# Dual effect of tacrolimus on mast cell-mediated allergy and inflammation through MAS-related G protein-coupled receptor-X2

Xueshan Du[1], Delu Che[1], Yi Zheng[1], Yong Hao[1], Tao Jia[1], Xinyue Zhang[1], Bin Peng[1], and Songmei Geng[1]

[1]Department of Dermatology The Second Affiliated Hospital of Xi'an Jiaotong University Xi'an China

June 1, 2022

## Abstract

**Background:** Topical tacrolimus has been widely used in the treatment of inflammatory and immune dermatoses for its immunosuppression effect. However, a transient irritation like itching, burning induced by tacrolimus is common when initial application, which is similar to pseudo-allergic reaction. MAS-related G protein-coupled receptor-X2 (MRGPRX2) in mast cells (MCs) mediates drug-induced pseudo-allergic reaction and IgE-independent pruritis in chronic skin diseases. Whether MRGPRX2 participates in above tacrolimus adverse reaction should be addressed. Further, immunosuppression mechanism of tacrolimus on MCs is greatly ignored. **Methods:** Wild-type (WT) mice, kit $^{w\text{-}sh/w\text{-}sh}$ mice and MrgprB2 deficient (MUT) mice were applied to explore the mechanism of initial irritant reaction and immunosuppression of tacrolimus on the skin MrgprB2 in MCs *in vivo*. LAD2 cells and MRGPRX2-knockdown LAD2 cells were used to confirm the regulation of MRGPRX2 by tacrolimus *in vitro*. **Results:** Tacrolimus could trigger IgE-independent dermatitis when initial application through MrgprB2-related MCs degranulation. Using FK-DNS, a fluorescently labeled tacrolimus, we found tacrolimus could bind to MRGPRX2 directly. Interestingly, after long-term tacrolimus treatment, the initial itching and inflammatory reaction faded away without IgE change. Hence, longstanding treatment with tacrolimus suppressed MRGPRX2/B2 expression and decreased inflammatory cytokines release. **Conclusion:** Our study provides for the first time a novel target for tacrolimus, demonstrating that short-term tacrolimus treatment induces pseudo-allergic reaction via MRGPRX2/B2 in MCs, while long-term treatment dampens MRGPRX2/B2 expression, leading to decreased inflammatory cytokines release and immune cells recruitment, which may contribute to its potent immunosuppression effect in the treatment of inflammatory and immune skin diseases.

## Dual effect of tacrolimus on mast cell-mediated allergy and inflammation through MAS-related G protein-coupled receptor-X2

Xueshan Du[1, *], Delu Che[1,2, *], Yi Zheng[1], Yong Hao[3,1], Tao Jia[1], Xinyue Zhang[1], Bin Peng[1, #], Songmei Geng[1,2, #]

[1] Department of Dermatology, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

[2] Center for Dermatology Disease, Precision Medical Institute, Xi'an, China.

[3] Department of Dermatology, The Second Affiliated Hospital of Baotou Medical College, Baotou, Inner Mongolia, China

*These authors contribute equally to this work

**Corresponding author:** Bin Peng, Tel: +86 186 2969 1372,*1200006589@xjtu.edu.cn*; Songmei Geng, Tel: +86 130 6042 3612, E-mail:*gsm312@yahoo.com.*

**Short title:** Tacrolimus regulates MAS-related G protein-coupled receptor-X2 in allergy and inflammation

**Acknowledgement:** This work was supported by the National Natural Science Foundation of China (Grant number: 82073451). And Key R&D plan of Shaanxi Province (Grant number: 2020SF-175). We also gratefully acknowledge the support of Research Fund of the Second Affiliated Hospital of Xi'an Jiaotong University and Chinese Association of Rehabilitation Medicine (Grant number: D201901).

**Abstract (246)**

**Background:** Topical tacrolimus has been widely used in the treatment of inflammatory and immune dermatoses for its immunosuppression effect. However, a transient irritation like itching, burning induced by tacrolimus is common when initial application, which is similar to pseudo-allergic reaction. MAS-related G protein-coupled receptor-X2 (MRGPRX2) in mast cells (MCs) mediates drug-induced pseudo-allergic reaction and IgE-independent pruritis in chronic skin diseases. Whether MRGPRX2 participates in above tacrolimus adverse reaction should be addressed. Further, immunosuppression mechanism of tacrolimus on MCs is greatly ignored.

**Methods:** Wild-type (WT) mice, $kit^{w\text{-}sh/w\text{-}sh}$mice and MrgprB2 deficient (MUT) mice were applied to explore the mechanism of initial irritant reaction and immunosuppression of tacrolimus on the skin MrgprB2 in MCs *in vivo* . LAD2 cells and MRGPRX2-knockdown LAD2 cells were used to confirm the regulation of MRGPRX2 by tacrolimus *in vitro* .

**Results:** Tacrolimus could trigger IgE-independent dermatitis when initial application through MrgprB2-related MCs degranulation. Using FK-DNS, a fluorescently labeled tacrolimus, we found tacrolimus could bind to MRGPRX2 directly. Interestingly, after long-term tacrolimus treatment, the initial itching and inflammatory reaction faded away without IgE change. Hence, longstanding treatment with tacrolimus suppressed MRGPRX2/B2 expression and decreased inflammatory cytokines release.

**Conclusion:** Our study provides for the first time a novel target for tacrolimus, demonstrating that short-term tacrolimus treatment induces pseudo-allergic reaction via MRGPRX2/B2 in MCs, while long-term treatment dampens MRGPRX2/B2 expression, leading to decreased inflammatory cytokines release and immune cells recruitment, which may contribute to its potent immunosuppression effect in the treatment of inflammatory and immune skin diseases.

**KEY WORDS:** Tacrolimus, MRGPRX2/B2, mast cell, inflammation, itching

**Introduction (3755)**

Tacrolimus, a 23-member macrolide produced by *streptomyces tsukabaensis,* is a potent immunosuppressant. It mainly suppresses the responsiveness of T lymphocytes to antigens by forming an FK506-binding protein/tacrolimus complex to inhibit calcineurin, as well as inhibits the release of inflammatory mediators from mast cells and basophils, and downregulates the expression of IL-8 receptor and FcεRI on Langerhans cell, which is important in many inflammatory diseases[1]. Currently, for its potent effects as immunosuppressant and anti-inflammation, topical tacrolimus has been used in the treatment of various chronic skin diseases, such as atopic dermatitis (AD), allergic contact dermatitis (ACD), lichen planus and psoriasis, etc[2]. Actually, tacrolimus could reduce the inflammatory reaction and chronic pruritus in AD and psoriasis.

MCs play important roles in allergic and inflammatory diseases[3,4]. Classically, IgE-dependent MC activation is the main mechanism in allergy. However, chronic pruritus in AD and psoriasis is mainly mediated by non-lgE dependent pathway[5]. Besides, more and more researchers have emphasized the importance of this non-lgE dependent pathway of MC stimulation and its role in persistent chronic skin inflammation and disease exacerbation[6]. Traditionally, tacrolimus could inhibit IgE-dependent MCs activation. Recently, it has been reported that topical tacrolimus could alleviate itch-scratch circle via blocking sensory nerve fibers elongation and desensitization as well as pruritus mediator IL-31 in chronic inflammatory pruritic skin disease[7-9] . However, the effect of tacrolimus on IgE-independent MCs activation has not been detected.

Notably, side effects such as mild to moderate skin irritation, itching, and burning induced by topical tacrolimus application often occur rapidly in the first few days and fade hereafter, which is similar to drug pseudo-allergy reaction[10,11]. Pseudo-allergic reaction often manifests as transient itching, skin flushing and vascular permeability in a dose-dependent manner[12,13].

Mas-related G protein-coupled receptor-X2 (MRGPRX2), expressed on mast cells, has been identified as a main receptor mediating pseudo-allergic reaction[14]. MRGPRX2 (or its murine equivalent MrgprB2) can be activated by multiple agonists such as mastoparan, substance P (SP) , compound 48/80 (C48/80), and some FDA approved drugs[15]. Compared to IgE dependent signaling pathway, MRGPRX2 activates MCs degranulation in a non-IgE dependent manner with more tryptase released instead of histamine[16]. Growing findings indicate MRGPRX2 on MCs may play a pivotal role in the pathological process of chronic inflammatory and pruritic skin diseases[14,17]. Recently reinforced by insights into the structure of MRGPRX2, MRGPRX2 can be identified as a crucial therapeutic target for chronic inflammatory dermatitis and itch[18].

