Published in final edited form as:
Cell Calcium. 2011 November ; 50(5): 473–480. doi:10.1016/j.ceca.2011.08.003.

Examination of the role of TRPM8 in human mast cell activation and its relevance to the etiology of cold-induced urticaria

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Abstract

Mast cells are considered the primary initiators of allergic diseases as a consequence of the release of multiple inflammatory mediators on activation. Although predominately activated through antigen-mediated aggregation of IgE-occupied-FcεRI, they can also be induced to release mediators by other receptors and environmental stimuli. Based on studies conducted in the RBL 2H3 rodent mast cell line, the transient receptor potential melastatin 8 (TRPM8) cation channel has been implicated in the activation of mast cells in response to cold and, by inference, the development of urticaria. Here we investigated the expression and role of TRPM8 receptor, in both human and mouse non-transformed cells, with the aim of exploring the potential link between TRPM8 and the pathology of cold urticaria in humans. Although expressed in mouse mast cells, we found no evidence of TRPM8 expression in human mast cells or functional mutations in *trpm8* in cold urticaria patients. Furthermore, neither mouse nor human primary cultured mast cells degranulated in response to cold challenge or TRPM8 agonists and mast cell reactivity was unaffected in *trpm8*$^{-/-}$ mice. From these data, we conclude that TRPM8 is unlikely to directly regulate mast cell activation in cold urticaria. Thus, alternative mechanisms likely exist for the pathogenesis of this disease.

Keywords

TRPM8; calcium; mast cells; cold-induced urticaria; FcεRI; degranulation

1. Introduction

Mast cells are cells of hematopoietic origin that contribute to innate and adaptive immune defense responses (1,2). Nevertheless, these cells, can adversely affect surrounding tissues

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Conflict of Interest Statement We declare no competing interests

by releasing both stored and *de novo* synthesized inflammatory mediators, thus contributing to allergic disorders such as asthma and anaphylaxis (3). Activation of mast cells can result from engagement of various cell surface receptors (4) or through the influence of a variety of physical stimuli. The high affinity receptor for IgE (FcεRI) is recognized as the principle receptor responsible for eliciting antigen-dependent mast cell activation (5), although other types of receptors can markedly modulate mast cell activation. These include pathogen-recognizing Toll-like receptor family members and receptors for endogenous factors such as PGE_2 (via the EP3 receptor), adenosine (via the A2b and A3 receptors), Il-33 (via the ST2 receptor) and stem cell factor (SCF) (via KIT) (5). In addition, mast cells are known to be activated in certain patients by such physical stimuli as exposure to cold or warm temperatures or vibration, a condition known as physically-induced urticaria (6).

Based on the ability of mast cells to degranulate in response to cold (7, 8), and the presence of elevated levels of mast cell-derived mediators in the plasma of patients with cold urticaria (9,10,11,12,13), mast cell activation has been implicated in the initiation of the symptomology associated with this condition. Cold-induced urticaria is typified by erythematous, circumscribed and pruritic wheals as a consequence of exposure to cold air or water, or even syncope on more extensive skin exposure to cold challenge. However the mechanisms by which cold exposure leads to chronic urticaria through mast cell activation are largely unknown. Recently, it has been proposed, that TRPM8, a temperature-sensitive calcium permeable cation channel, may be the regulator of such responses (14). TRPM8, which is primarily expressed in neuronal tissues but has also been reported in other cells types (15,16) including mast cells, is activated by low temperatures (<30°C), and by binding menthol and the synthetic cooling compounds, WS-12 and icilin (17) thus allowing Ca^{2+} flux from external and intracellular sources.

A potential role for the TRPM8 channel in chronic urticaria was proposed on the basis of the observation that TRPM8 was not only expressed in the rat basophilic leukaemia cell line (RBL 2H3), a model for mast cell function, but could be activated by menthol or by exposure to cold temperature to elicit increased calcium influx and induction of mediator release (14). RBL 2H3 cells are however a tumor cell line which may not truly reflect the function of non-transformed human mast cells. We have, therefore re-investigated the role of TRPM8 in the activation of both primary human and mouse mast cells and determined whether polymorphisms in TRPM8 may be associated with cold urticaria in a human patient population.

Here we report that, unlike rodent mast cells, human mast cells do not appear to express TRPM8 nor do they respond to its known activators. Furthermore we found no mutations predicted to affect function in the *trpm8* gene in peripheral blood cells from patients with cold urticaria when compared to normal subjects. In addition, mast cells derived from human peripheral blood cells and/or mouse bone marrow failed to respond to TRPM8 agonists or cold exposure. Finally, when expressed in mouse mast cells, TRPM8 neither altered mast cell activation or mast cell driven allergic responses. We conclude, therefore, that TRPM8 has no, or minimal, role in mast cell activation by antigen or mast cell-driven responses including the development of cold urticaria and anaphylaxis.

2. Materials and methods

2.1. Chemicals and tissue culture reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. Recombinant human (rH) and mouse (rM) SCF and IL-3, and human IL-6 were purchased from PepcoTech (Rocky Hill, NJ). With the exception of STEMPRO-34 SFM which was

purchased from Invitrogen (Carlsbad, CA), cell culture reagents were from Mediatech (Manassas, VA).

