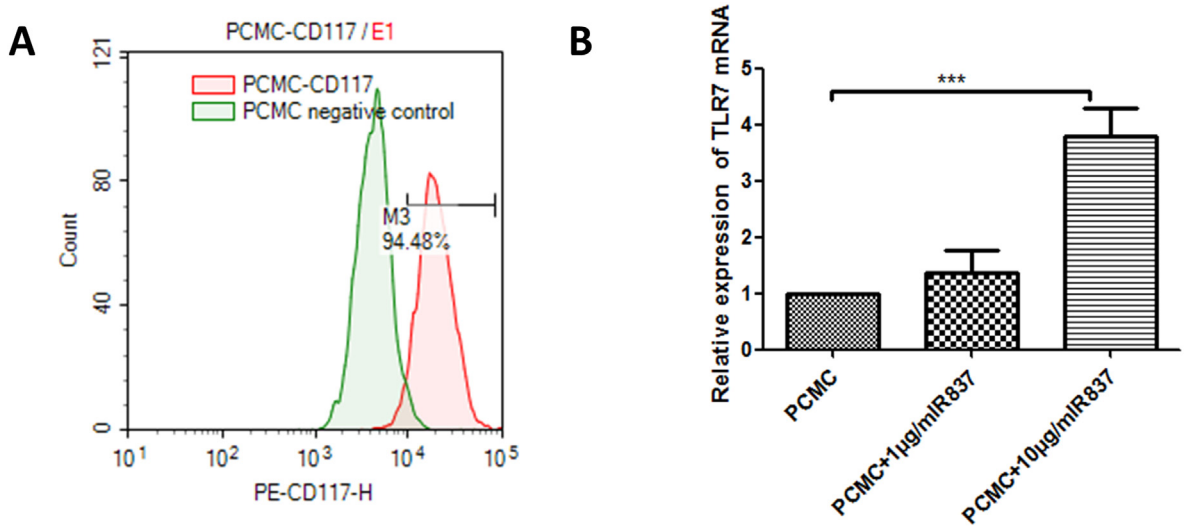

Fig. 5. CD117 and TLR7 mRNA detection of PCMCs.
A. PCMCs were stained with PE-conjugated anti-mouse CD117 or isotype control and analyzed by flow cytometry. B. Detection of TLR7 mRNA in PCMCs. The RNA of R837-treated or non-treated PCMCs was isolated, reverse transcribed and amplified using quantitative RT-PCR. The relative mRNA expression was calculated by using the $2^{-\Delta\Delta CT}$ method and the expression level of untreated cells was set as 1. (n = 3, ***P < 0.001).

