Roles of TRPM4 in immune responses in keratinocytes and identification of a novel TRPM4-activating agent

Kaori (Otsuka) Saito,[a,b,c]* Fumitaka Fujita,[a,b,c] Manami Toriyama,[a,c,d] Ratna Annisa Utami,[e] Zhihan Guo,[a] Masato Murakami,[a,f] Hiroko Kato,[a,c] Yoshiro Suzuki,[g,h,i,j] Fumihiro Okada,[b] Makoto Tominaga[g,h,i] Ken J Ishii[c,k]*

[a] *Laboratory of Advanced Cosmetic Science, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6, Yamadaoka, Suita, Osaka 565-0871, Japan*

[b] *Fundamental Research Institute, Mandom Corp., 5-12, Juniken-Cho, Chuo-ku, Osaka 540-8530, Japan*

[c] *Laboratory of Mock Up Vaccine, Center for Vaccine and Adjuvant Research (CVAR), National Institutes of Biomedical Innovation, Health and Nutrition (NBIOHN), 7-6-8, Asagi, Saito, Ibaraki-City, Osaka 567-0085, Japan*

[d] *Graduate School of Science and Technology, Nara Institute of Science and Technology, 8916-5, Takayama-Cho, Ikoma, Nara 630-0192, Japan*

[e] *School of Pharmacy, Institut Teknologi Bandung, Jl. Ganesha No. 10, Bandung 40132, Indonesia*

[f] *Technical Development Center, Mandom Corp., 5-12, Juniken-Cho, Chuo-ku, Osaka 540-8530, Japan*

[g] *Thermal Biology Group, Exploratory Research Center on Life and Living Systems National Institutes of Natural Sciences, 5-1, Aza-higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan*

[h] *Division of Cell Signaling, National Institute for Physiological Sciences, National Institutes of Natural Sciences, 5-1, Aza-higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan*

[i] *Department of Physiological Sciences, SOKENDAI (The Graduate University for Advanced Studies), 5-1, Aza-higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan*

[j] *Department of Physiology, Iwate Medical University, 1-1-1, Idaidori, Yahaba-cho, Shiwa-gun, Iwate 028-3694, Japan*

[k] *Division of Vaccine Science, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*

* Correspondence: Kaori Otsuka Saito and Ken J Ishii

E-mail address: saito-ka@phs.osaka-u.ac.jp(KOS); kenishii@ims.u-tokyo.ac.jp(KJI)

These authors contributed equally to this work.

Highlights

- TRPM4 activation resulted in the decrease of cytokine production in keratinocytes.
- Aluminum potassium sulfate suppressed the cytokine production in HaCaT cells.
- Aluminum potassium sulfate evoked TRPM4-mediated currents.
- Aluminum potassium sulfate was identified as a new TRPM4 agonist.

Abstract

The skin is a protective interface between the internal organs and environment and functions not only as a physical barrier but also as an immune organ. However, the immune system in the skin is not fully understood. A member of the thermo-sensitive transient receptor potential (TRP) channel family, TRPM4, which acts as a regulatory receptor in immune cells, was recently reported to be expressed in human skin and keratinocytes. However, the function of TRPM4 in immune responses in keratinocytes has not been investigated. In this study, we found that treatment with BTP2, a known TRPM4 agonist, reduced cytokine production induced by tumor necrosis factor (TNF) α in normal human epidermal keratinocytes and in immortalized human epidermal keratinocytes (HaCaT cells). This cytokine-reducing effect was not observed in TRPM4-deficient HaCaT cells, indicating that TRPM4 contributed to the control of cytokine production in keratinocytes. Furthermore, we identified aluminum potassium sulfate, as a new TRPM4 activating agent. Aluminum potassium sulfate reduced Ca^{2+} influx by store-operated Ca^{2+} entry in human TRPM4-expressing HEK293T cells. We further confirmed that aluminum potassium sulfate evoked TRPM4-mediated currents, showing direct evidence for TRPM4 activation. Moreover, treatment with aluminum potassium sulfate reduced cytokine expression induced by TNFα in HaCaT cells. Taken together, our data suggested that TRPM4 may serve as a new target for the treatment of skin inflammatory reactions by suppressing the cytokine production in keratinocytes, and aluminum potassium sulfate is a useful ingredient to prevent undesirable skin inflammation through TRPM4 activation.