2.2. Mice

For initial experiments, we obtained mice with a C57BL/6 background from The Jackson Laboratory (Bar Harbor ME). *Trpm*$^{-/-}$ mice with C57BL/6 background were kindly provided by Dr. Ajay Dhaka (University of Washington, Seattle) courtesy of Dr. Ardem Patapoutian (The Scripps Research Institute, La Jolla, CA). Animals were bred in the animal care facility at NIH and were used in these studies when 8–16 wks old. The genotype of these mice was confirmed by RT-PCR, using total RNA extracted from cells using the RNeasy total RNA isolation kit (Qiagen, Valencia Ca) or from ear biopsies using, REDRxtract-N-AmpTissue PCR Kit, (Sigma-Aldrich). To confirm the genotype, we used three different primers (Invitrogen): a) forward primer: 5′ GGG ATG TCA TAG TGC TGA AAG GCA GA 3′, b) Rev number 1 5′ CCG GGT GCT GCC CAT AGT ACC ATT TC 3′ and c) Rev number 2 5′ GGT GCA GAT GAA CTT CAG GGT CAG CT 3′. With primers a and b we identified *trpm8* $^{-/-}$ (fragment size 360 bp) and *trpm8* $^{+/+}$ (wild type) with primers a and c (fragment size 295 bp). PCR program [95 °C 30 s, 55°C 30 s, 72°C 1 min] for a total of 30 cycles. The positive control was mouse brain total RNA (Clontech).

2.3. Culture of HuMCs and mouse BMMCs

HuMCs were developed from CD34^{+} peripheral blood progenitor cells obtained from normal volunteers following informed consent on a NIH clinical protocol (09-I-0126) approved by the NIAID IRB. The cells were cultured for one wk in STEMPRO-34 containing STEMPRO supplement (Invitrogen), l-Glutamine (2 mM), penicillin/, streptomycin (100 U/μg/ml), rH IL-3 (30 ng/ml), rH IL-6 (100 ng/ml), and rH SCF (100 ng/ml). After the first wk, rH IL-3 was removed. The cells were subsequently cultured for 7–10 wk as described (18) at which time the cultures were greater than 99% HuMCs and were used for the studies reported (18) herein. LAD2 were similarly cultured but the media had no IL-6 present.

Mouse studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee at NIH. BMMC were developed from bone marrow, obtained by flushing the femurs, of WT and *trpm8*$^{-/-}$ mice. The cells were cultured as described (19) in RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μM beta-mercaptoethanol and rMuIL3 (30 ng/ml). The medium was changed twice a week, and cells were studied at 4−6 wks at which point >95% of cells revealed characteristic mast cell morphology and high surface expression of FcεRI and CD117.

2.4. Trpm8 genogram

Genomic DNA was isolated from whole blood samples on the Autopure LS robotic workstation (Qiagen Corporation, Germantown MD). Primer pairs to generate overlapping amplimers for sequencing of TRPM8 were designed using a Perl script modified from Primer3 (20). The included region for primer design was 5Kb 5′ to 5Kb 3′ of the gene. Primer pairs were designed on sequences with repeats masked to N and SNPs masked to lower case. The average amplicon length was 500 nucleotides with a 100 nucleotide average overalp. Primers were obtained from IDT (Coralville, IA). Liquid handling steps were automated on the BioMek FX robot (Beckman Coulter, Brea CA). Cycle sequencing reactions were performed at 1/64 Big Dye reaction scale (cycle sequence version v1.1, Applied Biosystems, Carlsbad CA). Thermocycling steps were run on MJ Tetrads (BioRad, Hercules CA). A magnetic bead purification system was used for post-PCR and cycle sequencing reaction cleaning (Agencourt Bioscience, Beverly MA). Bead cleaned

sequencing reactions were run on 48 capillary ABI 3730 sequencers (Applied Biosystems). Both strands were sequenced. Sequence data files were uploaded into the PolyPhred program (21) for quality analysis and polymorphism detection.

2.5. Quantitative real-time PCR

Expression of human TRPM8 transcripts relative to human GAPDH was measured in whole blood from 6 normal donors and 6 urticaria patients. Total RNA was isolated from 2.5 ml blood stored in PaxgeneRNA (QIAGEN; Valencia, CA) tubes according to manufactures instructions. Two mg of RNA was used for reverse transcription reaction using random hexamers and Superscript III reverse transcriptase (Invitrogen). Human brain total RNA (Clontech) was used as the positive control. One ml of the resulting cDNA was used for Real Time PCR using the following primers:

TRPM8 sense: 5′-ACCTGCTGGTCGCCATGTTT-3′

TRPM8: antisense 5′-GGATATTGAGGCGGCTGCAGTA-3′

GAPDH sense: 5′-CAGCCTCAAGATCATCAGCA-3′

GAPDH antisense: 5′-TGTGGTCATGAGTCCTTCCA-3′

The reaction mixture consisted of 10% volume of cDNA in a total volume of 15 ml that included SYBR Green master mix (SABiosciences, Frederick, MD) and 175 nM primers.

