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Original Article

Thimerosal induces skin pseudo-allergic reaction via Mas-related G-protein coupled receptor B2

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ABSTRACT

Background: Thimerosal has been used as a preservative in many products which may cause contact dermatitis. It is the second most common allergen in positive patch test reactions, though being a clinical irrelevant allergen. Thimerosal-induced contact dermatitis is generally considered to be a delayed-type hypersensitivity reaction, but it is difficult to explain the fact that most patients develop an allergic reaction upon first encounter with thimerosal. Recent studies have demonstrated the association between Mas-related G protein coupled receptor X2 (MRGPRX2) and pseudo-allergic reactions which occur at the first contact with stimulation. This suggests the possibility that thimerosal may cause contact dermatitis via MRGPRX2 mediated mechanism.
Objectives: To investigate the role of Mas-related G-protein coupled receptor B2 (MrgprB2)/MRGPRX2 in contact dermatitis induced by thimerosal.
Methods: Thimerosal induced pseudo-allergic reactions via MrgprB2/ MRGPRX2 were investigated using a novel skin pseudo-allergic reaction mouse model, footpad swelling and extravasation assays *in vivo* and mast cell degranulation assay *in vitro*.
Results: Thimerosal induced contact dermatitis in dorsal skin and footpad swelling in wild-type mice, but had no significant effect in MrgprB2-knockout mice. Thimerosal-induced dermatitis is characterized by infiltration of inflammatory cells and elevation of serum histamine and inflammatory cytokines, rather than elevation of serum IgE level. Thimerosal increased the intracellular Ca^{2+} concentration in HEK293 cells overexpressing MrgprB2/MRGPRX2. Downregulation of MRGPRX2 resulted in the reduced degranulation of LAD2 human mast cells.
Conclusions: MrgprB2 mediates thimerosal-induced mast cell degranulation and pseudo-allergic reaction in mice. MRGPRX2 may be a key contributor to human contact dermatitis.

1. Introduction

A variety of ingredients could induce eczematous pruritic allergic reactions known as contact dermatitis, the prevalence of which is about 27.0% in European general population [1]. Thimerosal is a well-established antiseptic and antifungal agent. It has been used as a preservative in vaccines, immunoglobulin preparations, cosmetics, antivenins, ophthalmic and nasal products, and tattoo inks [2,3]. Most clinically relevant allergic reactions to thimerosal occur with cosmetics use [4–6] or after contact with ophthalmic preparations, which results in mostly a facial dermatitis [7]. While thimerosal was the second and third most common allergen in terms of positive patch test reactions in adults [8] and children [9], respectively, there was great concern

Abbreviations: CXCL2, chemokine C-X-C motif ligand 2; EBD, Evans blue dye; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; MrgprB2, Mas-related G-protein coupled receptor B2; MRGPRX2, Mas-related G-protein coupled receptor X2.

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about its clinical relevance, with only 16.8% of patients with sensitivity to thimerosal was considered relevant to their dermatitis, ranking thimerosal last in relevance among the 50 allergens tested by the North American Contact Dermatitis Group (NACDG) [10,11].

Mast cells play a critical role as regulators of allergic diseases due to their wide distribution in various tissues [12]. A recent study showed that MRGPRX2 expression in skin mast cells is at high levels in patients with chronic urticarial [13], which is a common allergic disease and is associated with mast cell degranulation. And numerous studies have demonstrated the association between MRGPRX2 and pseudo-allergic reaction [14–16], which has a close relationship with or similarity to allergic symptoms but without an underlying antigen-specific sensitization and IgE level elevation [14].

MRGPRX2, a member of the Mas-related gene family, was found to be expressed in sensory neurons of dorsal root ganglia, mast cells, and keratinocytes [17]. Its ortholog in mouse is MrgprB2. MRGPRX2 and MrgprB2 are expressed at high levels in mast cells in human and mouse skin, respectively [13,14]. Previous studies have shown that mouse MrgprB2 and the human MRGPRX2 are activated not only by basic secretagogues and neurokinins [17,18], but also a number of small-molecule drugs (such as antimicrobials, mivacurium) [18,19], producing direct mast cell degranulation and pseudo-allergic reactions. However, whether thimerosal can directly activate mast cells to induce contact dermatitis has not been reported.

In this study, we investigated whether thimerosal induces pseudo-allergic reactions directly through MrgprB2 and MRGPRX2 on mast cells *in vitro* and *in vivo*. This study may improve our understanding about the mechanism of allergic reaction to certain allergens, thus may help develop new therapeutic strategies for contact dermatitis.

2. Materials and methods

2.1. Drugs and reagents

Thimerosal was purchased from Meilun Biotechnology Co., LTD. Compound 48/80 and histamine were purchased from Sigma-Aldrich (St. Louis, USA). Histamine·2HCl (A, A, B, B-D4, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). Mass spectrometry-grade formic acid was obtained from Sigma. HPLC-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Pittsburgh, USA). Fluo-3, AM ester and Pluronic F-127 were obtained from Biotium (California, USA). *P*-Nitrophenyl *N*-acetyl-β-D-glucosamide and Triton X-100 reagents were procured from Sigma Aldrich Co., LLC (St. Louis, USA) and were prepared at the proper concentrations prior to use. All aqueous solutions were prepared using ultrapure water produced by an MK-459 Millipore Milli-Q Plus ultra-pure water system.