Keywords: transient receptor potential melastatin 4 channel; skin; cytokines; aluminum compounds; inflammation, keratinocytes

Introduction

The immune system is essential for protecting the human body against attacks by harmful pathogens. As our major barrier to the outside world, the skin functions as more than a physical barrier: it is an immune organ. However, the function of the immune system in the skin remains unclear. Keratinocytes initiate cell-mediated immune responses in the skin by producing cytokines, such as interleukin (IL)-1, IL-6, IL-10, and tumor necrosis factor (TNF) α [1, 2]. IL-1α initiates immune reactions [3], enhances fibroblast migration and proliferation [4, 5], and promotes keratinocyte growth via autocrine signaling [6, 7]. These processes are important for protecting the human body against harmful attacks and for helping to repair the skin following injury; however, they are also activated under certain pathological conditions. Therefore, it is important to properly regulate immune responses in keratinocytes in order to maintain the health of the skin.

In recent years, increasing evidence has demonstrated that some members of the transient receptor potential (TRP) channel family have functional roles in the immune system [8, 9]. TRP channels are transmembrane ion channels and act as sensors for thermal, mechanical, chemical, and environmental stimuli. In the human body, there are 11 thermo-sensitive TRP channels (thermo-TRPs) [10]. Thermo-TRPs are also activated by specific compounds [10], and some of these channels are expressed in immune cells and mediate immune reactions [9]. We hypothesized that thermo-TRPs expressed in keratinocytes may contribute to immune reactions in the skin. In keratinocytes, expression of TRPV3 [11], TRPV4 [12], and TRPM4 [13] has previously been reported. Among these TRP channels, TRPM4 is known to be involved in modulating the immune responses in several cell types.

TRPM4 was first cloned and characterized in 2001 [14]. The full-length *TRPM4* gene was cloned and characterized in 2002, and its wide distribution was observed across numerous tissues, including the brain, muscle, and heart [15]. TRPM4 is activated by warm temperatures of 15–35°C [10], and its expression has been reported in various immune cells, such as T cells, dendritic cells, monocytes/macrophages, and mast cells [9]. In Jurkat T cells, molecular inhibition of endogenous TRPM4 enhances Ca^{2+} signaling and results in increased IL-2 production [16]. Lack of TRPM4 expression affects the macrophage population within bacteria-infected peritoneal cavities, and deletion of the *Trpm4* gene results in severe septic shock caused by cecal ligation and puncture and dramatically decreases the survival rate in a mouse model of sepsis [17]. However, the roles of TRPM4 in the skin and skin cells have not been investigated until recently. Wang et al. first reported TRPM4 protein expression in human skin and keratinocytes in 2019 [13]. Moreover, gain-of-function mutation in TRPM4 exhibit enhanced proliferation of keratinocytes [13, 18]. It also shows progressive symmetric erythrokeratodermia (PSEK), which shares similar clinical and histopathological features with psoriasis in human [13] and exhibits enhanced susceptibility to imiquimod-induced psoriasiform dermatitis in mice [18]. Therefore, we hypothesized TRPM4 may also have roles in the immune response of keratinocytes.

In this study, we investigated the effects of TRPM4 activation on cytokine production in normal human epidermal keratinocytes (NHEKs) and HaCaT cells. Our results showed that BTP2, a TRPM4 agonist, significantly suppressed cytokine production; this suppression was not observed in TRPM4-deficient HaCaT cells. We further identified a new TRPM4 agonist, aluminum potassium sulfate from the Japanese traditional spa water which is known to ameliorate the skin disease. Aluminum potassium

sulfate is thought to improve the inflammatory condition of the skin by suppressing the cytokine production in keratinocytes through TRPM4 activation.