Together, it seems paradoxical that tacrolimus induces initial inflammation meanwhile as an immunosuppressant suppressor. Our hypothesis is that MRGPRX2 in MCs is a vital target for tacrolimus dual effect to activate and suppress the MCs mediated inflammation in skin with time dependent way.

## Methods

### Mouse model

Male C57BL/6 mice aged 8 weeks were purchased from the Experimental Animal Center of Xi'an Jiaotong University. Mast Cell-Deficient C57BL/6 STOCK $Kit^{W\text{-}sh/W\text{-}sh}$mice and MrgprB2 deficient (MUT) mice were purchased from the Model Animal Research Center of Nanjing University. The mice were housed in individual cages in a colony room with 20–25 , a relative humidity of 40%, and were fed standard dry food twice per day.

Adult male mice weighing 18-22 g were anesthetized with an intraperitoneal injection of pelltobarbitalum natricum. Dorsal skin was shaved off a day before the experiment start, and the mice were randomly divided into different groups (6 mice per group).

For tacrolimus short-term application, experimental groups were treated with topical 0.03%, 0.1%, 0.3% tacrolimus prepared by glycerine respectively twice a day, while glycerine as solvent was given to control group. Scratching bouts were counted, skin and serum samples of each treated and control mouse were collected at the end of 1 day. For tacrolimus long-term application, experimental groups were treated with topical 0.03% twice a day. C48/80 was used for subcutaneous injection on day 7 and 11, scratching bouts were counted later and skin samples were collected respectively. The experimental protocols involving mice were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-711).

Finally, serum and skin samples were further used to detect inflammatory cytokines release and MrgprB2 mRNA expression. What's more, H&E staining and avidin staining were performed using collected skin samples.

### Hind paw swelling and extravasation

The experiment was performed as described previously[19]. Briefly, male C57BL/6 mice, MUT and $Kit^{W\text{-}sh/W\text{-}sh}$ mice were anesthetized with pelltobarbitalum natricum. Fifteen minutes after the induction of anesthesia, each mouse was injected intravenously (i.v.) with 0.2 ml Evans blue dye in saline. Then, 5 μl of tacrolimus (31.25, 62.5, 125 μM) or C48/80 (30 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 further analysis.

### Cell lines and cell culture

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 . MrgprB2-expressing HEK293 cells and NC-HEK293 cells were constructed by HIV-1-based lentiviral vectors. Both the cells cultured by DMEM. Human mast cell line-1 (HMC-1) was purchased from BLUEFBIO (Shanghai, China).

$1 \times 10^6$ LAD2 cells were incubated in a 96-well late overnight at 37  with 5% $CO_2$, then the culture medium was removed. For tacrolimus short-term use, LAD2 cells (and MRGPRX2-knockdown LAD2 cells) were treated with 25-, 50-, 100 uM tacrolimus diluted in Tyrode's solution for 30 minutes, and 30 μg/ml C48/80 was used to set positive control. For tacrolimus long-term use, LAD2 cells were treated with 0.5 nM tacrolimus or vehicle respectively for 3 d. Then, cells and culture medium were collected.

Cells were used to detect intracellular calcium mobilization assay, western blot and real time qPCR analysis. Moreover, culture medium was used to test β-hexosaminidase release and inflammatory cytokines release.

**Small interfering (si)RNA transfection of LAD2 cells**

The siRNAs targeting MRGPRX2 leading to specific knockdown or 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-ACGUGACACGUUCGGAGAATT-30; MRGPRX2 knockdown siRNA, forward, 50-GUACAACAGUGAAUG GAAATT-30, and reverse, 50-UUUCCAUUCACUGUUGUACTT-30. For transfection, the siRNAs were delivered using Lipofectamine® 2000 transfection reagent in accordance with the manufacturer's instructions. Cells were then used for the β-hexosaminidase assay, tryptase and cytokines release assay.

**Molecular docking**

Molecular docking study was carried out using the SYBYL-X 2.0 version to explore the binding mode of tacrolimus with MRGPRX2. The Xray crystal structure of MRGPRX2 (PDB code: 7S8N) was downloaded from PDB Bank. Water molecules were removed, and hydrogen was added. Tripos force field and Pullman charge were applied to minimize the molecular energy. Each drug molecule was depicted by the Sybyl/Sketch module (Tripos Inc.), optimized by Powell's method with the Tripos force field with convergence criterion at 0.005 kcal/(Å mol), and assigned using the Gasteiger–Hückel method.

**FK506-DNS synthesis**

FK506-DNS, a novel derivative of FK506, was constructed as previous studies[20]. A dansyl moiety was covalently bound via cadaverine to the C22 position of the FK506 molecule in FK506-DNS. Detailed synthetic procedure was shown in Figure S1.

**Statistical analysis**

All the experiments were repeated at least 3 times. Data are expressed as mean ± SEM 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 $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

Details about other methods including reagents, scratching bouts, histological analysis, β-hexosaminidase assay, intracellular calcium mobilization assay, ELISA, western blot and real time qPCR analysis are provided in the Data S1

**Results**

**Tacrolimus could induce dermatitis mediated mainly by lgE-independent MCs degranulation *in vivo***

WT mice were applied by different concentrations of tacrolimus and vehicle for 1 day on dorsal skin to mimic tacrolimus clinical application. Although no visible erythema and scaling difference were observed between control group and tacrolimus treated groups (Figure 1A), skin inflammatory cells infiltration (Figure 1B) and degranulation of MCs were more significant in treated groups (Figure 1C). Moreover, scratching bouts increased dose-dependently in tacrolimus group (Figure 1D). Nevertheless, this rapid MCs activation wasn't accompanied by remarkable elevated IgE levels between control group and treated groups (Figure 1D). However, the levels of tryptase, MCP-1 and TNF-α were upregulated dose-dependently (Figure 1E). Hence, initial topical tacrolimus application induced itching and inflammatory reaction without IgE change.

MCs play an important role in inflammation and pruritus. In order to further verify effect of tacrolimus on MCs, hind paw swelling and extravasation experiment was used. Some researchers have found even 0.01 % (125 μM) tacrolimus could enhance neuropeptide (SP) homogenate concentrations in healthy tissue[21]. Hence, descending concentrations of tacrolimus solution were used in hind paw swelling and extravasation experiment. We found an obvious swelling in left hind paw injected with tacrolimus or C48/80 in WT mice, compared with right hind paw treated with saline solution (Figure 1F). Besides, paw thickness increase rate for tacrolimus injection was dose-dependent, and tacrolimus increased vascular permeability *in vivo* , as assayed by Evans Blue dye (EBD) extravasation, which was also dose-dependent (Figure 1G). Here, we demonstrated that tacrolimus could activate MCs*in vivo* .

$Kit^{W\text{-}sh/W\text{-}sh}$ mice, MCs deficient mice, were used to explore the role of MCs in tacrolimus mediated dermatitis, which were applied with tacrolimus on dorsal skin for one day. There were no obvious erythema and scaling in $Kit^{W\text{-}sh/W\text{-}sh}$ mice group (Figure 2A). Also, skin inflammatory cells infiltration (Figure 2B), scratching behaviors, and the levels of tryptase, MCP-1 and TNF-α in serum didn't increase after one-day tacrolimus application in $Kit^{W\text{-}sh/W\text{-}sh}$ mice group compared with WT mice (Figure 2C). There was no significant different IgE level in serum between any two groups (Figure 2C). Moreover, in hind paw swelling and extravasation experiment, $Kit^{W\text{-}sh/W\text{-}sh}$ mice showed no prominent difference in paw swelling and EBD exudation between left (injected tacrolimus) and right (injected saline solution) hind paws of themselves, whereas they both showed decreased paw swelling and extravasation when compared to WT mice (injected tacrolimus) (Figure 2D).

Collectively, topical tacrolimus application triggered dermatitis and transient itching via non-IgE dependent MCs activation.

**MRGPRB2/X2 participates in tacrolimus induced MCs activation**

Further, we set out to detect whether MrgprB2 participates in MCs activation induced by tacrolimus as mentioned above. MrgprB2 deficient mice (MUT mice) were employed here, in hind paw swelling and extravasation experiment, MUT mice showed no significant difference in paw swelling and EBD exudation between left (injected tacrolimus) and right (injected saline solution) hind paws of themselves in hind paw swelling and extravasation experiment, whereas they both exhibited decreased paw swelling and extravasation when compared to WT mice (injected tacrolimus) (Figure 3A). Therefore, we concluded that tacrolimus could activate MrgprB2 leading to local inflammatory reaction induced by MCs.