2.2. Mice

Mast Cell-Deficient C57BL/6 STOCK Kit$^{W-sh/W-sh}$ mice [20] were purchased from the Model Animal Research Center of Nanjing University. C57BL/6 MrgprB2-knockout mice were provided as a kind gift from the laboratory of Xinzhong Dong at Johns Hopkins University. Male C57BL/6 mice aged 8 weeks were purchased from the Experimental Animal Center of Xi'an Jiaotong University. The mice were housed in individual cages in a large colony room with 20–25 °C, a relative humidity of 40% and a day-night cycle of 12/12 h, and free access to water and were fed standard dry food twice per day. All animal experiments involving equal treatments were conducted by researchers who were blinded to the conditions.

2.3. Ethics statement

The experimental protocols involving mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2011-0045). 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. All animals were under pentobarbital sodium anesthesia when undergoing any operation.

2.4. Cell lines

MRGPRX2-HEK293 cells were constructed by HIV-1-based lentiviral vectors, and MrgprB2-HEK293 cell line was provided from the laboratory of Xinzhong Dong at Johns Hopkins University, both cells cultured by DMEM with 10% FBS. The Laboratory of Allergic Disease 2 (LAD2) human mast cells were kindly provided by A. Kirshenbaum and D. Metcalfe (NIH, USA). The cells were cultured in StemPro-34 medium supplemented with 10 ml/l StemPro nutrient supplement, 2 mM L-glutamine, 100 ng/ml human stem cell factor (hSCF), and 1:100 penicillin-streptomycin in an atmosphere containing 5% CO_2 at 37 °C.

2.5. Contact dermatitis mice model

8-Week-old male C57BL/6 mice and MrgprB2-knockout mice were anesthetized with an intraperitoneal injection of 80 mg/kg 1% pelltobarbitalum natricum. Dorsal skin was shaved off a day before the experiment start, with an area about 1.5 cm*2 cm. Thimerosal was prepared by vaseline to the concentration of 0.5%. Thimerosal was applied twice a day for 3 days. Skin and serum samples of each treated and control mouse were collected at the end of each day.

2.6. Histological analysis

The dorsal skin was washed with PBS and fixed with 4% formaldehyde for 48 h and subjected to H&E staining. Stained slides were 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, followed by incubation with 1/500 FITC-avidin for 45 min. Sections were washed 3 times with PBS, and a drop of Fluoro-mount G (Southern Biotech, AL. U.S.A) was added. Images were taken using a confocal scanning laser microscope (Nikon, Tokyo, Japan).

2.7. Analysis of mouse serum samples

Mouse IgE, TNF-α, MCP-1 and CXCL2 levels were determined by ELISA Kits purchased from Excell Bio CO., LTD (Beijing, China) according to provided instructions. An LCMS 8040 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) was used for the LC-ESI-MS/MS method. Histamine·2HCl was used as interior label. Histamine was evaluated with the system by employing an HILIC column (Venusil HILIC, 2.1 mm × 150 mm, 3 μm, Agela Technologies, Tianjin, China) and an isocratic elution with acetonitrile-water containing 0.1% formic acid and 20 mM ammonium formate (77:23, v/v) at a flow rate of 0.3 ml/min.

2.8. Hind paw swelling and extravasation

Male C57BL/6 mice, C57BL/6 STOCK Kit$^{W-sh/W-sh}$ mice and C57BL/6 MrgprB2-knockout mice were anesthetized with an intraperitoneal injection of 80 mg/kg 1% pelltobarbitalum natricum. Hind paw swelling and extravasation assay was performed as described previously [18]. Briefly, each mouse was injected intravenously (i.v.) with Evans blue dye in saline. Five microliters

of thimerosal (1.25, 2.5, 5 μg/ml) was administered into the left paw, saline into the right paw as a blank control. Fifteen minutes later, the paw thickness was measured again and mice were sacrificed for analysis.

2.9. β-Hexosaminidase assay

LAD2 cells were incubated in a 96-well plate overnight at 37 °C with 5% CO_2. Varying (1.25, 2.5, 5 μg/ml) concentrations of thimerosal were added into corresponding well, 30 μg/ml Compound 48/80 was used as a positive control, and Tyrode's solution buffer only was a negative control. Beta-hexosaminidase release assay was performed as described previously [18].

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

A SMART pool of double-stranded siRNAs targeting MRGPRX2 and nonspecific siRNAs were obtained from Shanghai GenePharma Co., Ltd. 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′-GUACAACAGUGAAUGGAAATT-3′ and reverse, 5′-UUUCCAUUCACUGUUGUACTT-3′ for MRGPRX2; and forward, 5′-UUCUCCGAACGUGUCACGUTT-3′ and reverse, 5′-ACGUGACAC-GUUCGGAGAATT-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. The cells were incubated for 48 h to allow the knockdown of MRGPRX2. These cells were then used for the β-hexosaminidase assay and histamine release assay.