Materials and Methods

Cell culture and treatment

Neonatal normal human epidermal keratinocytes (NHEKs; NHEK-Neo; batch no. 00192907; Lonza) were cultured in KGM-Gold BulletKit (Lonza). HaCaT keratinocytes and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Inc.) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin/100 μg/mL streptomycin (Penicillin-Streptomycin Mixed Solution; Nacalai Tesque, Inc.), and 2 mM L-glutamine (Glutamax; Life Technologies). NHEKs and HaCaT cells were treated with TNFα (20 ng/mL) and the known TRPM4 agonist BTP2 (100 nM), or aluminum potassium sulfate (1 mM). Aluminum potassium sulfate was dissolved with DMEM and sterilized with filtration after adjustment of the pH to 7.4 using NaOH.

Western blot analysis

Cells were lysed with RIPA buffer (Santa Cruz Biotechnology, Inc.) containing 1 tablet/10 mL cOmplete mini (Roche Diagnostics K.K.), PhosSTOP (Roche Diagnostics K.K.), and 1 mM dithiothreitol (DTT; Sigma-Aldrich, Inc.). Cell lysates were incubated for 30 min at 4°C and then centrifuged at 16,000 × *g* for 20 min. Supernatants were collected, and protein (50 μg) was loaded on 10.5% TGX gels (Bio-Rad Laboratories, Hercules, CA, USA) and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories). Proteins were identified using anti-TRPM4 antibodies (cat. No. TA500381; 1:1000; Origene Technologies, Inc. USA) and anti-β-actin mouse monoclonal antibodies (cat. No. 3700; 1:1000; Cell Signaling Technology, Danvers, MA, USA).

Cytokine protein expression

NHEKs and HaCaT cells were treated with TNFα, and BTP2 or aluminum potassium sulfate as described above. After 48 h, supernatants were harvested, and cells were lysed as described above. The concentrations of IL-1α in the cell lysate and IL-6 in the supernatant were determined using enzyme-linked immunosorbent assays with IL-1α/IL-1F1 and IL-6 Human DuoSet Kits (R&D Systems, Inc.), respectively.

Cytokine gene expression

HaCaT cells and TRPM4-deficient HaCaT cells were treated with TNFα and BTP2 as described above. After 3 h, cells were lysed with a Real-Time Ready Cell Lysis Kit (Roche Diagnostics K.K.), and the expression levels of *IL-1A* and *IL-6* mRNAs were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) with a PrimeScript One-step RT-PCR kit (TaKaRa Bio, Inc.).

Transfection

One μg TrueClone human full-length TRPM4 plasmid (SC123991) in OPTI-MEM medium (Life Technologies) was transfected into HEK293T cells using Lipofectamine Plus Reagent (Life Technologies). Following incubation for 3–4 h, cells were reseeded onto coverslips and incubated overnight at 37°C in an atmosphere containing 5% CO_2.

Ca^{2+} measurement

Ca^{2+} imaging was performed 1 day after transfection. HEK293T cells overexpressing TRPM4 were loaded with the Ca^{2+} indicator Fura-2-AM (Molecular Probes, Invitrogen Corp.). HEK293T cells on coverslips were mounted in open chambers and superfused with a standard Ca^{2+}-free bath solution (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$,10 mM HEPES, and 10 mM glucose, pH 7.4). To deplete the endoplasmic reticulum (ER) Ca^{2+} store, 1 μM thapsigargin was added to a Ca^{2+}-free bath solution. After store depletion,

Ca^{2+} influx was triggered by replacement of the Ca^{2+}-free bath solution with 2 mM of a Ca^{2+}-containing bath solution. Cytosolic free Ca^{2+} concentrations were measured by dual-wavelength Fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm. The Fura-2 ratio image was calculated and acquired using the IP-Lab imaging processing system (Scanalytics Inc., Fairfax, VA, USA). Ionomycin (25 μM), a Ca^{2+} ionophore that enhances Ca^{2+} influx, was used to confirm cell viability in vector-transfected cells. $[Ca^{2+}]_i$ was normalized to maximum $[Ca^{2+}]_i$ induced by 25 μM ionomycin.