2.6. RT-PCR

RT-PCR for *trpm8* performed on HuMCs employed the following primers (Invitrogen). Forward primer: CAG ACC CCT GGG ACA TGG TGG ATG; reverse primer: GCC TTT CAA GGT TGC ATT TTG GGC GAC; PCR program (95°C, 30secs; 55°C 30secs; 72°C, 1 min; total 30 cycles). The fragment size was 584 bp. As a positive control we used human brain total RNA (Clontech, Mountain View, CA). For the mouse studies we employed the following primers (also from Invitrogen): Forward primer: 5′ GTG TCT TCT TTA CCA GAG ACT CCA AGG C 3′; reverse primer: 5′ TGC CAA TGG CCA CGA TGT TCT CTT CTG A 3′. PCR program (95 °C, 30 s; 55°C, 30 s; 72°C, 1 min; total of 30 cycles). The fragment size was 479 bp. The positive control was mouse brain total RNA (Clontech).

2.7. Cell activation, degranulation, and cytokine production

For degranulation, cytokine release, and signaling studies, BMMCs were sentisitized overnight with anti mouse monoclonal dinitrophenyl (DNP)-IgE (100 ng/ml) (Sigma) in IL-3-free RPMI medium. The following day, the cells were washed with HEPES buffer (10 mM HEPES pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4 $7H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2$ $2H_2O$) containing 0.04% BSA (Sigma). For degranulation experiments, cells were aliquoted (2×10^4 cells/well) into individual wells of a 96-well plate and triggered in the same buffer with antigen (0–100 ng/ml) for 30 min. In some of the degranulation experiments, BMMC were preincubated with different concentrations of menthol (Sigma) for 30 min or 6 h before antigen. Menthol used in experiments was dissolved in DMSO and subsequently diluted at least 1000× for different experimental conditions. We observed that menthol concentrations ≥1 mM induced significant cytotoxicity (data not shown). For all experiments we have used 100 μM menthol. WS-12 used as a TRPM8 agonist in some experiments was obtained from Sigma. Degranulation was monitored by the release of β-hexosaminidase into the supernatants and calculated as a percentage of the total content (cells and media) found in the supernatants after cell activation (22).

For cytokine release studies, cells were sensitized as above, washed with IL-3-free RPMI media, then the cells (5×10^5) were triggered in this media for 6 h with antigen (10 ng/ml) and/or SCF (10 ng/ml). Cytokines were measured in cell culture supernatants by mouse TNF-α, IL-6, and GM-CSF Quantikine ELISA kits (R&D Systems, Minneapolis, MN).

2.8. Immunoblotting

For signaling studies in which we examined protein phosphorylation by immunoblot analysis, cells were prepared as for degranulation and triggered (1×10^6 cells/100 μl) in 1.5 ml polyethylene screw cap tubes. The reactions were terminated by adding Lysis buffer (23). The proteins were separated by electrophoresis on 4–12% NuPage BisTris gels (Invitrogen). Following membrane transfer, proteins were probed using the following Abs: anti-β actin mAb (clone AC-15) (Sigma), anti-phospho-$PLC_{\gamma1}$ (Tyr(P)-783) pAb (Biosourse), anti-phospho-PLC_{Y2} (Tyr(P)-759) pAb, anti-phospho-AKT (Ser(P)-473) pAb, anti-phospho-ERK1/2 (Thr(P)-202, anti-phospho-LAT (Tyr(P)-171), anti-phospho-JNK (Thr(P)-183/Tyr(P)185), anti-phospho-p38 (Thr(P)-180/Tyr(P)182) (Cell Signaling), and anti-phospho BTK (Tyr(P)-551) pAb (BD Pharmigen). Immunoreactivity was determined following incubation with a secondary Ab (anti mouse IgG peroxidase) and then by ECL (Sigma). To normalize the protein loading, identically loaded samples are probed for β-actin. Protein phosphorylation was determined by scanning the ECL films using a Quantity One scanner (Bio-Rad).

2.9. Toluidine blue staining

Approximately 1×10^5 cells were suspended in Hepes / 20% BSA, then attached to slides by cytospin, fixed in Mota's fixative (20% ethanol, 2.5% lead acetate, 2% acetic acid, in water solution), and stained in toluidine blue solution (30% ethanol, 0.13% toluidine blue, 1% HCl, pH 4) as described (24).

2.10. Flow cytometry

FITC-conjugated rat-anti mouse IgE, PE-conjugated rat-anti mouse CD117, as well as rat IgE, FITC IgE control and PE-conjugated rat IgG2bk isotype control were purchased from BD Biosciences. Cellular staining was performed in PBS, BSA 0.1%, buffer, for 1 h. The cells were washed in the same buffer and analyzed with FACSCalibur Analytic Flow Cytometer (Becton Dickinson).

2.11. Measurement of intracellular calcium

BMMC were loaded with 0.5 μM Fura-2 AM (Molecular Probes, Eugene, OR) in HEPES buffer containing 0.4% BSA and 0.3 mM sulfinapyrazole (25) at 37 °C for 30 min. After incubation, cells were rinsed and re-suspended in the same buffer without Fura-2 AM and then placed in 96-well black culture plate (1×10^4 cells/100 μl/well) (Culture plate-96F, PerkinElmer Life Sciences). The intensity of fluorescence was measured using FlexStation II (Molecular Devices) at two excitation wave lengths (340 and 380 nm) and an emission wavelength of 510 nm. The ratio of the fluorescence readings was calculated following subtraction of the fluorescence of the cells that had not been loaded with Fura-2 AM.