2.11. Cytotoxicity assay

The plates were pre-incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2 before LAD2 cells were seeded into 96-well plates at a density of 5×10^3 cells per well. The plate was centrifuged at $200 \times g$ for 5 min at 4 °C and medium was removed. Cell viability was determined using Abbkine-Cell Counting Kit assays (California, USA). Each well was treated with 100 μl of thimerosal at different concentrations (0, 1.25, 2.5, 5, 10, 20 μg/ml) prepared by medium and incubated for 12 h in a humidified incubator at 37 °C, 5% CO_2. Next, 10 μl of Cell Counting Kit solution was added to each well followed by incubation for 2 h. Further, the relative cell viability was assessed by detection of absorbance at 450 nm using a microplate reader (Bio-Rad, Carlsbad, CA, USA). Survival rate of LAD2 cells was calculated as:

$$[(OD_{Treated} - OD_{Blank})/(OD_{Control} - OD_{Blank})] \times 100\%.$$

2.12. Intracellular calcium mobilization assay

MRGPRX2-HEK293 cells and MrgprB2-HEK293 cells were plated at 1×10^4 cells per well in a 96-well plate and incubated overnight at 37 °C with 5% CO_2. The incubation buffer consisted of 1 μl of Fluo-3, 3 μl of Pluronic F-127 and 996 μl of calcium imaging buffer (CIB). All drug substances were diluted to the required concentrations in CIB (125 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM HEPES, 20 mM glucose, 1.2 mM $NaHCO_3$ and 20 mM sucrose, with the pH adjusted to 7.4 using NaOH). The cells were then washed twice in 100 μl of CIB. Next, 100 μl of incubation buffer was added, and the plate was incubated for 30 min at 37 °C with 5% CO_2. Then, the cells were washed twice with CIB. The cells were used immediately for imaging. For the calcium imaging, the cells were magnified 200 times, and one photo per second was obtained under blue light. Cells were identified as responding if the calcium rose by at least 50% after the substances were injected.

2.13. Statistical analysis

Experiments were repeated 3 times. Data are presented as mean ± S.E.M. and analyzed using two-tailed unpaired student's *t*-test. An independent samples analysis of variance was used to determine statistical significance in comparisons of the data using the SPSS software. Differences were considered significant at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

3. Results

3.1. Thimerosal induces skin pseudo-allergic reaction in mice which is IgE-independent

Wild-type mice were topically treated with thimerosal or vaseline for 3 days, and excised skin and blood samples were collected each day. When thimerosal was applied to dorsal skin of wild-type mice to mimic the patch test practice in human, skin allergy was observed, with the most obvious redness and swelling on day 3 (Fig. 1A). While there was no obvious abnormality in control mice, profound inflammatory cell infiltration and vasodilation were observed in the skin histopathology of wild-type mice after 3 days of thimerosal treatment. Infiltrating inflammatory cells are mainly neutrophils, and a small number of eosinophils were also seen (Fig. 1B). Though there was no significant change in the number of mast cells, degranulation of mast cells increased dramatically in thimerosal treated skin when compared with control skin (Fig. 1C). Intriguingly, while serum histamine increased following thimerosal application in a time-dependent manner, there was no significant difference in IgE levels between thimerosal treated mice and control mice (Fig. 1D, E).

3.2. Pseudo-allergic reaction induced by thimerosal in mice is dose-dependent

To further investigate the pseudo-allergic reaction induced by thimerosal, we injected varying concentrations (0.62520 μg/ml) of thimerosal into the footpad of wild-type mice to see whether it could cause vasodilation and swelling. As shown in Fig. 2A, the swelling was observed in 15 min after the injection, which occurred in a dose-dependent manner (Fig. 2A, B). A significant swelling was observed from thimerosal at 2.5 μg/ml when compared to physiological saline solution (negative control), although much lower than compound 48/80 (positive control). The Evans blue dye (EBD) exudation also indicated that the vasodilation reaction is in a dose-dependent manner (Fig. 2C). These findings indicated that thimerosal could induce local pseudo-allergic reactions in wild-type mice.

3.3. MrgprB2-mediated mast cells activation contributes to pseudo-allergic reaction induced by thimerosal

To study the function of mast cell in pseudo-allergic reaction induced by thimerosal, we used a mast cell-deficient mice in this study. The footpad swelling and EBD exudation of the $Kit^{W-sh}/^{W-sh}$ mice induced by thimerosal were similar to those observed in the vehicle control (Fig. 3A), which indicated that mast cells play a major role in thimerosal-induced pseudo-allergic reaction. Since MrgprB2 is homologous to MRGPRX2 on human mast cell, a comparison between wild-type mice and MrgprB2-knockout mice was performed to further elucidate the target involved in thimerosal-induced pseudo-allergic reaction. The footpads of MrgprB2-knockout mice injected with thimerosal did not swell