Electrophysiology

Patch-clamp recordings were performed 1 day after transfection. For inside-out single-channel recordings, the bath solution contained 140 mM KCl, 5 mM EGTA, and 10 mM HEPES at pH 7.4 (adjusted with KOH), and the pipette solution was the same as the bath solution used for the fluorescence measurement. Data from inside-out single-channel voltage-clamp recordings were sampled at 10 kHz and filtered at 5 kHz with a low-pass filter for analysis (Axon 200B amplifier with pCLAMP software). The pipette potential was held at -60 mV. All experiments were performed at room temperature.

Statistics

Statistical analysis was performed using GraphPad PRISM 7 software.

Results

Effects of TRPM4 activation on cytokine production in keratinocytes

First, we confirmed the expression of TRPM4 protein in keratinocytes. We detected clear bands at approximately 138 kDa in the crude protein extracts from NHEKs and HaCaT cells (Fig 1A), as previously reported [13]. To investigate whether TRPM4 influenced immune responses in keratinocytes, we next determined cytokine production levels in keratinocytes. The production of IL-1α and IL-6 protein was induced by TNFα treatment in NHEKs (Fig 1B, C). Treatment with the known TRPM4 agonist BTP2 [19] significantly reduced IL-1α and IL-6 levels in NHEKs stimulated with TNFα (Fig 1B, C).

BTP2 not only activates TRPM4 but also interferes with other Ca^{2+} channels, including calcium release-activated calcium (CRAC) channels [20]. To confirm that the cytokine suppression was actually related to TRPM4, we established a *TRPM4*-knockout HaCaT cell line with CRISPR/Cas9 gene targeting technology. Sanger sequencing revealed that the TRPM4-deficient clone presented a homozygous deletion of 1 base pair (bp) in exon 2 (Fig. S1B). This genetic alteration resulted in a frameshift. Deficiency of the TRPM4 protein was validated by western blot analysis (Fig 2A). Production of *IL-1A* and *IL-6* mRNA transcripts was induced by TNFα treatment in both HaCaT cells and TRPM4-deficient HaCaT cells in a similar manner (Fig 2B–E). However, production of *IL-1A* and *IL-6* mRNA transcripts was decreased by BTP2 treatment in a concentration-dependent manner in HaCaT cells (Fig 2B, C), but not in TRPM4-deficient HaCaT cells (Fig 2D, E). Moreover, IL-1α and IL-6 protein production was induced by TNFα and

decreased by BTP2 treatment in HaCaT cells (Fig 3A, B), but not in TRPM4-deficient HaCaT cells (Fig 3C, D). These results suggested that TRPM4 contributed to the control of cytokine production in keratinocytes.

Effects of aluminum potassium sulfate on cytokine production in keratinocytes

Aluminum potassium sulfate is the main component of Japanese traditional Myoban-onsen spa water [21], which is used as a traditional remedy to ameliorate skin disease. To address the question whether sulfate salt of aluminum may suppress the immune reaction or not, we confirmed the effect of aluminum potassium sulfate on cytokine production in HaCaT cells. Treatment with aluminum potassium sulfate (1 mM) significantly reduced the production of IL-1α and IL-6 proteins induced by TNFα in HaCaT cells (Fig 4A, B) indicating that aluminum potassium sulfate regulate immune responses in keratinocytes.