Besides, MUT mice were also applied with tacrolimus on dorsal skin to mimic tacrolimus clinical use. After one day application of tacrolimus on dorsal skin of WT mice and MUT mice, no visible erythema and scaling were observed in all groups (Figure 3B). However, inflammatory cells infiltration was more obvious in WT group compared with MUT mice (Figure 3C). With avidin staining, no obvious MCs degranulation could be observed in MUT mice (Figure 3D). Significantly, MUT mice didn't show increased scratching behaviors, as well as serum tryptase, MCP-1 and TNF-α after one-day tacrolimus application (Figure 3E). IgE level in serum didn't differ significantly between any two groups (Figure 3E). Hence, MrgprB2 plays a vital role in initial dermatitis and MCs stimulation triggered by topical tacrolimus.

Considering the rapid response of MrgprB2 to tacrolimus leading to MCs degranulation *in vivo* , we set out to further explore whether short-term tacrolimus exposure could activate MCs via MRGPRX2 *in vitro* . We found calcium influx of LAD2 cells, the early phase of MCs activation, could be altered by short-term

tacrolimus priming (Figure 4A), C48/80 (a MRGPRX2 agonist) as a positive group (Figure 4B). Further studies revealed that β hexosaminidase release increased (Figure 4C), as well as tryptase, MCP-1 and IL-8 increased dose-dependently after treated with tacrolimus in LAD2 cells for 30 minutes (Figure 4D). Furthermore, we treated MrgprB2-HEK293 cells with tacrolimus, which resulted in increased intracellular $Ca^{2+}$ concentration (Figure 4E), but tacrolimus did not alter the intracellular $Ca^{2+}$ concentration in normal control (NC)-HEK293 cells (Figure 4F). Intriguingly, lower release of inflammatory mediators (β hexosaminidase, tryptase, MCP-1 and IL-8) was observed obviously in MRGPRX2-knockdown group compared to NC-LAD2 cells (Figure 4G, H). Thus, tacrolimus mimics the behavior of C48/80 in LAD2 cells, supporting action at the same receptor MRGPRX2.

**Tacrolimus could interact directly with MRGPRX2 to active MCs**

To detect whether MRGPRX2 is the target of tacrolimus, molecular docking was implemented. Strikingly, tacrolimus can directly bind to MRGPRX2 by GLU164 residue (Figure 5A). FK-DNS, a fluorescently labeled tacrolimus, was constructed (Figure S1), and only when it binds to MRGPRX2 will it show fluorescence. The detailed information about FK-DNS were shown in Supplementary materials. Similar to tacrolimus, FK-DNS was able to activate LAD2 cells to release β hexosaminidase dose-dependently after short-term treatment and has the similar effect on MRGPRX2 expression after long-term treatment (Figure S2).

To explore whether tacrolimus could interact with MRGPRX2 directly or not, LAD2 cells, MRGPRX2-knockdown LAD2 cells and HMC-1 which expresses no MRGPRX2 were employed. Treated with FK-DNS, NC-LAD2 cells showed significant increased intracellular $Ca^{2+}$concentration, while calcium influx increased slightly and barely in MRGPRX2-knockdown LAD2 cells and HMC-1 respectively (Figure 5B). Through calcium images, we discovered that FK-DNS was able to bind to NC-LAD2 cells after 1-min exposure, which manifesting as weak fluorescence intensity on the cell membrane, while visible fluorescence was not shown in MRGPRX2-knockdown LAD2 cells and HMC-1 groups. After 10-min tacrolimus treatment in LAD2 cells group, we observed intense fluorescence was inside the cell membrane. In MRGPRX2-knockdown LAD2 cells and HMC-1 groups, only slight fluorescence was found inside the membrane for good lipid solubility of tacrolimus (Figure 5B).

Then, we determined to address the mechanism behind this phenomenon, and it performed that the rapid response to tacrolimus made phosphorylated PLCγ1, PKC and ERK1/2 expression increase in LAD2 cells compared with vehicle treated group. Meanwhile, the quantification of their expression had massive significance (Figure 5C).

**Initial MCs activation disappeared with decreased MrgprB2 expression after tacrolimus long-term treatment**

To mimic tacrolimus clinical long-term use, 0.03% tacrolimus cream were applied on dorsal skin of mice twice per day. Strikingly, the earlier irritant manifestations (scratching and inflammatory cells infiltration) fade away with no skin IgE level change (Figure 6A, B and C). The levels of skin TNF-α, MCP-1, IL-8 and tryptase on day 7 and 11 are with no significant difference in the absence of additional C48/80 stimulation (Figure 6D). To assess the status of MC at this time, avidin staining was used, which showed no remarkable distinct in MCs infiltration and degranulation compared to control group (Figure 6E). Surprisingly, skin MrgprB2 expression was massively downregulated after 7-day and 11-day tacrolimus topical use *in vitro* (Figure 7F). Hence, we speculated that suppressed MrgprB2 expression may be involved in disappearance of initial MCs stimulation after longer tacrolimus treatment.

**Tacrolimus inhibited MRGPRX2/B2-elicited MCs activation and inflammatory reaction**

In order to further assess MrgprB2-elicited degranulation after tacrolimus long-term treatment, C48/80 was used for subcutaneous injection. Surprisingly, scratching bouts has been largely reduced after 7- and 11-day exposure to tacrolimus when compared to vehicle group (Figure 7A) with no significant difference on IgE levels (Figure 7C). In order to investigate the effect of chronic tacrolimus application on the inflammatory reaction by MrgprB2 activation, we detected the skin inflammatory cell infiltration by H&E staining of dorsal

skin, which showed reduced inflammatory cells treated by C48/80 in tacrolimus group, and the number of aggregated inflammatory cells both decreased on day 7 and day 11 (Figure 7B). Moreover, MrgprB2-elicited MCs degranulation was exhibited by avidin immunofluorescence staining (Figure 7D). We found that the number of local MCs has not changed after tacrolimus long-term application. Conversely, MCs degranulation and the rate of MCs degranulation treated by C48/80 has been significantly suppressed by tacrolimus (Figure 7D). Further, we detected the skin inflammatory cytokines released by MCs. We discovered that the MrgprB2-triggered MCs degranulation was severely hampered by long-term tacrolimus use, as exhibited by the decreased levels of skin tryptase, IL-8, TNF-α,and MCP-1 (Figure 7E), and decreased mRNA expression similarly (Figure 7F). Consistent with previous results, long-term tacrolimus application eliminates MrgprB2 *in vivo* , and thereby restrains MrgprB2-triggered MCs degranulation.

What's more, our study measured long-term pretreatment of tacrolimus to modify the capacity of LAD2 cells degranulation induced by C48/80*in vitro* . When LAD2 cells were incubated with tacrolimus for 3 d, MRGPRX2 mRNA (Figure 8A) and C48/80 stimulated release of β-hexosaminidase (Figure 8B) from LAD2 (10 μg/L) decreased significantly. On day 3, MRGPRX2 protein expression diminished dramatically (Figure 8C). Similarly, release of tryptase, TNF-α, MCP-1 and IL-8 from LAD2 cells by different concentrations of C48/80 reduced dramatically (Figure 8D).

Collectively, chronic tacrolimus treatment could inhibit MCs induced inflammatory reaction by dampening MGRPRX2/B2 *in vitro* and*in vivo* .

## Discussion

MRGPRX2, recently discovered receptor, appears to be observed predominantly in skin MCs, while other members of MRGPR family are found in peripheral neurons mostly[22]. Unusually, MRGPRX2 can be activated broadly by several ligands which include both peptides and small molecules such as antibiotics, neuropeptides and anesthetics. Possibly, C48/80 is the most potent of the small molecules and non-peptidergic MRGPRX2 agonists[23]. These agonists of MRGPRX2 are able to stimulate tissue-resident MCs which are found in close proximity to nerve endings and blood vessels. Interestingly, MCs activation induced by MRGPRX2 is non-IgE dependent. And then MCs activation leads to enhanced inflammatory reaction, as well as engagement in a bidirectional loop with sensory nerve by releasing MCs-derived cytokines[24]. Currently, MRGPRX2 has been identified as a pruritogenic receptor[18]. As a result, MRGPRX2-stimulation will result in inflammatory reaction and pruritus such as pseudo-allergic reaction (transient side effects by first-time drug therapy), as well as many chronic, inflammatory and pruritus dermatoses with over-expressed MRGPRX2[14,25].