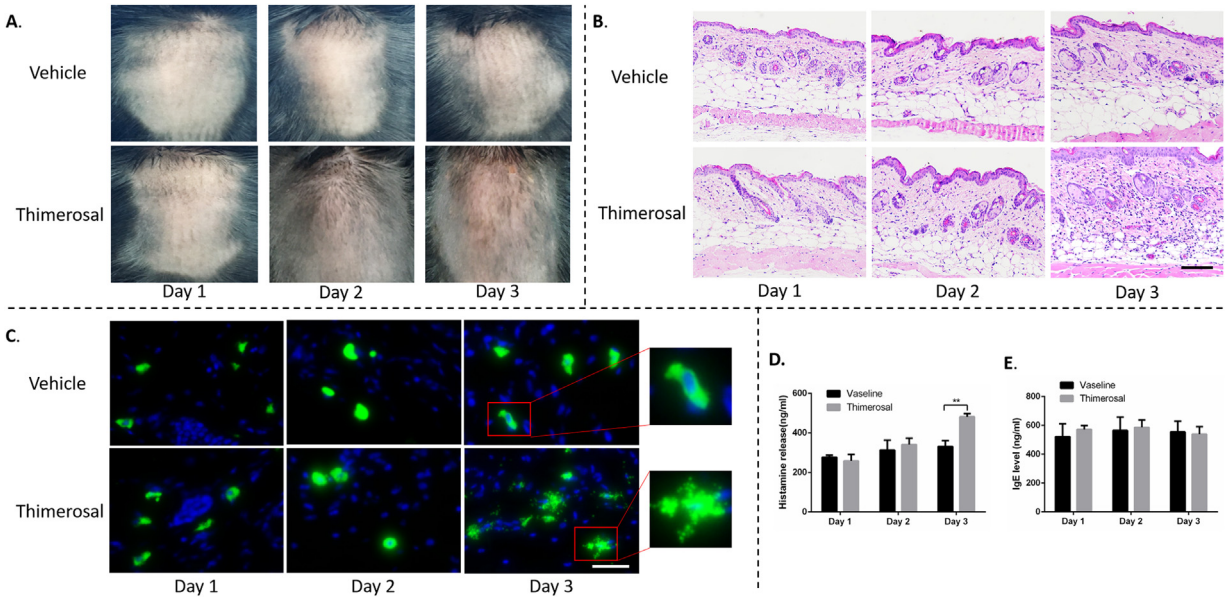

Fig. 1. Thimerosal induced IgE-independent skin pseudo-allergic reaction in mice. Thimerosal prepared by vehicle was set as treated group, and vehicle only was set as control group. All experiments were repeated 3 times, 6 mice per group. (A) Representative images of skin pseudo-allergic reaction after thimerosal application. (B) Thimerosal increased permeability of the vessel and inflammatory cell infiltration analyzed by H&E staining. Scale bar, 100 μm. (C) Thimerosal induced skin MCs degranulation analyzed by avidin-FITC staining: an inactive mast cell appears as a well-defined round or oval cell, and a degranulated mast cell appears as a cluster of scattered small dots. Scale bar, 20 μm. (D) Thimerosal increased histamine release in serum. (E) Thimerosal did not increase IgE level in serum.

and showed no obvious EBD exudation or local pseudo-allergic reactions when compared to WT mice (Fig. 3B).

3.4. Skin pseudo-allergic reaction induced by thimerosal was reduced in MrgprB2 knockout mice

To further investigate whether thimerosal induced skin pseudo-allergic reaction through MrgprB2, thimerosal was topically applied to dorsal skin of MrgprB2-knockout mice for consecutive 3 days. Compared to wild-type mice, infiltrated inflammatory cells and vasodilation were much lower in the skin of MrgprB2-knockout mice, degranulation of mast cells reduced as well (Fig. 4A). Similarly, the serum histamine was reduced in MrgprB2-knockout mice compared to that in WT mice, while the IgE level has no difference between them (Fig. 4B, C). The levels of inflammation related cytokines, including TNF-α, MCP-1, and CXCL2, in serum of MrgprB2-knockout mice were much lower than those of WT mice (Fig. 4D–F), which indicated a reduced skin