Aluminum potassium sulfate activated TRPM4

We hypothesized that the observed regulation of immune responses by aluminum potassium sulfate was mediated through TRPM4 activation. Although TRPM4 is a Ca^{2+}-impermeable channel [15], it influences Ca^{2+} uptake of the cells. Therefore, we investigated the effects of TRPM4 activation on store-operated Ca^{2+} entry (SOCE) in HEK293T cells expressing TRPM4 as previously reported [20, 22]. After depletion of Ca^{2+} stores using thapsigargin, and exchanging the extracellular solution to a 2 mM Ca^{2+}-containing bath solution facilitated Ca^{2+} influx through CRAC channels across the plasma

membrane, resulting in elevation of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$; Fig 4C). Treatment with 10 μM U73122, a known TRPM4 agonist, significantly suppressed the elevation of $[Ca^{2+}]_i$ induced by extracellular Ca^{2+} (Fig 4C, D), indicating that Ca^{2+} influx was decreased by TRPM4 activation. Next, we investigated the effects of aluminum potassium sulfate on Ca^{2+} influx through TRPM4 activation. Aluminum potassium sulfate has a low pKa value of −3; therefore, the pH of its aqueous solution is acidic. Because an external acidic pH suppresses Ca^{2+} entry by SOCE [23], the pH of the medium dissolving aluminum potassium sulfate was adjusted to 7.4 before the analysis. Treatment with 1 mM aluminum potassium sulfate significantly decreased the Ca^{2+} influx, suggesting that aluminum ions may activate TRPM4 (Fig 4D).

The decline in $[Ca^{2+}]_i$ was thought to result from the decrease in the driving force for Ca^{2+} influx due to TRPM4 activation; however, direct evidence for this cannot be achieved by Ca^{2+} measurements. Therefore, patch-clamp recordings were further performed to directly observe TRPM4 channel opening. Bath application of 3 μM Ca^{2+} evoked the single channel currents in the inside-out patches excised from HEK293T cells expressing hTRPM4 (Fig 5A). Moreover, 100 μM 9-phenanthrol, a known TRPM4 antagonist, robustly blocked this activation current (Fig 5A). Under these conditions, application of aluminum potassium sulfate (1 mM in pipette solution) also evoked the single channel current that was inhibited by 100 μM 9-phenanthrol under Ca^{2+}-free conditions in hTRPM4-transfected HEK293T cells (Fig 5B), but not in mock plasmid-transfected cells (Fig 5C). Furthermore, aluminum potassium sulfate in the bath solution also evoked the single channel current, which was inhibited by 9-phenanthrol in an inside-out configuration (Fig 5D). These results strongly indicated that aluminum potassium sulfate activated TRPM4.

Discussion

In this study, we first found that TRPM4 contributed to regulation of the immune reaction in keratinocytes. TRPM4 expression in skin and keratinocytes was reported for the first time in 2019, and TRPM4 was shown to affect the proliferation and differentiation of keratinocytes [13, 18]. Furthermore, TRPM4 is expressed in various types of immune cells [9] and is thought to modulate the excessive immune reaction in immune cells. However, the role of TRPM4 in the immune reaction of skin cells has not been investigated.

Recently, some members of the thermo-TRP family have been reported to have functional roles in the immune system [8, 9]. The warm temperature-sensing TRPV3 and TRPV4 are known to be expressed in keratinocytes [11, 12]. Because skin inflammatory diseases, such as atopic dermatitis and psoriasis, show seasonal aggravation in the winter [24, 25], the inflammatory reaction in the skin is thought to be moderated by warm temperature. However, TRPV3 activation results in the release of various inflammatory mediators in keratinocytes such as prostaglandin E2 [26], IL-1α, IL-6 and IL-8 [27, 28]. Moreover, the contribution of TRPV4 to the immune reaction is unknown. Based on our results, TRPM4 may contribute to amelioration of skin conditions in the context of warm temperature by suppressing cytokine production in keratinocytes.