2.12. Chemotaxis

Chemotaxis studies were performed as described (26). Cells were starved in cytokine free media overnight. The day after, the cells were washed and re-suspended in media with SCF or menthol. For the migration assays, we used 24 well Costar 3421 transwell and 500 000 cells/well.

2.13. In vivo studies, induction of anaphylaxis

Mice were sensitized intravenously/retro-orbital plexus with 3 μg anti-DNP IgE mAb (generous gift from Dr. Juan Rivera NIAMS, NIH). Approximately 24 h after sensitization, anaphylaxis was induced by injection 200 μg of DNP-HSA (Sigma) in PBS. Changes in core body temperature were measured every 5 min for 2 h using an implantable electronic transponder (IPTT-300, Bio Medic Data Systems).

To examine passive cutaneous reactions, mice were injected intradermally with IgE anti-DNP-HSA (75 ng) in 30μl of saline in one ear. A sham injection with saline was performed in the other ear. Approximately 24 h after sensitization, mice were challenged intravenously/retro-orbital plexus with 200 μg DNP-HSA in 200 μl of a saline solution containing 0.5% (weight/volume) Evans blue. Thirty min after the DNP-HSA injection, mice were sacrificed, and the ears were collected. Minced ear tissues were incubated in 500 μl of formamide at 55 °C for 2 h to extract the Evans blue dye. Tubes were centrifuged and 200 μl of the supernatant transferred to a 96 well plate to read absorbance at 620 nm.

3. Results

3.1. The expression and function of TRPM8 in human mast cells and BMMCs

We initially examined the expression of *trpm8* mRNA by RT-PCR in human peripheral blood-derived mast cells (HuMCs) and mouse bone marrow-derived mast cells (BMMCs) using comparative expression in human brain tissue as a positive control. As shown in Figure 1A, *trpm8* was expressed in human brain but it was not detected in either the primary cultured HuMCs or the LAD2 human mast cell line. Furthermore, we found no evidence of TRPM8 expression following incubation (6 h) of the cells with 250 μM menthol or with reduced ambient temperatures (32°C) (data not shown). In contrast, TRPM8 transcripts were detected in the mouse BMMCs.

Given that the levels of TRPM8 mRNA in the HuMCs could be below the limit of detection, we examined whether the TRPM8 agonists, menthol and WS-12, could regulate HuMC activation. For these experiments, we co-challenged HuMc, sensitized with biotinylated human IgE (bio-IgE), with fixed concentrations of the TRPM8 agonists in the presence or absence of increasing concentrations of cross-linker, streptavidin (SA). As shown in Figure 1B we observed no effects of the TRPM8 agonists on HuMC degranulation, either in the absence or presence of Fc RI-cross linking. Extended periods of incubation for up to 6 h with the TRPM8 agonists also did not elicit degranulation (data not shown).

Although these data suggest that functional TRPM8 receptors are not expressed in normal human mast cells, the possibility remains that a mutated form of TRPM8 is expressed in mast cells from patients with cold urticaria. We initially conducted real time quantitative PCR on RNA extracted from whole blood from 6 normal controls and 6 patients with cold urticaria. Although we could detect message in human brain extract, which served as a positive control, we were unable to detect message in normal donor and patient samples. Regardless, we sequenced *trpm8* genomic DNA from 8 patients with cold urticaria and 10 controls. Although many polymorphisms were detected, we found no alterations that would disrupt gene regulation or function in any individual (Figure 1C). Taken together, these data demonstrate that TRPM8 could not influence degranulation in human mast cells.

Despite the apparent absence of TRPM8 in HuMCs, we could not rule out the possibility of cryptic upregulation of TRPM8 expression in HuMCs under pathological conditions. Therefore, it was of interest to investigate the role of endogenous TRPM8 in mouse BMMCs where we observed TRPM8 expression

3.2. Menthol does not induce degranulation or calcium flux in primary culture mouse mast cells

To determine whether TRPM8 has indeed a regulatory role in mouse BMMCs, we examined the effects of the TRPM8 agonists, menthol, and WS-12 on BMMC activation. As shown in Figure 2A, menthol at concentrations up to 100 μM failed to produce an increase in cytosolic calcium levels in the BMMCs even though antigen-induced Fc RI aggregation evoked such a response. In addition, antigen-stimulated degranulation was unaffected by the presence of increasing concentrations of menthol (10–100 μM), when added to the cells 30 min prior to antigen, and by itself menthol did not induce degranulation (Figure 2B). Furthermore, menthol and its counterpart, WS-12, had minimal inconsistent effect (menthol: slightly lower; WS-12: slightly higher, at higher concentrations) when responses were augmented by co-stimulation of cells with antigen and SCF (Figure 2C). Therefore, taken as a whole, the data indicate that TRPM8 did not appear to modulate the calcium signal or degranulation in either BMMC or HuMCs.