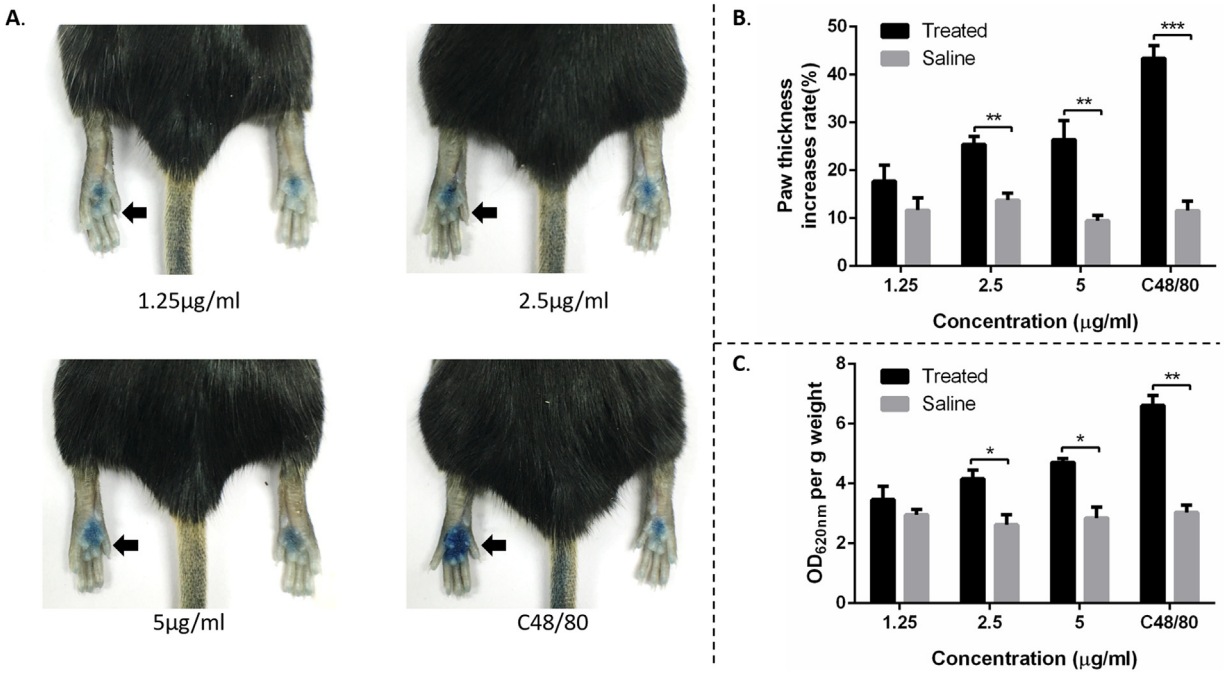

Fig. 2. Thimerosal increased pseudo-allergic reactions in wild type mice. Pseudo-allergic reaction of mice which were treated with 1.25, 2.5, 5 μg/ml of thimerosal, and 30 μg/ml of C48/80 as positive control. (A) Representative images of EBD extravasation 15 min after intraplantar injection of different concentrations of thimerosal (left, arrowhead) or saline (right). (B–C) The quantification of EBD leakage and the paw thickness increase after 15 min. (n = 6 per group).

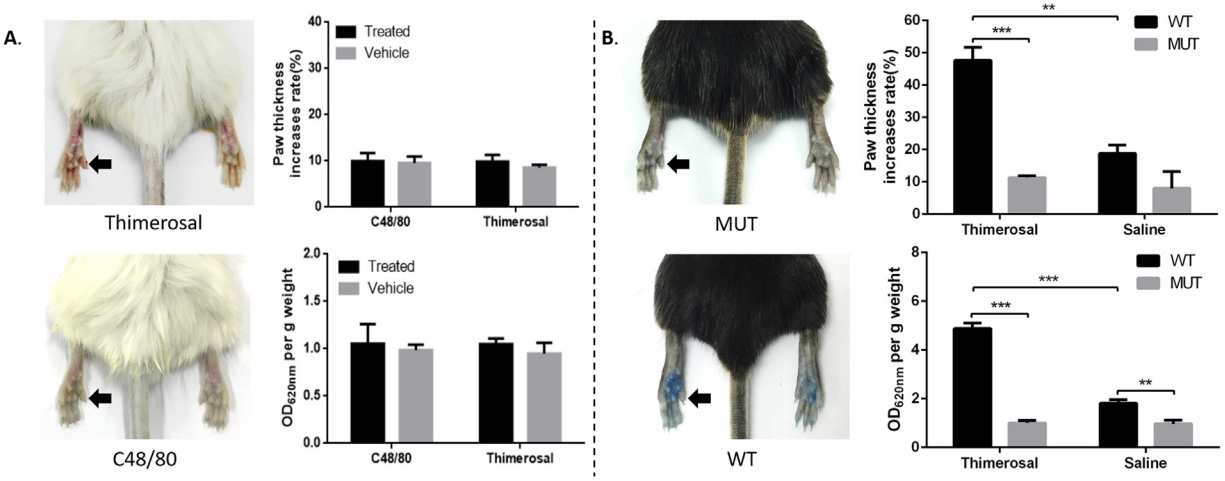

Fig. 3. MrgprB2 mediated pseudo-allergic reaction induced by thimerosal. kit$^{W-sh/W-sh}$ mice and MrgprB2-/- (MUT) mice were injected with 5 μg/ml of thimerosal in the left paw (arrowhead) and saline was administered in the right paw as a negative control, 30 μg/ml C48/80 as positive control. (A) Thimerosal could not induce pseudo-allergic reaction in kit$^{W-sh/W-sh}$ mice. (B) Thimerosal could not induce pseudo-allergic reaction in MrgprB2-/- (MUT) mice. (n = 6 per genotype).

pseudo-allergic reaction in MrgprB2-knockout mice. Collectively, these results strongly suggest that thimerosal induces pseudo-allergic reaction through MrgprB2 in mice.