In this study, we clearly showed that TRPM4 activation resulted in decreased cytokine production induced by TNFα in keratinocytes. On the other hand, Yamada et al. reported that gain-of-function mutations of TRPM4 predisposed mice to psoriasiform dermatitis induced by imiquimod in 2022 [18]. Psoriasis is an unconventional autoimmune disease. Enhanced dendritic cell migration in TRPM4 gain-of-function mice is considered to contribute to accumulation of Il-17A producing γδT cells and lead to IL-

17A mediated dermatitis [18]. The function of TRPM4 in these cells under such autoimmune disease condition is probably different from the TRPM4 function in keratinocytes under conventional immune reaction.

The mechanism(s) how TRPM4 affects cytokine production in keratinocytes are not clarified yet. In T cells, TRPM4 is known to mediates cell membrane depolarization, and decreases IL-2 production by decreasing the driving force for Ca^{2+} influx through store-operated Ca^{2+} channels [15, 16]. By contrast, in keratinocytes, ultraviolet B - induced IL-1α secretion is triggered by intracellular Ca^{2+} signals via sequential generation of the Ca^{2+} messenger inositol 1,4,5-trisphosphate (InsP3), nicotinic acid adenine dinucleotide phosphate, and cyclic ADP-ribose [29]. In addition, protease-activated receptor-2 activation stimulates the activity of phospholipase C (PLC) and InsP3-mediated Ca^{2+} release from the ER [30, 31], the activation of the transcription factor nuclear factor-κB [31, 32], and the Ca^{2+}-dependent production of IL-1α, TNFα, and thymic stromal lymphopoietin [32]. Based on these data, decreased Ca^{2+} entry through SOCE by activation of TRPM4 may result in decreased production of these cytokines in keratinocytes.

To identify TRPM4 agonists from natural resources, we have focused on metal ions because TRP channels are known to be activated by some metal ions. TRPA1 is activated by Zn^{2+}, Cu^{2+}, and Cd^{2+} [33], whereas TRPV1 is activated by Zn^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, and Ni^{2+} [34- 39]. Moreover, Zn^{2+} also interacts with TRPM2 [40] and TRPM5 [41]. Myoban-onsen is a type of traditional Japanese spa experience that claims to ameliorate skin diseases and to improve the health status of the skin. Myoban-onsen spa water contains aluminum potassium sulfate as a main component [21], and its incrustation is certified as a quasi-drug by the Japanese Ministry of Health, Labour, and Welfare.

Therefore, we considered the possibility that aluminum ions may interact with TRPM4. In this study, we showed that treatment with aluminum potassium sulfate actually reduced TNFα-induced cytokine production in HaCaT cells (Fig 4A, B). To investigate the effects of aluminum potassium sulfate on Ca^{2+} influx, we performed Ca^{2+} imaging. Because BTP2 is a widely used TRPM4 agonist [19] and an inhibitor of SOCE [20], we selected another TRPM4 agonist U73122 for the Ca^{2+} measurement experiment. U73122 was first established as a selective pharmacological inhibitor of PLC [42, 43], however, it directly modulates TRPM4 currents [44]. Intracellular Ca^{2+} concentrations were decreased with U73122 treatment (Fig 4C, D), indicating that TRPM4 activation resulted in a decrease in Ca^{2+} influx via SOCE. Aluminum potassium sulfate (1 mM) significantly reduced the $[Ca^{2+}]_i$ in TRPM4-expressing HEK293T cells (Fig 4D), indicating possible TRPM4 activation.