3.3. Mast cell activation is not reduced in trpm8$^{-/-}$ mast cells

To exclude the possibility that TRPM8 is inherently activated upon Fc RI aggregation, which could mask the effects of menthol activation of TRPM8 on the calcium response or degranulation, experiments were conducted with BMMCs derived from *trpm8*$^{-/-}$ mice and the responses compared to those obtained in BMMCs derived from wild type (WT) mice. Flow cytometry performed on BMMCs from both sets of mice showed identical expression of FcεRI and the SCF receptor, KIT (Fig. 3A). Gross morphology and granularity of the cells, as determined by toluidine blue staining, was also indistinguishable between the WT and *trpm8*$^{-/-}$ derived BMMCs (Figure 3B).

When challenged with antigen, SCF or the combination thereof, WT and *trpm8*$^{-/-}$ BMMCs exhibited no difference in the calcium signal (Figure. 3C). Similarly, there were no obvious defects in other signals, critical for mast cell activation, in the *trpm8*$^{-/-}$ BMMCs with these stimulants (Figure 3D). Degranulation in response to antigen, in the absence or presence of SCF, was also unaffected by *trpm8* deficiency (Figure 4A). Furthermore, there were only slight reductions in the release of TNF-α (Figure 4B) and IL-6 (Figure 4C) in the *trpm8*$^{-/-}$ BMMCs and TRPM8 agonists failed to stimulate chemotaxis in either the WT or *trpm8*$^{-/-}$ BMMC cultures (Figure 4D), in contrast to SCF which was used as a positive control.

Collectively, these results indicate that endogenous TRPM8 in the mouse BMMCs had little regulatory capacity in the stimulation or modulation of mast cell responses.

3.4. Deficiency in trpm8 does not reduce in vivo anaphylaxis responses

To further verify that TRPM8 does not regulate mast cell activation, we utilized *in vivo* mouse models of anaphylaxis. In the first model, WT and *trpm8*$^{-/-}$ mice were passively sensitized with mouse monoclonal IgE targeted against dinitropenol (DNP) followed by i.v. injection of the antigen (DNP-human serum albumin [HSA]). This induced a marked drop in core body temperature in both the WT and *trpm8*$^{-/-}$ mice, up to 40 min after antigen challenge which gradually reversed with full recovery of the body temperature within 120 min. Contrary to what would be expected if TRPM8 played an active role in enhancing mast cell degranulation, the anaphylactic response in the *trpm8*$^{-/-}$ mice was not diminished but rather augmented (Figure 5A). To confirm that TRPM8 is not required for mast cell-driven responses in vivo, we additionally examined comparative passive cutaneous anaphylactic (PCA) responses in the *trpm8*$^{-/-}$ and WT mice. As shown in Figure 5B, there were no significant differences in the antigen-induced PCA responses between the two sets of mice, providing support for the conclusion that mast cell-mediated responses are not directly influenced by the expression and activation of TRPM8.

4. Discussion

In this study, we investigated whether mast cells are activated by cold challenge and the potential role of the TRPM8 cation channel in such activation. The studies made use of the TRMP8 agonists, menthol and WS-12 (Figs. 1, 2 and 4), and BMMCs from *trpm8* knockout mice (Figs. 3–5). The three major findings were these: First, primary mouse and human mast cells in culture failed to respond to cold challenge or the TRPM8 agonists (Fig. 1 and data not shown). Second, neither TRPM8 agonists nor TRPM8-deficiency altered mast cell responses to antigen stimulation *in vivo* (Fig. 5) and *in vitro* with respect to signaling (Fig. 3), degranulation (Figs. 1, 2, and 4), cytokine production, and chemotaxis (Fig. 4). Third, human mast cells derived from peripheral blood cells were deficient in functional TRPM8 in normal subjects (Fig. 1A, B and D). Furthermore, there were no discernable mutations or polymorphisms in the *trpm8* gene of cold urticaria patients that would impact on TRPM8 function (Fig. 1C).

One possible implication of the above findings is that eruption of lesions in these patients is not a consequence of direct activation of mast cells by cold temperature but is dependent on additional mechanisms that lead to mast cell activation. A role for IgE in the pathogenesis of cold urticaria has been proposed on the basis of serum transfer studies (27,28) and successful treatment with the anti-IgE drug omalizumab (29). If an IgE-dependent mechanism is operative in a substantial proportion of patients, as may be the case (27), TRPM8 is unlikely to be involved because of the absence of functional TRPM8 in human mast cells, at least in cell culture, and the lack of effect of TRPM8 agonists on IgE-dependent activation of mast cells by antigen. It should be noted, however, that the exact mechanism by which IgE provokes urticarial lesions, and thus the relevance to antigen-induced activation, are unclear.

Although mast cells do not appear to be directly activated by TRPM8 stimulation, whether by cold or TRPM8 agonists, TRPM8 could conceivably have an indirect role by acting, for example, as cold receptors (30,31) for mediating release of neuropeptides from adjacent sensory neurons. Neuropeptide-containing neurons have direct anatomical contact with mast cells (32) and the absence of sensory neurons diminishes mast cell-initiated cutaneous inflammation (33). Human mast cells express receptors for neuropeptides including neurokinin-1, substance P, somatostin, vasoactive intestinal peptide, and the calcitonin-receptor-like receptor for calcitonin gene-related peptide and are known to be activated by most of these peptides (34,35,36). Release of cytokines and other mast cell mediators are thought to induce further release of neuropeptides from sensory neurons in a bidirectional manner to further enhance activity and recruitment of mast cells in skin lesions of patients with mastocytosis (37).