3.5. Thimerosal induced human mast cell degranulation via MRGPRX2 in a dose-dependent manner

Further experimental study revealed that varying concentrations (1.25, 2.5, 5 μg/ml) of thimerosal increased the intracellular Ca^{2+} concentration in HEK293 cells overexpressing MrgprB2/MRGPRX2 (Fig. 5A, B). Interestingly, the intracellular Ca^{2+} concentration in MrgprB2-HEK293 cells is much higher than that in MRGPRX2-HEK293 cells upon the same stimulation (Fig. 5B), this may implicate that mice are more sensitive to thimerosal than humans. Given that thimerosal may cause apoptosis in human neurons and fibroblasts [21], we conducted cytotoxicity assay which indicated that no cytotoxic effect on LAD2 cells under the treatment of thimerosal at concentrations of 1.25, 2.5, and 5 μg/ml (Fig. 5C). Moreover, thimerosal induced LAD2 cells degranulation *in vitro* under certain concentration, which led to the dose-dependent release of β-hexosaminidase (Fig. 5D). Transfection with siRNA was used to verify the target of thimerosal in human mast cells. MRGPRX2 expression in LAD2 cells was down-regulated by siRNA, compared to LAD2 cells transfected with nonfunctional siRNA. At the same concentration, the release rate of β-hexosaminidase by MRGPRX2-knockdown LAD2 cells was much

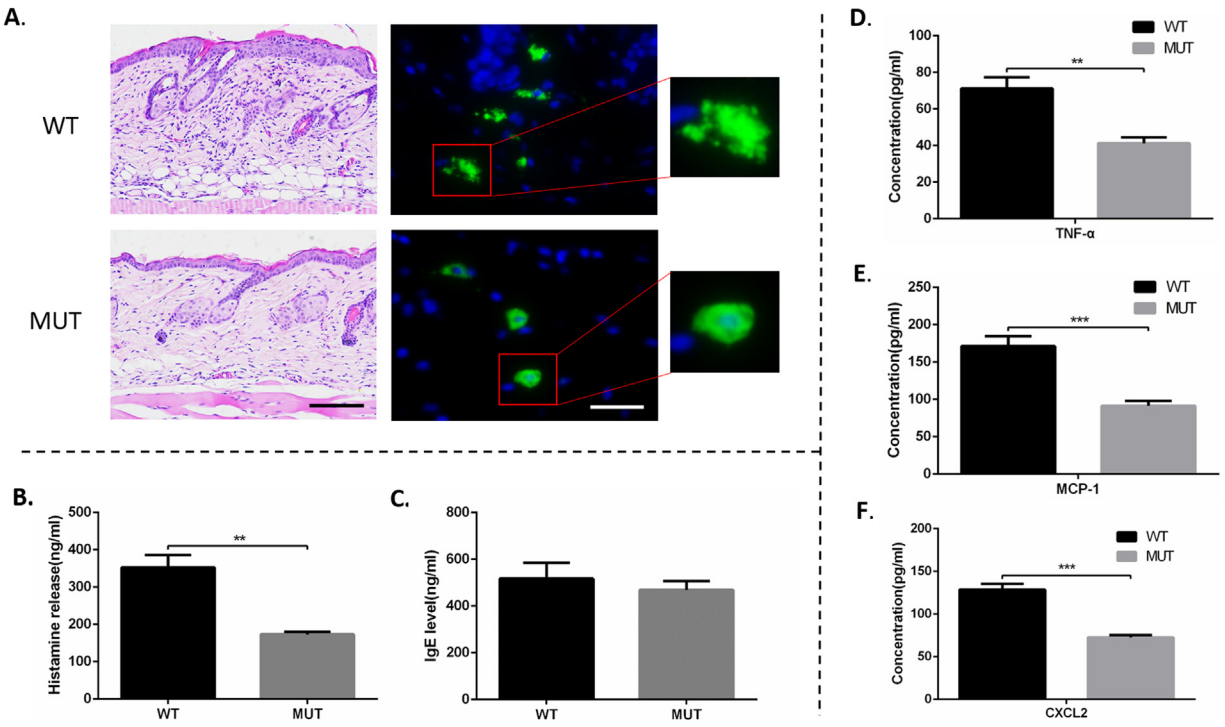

Fig. 4. Skin pseudo-allergic reaction reduced in MrgprB2 knockout mice. Wild-type (WT) and MrgprB2-/- (MUT) mice treated with thimerosal. (A) Thimerosal could not increase permeability of vessels and mast cells degranulation in MUT mice. Black scale bar, 100 μm. White scale bar, 20 μm. (B) Serum histamine of MUT mice was significantly lower than the WT mice. (C) Serum IgE of MUT mice had no difference with the WT mice. (D–F) Serum TNF-α, MCP-1 and CXCL2 of MUT mice were significantly lower than WT mice. (n = 6 per genotype).