Because TRPM4 is a Ca^{2+}-impermeable ion channel, TRPM4 activation cannot be observed directly by Ca^{2+} measurement. Patch-clamp recordings can be used to directly demonstrate TRPM4 channel opening by detecting currents across the cell membrane. Single-channel recordings of TRPM4 were reported in both 2016 and 2017 [45, 46]; these recordings have been used to identify genuine TRPM4 activation currents because the current is measured on only the cell membrane attached to the pipette without the cell body including ER, whereas recordings in whole-cell mode cannot exclude the effects of SOCE. Aluminum potassium sulfate evoked TRPM4-mediated currents in single-channel recordings, thereby demonstrating that it truly activated TRPM4 (Fig 5B). Aluminum potassium sulfate evoked TRPM4-mediated currents when applied in both pipette (Fig 5B) and bath (Fig 5D) solutions, indicating the possibility that impermeable aluminum may directly interact with the transmembrane region of TRPM4 through the

cell membrane.

Aluminum compounds (alum) have been widely used as vaccine adjuvants since the 1930s. Alum triggers IL-1β production and activates caspase 1 [47] through inflammasomes containing NLRP3 [48]. Alum causes release of host DNA at the injection site which acts as an endogenous immunostimulatory signal mediating alum-adjuvanted immunization [49, 50]. Both alum adjuvants, i.e., aluminum hydroxide and aluminum phosphate, are thought to form a colloidal-structure when injected in vivo. By contrast, aluminum potassium sulfate reduces the allergic reaction of the skin to allergens, as tested after an 8.75% diluent was mixed with allergens at a ratio of 10% [51]. Moreover, Myoban-onsen has long been used as a traditional remedy for skin diseases. It is considered that free aluminum ions exist in the spa water, because the pH of the Myoban-onsen is highly acidic [52]. Considering these findings and our results, we propose that free aluminum ions may have suppressive effects on inflammatory reactions, whereas colloidal aluminum could show adjuvanticity. The skin-improving effects of the Myoban-onsen spa may be caused in part by TRPM4 activation induced by the aluminum potassium sulfate.

In this study, we demonstrated that TRPM4 may become a new therapeutic target to ameliorate skin conditions. Furthermore, we identified a new TRPM4 agonist, aluminum potassium sulfate, which directly activated TRPM4 and suppressed cytokine production in keratinocytes. Alleviation of skin conditions and prevention of skin problems may be achieved using this new approach, which modulates cytokine production in keratinocytes through TRPM4 activation.

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Fig 1. TRPM4 expression and effect of activation on IL-1α and IL-6 production in keratinocytes. Western blot analysis of TRPM4 in keratinocytes (A). The cell lysate from hTRPM4-expressing HEK293T cells was used as an hTRPM4 control (A). Effects of TRPM4 activation on IL-1α (B) and IL-6 (C) protein production in NHEKs. NHEKs were treated with 20 ng/mL TNFα and 100 nM BTP2 for 48 hours. The concentrations of IL-1α in the cell lysate and IL-6 in the supernatant were determined. Relative production levels were calculated by setting the control value to 1.0. Results are presented as means ± standard errors of the means of six replicates; *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, one-way analysis of variance with Dunnett's test. Treatment with 100 nM of the TRPM4 agonist BTP2 significantly reduced IL-1α and IL-6 protein expression in NHEKs.

Fig 2. Effect of TRPM4 knockout on *IL-1A* and *IL-6* gene expression in HaCaT cells. Western blot analysis of TRPM4 in TRPM4-deficient HaCaT keratinocytes (A). The cell lysate from hTRPM4-expressing HEK293T cells was used as an hTRPM4 control (A). Effects of TRPM4 activation on *IL-1A* (B, D) and *IL-6* (C, E) gene expression in HaCaT cells. HaCaT cells and TRPM4 deficient HaCaT cells were treated with 20 ng/mL TNFα and BTP2 for 3 hours. mRNA levels were analyzed by quantitative real-time PCR. Expression of target genes was normalized to the expression of reference gene, GAPDH. Relative expression levels were calculated by setting the control value to 1.0. Results are presented as means ± standard errors of the means of six (B, C) or five (D, E) replicates; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, one-way analysis of variance with Dunnett's test. TNFα (20 nM) was applied to induce cytokine production. Treatment with the TRPM4 agonist BTP2 significantly reduced *IL-1A* and *IL-6* mRNA levels in HaCaT cells (B, C). This suppression was not observed in the TRPM4-deficient HaCaT cells (D,

E).