Nevertheless, our data also suggest that TRPM8 has little or no direct role in mast cell degranulation by antigen in mouse and human primary mast cell cultures although a recent study in the RBL-2H3 mast cell line implies otherwise (14). In the latter study, RBL-2H3 cells were found to express TRPM8, as do mouse BMMC but not human mast cells (our study), and be activated by menthol (ED_{50} ~40 mM) and cold temperature (10°C). Apart from concerns about the relatively high concentration of ethanol (5%) needed for application of menthol and a contrary report that low temperatures (down to 15°C) do not activate but instead suppress responses of RBL-2H3 cells to antigen (38), the apparent discrepancy in findings could reflect differences between our primary mast cell lines and the transformed RBL-2H3 tumor cell line. Due to the slight reduction in cytokine production, in the *trpm8*$^{-/-}$ BMMCs when co-stimulated with antigen and SCF, we cannot rule out a potential contributory role of TRPM8 to cytokine generation, although the mechanism by which this

would occur is unclear. Nonetheless, based on the data as a whole, we conclude that TRPM8 is an unlikely candidate in the pathogenesis of cold urticaria in humans.

In summary, there are compelling reasons to suspect TRPM8 as a primary candidate in the pathogenesis of cold urticaria whether by enabling direct or indirect activation of mast cells in response to cold challenge. TRPM8 is a major sensor and signal transducer of cold environmental temperature and is thought to be involved in chronic neuropathic inflammatory disease (39). At least in one initial report TRPM8 is expressed in RBL 2H3 cells (14). However, we find that TRPM8 is not a ubiquitously expressed in all mast cell types and is absent in human mast cells. A caveat is that, in culture, human mast cells may lose the ability to express TRPM8 although mouse BMMC appear to retain this ability. But even in BMMC, stimulation with menthol appeared to have no functional consequences and therefore the role, if any, of TRPM8 in mast cells remains unclear. As noted earlier, our results do not preclude a neuropathic TRPM8 pathway for cold urticaria but if so there was no obvious genetic defect related to *trpm8* in patients with this disease. The significant delay in recovery of a passive systemic anaphylaxis reaction in *trpm8*$^{-/-}$ mice may, however, suggest that TRPM8 expressed on other cell types apart from mast cells may confer a partial protection against such anaphylaxis, potentially through influencing vascular tone.

Acknowledgments

Financial support for this work was provided by the Division of Intramural Research of NIAID and NHLBI within the National Institutes of Health. The authors would like to thank Joel Abramowitz (NIEHS, NIH) who coordinated the effort and Dr. Ajay Dhaka (University of Washington, Seattle) and Dr. Ardem Patapoutin (The Scripps Research Institute) for supplying the *trpm8*$^{-/-}$ mice; and Dr. Kirshenbaum and the LAD clinical staff for the provision of CD34+ cells.

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- TRPM8 has been implicated in mast cell activation in cold induced urticaria.
- No evidence of TRPM8 expression was observed in normal human mast cells.
- Additionally, no functional mutations in *trpm8* were seen in cold urticaria patients.
- Mast cells responses were unaffected by cold challenge, TRPM8 agonists, and in *trpm8*$^{-/-}$ mice.
- The results indicate that alternative mechanisms regulate mast cell activation in cold urticaria.

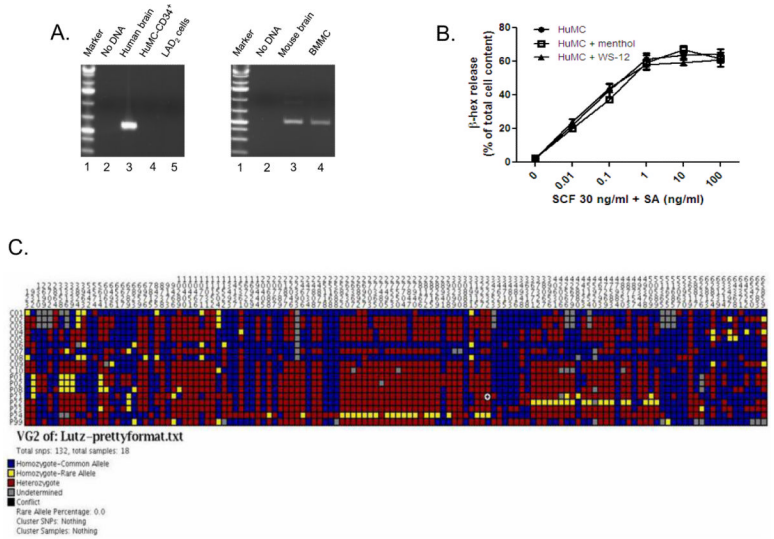

Fig. 1.