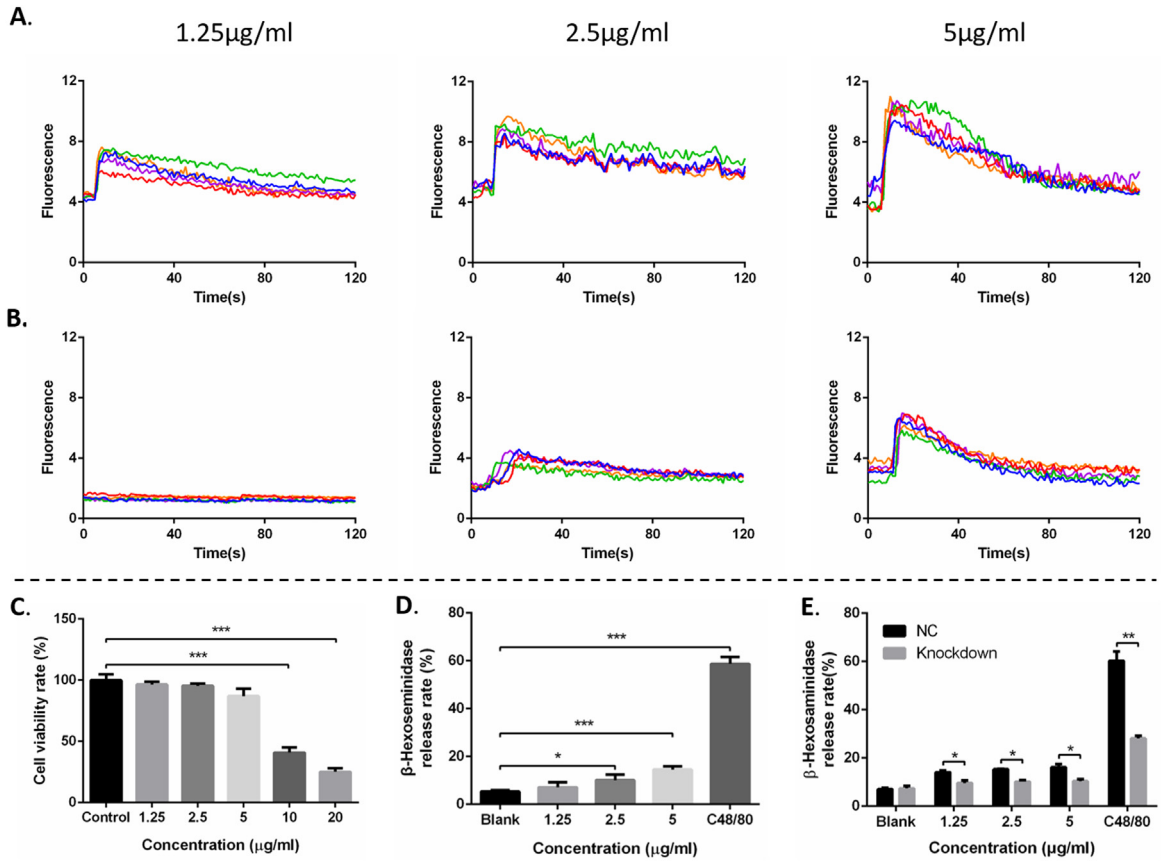

Fig. 5. Thimerosal induced mast cell degranulation via MRGPRX2. (A–B) Thimerosal increased intracellular calcium concentrations in HEK cells overexpressing MrgprB2 (A) or MRGPRX2 (B) dose-dependently. Example traces of intracellular calcium concentrations measured by Fluo-3 imaging are shown with each color line representing an individual cell. (C) Thimerosal showed no cytotoxic effect on LAD2 cells in the concentration of 1.25, 2.5, 5 μg/ml in 12 h. (D) β-hexosaminidase release from LAD2 cells treated with different concentrations of thimerosal. (E) β-hexosaminidase release in NC-LAD2 and MRGPRX2-knockdown LAD2 cells treated with thimerosal, the dose was set as 1.25, 2.5, 5 μg/ml and Tyrode solution buffer was set as negative control, 10 μg/ml of C48/80 as positive control.

lower than that by NC-LAD2 cells, with a significant decrease in thimerosal-induced degranulation (Fig. 5E). The knockout of MrgprB2/MRGRPX2 renders mast cells less responsive to the stimulation. As a ligand for MrgprB2/MRGRPX2 and functioning through MrgprB2/MRGRPX2 pathway, positive control C48/80 also showed significantly reduced response (Fig. 5E).

4. Discussion

Mast cell plays a crucial role in allergic diseases, when mast cell is activated, it releases a variety of chemokines, cytokines, and growth factors, which cause clinical allergic symptoms [16]. The differences in clinical allergic symptoms depends on different activation routes of mast cells. In this study, we constructed a skin pseudo-allergic mouse model using MrgprB2-knockout mice, demonstrating that thimerosal causes skin pseudo-allergic reactions in mice by activating MrgprB2 on mast cells. In addition, we found that thimerosal could also activate LAD2 human mast cell line via MRGPRX2, and the degranulation of these cells.