Fig 3. Effect of TRPM4 knockout on IL-1α and IL-6 production in HaCaT cells. Effects of TRPM4 activation on IL-1α (A, C) and IL-6 (B, D) protein production in HaCaT cells (A, B) and in TRPM4-deficient HaCaT cells (C, D). HaCaT cells and TRPM4 deficient HaCaT cells were treated with 20 ng/mL TNFα and 100 nM BTP2 for 48 hours. The concentrations of IL-1α in the cell lysate and IL-6 in the supernatant were determined. Relative production levels were calculated by setting the control value to 1.0. Results are presented as means ± standard errors of the means of six replicates; $^{**}p < 0.01$, $^{****}p < 0.0001$, one-way analysis of variance with Dunnett's test. Treatment with 100 nM of the TRPM4 agonist BTP2 significantly reduced IL-1α and IL-6 protein expression in HaCaT cells (A, B). This suppression was not observed in the TRPM4-deficient HaCaT cells (C, D).

Fig 4. Effect of aluminum potassium sulfate on IL-1α and IL-6 production and intracellular Ca^{2+} concentration. Effects of aluminum potassium sulfate on IL-1α (A) and IL-6 (B) protein production in HaCaT cells. HaCaT cells were treated with 20 ng/mL TNFα and 1 mM aluminum potassium sulfate for 48 hours. The concentrations of IL-1α in the cell lysate and IL-6 in the supernatant were determined. Relative production levels were calculated by setting the control value to 1.0. Results are presented as means ± standard errors of the means of six replicates; $^{****}p < 0.0001$, one-way analysis of variance with Dunnett's test. Treatment with 1 mM aluminum potassium sulfate significantly reduced IL-1α and IL-6 protein expression in HaCaT cells (A, B). Representative Ca^{2+} traces from hTRPM4-expressing HEK293T cells treated with or

without 10 μM U73122 (C). Thapsigargin (1 μM) was added to deplete stored Ca^{2+} to open plasma membrane store-operated Ca^{2+} channels, and addition of external Ca^{2+} (2 mM) caused Ca^{2+} influx through store-operated channels. Ca^{2+} signals were normalized to ionomycin (C). Average Ca^{2+} responses of hTRPM4-expressing HEK293T cells normalized to ionomycin were decreased with 10 μM U73122 or 1 mM aluminum potassium sulfate treatment after depletion of stored Ca^{2+} by thapsigargin (D). Means ± standard errors of the means; $^{**}p < 0.01$, $^{****}p < 0.0001$, control versus treatment; one-way analysis of variance with Dunnett's test.

Fig 5. Direct activation of TRPM4 by aluminum potassium sulfate. Representative traces of the inside-out patches excised from HEK293T cells expressing hTRPM4 or mock plasmid-transfected cells. Bath application of 3 μM Ca^{2+} activated the current in HEK293T cells expressing hTRPM4 (A). Application of 1 mM aluminum potassium sulfate in pipette solution activated the current in HEK293T cells expressing hTRPM4 (B) but not in mock plasmid-transfected cells (C). Bath application of 9-phenanthrol (100 μM) suppressed the current evoked by intercellular Ca^{2+} (A) and aluminum potassium sulfate (B). Bath application of 1 mM aluminum potassium sulfate activated the current in HEK293T cells expressing hTRPM4; the current was inhibited by 9-phenanthrol (100 μM) (D).

Supporting Information

S1 Fig. Generation of HaCaT cell clones with targeted deletions in TRPM4 using CRISPR/Cas9. (A) Schematic representation of the gene structure around the target site. (B) Sequence of the TRPM4-deficient clone.