(A) *Trpm8* expression in human and mouse mast cells. Left Panel: RT-PCR reveals no detectable expression of *trpm8* in HuMC and LAD2 mast cells compared to the positive control (human brain total RNA; lane 3; predicted size 584 bp). Right panel: RT-PCR reveals the expression of *trpm8* in Mouse BMMC (predicted size 479 bp) and in the positive control (mouse brain total RNA; lane 3). (B) Lack of effect of the TRPM8 agonists, menthol and WS-12 on HuMC degranulation. After pre-incubation with menthol (100 μM) or WS-12 (50 μM) for 30 min, Biotinylated-IgE-sensitized cells were treated with a fixed concentration of SCF (30 nM) and increasing concentrations of streptavidin (SA) for additional 30 min to determine β-hexosaminidase release. The data are presented as means ± S.D. of (n=2) separate experiments conducted in duplicate. (C) Visual Genogram, prettybase file and polymorphism spreadsheet of 7 patients with CU and 10 negative control patients. Patients are designated with “P” and controls with a “C”. Sequencing was conducted across the entire gene using overlapping PCR fragments sequenced in both directions. No alterations were found that would disrupt gene function in any individual.

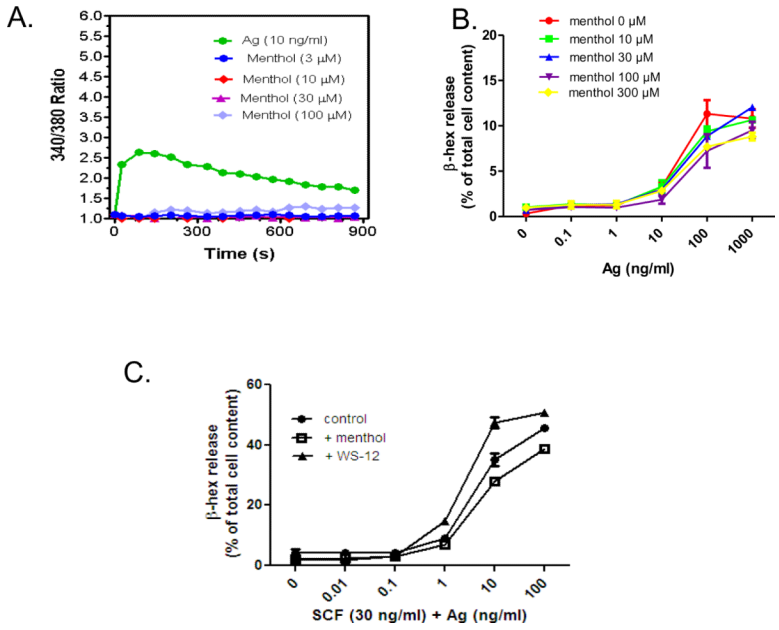

Fig. 2.

(A) Lack of effect of menthol on calcium flux in mouse BMMCs. Anti-DNP-IgE-sensitized cells were loaded with Fura 2 then challenged with DNP-HSA (Ag) or menthol as indicated. (B) Menthol has no effect on BMMC degranulation either in the presence or absence of antigen. Anti-DNP-IgE-sensitized cells were pre-incubated in the presence of different concentrations of menthol (10, 30, 100, 300 μM) for 30 min. Subsequently Ag was added for an additional 30 min and β-hexosaminidase release was measured. The data are presented as means ± S.D. of (n=3) separate experiments conducted in duplicate. (C) The enhanced degranulation in response to SCF was not markedly modified by the addition of either menthol or WS-12. Anti-DNP-IgE-sensitized BMMC were pre-incubated with menthol (100 μM) or WS-12 (50 μM) for 30 min, and the combination of SCF (30ng/ml) and DNP-HSA (Ag) for a further 30 min. The data are presented as means ± S.D. of (n=2) separate experiments conducted in duplicate.

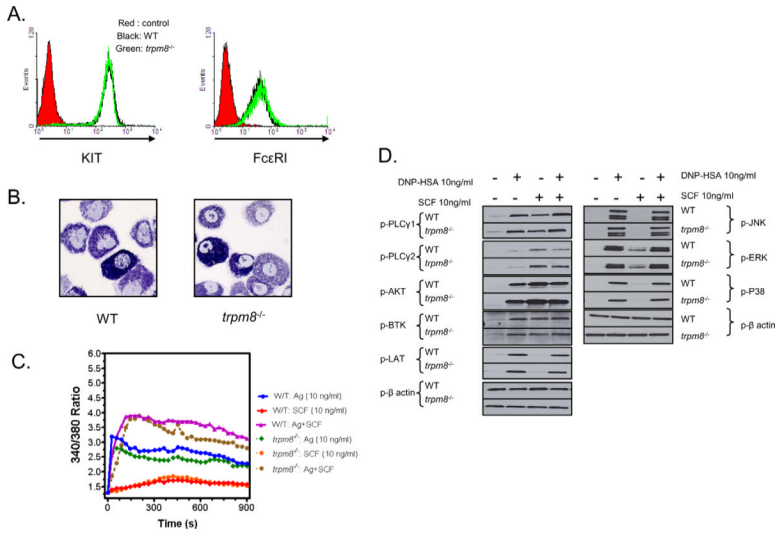

Fig. 3.