Tacrolimus is an immunosuppressant, and topical tacrolimus has been widely used in the treatment of chronic and inflammatory skin diseases such as AD, rosacea, contact dermatitis, and psoriasis for its potent anti-inflammation effect[26]. Besides, refractory itching of these diseases, reported to be IgE-independent, can be alleviated by topical tacrolimus therapy[27]. However, initial skin irritation induced by first-time tacrolimus use is common and can't be ignored. We therefore investigated the underlying mechanism of side effects induced by short-term tacrolimus application and suppressed inflammatory reactions after chronic tacrolimus exposure, placing particular emphasis on MRGPRX2.

Intriguingly, we discovered that dose-dependent inflammatory reaction and scratching behavior could be observed in WT mice instead of $Kit^{W\text{-}sh/W\text{-}sh}$ mice via non-IgE pathway after tacrolimus short-term application. This transient skin irritation *in vivo* is similar to initial side effect of tacrolimus clinical topical application, which may be speculated as a non-IgE dependent pseudo-allergic reaction. Actually, IgE-independent MCs activation plays a vital role in the underlying mechanism of initial side effect of topical tacrolimus application. And we confirmed that tacrolimus could act on MCs in a IgE-independent pathway.

We set out to further explore this non-IgE dependent MCs activation by tacrolimus, and we found short-term tacrolimus exposure causes MCs activation via MRGPRX2/B2 *in vivo* and *in intro* . Therefore, we speculated that MRGPRX2/B2 could be a novel target for tacrolimus. We discovered tacrolimus could bind to MRGPRX2 directly by molecular docking. To further clarify how tacrolimus regulates MRGPRX2,

FK-DNS, a fluorescently labeled tacrolimus, was constructed. We found FK-DNS, having similar function to tacrolimus on MRGPRX2, can bind to MRGPRX2 directly exhibiting green fluorescence, and later move into cell to modulate MRGPRX2. Hence, tacrolimus was acknowledged to regulate MCs activation via MRGPRX2/B2 directly.

Addressing the MRGPRX2 intracellular signaling events elicited by short-term tacrolimus in MCs, upregulated phosphorylated PLCγ1, PKC, ERK1/2 and P38 expression were verified. ERK and p38, part of MAPK family, mediate intracellular signaling and play important roles in producing proinflammatory cytokines, which demonstrates the modulatory role of tacrolimus on MRGPRX2 in inflammation pathology[28,29]. Here, inflammatory cytokines, tryptase, IL-8, TNF-α and MCP-1, released from MCs upregulated by tacrolimus short-term treatment in our study, will result in inflammatory cells recruitment, inflammatory reaction, and pruritus, even burning[16,30,31]. Collectively, tacrolimus could regulate MRGPRX2-mediated inflammatory respond.

Initial inflammatory reaction faded away and MCs activation induced by long-term tacrolimus decreased when compare to short-term tacrolimus treatment. Strikingly, we found that chronic tacrolimus treatment can suppress MRGPRX2/B2-elicited activation by dampening expression of MRGPRX2/B2. Consequently, we discovered MRGPRX2/B2-elicited MCs activation and inflammatory mediators, IL-8, TNF-α, MCP-1 and tryptase, released decreased in the presence of long-term tacrolimus. Besides, the infiltration of inflammatory cells also decreases. Similarly, the inhibitive effect of MRGPRX2/B2 expression was increased by tacrolimus with respect to time. Possibly, these data suggests that tacrolimus may also inhibit inflammatory response of other immune cells involved in MRGPRX2/B2-elicited pathology by suppressing inflammatory cytokines release. What's more, we speculated dampened MRGPRX2 expression plays an important role in the disappearance of side effects of tacrolimus application.

Moreover, tryptase, main mediators of non-IgE dependent pruritis, drastically reduces from MRGPRX2/B2-stimulated degranulation after long-term tacrolimus treatment in our study. The declined tryptases release potentially contributes to itching suppression, for the receptors of which (Protease activated receptor 2, PAR2) present on itch-sensory neurons[32]. PAR2 activated by tryptase may be a mechanism by which tacrolimus inhibits MRGPRX2-stimulated neurogenic itching and inflammation[32].

In addition to its function on MRGPRX2 which has not been reported before, topical tacrolimus has additional mechanisms to alleviate chronic inflammation and pruritus[33]. Some studies raised the possibility that tacrolimus can act on the sensory nerves and cause desensitization[33]. Moreover, neuroimmune has been given more attention in chronic inflammation and pruritus, and MRGPRX2 has its potential role in neurogenic inflammation and itch[14]. For example, SP, a vital factor in neurogenic inflammation and pruritis, has been recently implicated in correlating with MRGPRX2/B2 activation in many chronic skin diseases[34,35]. Interestingly, skin SP immunoreactive cell counts decreased significantly after tacrolimus treatment in AD patients[36]. Hence, tacrolimus may modulate neuroimmune clusters by MRGPRX2/B2 signaling in skin physiological and itching processes[37]. In the future, in pathology of neurogenic inflammation and itching, inhibition MRGPRX2 and its signaling may be a potential therapeutic strategy for tacrolimus.

In summary, tacrolimus can regulate IgE-independent MCs activation and MRGPRX2 is a novel target of tacrolimus. Inhibition of MRGPRX2 activation by short-term tacrolimus may play an important role in initial side effects induced. Chronic tacrolimus treatment may have potential to be utilized in the resolution of chronic inflammatory reaction related to MRGPRX2 particularly.

**Reference**

1. Pustisek N, Lipozencić J, Ljubojević S. Tacrolimus ointment: a new therapy for atopic dermatitis–review of the literature. *Acta Dermatovenerol Croat.* 2002;10(1):25-32.

2. Beck LA. The efficacy and safety of tacrolimus ointment: a clinical review. *J Am Acad Dermatol.* 2005;53(2 Suppl 2):S165-170.

3. Goretzki A, Lin YJ, Schülke S. Immune metabolism in allergies, does it matter?-A review of immune

metabolic basics and adaptations associated with the activation of innate immune cells in allergy.*Allergy.* 2021;76(11):3314-3331.

4. Jiménez M, Cervantes-García D, Córdova-Dávalos LE, et al. Responses of Mast Cells to Pathogens: Beneficial and Detrimental Roles.*Front Immunol.* 2021;12:685865.

5. Trier AM, Kim BS. Structural insights into MRGPRX2: a new vision of itch and allergy. *J Allergy Clin Immunol.* 2022.

6. Voss M, Kotrba J, Gaffal E, et al. Mast Cells in the Skin: Defenders of Integrity or Offenders in Inflammation? *Int J Mol Sci.*2021;22(9).

7. Samukawa K, Izumi Y, Shiota M, et al. Red ginseng inhibits scratching behavior associated with atopic dermatitis in experimental animal models. *J Pharmacol Sci.* 2012;118(3):391-400.

8. Pereira U, Boulais N, Lebonvallet N, et al. Mechanisms of the sensory effects of tacrolimus on the skin. *Br J Dermatol.*2010;163(1):70-77.

9. Murota H, El-latif MA, Tamura T, et al. Olopatadine hydrochloride decreases tissue interleukin-31 levels in an atopic dermatitis mouse model. *Acta Derm Venereol.* 2014;94(1):78-79.

10. Cury Martins J, Martins C, Aoki V, et al. Topical tacrolimus for atopic dermatitis. *Cochrane Database Syst Rev.* 2015(7):CD009864.

11. Ashcroft DM, Dimmock P, Garside R, et al. Efficacy and tolerability of topical pimecrolimus and tacrolimus in the treatment of atopic dermatitis: meta-analysis of randomised controlled trials. *BMJ.*2005;330(7490):516.

12. McNeil BD, Pundir P, Meeker S, et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions.*Nature.* 2015;519(7542):237-241.

13. Meixiong J, Anderson M, Limjunyawong N, et al. Activation of Mast-Cell-Expressed Mas-Related G-Protein-Coupled Receptors Drives Non-histaminergic Itch. *Immunity.* 2019;50(5):1163-1171 e1165.

14. Subramanian H, Gupta K, Ali H. Roles of Mas-related G protein-coupled receptor X2 on mast cell-mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases.*J Allergy Clin Immunol.* 2016;138(3):700-710.

15. Arifuzzaman M, Mobley YR, Choi HW, et al. MRGPR-mediated activation of local mast cells clears cutaneous bacterial infection and protects against reinfection. *Sci Adv.* 2019;5(1):eaav0216.

16. Meixiong J, Anderson M, Limjunyawong N, et al. Activation of Mast-Cell-Expressed Mas-Related G-Protein-Coupled Receptors Drives Non-histaminergic Itch. *Immunity.* 2019;50(5):1163-1171.e1165.