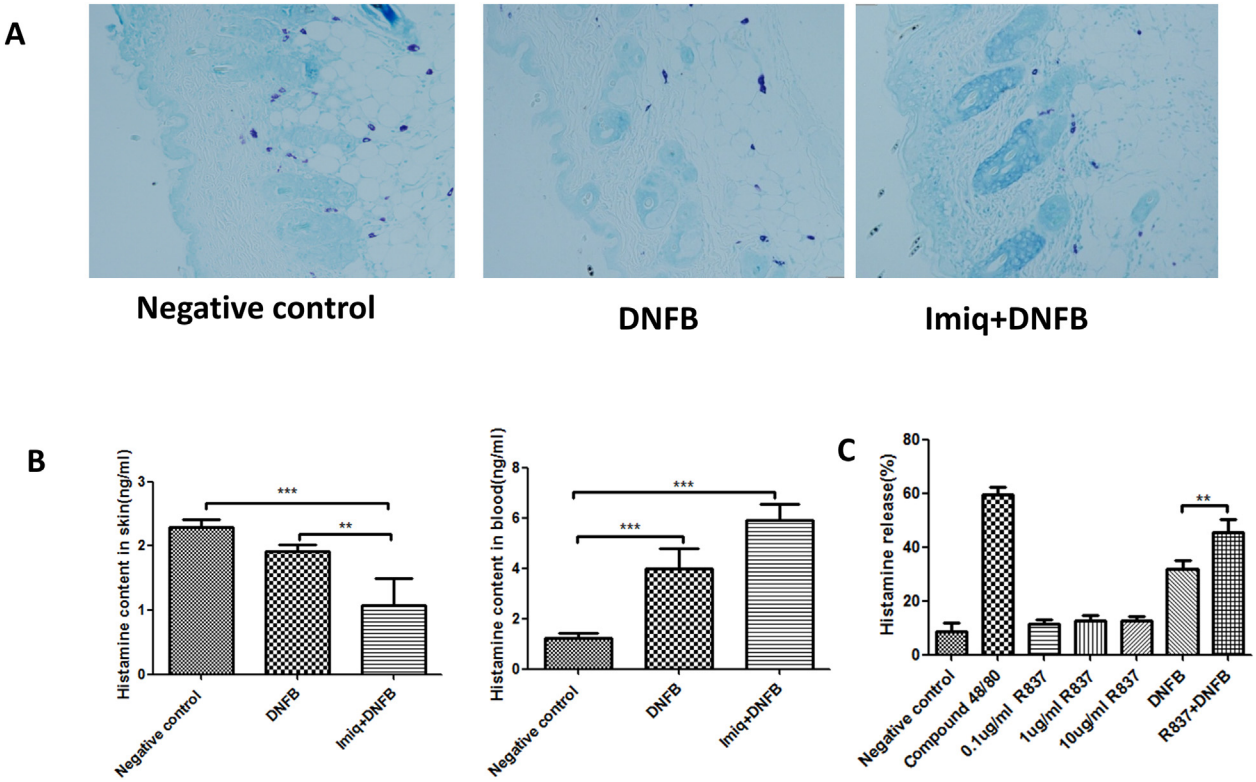

Fig. 6. Effect of Imiq on DNFB mediated mast cell degranulation.
A. Mice were sensitized with DNFB after treatment with Imiq or vehicle cream. One hour after DNFB sensitization, local skin paraffin sections were prepared and stained with toluidine blue. B. The histamine levels in the supernatant of homogenized skin and in blood were detected by ELISA. C. Histamine release of PCMCs stimulated with Compound 48/80, the indicated concentration of R837 or DNFB and R837 + DNFB. (n = 3–5, **P < 0.01, ***P < 0.001).

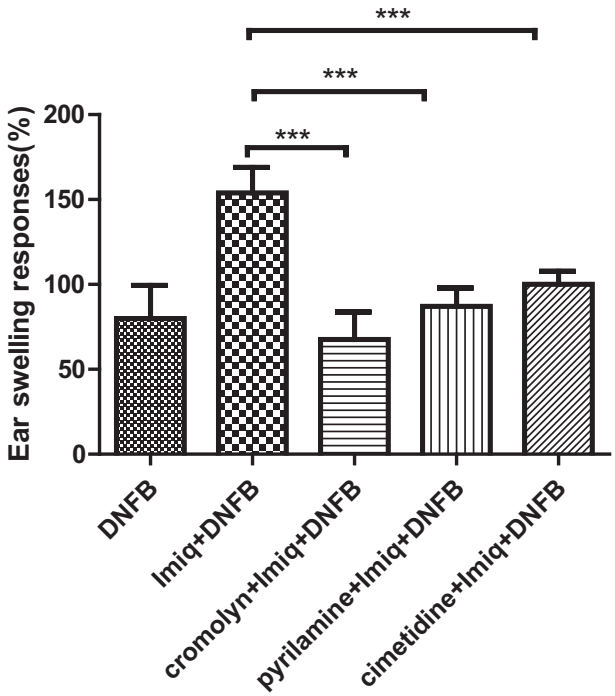

Fig. 7. Effect of cromolyn, pyrilamine and cimetidine on Imiq-involved CHS.
Imiq treated mice were i.p. injected with cromolyn, pyrilamine or cimetidine 90 min before DNFB sensitization. Ear thickness was measured 48 h after DNFB challenge. (n = 4, ***P < 0.001).