Thimerosal is one of the most common contact allergens which may cause allergic contact dermatitis. It is generally thought that allergic contact dermatitis is caused by type IV delayed-type hypersensitivity responses to antigens that come into contact with the skin [22,23]. Contact hypersensitivity mouse model was widely used to study the allergic contact dermatitis, in which the potent compounds are applied directly to the shaved abdominal skin of the experimental mouse over a period of 1 or 2 days (sensitization phase), then an elicitation response is induced by application of the same compound to the ear or footpad after 5–7 days [23,24]. To determine if thimerosal could activate mast cell directly, which may implicate an immediate hypersensitivity reaction, we developed a novel dermatitis mouse model. In our model, 0.5% thimerosal was applied to non-sensitized mouse dorsal skin for consecutive 3 days to mimic the patch test practice in human. Our results indicated that mouse skin is allergic to thimerosal. Even though serum histamine was significantly increased, serum IgE was not significantly changed. Given the allergic reaction to thimerosal at the first encounter, the comparable IgE level to normal control, and dose-dependent reaction in local swelling, we believe that thimerosal could induce pseudo-allergic reaction in mouse.

The mechanism of pseudo-allergic reaction is not yet completely understood. Most of the time pseudo-allergic reaction is a candidate for consideration in IgE-independent allergic reactions [14,25], but given the symptoms of pseudo-allergy, the most likely explanation could be the hypothesis that certain antigens activate the effector cells of allergic inflammation, such as mast cells, without evidence of antigen-specific IgE, IgG or T cells [26]. Recent studies linked the pseudo-allergic reaction to a single receptor on mast cell, known as MRGPRX2 in human. MRGPRX2 is mostly associated with the tryptase/chymase positive MCs (MC_{TC}) that are predominant in the skin [14,15,27]. MRGPRX2 can be activated by various peptides encompassing drugs, antimicrobial peptides, and neuropeptides like Substance P (SP), making the receptor quite universal [17,19,28]. While thimerosal caused footpad swelling under local

injection in wild-type mice, we found that neither mast cell-deficient mice nor MrgprB2-knockout mice responded to local thimerosal injection. These results indicate that MrgprB2 may be a target in thimerosal induced allergic reaction in mice. When thimerosal was topically applied to MrgprB2-knockout mice, infiltrated inflammatory cells, vasodilation, and mast cells degranulation were reduced than in wild-type mice. Consistent with this, the serum histamine, inflammation related factors including TNF-α, MCP-1, and CXCL2 (the functional homologues of human IL-8 in mouse [29]) in MrgprB2-knockout mice were much lower as well. It has been demonstrated that lack of MrgprB2 does not affect the degranulation and cytokine production ability of mast cells via IgE-mediated signaling [14]. Taken together, these results strongly suggest that thimerosal induces pseudo-allergic reaction through MrgprB2 in mice. To further study whether MRGPRX2 on human mast cells plays a similar role, we used HEK293 cells overexpressing MrgprB2/MRGPRX2 to see intracellular calcium mobilization, and found that thimerosal increased the intracellular Ca^{2+} concentration in a dose-dependent manner in these cells. Moreover, thimerosal induced LAD2 human mast cell degranulation *in vitro* under certain concentrations, leading to the dose-dependent release of β-hexosaminidase, and a consistent trend was observed in MRGPRX2 down-regulated LAD2 cells, which indicated that thimerosal may induce pseudo-allergic reaction in human via MRGPRX2. In addition, mercury was reported to induce inflammatory mediator release from human mast cells [30]. This, combined with our results, suggests that all mercury compounds may have the potential to induce mast cell-related allergic reactions.

Despite the positive patch test reaction prevalence is as high as 5% [1], thimerosal has been removed from the NACDG and Mayo Clinic standard series because of its high clinical irrelevance [11,31,32]. One possible explanation is that patients who test positive to thimerosal have either been exposed to products containing thimerosal as a preservative or have been asymptomatically sensitized to thimerosal as a result of vaccinations [2,33,34]. Our results support another possibility that thimerosal can induce skin pseudo-allergic reaction at the first encounter by activating mast cells. This explanation doesn't account for all patients with sensitivity to thimerosal, as there are some who are not allergic at the first encounter and only develop symptoms at a later encounter. We don't know what percentage of patients with sensitivity to thimerosal experienced pseudo-allergy, and whether they experienced only pseudo-allergic reaction or presented other symptoms. These issues will be further investigated using clinical samples.

In summary, we developed a new contact dermatitis mouse model and demonstrated that MrgprB2 on mast cells may play an important role in thimerosal-induced dermatitis in mice. Although dermatitis occurred in mice after the first exposure to thimerosal, we chose not to use "irritant contact dermatitis" because of the involvement of mast cells. In our model, thimerosal causes dermatitis by a so called pseudo-allergic reaction instead of generally believed type IV delayed-type hypersensitivity responses. We also found that thimerosal can activate LAD2 human mast cell via MRGPRX2 leading to mast cell degranulation, which may indicate that thimerosal can cause pseudo-allergic reactions in human via MRGPRX2. Based on our results, we hypothesized that there may be two mechanisms that cause contact dermatitis in general population, namely pseudo-allergic reaction and delayed-type hypersensitivity response. But it is not clear whether these two mechanisms are co-existing in the same individual who is allergic to thimerosal. Ourinvestigation provides a novel approach to explore contact dermatitis, and demonstrates that targeting MRGPRX2 is promising for the treatment of contact dermatitis.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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