17. Ogasawara H, Noguchi M. Therapeutic Potential of MRGPRX2 Inhibitors on Mast Cells. *Cells.* 2021;10(11).

18. Cao C, Kang HJ, Singh I, et al. Structure, function and pharmacology of human itch GPCRs. *Nature.* 2021;600(7887):170-175.

19. Liu R, Che D, Zhao T, et al. MRGPRX2 is essential for sinomenine hydrochloride induced anaphylactoid reactions. *Biochem Pharmacol.*2017;146:214-223.

20. Cañadas O, Sáenz A, Orellana G, et al. Equilibrium studies of a fluorescent tacrolimus binding to surfactant protein A. *Anal Biochem.* 2005;340(1):57-65.

21. Ständer S, Ständer H, Seeliger S, et al. Topical pimecrolimus and tacrolimus transiently induce neuropeptide release and mast cell degranulation in murine skin. *Br J Dermatol.*2007;156(5):1020-1026.

22. Ray P, Torck A, Quigley L, et al. Comparative transcriptome profiling of the human and mouse dorsal root ganglia: an RNA-seq-based resource for pain and sensory neuroscience research. *Pain.*2018;159(7):1325-1345.

23. McNeil BD. Minireview: Mas-related G protein-coupled receptor X2 activation by therapeutic drugs. *Neuroscience Letters.* 2021;751.

24. Roy S, Chompunud Na Ayudhya C, Thapaliya M, et al. Multifaceted MRGPRX2: New insight into the role of mast cells in health and disease.*J Allergy Clin Immunol.* 2021;148(2):293-308.

25. Shtessel M, Limjunyawong N, Oliver ET, et al. MRGPRX2 Activation Causes Increased Skin Reactivity in Patients with Chronic Spontaneous Urticaria. *J Invest Dermatol.* 2021;141(3):678-681.e672.

26. Ohtsuki M, Morimoto H, Nakagawa H. Tacrolimus ointment for the treatment of adult and pediatric atopic dermatitis: Review on safety and benefits. *J Dermatol.* 2018;45(8):936-942.

27. Takeuchi S, Saeki H, Tokunaga S, et al. A randomized, open-label, multicenter trial of topical tacrolimus for the treatment of pruritis in patients with atopic dermatitis. *Ann Dermatol.*2012;24(2):144-150.

28. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim Biophys Acta.* 2010;1802(4):396-405.

29. Gilfillan AM, Tkaczyk C. Integrated signalling pathways for mast-cell activation. *Nat Rev Immunol.* 2006;6(3):218-230.

30. Zelová H, Hošek J. TNF-α signalling and inflammation: interactions between old acquaintances. *Inflamm Res.* 2013;62(7):641-651.

31. Doszhan A, Bektayeva R, Imanbayeva N, et al. [THE ROLE OF INTERLEUKIN 8 / CXCL8 IN THE IMMUNOPATHOGENESIS AND CARCINOGENESIS OF INFLAMMATORY BOWEL DISEASES (REVIEW)]. *Georgian Med News.*2019(296):150-153.

32. Choi JE, Di Nardo A. Skin neurogenic inflammation. *Semin Immunopathol.* 2018;40(3):249-259.

33. Nakahara T, Morimoto H, Murakami N, et al. Mechanistic insights into topical tacrolimus for the treatment of atopic dermatitis. *Pediatr Allergy Immunol.* 2018;29(3):233-238.

34. Azimi E, Reddy VB, Shade KC, et al. Dual action of neurokinin-1 antagonists on Mas-related GPCRs. *JCI Insight.* 2016;1(16):e89362.

35. Thapaliya M, Chompunud Na Ayudhya C, Amponnawarat A, et al. Mast Cell-Specific MRGPRX2: a Key Modulator of Neuro-Immune Interaction in Allergic Diseases. *Curr Allergy Asthma Rep.* 2021;21(1):3.

36. Kim HO, Lee CH, Ahn HK, et al. Effects of tacrolimus ointment on the expression of substance P, nerve growth factor, and neurotrophin-3 in atopic dermatitis. *Int J Dermatol.* 2009;48(4):431-438.

37. Corbière A, Loste A, Gaudenzio N. MRGPRX2 sensing of cationic compounds-A bridge between nociception and skin diseases? *Exp Dermatol.* 2021;30(2):193-200.

**Figure legends**

**Figure 1 Transient inflammatory reaction and non-IgE related itching were triggered by short-term tacrolimus application *in vivo*.** WT mice were applied with different concentrations of tacrolimus for one day. Clinical photographs**(A)** and Haematoxylin and eosin (H&E) staining **(B)** of skin lesions. Scale bar, 50 μm. **(C)** Immunofluorescence staining of avidin in WT mice. Scale bar, 20 μm. Red arrow points to one of the undegranulated MCs, white arrows point to degranulated MCs. **(D)**Scratching bouts in 20 min and level of serum IgE (mean ± SEM, n = 6).**(E)** Levels of tryptase, TNF-α and MCP-1 in serum (mean ± SEM, n = 6). In hind paw swelling and extravasation experiment, **(F)**typical hind paw photos of WT mice after 15-minutes injection with tacrolimus and C48/80. **(G)** Paw thickness increase rate and $OD_{620nm}$ per g weight of WT mice (mean ± SEM, n = 6). All experiments were repeated 3 times. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***$p$¡0.005) by two-tailed paired Student's t-test.

**Figure 2 $Kit^{W\text{-}sh/W\text{-}sh}$ mice showed no inflammatory reaction induced by tacrolimus**

$Kit^{W\text{-}sh/W\text{-}sh}$mice were treated with tacrolimus on dorsal skin for one day. Clinical photographs **(A)** and Haematoxylin and eosin (H&E) staining**(B)** of skin lesions. Scale bar, 50 μm. **(C)** Scratching bouts in 20 min and levels of IgE,tryptase, TNF-α and MCP-1 in serum (mean ± SEM, n = 6). **(D)** In hind paw swelling and extravasation experiment, typical hind paw photos, paw thickness increase rate and $OD_{620nm}$ per g weight of $Kit^{W\text{-}sh/W\text{-}sh}$mice and WT mice (mean ± SEM, n = 6). All experiments were repeated 3 times. Statistical significance was accepted at *$p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005)* by two-tailed unpaired Student's t-test.

**Figure 3 Immediate effect of tacrolimus primed MrgprB2-elicited MCs activation *in vivo*.**

In hind paw swelling and extravasation experiment, **(A)** typical hind paw photos, paw thickness increase rate and $OD_{620nm}$per g weight of MUT mice and WT mice (mean +- SEM, n = 6). MUT mice were treated with tacrolimus on dorsal skin for one day. Clinical photographs**(B)** and Haematoxylin and eosin (H&E) staining **(C)** of skin lesions. Scale bar, 50 μm. **(D)** Immunofluorescence staining of avidin in MUT mice after 1-d tacrolimus application. Scale bar, 20 μm. Red arrow points to one of the undegranulated MCs, white arrows point to degranulated MCs. **(E)** Scratching bouts in 20 min and levels of IgE,tryptase, TNF-α and MCP-1 in serum (mean ± SEM, n = 6). All experiments were repeated 3 times. Statistical significance was accepted at *$p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005)* by two-tailed unpaired Student's t-test.

**Figure 4 Tacrolimus could activate MCs via MRGPRX2 *in vitro*.**

**(A)** Intracellular calcium concentrations of LAD2 cells treated by tacrolimus. **(B)** Intracellular calcium concentrations of LAD2 cells treated by C48/80. Human LAD2 cells were treated with tacrolimus for 30 minutes, and C48/80 was used to set positive control.**(C)** β hexosaminidase release rate, and **(D)** levels of tryptase, MCP-1 and IL-8 of LAD2 cells (mean ± SEM, n = 3). **(E)**Intracellular calcium concentrations of MrgprB2-HEK293 cells and**(F)** NC-HEK293 cells treated by tacrolimus. MRGPRX2-knockdown LAD2 cells were also treated by tacrolimus. **(G)** β hexosaminidase release rate, and **(H)** levels of tryptase, MCP-1 and IL-8 of NC-LAD2 cells and MRGPRX2-knockdown LAD2 cells (mean ± SEM, n = 3). All experiments were repeated 3 times. Statistical significance was accepted at *$p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005)* by two-tailed unpaired Student's t-test.