4. Discussion

CHS is a complex process involving many kinds of immunocyte interactions. The essential step in the sensitization phase for CHS is the migration of allergen-bearing cutaneous DCs into DLNs [1,2]. Mast cells and DCs locate in close proximity to environmental interfaces of the skin [26]. Mast cell-derived soluble factors such as TNF-α, histamine and lipid mediators have been shown to affect DC functions and Th polarization [16,27–32]. The DC-mast cell interaction plays an essential role in the sensitization phase of CHS [33]. Dudeck et al. confirmed that mast cell activation and histamine released by mast cells play an important role in CHS sensitization stage [18].

As a synthetic TLR7 agonist, Imiq displays potent antiviral and antitumor activities through direct and indirect action on skin and immune system. Imiq can act on TLR7 of monocytes, macrophages and DC cells, which induces secretion of IL-12, IL-1β, TNF-α and interferon type 1 involved in antiviral and antitumor effects [34–38]. Imiq cream has been approved to treat external genital and perinal warts in humans [34]. In contrast to their beneficial properties, Imiq has some side effects. Daily administration of Imiq can lead to skin inflammation and has been used to induce psoriasis-like or lupus like disease in mice [39,40]. Since skin is the common site of Imiq administration, it is likely that its usage could influence the outcome of antigen-specific skin inflammatory diseases, especially contact allergic dermatitis. So far, few studies addressed this question. In this report, we found Imiq enhanced DNFB mediated CHS. The splenic cell proliferation to DNBS in Imiq combined with DNFB treated mice was greater than that in mice of single DNFB treatment.

Traditionally, mast cells are considered critical effector cells in allergy by their potential to secrete preformed granule associated mediators such as histamine and serotonin and newly generated lipid mediators such as leukotrienes, prostaglandins and cytokines [41]. In

addition, mast cells express many receptors of pathogen recognition and play critical roles against some bacterial and virus infections [13]. Mast cells have been implicated in many other pathological responses such as rheumatoid arthritis and arteriosclerosis [42,43]. In this study, we found that PCMCs expressed TLR7 mRNA.TLR7 is the receptor of recognizing single stranded RNA and Imiq. Therefore, the enhancement of Imiq on CHS may relate to mast cell activation. We first studied the degranulation of skin mast cells by toluidine blue staining method. No obvious difference was found in the number of mast cells between skin samples of Imiq treated group and those in control group (Fig. 2B). Consistent with the result of microscopy stained with toluidine blue, no significant difference was found in histamine content in local skin and blood between Imiq treated mice and control mice (Fig. 2C). Therefore, the results suggested that Imiq treatment didn't induce histamine release from mast cells. We further examined the effect of Imiq on mast cells in DNFB-mediated CHS and found that DNFB sensitized skin and DNFB sensitized skin pretreated with Imiq demonstrated fewer mast cells compared with that from negative control (Fig. 6A). The reduced number of mast cells may due to the feature of toluidine blue staining method in which totally degranulated cells cannot be easily seen under microscope [44]. We verified the hypothesis by detecting histamine level in the blood and in the local skin of the three groups (Fig. 6B). Compared with the negative control group, the skin histamine content decreased in DNFB and Imiq + DNFB groups significantly. While the histamine level in blood increased significantly in DNFB and Imiq + DNFB groups. These results indicated an enhancement of DNFB-induced mast cell degranulation by Imiq pretreatment. In vitro PCMC degranulation assay demonstrated that DNFB induced histamine release by PCMCs, which is consistent with the study that DNFB directly induced degranulation of rat peritoneal mast cells [45]. Imiq alone could not induce PCMC degranulation, but adding it to DNFB solution promoted PCMC degranulation (Fig. 6C). Therefore, both in vivo and in vitro assays indicated that Imiq enhanced DNFB mediated mast cell degranulation. The signaling pathways of mast cell degranulation can be classified into two categories: IgE-dependent, tyrosine kinase cascade-mediated pathway and IgE-independent, Gi-mediated pathway. DNFB could activate phospholipase C via Gi activation and induce cytosolic Ca^{2+} mobilization, which are related with mast cell degranulation [45]. The combined treatment of DNFB and Imiq to PCMCs may form a positive feedback of signal transduction by TLR7 pathway and Gi- phospholipase C-Ca^{2+}pathway, which leads to enhanced mast cell degranulation and larger histamine secretion.