(A) BMMCs prepared from WT and *trpm8*$^{-/-}$ mice showed identical expression of both FcεRI and the SCF receptor, KIT. For the flow cytometry assay, BMMCs were incubated for 1 h in the presence of FITC-conjugated rat-anti mouse IgE (2.5 μg/ml), PE-conjugated rat-anti mouse CD117 (1 μg/ml), rat IgE FITC IgE (2.5 μg/ml) control or PE-conjugated Rat IgG2bκ isotype control (1 μg/ml). (B) Toluidine blue staining revealed no difference in morphology between the WT and *trpm8*$^{-/-}$ BMMCs. (C) Lack of difference in the calcium signals observed between BMMCs obtained from WT and *trpm8*$^{-/-}$ BMMCs. Anti-DNP-IgE-sensitized cells were challenged as indicated. The solid lines represent WT BMMCs and the dotted trpm8$^{-/-}$ BMMCs (D) *Trpm8* deficiency does not affect phosphorylation of the signaling molecules important for MC activation. Anti-DNP-IgE-sensitized BMMC were stimulated with DNP-HSA (10 ng/ml) and/or SCF (10 ng/ml) for 10 min. Phosphorylation of different signaling molecules was determined by immune-blot analysis as described in materials and methods. The blots are representative of three independent experiments.

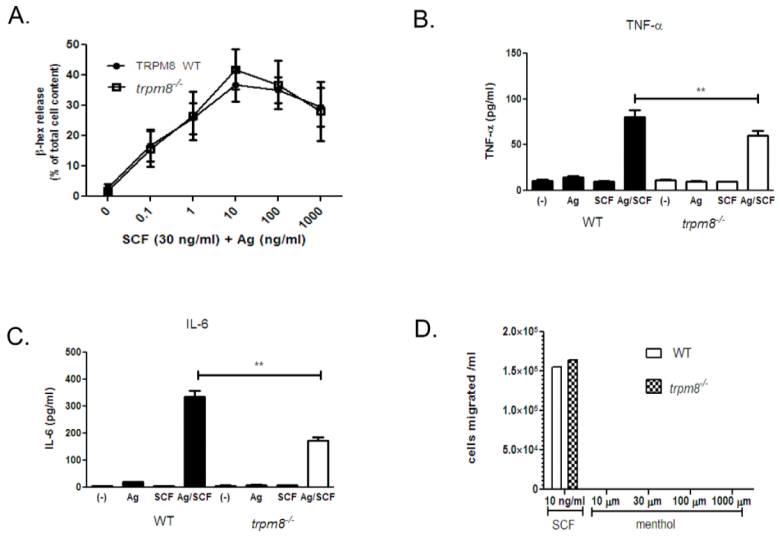

Fig. 4.

(A) *Trpm8* deficiency does affect degranulation. Anti-DNP-IgE-sensitized BMMC were challenged for 30 minutes with SCF (30 ng/ml) and DNP-HSA (10 ng/ml) and β-hexosaminidase release was measured. The data are presented as means ± S.D. of (n=3) separate experiments conducted in duplicate. (B) TNF-α and (C) IL-6 production in BMMCs from WT and *trpm8*$^{-/-}$. IgE sensitized cells were incubated with antigen (10 ng/ml) and SCF (10 ng/ml) individually or in combination for 6 h. The levels of TNF-α and IL-6 were determined by ELISA kit. Values are the mean ± SEM from 3 independent experiments. **, ($p<0.05$) for comparison with Ag/SCF alone by Student's *t* test (D) SCF-enhanced migration of BMMCs. BMMCs from wild type (WT) and *trpm8*$^{-/-}$ BMMCs suspended in RPMI media (top chamber) were allowed to migrate towards the agonist (bottom chamber) as discussed in “Materials and Methods”.

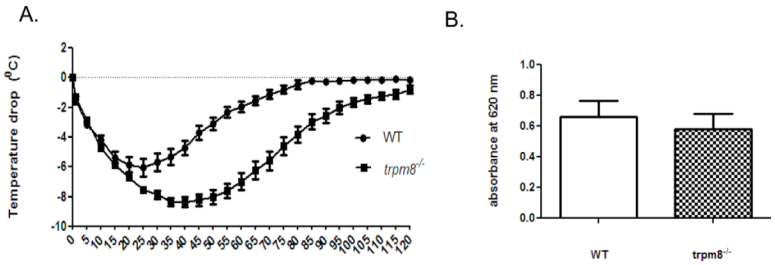

Fig. 5.

(A) Passive Systemic Anaphylaxis. Differences in body temperature induced by antigen (DNP-HAS) mediated anaphylactic shock in TRPM8 wildtype and knockout mice were monitored (N=9). Changes in body temperature were monitored for 2 h using an implantable electronic transponder as outlined in “Materials and Methods”. Statistical significance ($p<0.0001$) was determined using 2-way Anova followed by Bonferroni post-tests. (B) Passive Cutaneous Anaphylaxis: Antigen (DNP-HSA)-mediated vascular permeability differences in wild type (WT) and *trpm8*$^{-/-}$ mice are measured by Evans blue dye extravasation (N=3). The vascular permeability is calculated as the absorbance of dye at 620 nm as outlined in “Materials and Methods”. The values are represented as mean ±SEM.