**Figure 5 Tacrolimus interacted directly with MRGPRX2 and regulated MRGPRX2.**

**(A)** Molecular docking between tacrolimus and MRGPRX2.**(B)** Intracellular calcium concentrations of NC-LAD2 cells, MRGPRX2-knockdown LAD2 cells and HMC-1 cells treated by short-term tacrolimus treatment. Calcium images at different times of NC-LAD2 cells, MRGPRX2-knockdown LAD2 cells and HMC-1 cells were presented respectively, fluorescence intensity represents the intracellular $Ca^{2+}$ concentration. **(C)** Western blot analysis of the expression levels of P-PLCγ1, PLCγ, P-PKC, PKC, P-ERK, ERK, P-P38, P38 and GAPDH in LAD2 cells, and quantification of P-PLCγ1, PLCγ, P-PKC, PKC, P-ERK, ERK, P-P38 and P38 protein expression respectively by densitometric analysis. Experiments of (B) and (C) were repeated 3 times. Statistical significance was accepted at *$p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005)* by two-tailed unpaired Student's t-test.

**Figure 6 Initial inflammation induced by tacrolimus faded away and MrgprB2 expression was suppressed after tacrolimus long-term use.**

WT mice were applied with and vehicle on dorsal skin tacrolimus for 7 days and 11 days respectively. **(A)** Scratching bouts in 20 min on day 7 and 11 respectively (mean ± SEM, n = 6). **(B)** H&E staining of skin lesions. Scale bar, 50 μm. And inflammatory cell infiltration per field of H&E image on day 7 and 11 respectively (mean ± SEM, n = 6). **(C)** Level of skin IgE on day 7 and 11 respectively (mean ± SEM, n = 4). **(D)** Levels of skin TNF-α, MCP-1, IL-8 and tryptase on day 7 and 11 respectively (mean ± SEM, n = 6). **(E)** Immunofluorescence staining of avidin on day 7 and 11. Scale bar, 5 μm. MC infiltration per field, degranulation per field and the degranulation rate were counted in avidin staining images on day 7 and 11 respectively (mean ± SEM, n = 6). **(F)** Skin MrgprB2 relative mRNA expression on day 7 and 11 respectively (mean ± SEM, n = 6). All experiments were repeated 3 times. Statistical significance was

accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005) by two-tailed unpaired Student's t-test.

**Figure 7 Inflammatory reaction elicited by MrgprB2 could be suppressed by long-term tacrolimus treatment.**

WT mice were treated with tacrolimus and vehicle on dorsal skin respectively for 11 d. C48/80 was subcutaneously injected after 7- and 11- day exposure to tacrolimus. **(A)** Scratching bouts in 20 min on day 7 and 11 respectively (mean +- SEM, n = 6). **(B)** H&E staining of skin lesions. Scale bar, 50 μm. And inflammatory cell infiltration per field of H&E image on day 7 and 11 respectively (mean ± SEM, n = 6). **(C)** Level of skin IgE (mean ± SEM, n = 6).**(D)** Immunofluorescence staining of avidin. Scale bar,5 μm. Red arrow points to one of the undegranulated MCs, white arrows point to degranulated MCs. MC infiltration per field, degranulation per field and the degranulation rate were counted in avidin staining images on day 7 and 11 respectively (mean ± SEM, n = 6). **(E)** Levels of skin tryptase, IL-8, TNF-α and MCP-1 on day 7 and 11 respectively (mean ± SEM, n = 6). **(F)** Skin tryptase, IL-8, TNF-α and MCP-1 mRNA expression on day 7 and 11 respectively (mean ± SEM, n = 6). All experiments were repeated 3 times. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005) by two-tailed unpaired Student's t-test.

**Figure 8 MCs-mediated inflammation could be inhibited by long-term tacrolimus treatment via suppression of MRGPRX2 expression.**

Human LAD2 cells were treated with 0.5 nM tacrolimus and vehicle respectively for 3 d. **(A)** MRGPRX2 relative mRNA expression in LAD2 cells at day 1-3 respectively (mean +- SEM, n = 3). **(B)**β-hexosaminidase release rate of LAD2 cells activation by C48/80 at day 1-3 respectively (mean ± SEM, n = 3). **(C)** Western blot analysis of the expression levels of MRGPRX2 and β-actin in LAD2 cells on day 3, and quantification of MRGPRX2 protein expression respectively by densitometric analysis. **(D)** Different concentrations of C48/80 induced LAD2 release tryptase, TNF-α, IL-8 and MCP-1 on day 3 (mean ± SEM, n = 3). All experiments were repeated 3 times. Statistical significance was accepted at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$ and ***p¡0.005) by two-tailed unpaired Student's t-test.