Imiq may lead to CHS enhancement in many ways. We identified Imiq application induced local inflammation and increased the number of DCs in the DLNs. Larger number of DCs in T cell area increases the probability of DC–T cell encounter and delivers a sustained stimulation through interactions with T cells [46]. Imiq acts on TLR7 of DC and transduces activation signal into cells through MyD88 dependent pathway, which induces activation of nuclear transcription factor-κB and secretion of IL-12, thus promoting migration and maturation of DC and differentiation of CTL [47].

The research indicated that Imiq combined with DNFB treatment induced larger amount of histamine release from mast cells. We further examined the roles of histamine on Imiq enhanced DNFB-induced CHS in mice. Cromolyn is a mast cell stabilizer which can inhibit mast cell degranulation. Pyrilamine and cimetidine antagonize the action of histamine by interaction with H1Rand H2R respectively. Pretreatment of the animals with cromolyn, pyrilamine and cimetidine before DNFB sensitization resulted in a significant decline of ear thickness compared with non-treated group (Fig. 7). Therefore, the results indicated that mast cell degranulation and the released histamine involved in the process of Imiq enhanced CHS. The role of cromolyn is greater than that of pyrilamine and cimetidine. The different efficiency of mast cell stabilizer and histamine antagonists in attenuation of CHS indicated that other intermediates such as TNF-α, IL-1 β may play roles in CHS induction besides histamine [22,44].

Many studies have explored the effects of histamine and histamine receptor antagonists on adaptive immune response from different perspectives. Most of the studies focused on the effect of histamine on T cell differentiation during effector phase of the immune response and indicated that histamine played regulatory roles in allergic diseases. In this study, we focused on the effect of Imiq combined with DNFB on CHS during the sensitization phase. Injecting pyrilamine and cimetidine before DNFB sensitization led to the inhibitory effect on CHS. A prerequisite for immune response is the migration of Ag-bearing-DC homing to DLNs. It is postulated that topical application of Imiq plus DNFB led to increased histamine release from mast cells in local skin, which can quickly act on H1 and H2 receptors expressed on adjacent DC, promote DC migration and benefit T cell sensitization [16,18]. The application of pyrilamine and cimetidine before sensitization antagonized the effect of histamine on DC and affect the migration of local DC to DLN, which resulted in the weakening of CHS [16,18]. In this study, mice were treated only once with antagonists, and the results were different from that in previous studies in which mice were treated repeatedly [48] or in histamine receptor gene deficiency mice [49]. The results were consistent with the report that Roxatidine (another H2R antagonist) inhibited CHS [50]. Dawicki et al. reported that a H2 receptor-dependent pathway controlled the selective influx of both plasmacytoid and CD11b + DC into the lymph node [31]. It has been reported that cimetidine may display anti-inflammatory properties to overcome the harmful inflammatory responses [51,52]. Histamine binding to H1R on DC promotes Ag presentation, cytokine production and Th1 priming ability [27,53]. Usage of H1R antagonist pyrilamine attenuated Imiq involved CHS may relate to the inhibitory roles to Ag presentation, cytokine production and Th1 priming ability of DCs.

In conclusion, Imiq enhanced DNFB mediated CHS and augmented DC migration to DLNs. Mast cell stabilizer and histamine receptor antagonist counteracted the process. Our findings suggest that some TLR7 related agonists may augment the intensity of CHS reaction. The mechanisms underlying these effects may relate to enhanced DC homing to DLNs and histamine release by mast cells. Blocking the action of histamine in early time of allergen contact is beneficial to the alleviation of CHS.

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