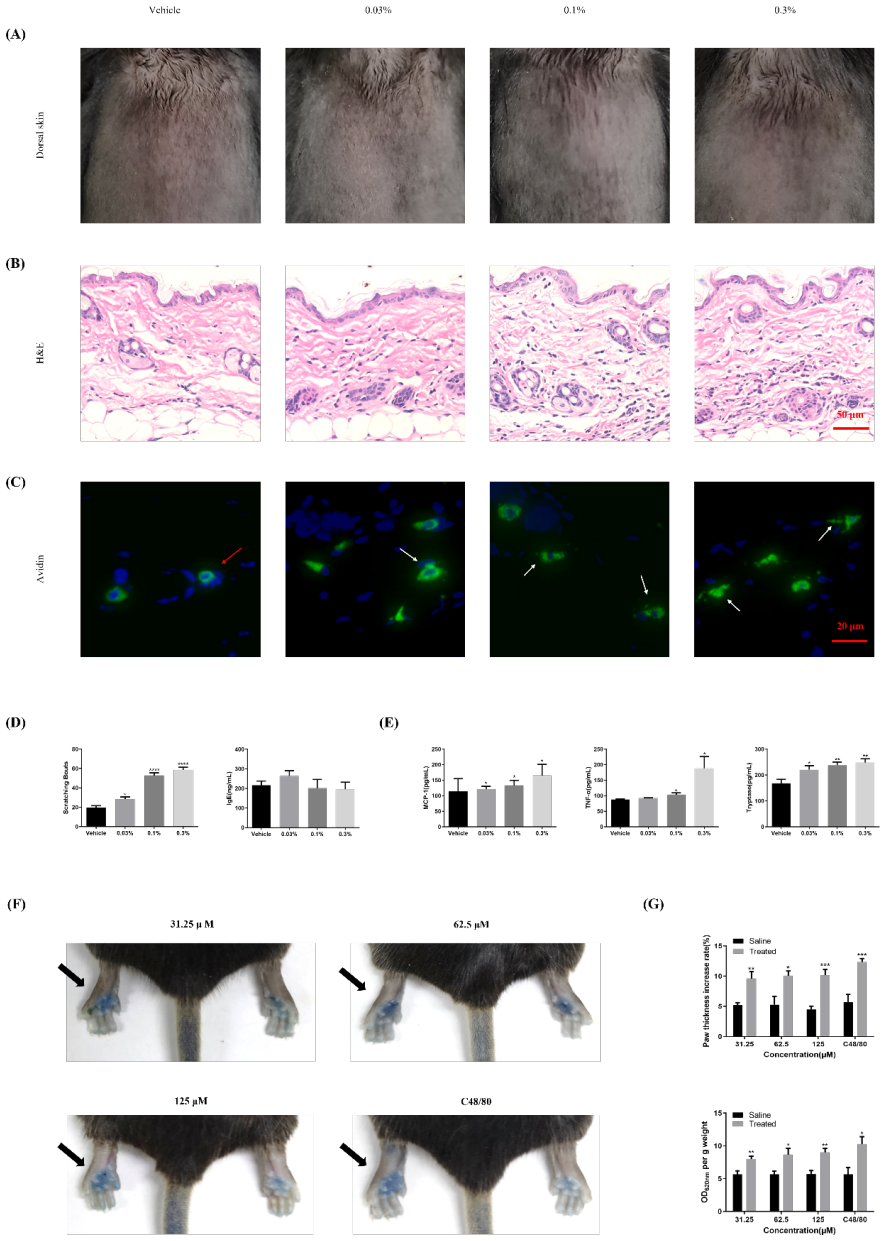


**Figure 1**

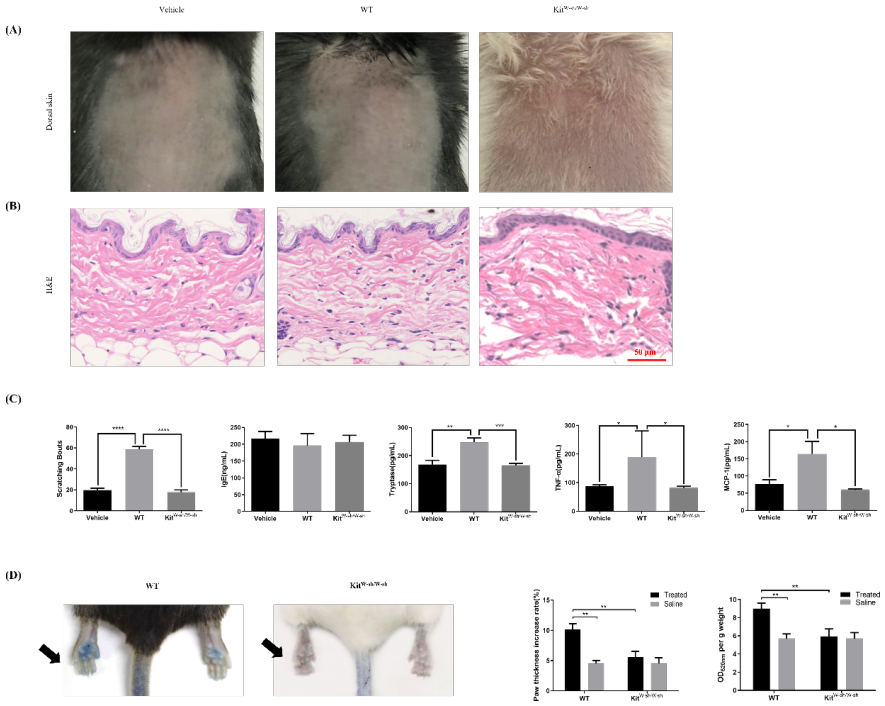


**Figure 2**

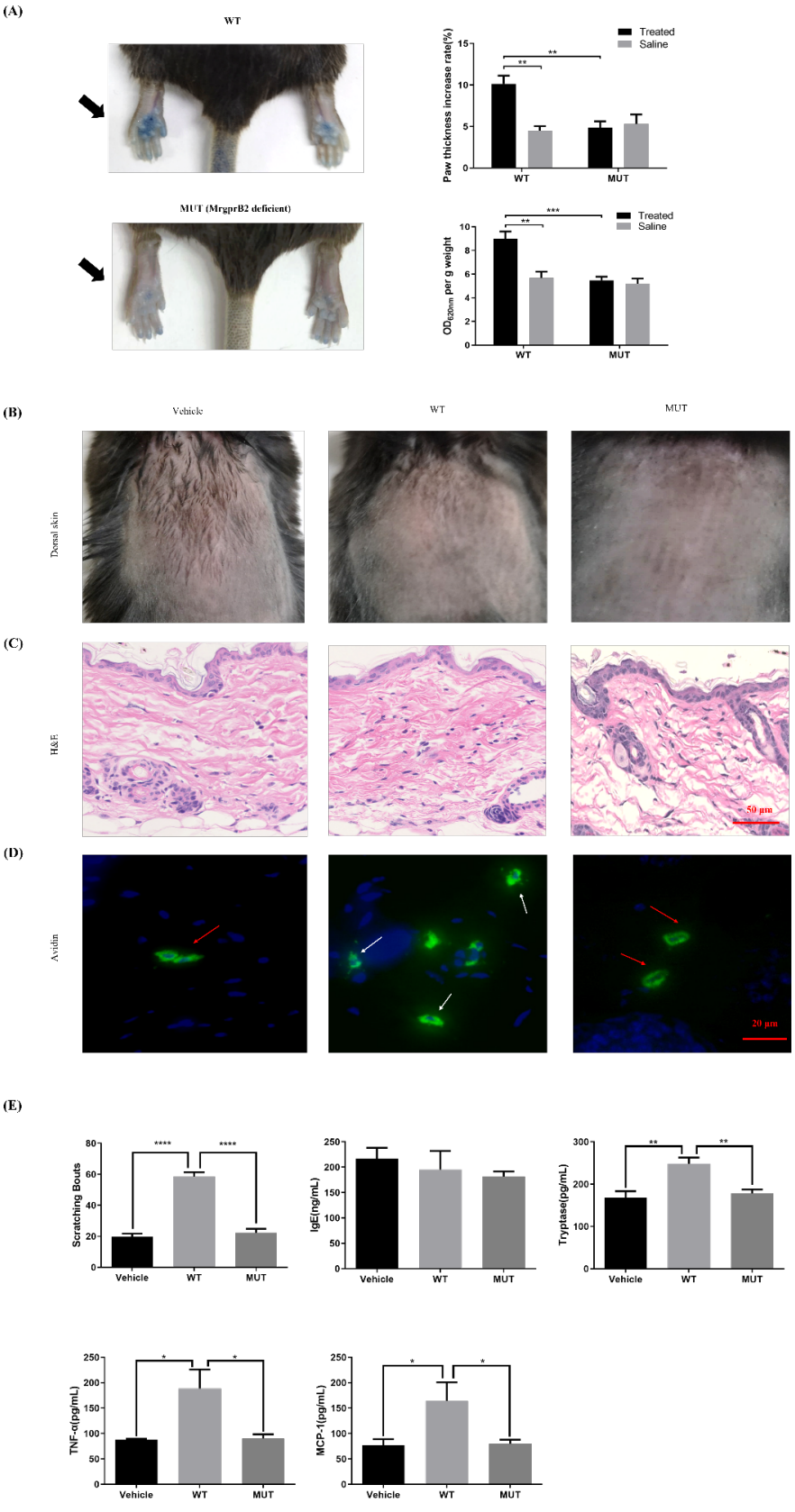

**Figure 3**

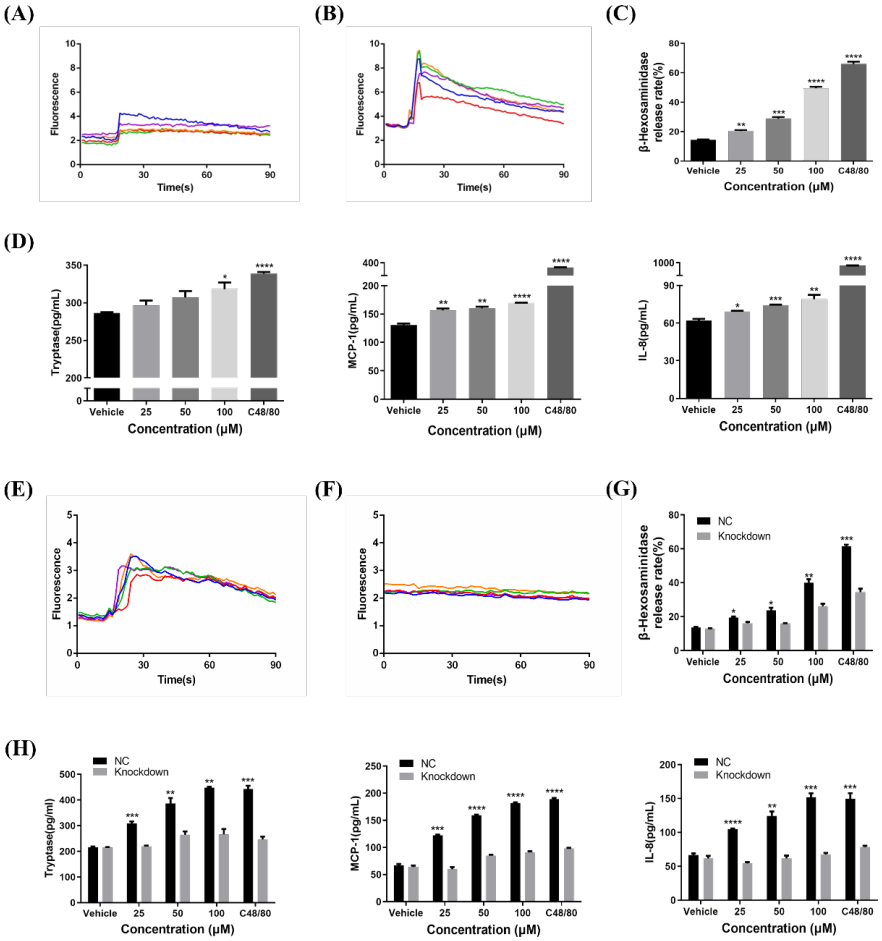


**Figure 4**

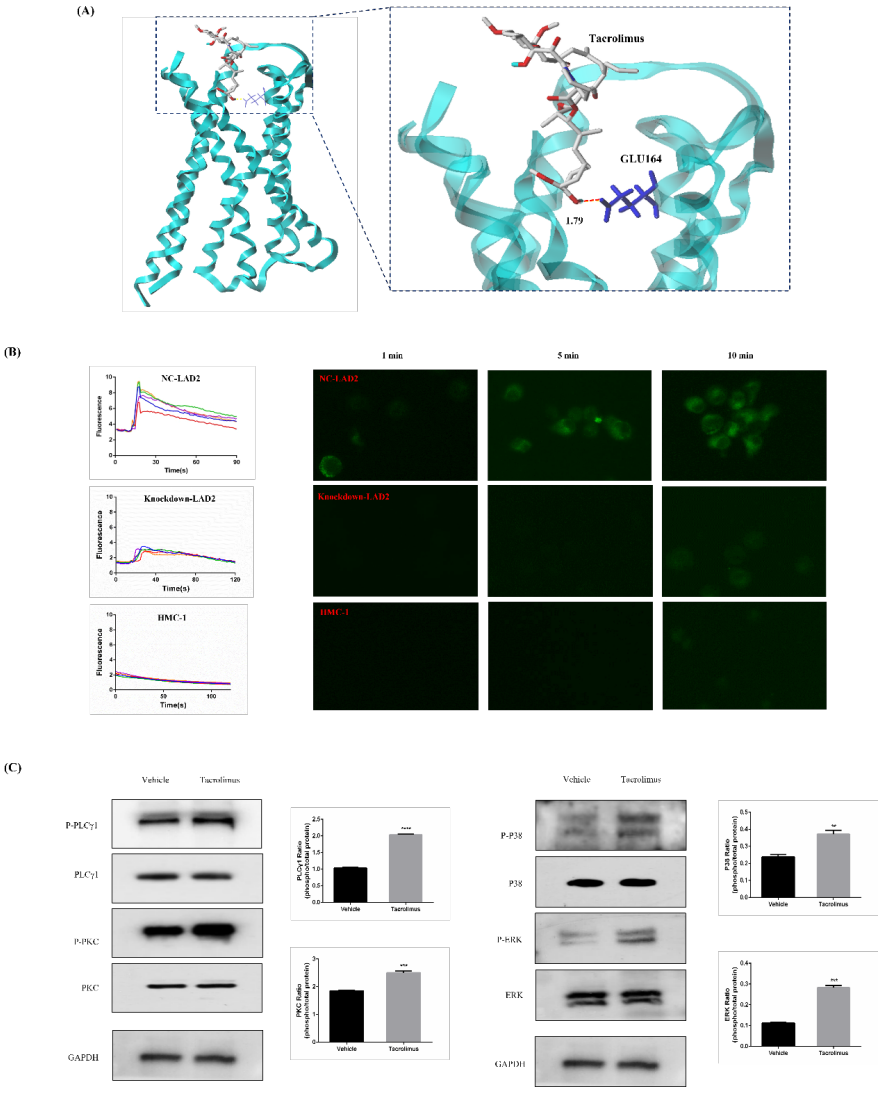


**Figure 5**

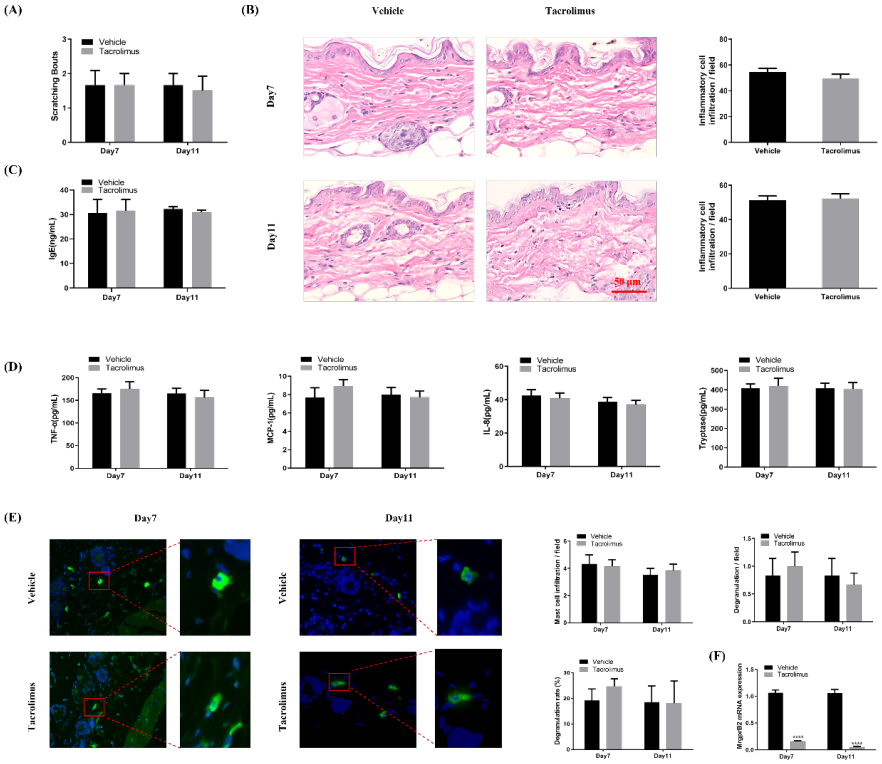


**Figure 6**

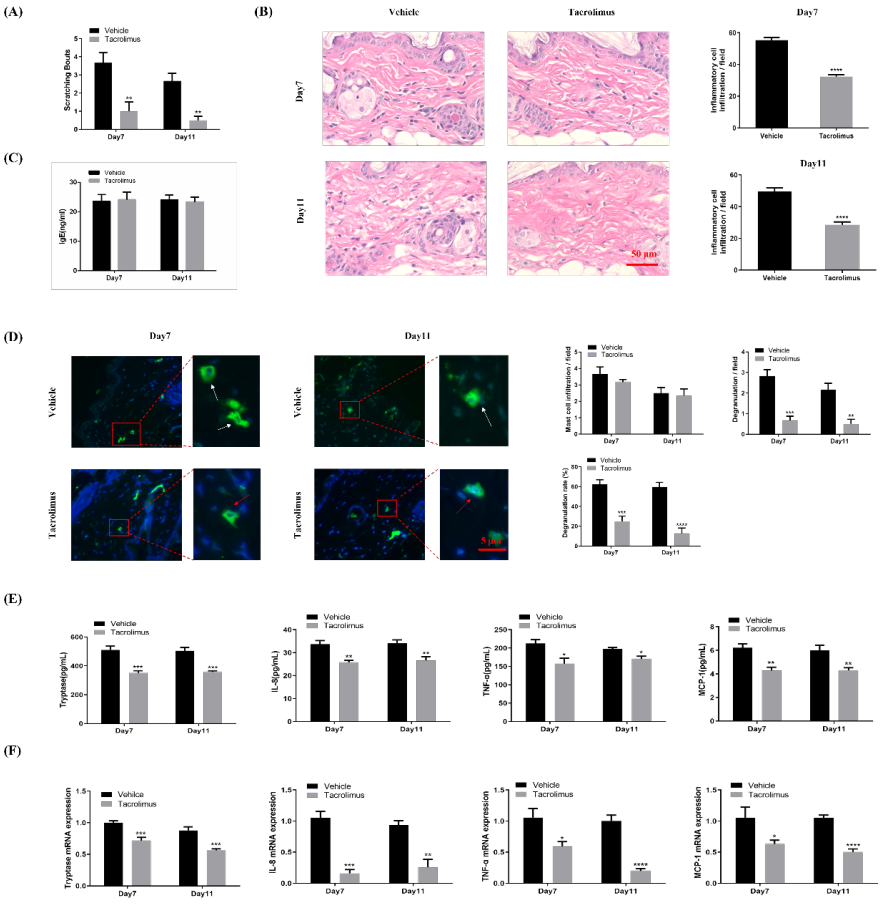


**Figure 7**

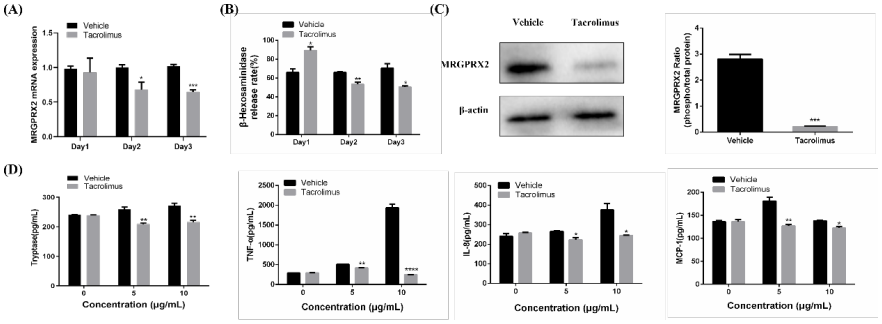


**Figure 8**